생활과학박사학위논문

Optimized Formation of Electrostatic Complexes Using Sodium Caseinate and Polysaccharides and Physicochemical Properties of Curcumin or Ellagic Acid-Incorporated Complex

카제인나트륨과 다당류를 이용한 Electrostatic Complex 제조 조건 설정과 Curcumin 또는 Ellagic Acid를 결합시킨 Complex의 이화학 특성

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조 현 노

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Abstract

Optimized Formation of Electrostatic Complexes Using Sodium Caseinate and Polysaccharides and Physicochemical Properties of Curcumin or Ellagic Acid-Incorporated Complex

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Bioactives such as polyphenols are known to have many health benefits including antioxidant and antiinflammatory activities. However, low solubility and stability of the polyphenols hinder their use in food matrixes. One of the ways to overcome these drawbacks is to bind these polyphenols to protein-polysaccharide electrostatic complexes. The ultimate objective of this study was to find optimum condition to form electrostatic complexes using sodium caseinate (NaCas) and polysaccharides, and to increase stability, solubility and utilization of curcumin and ellagic acid (EA) using the complexes.

The objective of study I was to find optimum condition to form electrostatic complexes between NaCas and polysaccharides. Also, NaCas and NaCas-polysaccharide electrostatic complexes were compared for their ability to bind and

stabilize curcumin. Despite many reported bioactivities of curcumin, its application is limited due to its low bioavailability, solubility and stability. Proteins have been reported to stabilize curcumin in aqueous media. Stabilization of curcumin could be enhanced when proteins form an electrostatic complex with polysaccharides. In this study, electrostatic complexes of NaCas were prepared using high-methoxyl pectin (HMP and NaCas-HMP) and carboxymethyl cellulose (CMC and NaCas-CMC). NaCas and polysaccharide ratio of 1:2 resulted in the lowest turbidity and sedimentation. The electrostatic complexes were more stable than native NaCas against changes in pH and ionic strength. Binding of curcumin to NaCas and the electrostatic complexes were confirmed by UV-vis and fluorescence spectra and Fourier transform infrared spectroscopy (FT-IR). The electrostatic complexes showed a higher binding constant and protected curcumin better than the native NaCas. This study suggests that the electrostatic complexes may be a superior carrier to NaCas in an acidic environment.

The objective of study II was to find optimum pH for the electrostatic complexes by evaluating effect of pH on encapsulation efficiency, particle size, zeta potential and heat stability and apply curcumin bound to the complexes as a food colourant to a model beverage. Effect of pH on the characteristics of the complex was evaluated, finding pH 4 was optimum. Zeta potential of NaCas-CMC (-33.59) was larger than that of NaCas-HMP (-22.19) at pH 4, implying higher colloidal stability. The complexes protected curcumin from heat treatment. Antioxidant activity of curcumin

bound to the complexes was similar to that of native curcumin. Incorporation of sucrose partially prevented freeze-drying-induced aggregation of the complex, especially for NaCas-HMP. In a model beverage, curcumin bound to the complexes showed higher colour stability. In vitro bioaccessibility of curcumin bound to NaCas-HMP (53.0%) and NaCas-CMC (51.6%) was higher than the native curcumin (21.4%). This study suggests that curcumin bound to the complexes, especially NaCas-HMP-bound curcumin may be used as a potential food colourant, where transparency is needed.

The objective of study III was to incorporate EA into NaCas and use EA-incorporated NaCas and polysaccharides to increase oxidative stability of emulsions. EA was incorporated into NaCas using a pH cycle method, a method involving higher solubility of EA and dissociation of NaCas in alkaline media. Fluorescence spectra showed interaction between NaCas and EA. FT-IR showed incorporation of EA into NaCas. EA-incorporated NaCas was used as an emulsifier to evaluate effect of EA on the oxidative stability of an emulsion. HMP or CMC was added to increase stability of the emulsion at acidic pH. A stable emulsion was formed when the ratio of NaCas to the polysaccharide was 1:1 at pH 4. EA did not affect creaming index of the emulsion. However, formation of lipid hydroperoxides in the NaCas-HMP and NaCas-CMC-stabilized emulsions with EA was reduced by 22.5% and 24.0%, respectively. Volatile lipid oxidation products were also produced less in the emulsions with EA than without it. These results suggest that the pH cycle method

may be used to incorporate EA into NaCas, which could be used as an emulsifier to increase oxidative stability of an emulsion.

Results of this study show that electrostatic complexes could be formed using NaCas and HMP or CMC, and curcumin and EA could be incorporated into the complexes. The complexes were able to bind and stabilize curcumin, and curcumin bound to the complexes could be used as a food colourant in food systems including beverages where transparency is preferred. Also, emulsions stabilized by EA-incorporated NaCas-polysaccharides showed higher oxidative stability than the emulsions without EA. These results suggest that the complex between NaCas and polysaccharides could be used to deliver water-insoluble polyphenols into food systems. Also, incorporated polyphenols could act as a food colourant or antioxidant in food matrixes, providing additional benefits.

KEYWORDS: Sodium caseinate, High-methoxyl pectin, Carboxymethyl cellulose, Electrostatic complex, Curcumin, Ellagic acid

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List of Abbreviations

ABTS: 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid) diammonium salt

CI: Creaming index

CMC: Carboxymethyl cellulose

DHA: Docosahexaenoic acid

DW: Distilled water

EA: Ellagic acid

EE: Encapsulation efficiency

EPA: Eicosapentaenoic acid

EtOH: Ethanol

FAME: Fatty acid methyl ester

FT-IR: Fourier transform infrared

GCMS: Gas chromatograph mass spectrometer

GRAS: Generally recognized as safe

HMP: High-methoxyl pectin

MUFA: Mono unsaturated fatty acids

NaCas: Sodium caseinate

PDI: Polydispersity index

PUFA: Polyunsaturated fatty acids

SFA: Saturated fatty acids

SPME: Solid-phase microextraction

Chapter 1

Introduction and Literature Review

1.1 Background

Proteins are widely used for encapsulation and delivery of bioactives as proteins are able to bind hydrophobic compounds, interact with other biopolymers, act as an emulsifier and are widely available (Zimet et al., 2008; Yang et al., 2013). Proteins have amphiphilic nature, which allows them to bind to hydrophobic polyphenols maintaining solubility (Santipanichwong at el., 2008). Among proteins, sodium caseinate (NaCas) is commonly used in drug delivery systems as NaCas is biocompatible, low in toxicity and widely available (Elzoghby et al., 2011). NaCas was used to stabilize a variety of bioactives including curcumin, bixin, thymol and folic acid (Sneharani et al., 2009; Esmaili et al., 2011; Zhang & Zhong, 2013; Pan et al., 2014; Penalva et al., 2015).

However, NaCas is unstable when pH is close to isoelectric point (pI) or when ionic strength is high (McClements, 2006). Stability of proteins against environmental stresses including pH and ionic strength could be increased by forming electrostatic complexes with polysaccharides (Santipanichwong et al., 2008). Previous papers reported that stabilization of bioactives by proteins could be enhanced by forming complexes with polysaccharides (Zimet et al., 2008; Yang et al., 2013; Li et al., 2015). When pH is below pI of the proteins, positively charged proteins interact with negatively charged polysaccharides to form complexes (Zimet et al., 2008). High-methoxyl pectin (HMP) and carboxymethyl cellulose (CMC) were reported to be able to stabilize NaCas in an acidic environment (Pereyra et al., 1997;

Nakamura et al., 2006). Therefore, it was hypothesized that HMP and CMC could be used to form electrostatic complexes with NaCas to increase stability of NaCas against environmental stresses including pH and ionic strength. The complexes could be used to incorporate bioactives or to stabilize emulsions in acidic environment where NaCas alone is not applicable. Many factors including pH and the ratio of proteins to polysaccharides should be optimized in order to form stable complexes without sedimentation or to form stable emulsions without phase separation (Zimet et al., 2008; de Kruif et al., 2004; Liu et al., 2012). The complexes formed could be used to bind, stabilize and increase solubility of water-insoluble polyphenols, increasing their utilization.

Curcumin is a yellow hydrophobic polyphenol from turmeric with various functionalities including antioxidant, anticancer and antiinflammatory activities (Wahlstrom & Blennow, 1978; Kuttan et al., 1985; Chan et al., 1998). However, application of curcumin is limited due to its low solubility and stability (Wang et al., 1997; Kaminaga et al., 2003; Yang et al., 2013). Due to its amphiphilic nature, NaCas has been used to bind and stabilize lipophilic compounds including curcumin (Pan et al., 2013). Many studies reported that NaCas could increase solubility and stability of curcumin through binding (Esmaili et al., 2011; Pan et al., 2014). As mentioned above, complexes between proteins and polysaccharides showed higher polyphenol-stabilizing ability than the protein counterparts (Yang et al., 2013; Li et al., 2015). Thus, it was hypothesized that curcumin-stabilizing ability of NaCas could be

enhanced by forming complexes with HMP or CMC. However, no study was done on stabilization of curcumin using electrostatic complexes between NaCas and HMP or CMC.

Ellagic acid (EA) is a polyphenol commonly found in berries and nuts (Daniel et al., 1989). Despite of its antioxidant and anticancer activities, utilization of EA is limited due to low solubility of EA in aqueous media and organic solvents (Bala et al., 2006). NaCas is known to dissociate in alkaline conditions (Vaia et al., 2006). Some polyphenols are insoluble in aqueous media but soluble in alkaline media due to deprotonation (Leung et al., 2008; Luo et al., 2015). These characteristics have been used to incorporate polyphenols into NaCas without using organic solvents (Pan et al., 2014; Luo et al., 2015). EA shows higher solubility in alkaline media, implying that EA could be incorporated into NaCas using a pH cycle method. Also, it was expected that EA-incorporated NaCas could be used as an emulsifier with antioxidant activity. However, incorporation of EA using a pH cycle method and efficacy of EA-incorporated NaCas on oxidative stability of emulsions have not been tested.

1.2 Objectives

Objective of this study was to find optimum condition to form electrostatic complexes using sodium caseinate (NaCas)-polysaccharides and to incorporate curcumin and ellagic acid (EA) into the complexes. The complexes formed were used to increase stability and utilization of curcumin (Part 1), and EA-incorporated

complexes were used as an emulsifier to increase oxidative stability of emulsion (Part 2). The specific objectives were:

Part 1

Study I

To find optimum condition to form electrostatic complexes between NaCas and HMP or CMC;

To compare HMP and CMC for their ability to form electrostatic complexes with NaCas;

To compare stability and characteristics of the complexes with NaCas; and

To compare NaCas and the complexes for their ability to bind and stabilize curcumin.

Study II

To evaluate effect of pH on the characteristics of the complexes to find optimum pH;

To compare antioxidant activity of curcumin bound to the complexes and native curcumin;

To find optimum condition to prevent or minimize freeze-drying-induced aggregation of the complexes; and

To evaluate stability and in vitro bioaccessibility of native curcumin and curcumin bound to the complexes in the model beverage.

Part 2

Study III

To find optimum condition to form emulsions stabilized by NaCas-polysaccharide;

To incorporate EA into NaCas using a pH cycle method;

To evaluate effect of EA incorporation on the physical stability of emulsions stabilized by NaCas-polysaccharide; and

To evaluate effect of EA incorporation on the oxidative stability of emulsions stabilized by NaCas-polysaccharide.

1.3 Literature review

1.3.1 Sodium caseinate

Bovine milk contains about 30 to 36 g/L proteins and the major milk protein is casein, which is about 79% of total proteins followed by whey protein with about 19% (Tavares et al., 2014). The composition of milk proteins is shown in Table 1.1 (Tavares et al., 2014). The caseins are proline-rich proteins with hydrophobic and hydrophilic domains and serine–phosphate residue is available in α_{s1}-casein, α_{s2}-casein and β-casein for calcium sequestration (Livney, 2010). Most of the caseins (about 95%) are self-assembled into casein micelles by hydrophobic interactions and calcium phosphate bridges with 50-500 nm in size (Livney, 2010; Elzohoby et al., 2011). The surface of casein micelle is covered with κ-casein which provides electrostatic and steric stabilization (de Kruif et al., 1996). The main function of casein micelle in milk is believed to deliver calcium, phosphate and protein from mother to neonate (Livney, 2010). Casein is commonly used in the form of NaCas, which has higher aqueous solubility than acid casein (Horne, 2002). Due to high hydrophobicity, NaCas has been used to encapsulate hydrophobic bioactives.

Table 1.1 Composition of bovine milk proteins

Proteins		Concentration in milk (g/kg)	Molecular weight (kDa)	Isoelectric point
Casein micelle		26	~10^5	4.6
Caseins fractions	α_{S1}	10.7	23.6	4.9
	α_{S2}	2.8	25.2	5.2
	β	8.6	24	5.4
	κ	3.1	19	5.6
Whey proteins	β -Lactoglobulin	3.2	18.3	5.2
	α-Lactalbumin	1.2	14.2	4.3-4.7
	Bovine serum albumin	0.4	66.3	5
	Lactoferrin	0.1	83	8.5

(Tavares et al., 2014)

1.3.2 Protein-polysaccharide interaction

Proteins and polysaccharides could have different interactions depending on the environmental conditions and nature of proteins and polysaccharides (Fig. 1.1; Ye, 2008). Proteins and polysaccharides could be co-soluble and may form a stable solution. If the proteins and polysaccharides have the same charge, then the biopolymers are incompatible and could be separated into protein-rich phase and polysaccharide-rich phase (Tolstoguzov, 1991). If the proteins and polysaccharides have different charges, then the complexes could be formed through electrostatic interaction (de Kruif et al., 2004). Complex formation occurs at the pH below isoelectric point of the proteins and above pK value of the anionic polysaccharides where proteins and anionic polysaccharides carry net positive and negative charges, respectively (de Kruif et al., 2004). Even if pH is above isoelectric point of the proteins, some electrostatic interactions could occur between charged patches of the proteins and polysaccharides (Doublier et al., 2000). If the charge of the proteins is neutralized by that of polysaccharides, insoluble complexes would be formed (Schmitt et al., 1998). When proteins or polysaccharides are in excess, charge will not be neutralized and soluble complexes could be formed (Schmitt et al., 1998).

Protein-polysaccharide electrostatic complexes could increase the stability of proteins against change in pH by preventing protein-protein interaction by providing electrostatic and steric repulsion (Fig. 1.2; McClements, 2006; Ye, 2008; Hosseini et al., 2015). The electrostatic complexes are reported to be more stable to changes in

pH and ionic strength than their protein counterparts. Ye et al. (2006) reported that the complexes of NaCas and gum arabic showed higher pH stability than NaCas. Also, the complexes of β-lactoglobulin and pectin were more stable to increase in NaCl concentration than β-lactoglobulin alone (Santipanichwong at el., 2008).

Moreover, electrostatic complexes showed superior stabilizing activity compared to their protein counterparts in many studies. Yang et al. (2013) reported that the complex between bovine serum albumin and iota-carrageenan showed higher stabilizing effect on curcumin than bovine serum albumin. Li et al. (2015) also showed that thermally induced complex between bovine serum albumin and iota-carrageenan had better stabilizing effect on (−)-epigallocatechin-3-gallate. The electrostatic complexes of β-lactoglobulin and pectin showed better stabilizing effect on n-3 polyunsaturated fatty acids and vitamin D_2 than β-lactoglobulin alone (Zimet et al., 2009; Ron et al., 2010).

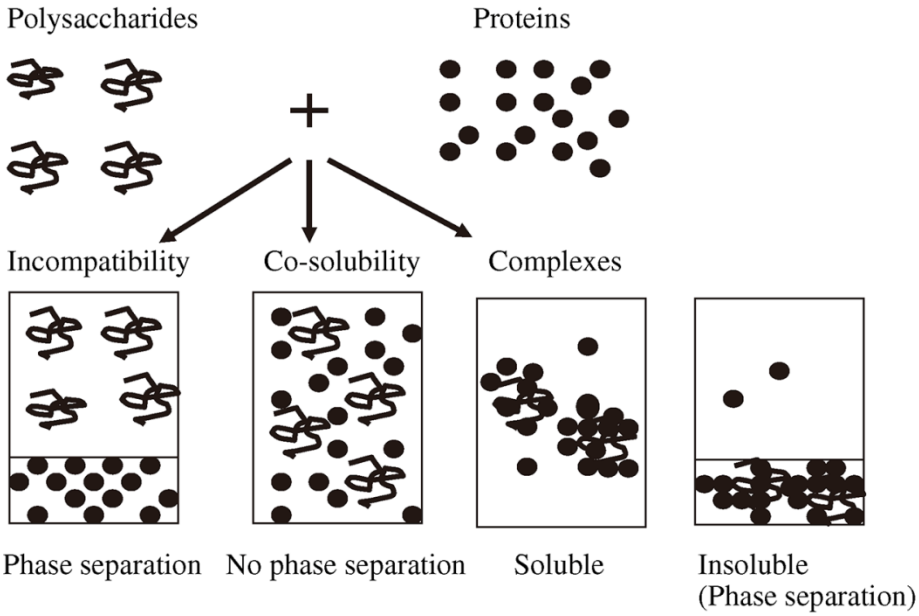

Fig. 1.1 Possible interactions in a mixture of proteins and polysaccharides

(Ye, 2008)

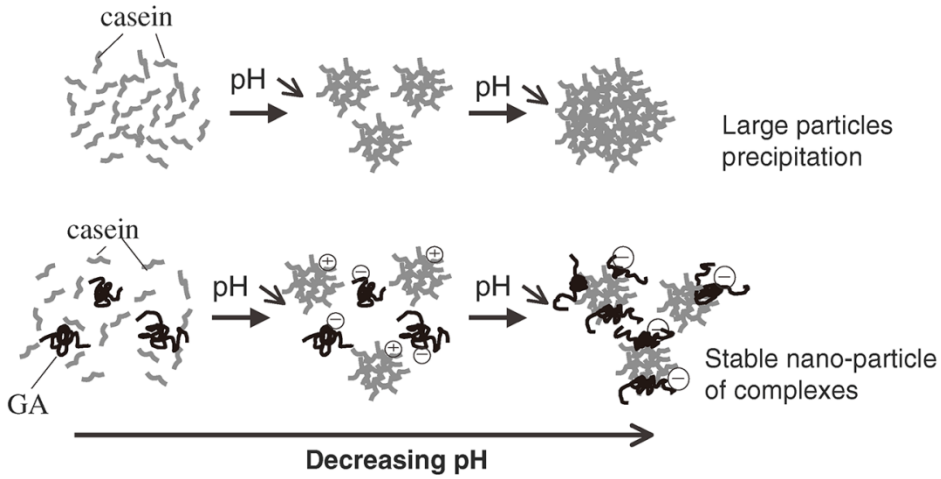

Fig. 1.2 Interaction and stabilization of caseins with gum arabic (GA) against change in pH

(Ye, 2008)

1.3.3 Curcumin

Turmeric, *Curcuma longa* L. (Zingiberaceae family) rhizomes, has been used as traditional medicine for centuries (Ammon & Wahl, 1991). The main active compounds in turmeric are curcuminoids (Maheshwari et al., 2006). Amount of curcuminoids in turmeric is about 3-5% (Goel et al., 2008). Curcuminoids are consist of curcumin (curcumin I; 77%), demethoxycurcumin (curcumin II; 17%), and bisdemethoxycurcumin (curcumin III; 3%) (Goel et al., 2008; Strimpakos & Sharma, 2008).

Curcumin is known to have antiinflammatory (Srimal & Dhawan, 1973), anticancer (Kuttan et al., 1985), antioxidant (Toda et al., 1985), wound healing (Sidhu et al., 1998) and antimicrobial effects (Negi et al., 1999). Bioavailability of curcumin is low. Wahlstrom et al. (1978) reported that when curcumin was orally administrated, about 75% of curcumin was excreted in the feces and a trace amount was detected in the urine. Low bioavailability of curcumin was believed to be due to its low solubility in water. Curcumin is practically insoluble in water at an acidic and neutral pH and soluble in organic solvents including dimethylsulfoxide, acetone and methanol (Prasad et al., 2014). Another reason for low bioavailability of curcumin is its low stability in gastrointestinal track (Wang et al., 1997). Curcumin is unstable at aqueous media, especially at neutral pH. At pH 7.2, more than 90% of curcumin was degraded in 30 min (Wang et al., 1997).

1.3.4 Ellagic acid

EA, a dimer of gallic acid, is found in the plant vacuole as free EA, EA derivatives, or bound forms as ellagitannins (ET) (Amakura et al., 2000). Amount of EA in various fruits and nuts after acid hydrolysis is shown in Table 1.2 (Daniel et al., 1989). ET are a complex class of polyphenols, consisting of hexahydroxydiphenoyl (HHDP) moieties esterified to a sugar, usually glucose (Landete, 2011). Due to the different possibilities for the linkage of HHDP residues with glucose moiety, and tendency to form dimeric and oligomeric derivatives, ET have much structural variability (Niemetz & Gross, 2005). When ET are hydrolyzed by acids or bases, HHDP group spontaneously rearranges to EA after hydrolysis of ester bonds (Clifford & Scalbert, 2000). Antioxidant, antimugatenic, anticancer and apoptosis inducing activities are reported for EA (Bala et al., 2006). However, efficacy of EA is low due to its low solubility. EA is almost insoluble in water and sparingly soluble in organic solvents (Bala et al., 2006).

Table 1.2 Amount of ellagic acid (EA) in various fruits and nuts after acid hydrolysis

Food	Genus species	µg EA/g, dry weight
Strawberries	*Fraqaria ananassa*	630 ± 90
Raspberries	*Rubus ideaus*	1500 ± 100
Blackberries	*Rubus ursinus*	1500 ± 140
Cranberries	*Vaccinium*	120 ± 4
Pecans	*Caryna illinoensis*	330 ± 0.3
Walnuts	*Juqlans niqra*	590 ± 1
Peanuts	*Anachis hypoqaea*	< 100[c]
Cashews	*Anacardium occidentale*	< 100
Red Apples	*Malus pumila*	< 100
Navel Oranges	*Citrus sinensis*	< 100
Pink Grapefruit	*Citrus paradisi* (pink)	< 100
White Grapefruit	*Citrus paradisi* (white)	< 100
Tangerine	*Citrus reticulata*	< 100
Tangelo	*Citrus tanqelo*	< 100
Peach	*Prunus perspica*	< 100
Brown Pear	*Prunus communis* (brown)	< 100
Green Pear	*Prunus communis* (green)	< 100
White Grape	*Vitis* (white)	< 100
Red Grape	*Vitis* (red)	< 100
Sour Cherry	*Prunus serotina* (sour)	< 100
Bing Cherry	*Prunus serotina* (bing)	< 100
Elderberry	*Sambucus*	< 100
Blue Plum	*Prunus domesticata*	< 100
Blueberries	*Vaccinium occidentalis*	< 100
Kiwi	*Actinidia chinensis*	< 100

Samples were extracted with methanol and hydrolyzed with trifluoroacetic acid.

[c] Ellagic acid was below the calibration range.

(Daniel et al., 1989)

Chapter 2

Formation of Electrostatic Complexes Using Sodium Caseinate with High-Methoxyl Pectin and Carboxymethyl Cellulose and Their Application in Stabilization of Curcumin (Part 1: Study I)

2.1 Introduction

Curcumin, a yellow hydrophobic polyphenol from turmeric, is known to have many physiological activities including anticancer, antiinflammatory and antioxidant activities without significant side effects (Wahlstrom & Blennow, 1978; Chan et al., 1998; Cheng et al., 2001; Rosa et al., 2014). However, application of curcumin is limited due to its low solubility in water (Kaminaga et al., 2003) and its bioavailability is also limited due to its low stability (Wang et al., 1997; Yang et al., 2013).

Proteins could be potential carriers for hydrophobic compounds including curcumin due to their amphiphilic nature. Moreover, proteins are advantageous compared to other carrier materials as they are nontoxic, cheap and widely available (Yang et al., 2013). Previous studies reported that casein, β-lactoglobulin, soy proteins and bovine serum albumin were able to increase solubility, stability and bioavailability of curcumin (Sneharani et al., 2009; Sneharani et al., 2010; Esmaili et al., 2011; Tapal & Tiku, 2012). However, proteins tend to form insoluble aggregates when pH is near the isoelectric point (pI) or when the ionic strength is high (McClements, 2006). The stability of proteins could be improved by forming electrostatic complex with polysaccharides (Santipanichwong et al., 2008). These electrostatic complexes are formed at pH below the pI of the proteins, where proteins carry net positive charges and anionic polysaccharides carry net negative charges (de Kruif et al., 2004).

Previous papers reported that casein micelles could be used to encapsulate curcumin (Sneharani et al., 2009; Esmaili et al., 2011). Also, Pereyra et al. (1997) reported that low and high-methoxyl pectins (LMP and HMP, respectively) were able to stabilize caseins in an acidic environment by adsorbing onto the surface of caseins and the effect was greater for HMP than LMP (Pereyra et al., 1997; Nakamura et al., 2006). Also, carboxymethyl cellulose (CMC) was reported to stabilize milk proteins in acidic conditions (Du et al., 2007; Wu et al., 2013). However, effect of these polysaccharides on binding and stabilization of curcumin to sodium caseinate (NaCas) has not been studied. In this study, two different polysaccharides, HMP and CMC, were compared for their ability to form electrostatic complex with NaCas in acidic conditions where NaCas is not applicable. Also, stability and characteristics of electrostatic complexes were measured and compared to those of the native proteins. Finally, NaCas and electrostatic complexes were compared for their ability to bind and stabilize curcumin.

2.2 Materials and methods

2.2.1 Materials

Food grade NaCas (protein content: 91% (wet basis); and moisture content: 5.6%) was kindly donated from Samik Dairy & Food Co. Ltd. (Seoul, Korea). Food grade HMP (degree of esterification: 71%) and CMC (degree of substitution: 1.0-1.2) were purchased from Esfood Co. Ltd. (Pocheon, Korea) and Ashland Chemical Co. (Changzhou, Jiangsu, China), respectively. Curcumin, ethanol (EtOH), HCl and

NaOH were from Samchun Chemicals (Seoul, Korea). Sodium chloride and phosphate buffered saline were from Junsei Chemical (Tokyo, Japan) and Bio-Rad (Richmond, CA, USA), respectively.

2.2.2 Formation of electrostatic complex

Solutions of NaCas, HMP and CMC were prepared separately by dispersing the powder in distilled water under continuous stirring for 2 hr and incubating the solutions at 4 °C overnight for complete hydration. The pH of the solutions was adjusted to 7 using HCl and NaOH. To determine optimum ratio of NaCas to HMP and CMC, the two solutions were mixed at various proportions to have the required concentrations of NaCas and the polysaccharides. The final concentrations of NaCas and the polysaccharides were 0.1% and 0 to 0.5%, respectively. Then the pH of the mixture was gradually reduced to 4 to form complex (Santipanichwong et al., 2008; Ron et al., 2010). pH of the solution was adjusted to 7, and then gradually reduced to 4 as a previous study showed that acidification after mixing resulted in lower turbidity and particle size (Bedie et al., 2008). During acidification, 0.1 N HCl (pH from 7 to 5), 0.4 N HCl (pH from 5 to 3) and 1 N HCl (pH from 3 to 2) were added dropwise under continuous stirring (Hosseini et al., 2013). The mixture was left at room temperature overnight, and the sedimentation and turbidity were measured. Sedimentation was measured by taking digital image. After vortexing, turbidity was measured using a spectrophotometer (Optizen 2020UV, Mecasys, Daejeon, Korea) with 1 cm pathlength cuvette at 600 nm. The complex composed of 0.1% NaCas and

0.2% HMP or CMC (NaCas-HMP and NaCas-CMC, respectively) was found to be the most stable with the lowest sedimentation and turbidity (Fig. 2.1). Thus this ratio was chosen for the rest of the study.

2.2.3 Effect of pH and NaCl on the stability of the complex

To evaluate pH stability, NaCas, NaCas-HMP and NaCas-CMC at pH 7 were gradually acidified to pH 2 as described above. Aliquots were taken for every 0.5 unit change in pH and stored at room temperature overnight before turbidity measurement. In order to evaluate the effect of NaCl on the stability of NaCas and the complexes, NaCas and the electrostatic complexes were prepared and adjusted to pH 3. This pH was chosen as turbidity of NaCas was too high when the pH was above 3, interrupting the turbidity measurement. Concentrated NaCl solution (17.5%, w/v) was added to achieve various concentrations of NaCl (0 – 250 mM). Distilled water was added to the samples to maintain constant concentrations of the solute. After incubation at room temperature overnight, digital images were taken and turbidity was measured. Turbidity of HMP and CMC was remained unchanged regardless of the NaCl concentration (data not shown).

2.2.4 Loading of curcumin

Stock solution of curcumin was prepared at the concentration of 1.0 mg/mL in EtOH. This concentration was chosen according to the previously published literature (Yang et al., 2013). Ten µL of curcumin stock solution was added per mL of the

solution of NaCas or electrostatic complex to have a final curcumin concentration of 10 μg/mL. Concentration of EtOH in the final solution was 1%, which is negligible. To measure encapsulation efficiency (EE), the mixture was centrifuged at 2,580 x *g* for 30 min at 4 °C. The pelleted free curcumin was quantified using standard curve constructed with curcumin in EtOH (Xu et al., 2014).

2.2.5 Particle size and zeta potential

The particle size, polydispersity index (PDI) and zeta potential of the NaCas and electrostatic complexes were measured using an electrophoretic light scattering spectrophotometer (ELSZ-1000, Otsuka Electronics, Osaka, Japan). NaCas, NaCas-HMP and NaCas-CMC were prepared at pH 3. All the measurements were done at 25 ˚C and the angles were 165˚ and 15˚ for particle size and zeta-potential, respectively.

2.2.6 UV-vis absorption and fluorescence spectra

Absorption spectra from 300 to 600 nm were measured (SpectraMAX, Molecular Devices, Sunnyvale, CA, USA) for the solutions prepared as described above. For fluorescence spectra, excitation wavelength was 424 nm and emission was recorded from 450 to 700 nm (CLARIOstar, BMG Labtech, San Francisco, CA, USA). For binding constant measurement, NaCas, NaCas-HMP and NaCas-CMC were mixed with various concentrations of curcumin (0 to 10 μg/mL). Fluorescence spectra were measured using FluoroMate (FS-2, Scinco, Seoul, Korea). Excitation and emission wavelengths were 280 nm and from 300 to 600 nm, respectively.

2.2.7 Fourier transform infrared spectroscopy

Fourier transform infrared (FT-IR) spectra of curcumin and NaCas, NaCas-HMP and NaCas-CMC with curcumin were obtained using a Nicolet 6700 FTIR spectrometer (Thermo Nicolet Corp., Madison, WI, USA). NaCas, NaCas-HMP and NaCas-CMC were prepared at pH 3 and then freeze-dried before the measurement. The range of wavelength was 4000-650 cm^{-1}. The number of scan was 32. Resolution was 8 cm^{-1}.

2.2.8 Stability of curcumin

Solutions of DW, NaCas, HMP, CMC and complexes at pH 3 were prepared as described above and mixed with curcumin stock solution to assess stability of curcumin. The solutions were incubated under dark at room temperature for 24 hr or at 60 °C for 6 hr. Initial intensity at 428 nm was set as 1 and change in intensity in relation to initial absorbance was recorded.

2.2.9 Statistical analysis

Statistical analysis using one-way analysis of variance (ANOVA) was conducted using SPSS 22 software (SPSS Inc., Chicago, IL, USA). Duncan's multiple range test ($P < 0.05$) was used to determine significance between the samples.

2.3 Results and discussion

2.3.1 Formation of electrostatic complex

Turbidity of NaCas was high at pH 4 (Fig. 2.1) since the pH was close to pI of NaCas (4.6; Zhang & Zhong, 2013a). Turbidity initially increased slightly when a low concentration (0.01%) of HMP or CMC was added. As the concentration of the polysaccharide increased, turbidity became lower. When the concentration was above 0.1 %, turbidity was maintained at a relatively low level. For CMC, turbidity increased when the CMC concentration was 0.5%. Therefore, the optimum concentration of NaCas to polysaccharides was chosen as 0.1% NaCas to 0.2% polysaccharides. Similar results were reported in previous reports (Bedie et al., 2008; Ron et al., 2010; Luo et al., 2015). It was reported that a lower concentration of polysaccharides may destabilize the mixture by charge neutralization and bridging flocculation (McClements, 2005).

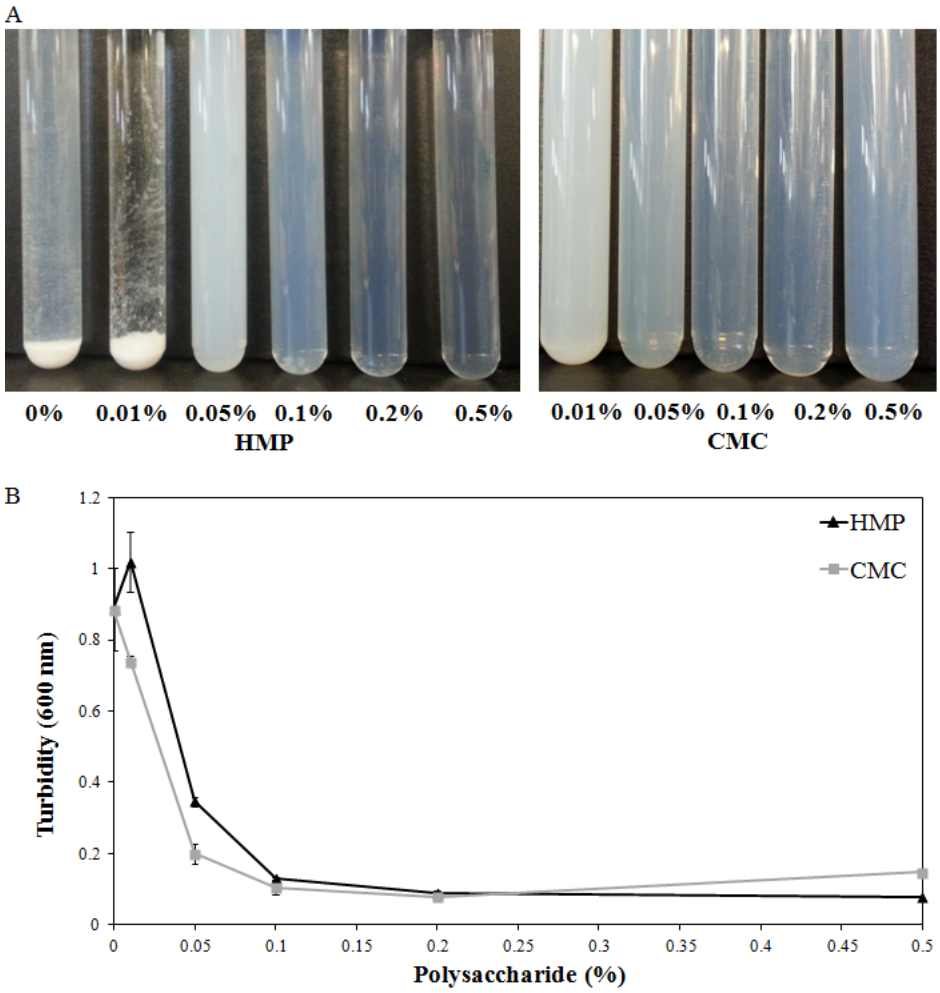

Fig. 2.1 Effect of polysaccharide concentration on the visual appearance (A) and turbidity (B) of NaCas-HMP and NaCas-CMC mixtures at pH 4

Values are means ± standard deviations (n = 3).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

2.3.2 pH stability of sodium caseinate and the complexes

The effect of pH on the turbidity of NaCas, NaCas-HMP and NaCas-CMC is shown in Fig. 2.2. For NaCas, increases in turbidity and sedimentation were observed when pH was close to pI of NaCas. Then the solutions became clear when the pH was further reduced to 3. For the complexes, a slight increase in turbidity was observed at the pH ranging from 6 to 3, implying the complex is formed in this pH range. Increase in turbidity indicates that the particles formed were large enough to scatter the light (Ducel et al., 2004). Surh et al. (2006) also reported that NaCas and HMP interacted at the pH from 3 to 5. This result indicates that electrostatic complexes could be utilized in food systems with a low or acidic pH where NaCas precipitates.

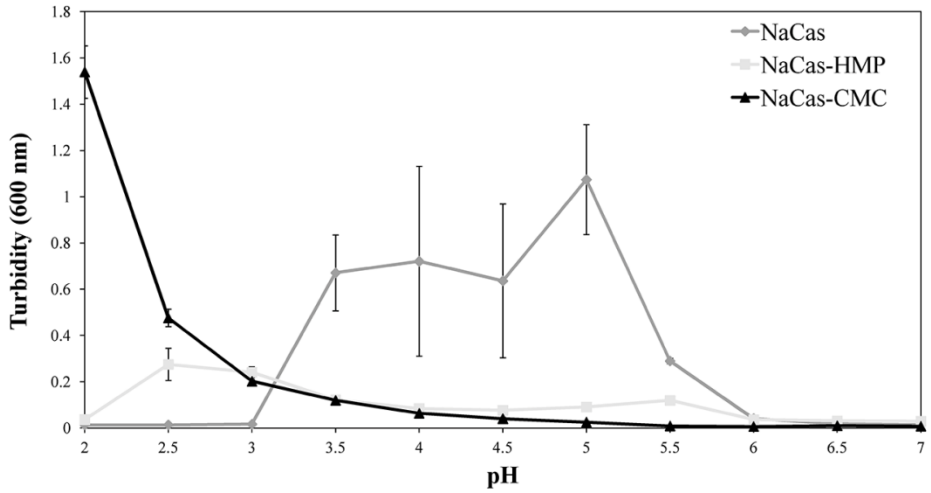

Fig. 2.2 Effect of pH on the turbidity of NaCas, NaCas-HMP and NaCas-CMC

Values are means ± standard deviations (n = 3).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

2.3.3 Salt stability of sodium caseinate and the complexes

Effect of NaCl on the stability of the solutions is shown in Fig. 2.3. Unlike experimental conditions, many environmental stresses including ionic strength are present in food system. Therefore, stability toward these environmental stresses is important when incorporating the electrostatic complex to a food system. The electrostatic complexes showed higher stability against ionic strength than NaCas as turbidity of NaCas increased sharply when the concentration of NaCl was above 50 mM. NaCl-induced precipitation was not observed in the electrostatic complexes. A similar result was found for electrostatic complex between β-lactoglobulin and beet pectin (Santipanichwong et al., 2008). Together with pH stability results, this result suggests that electrostatic complexes may be incorporated to a wider range of food systems compared to NaCas.

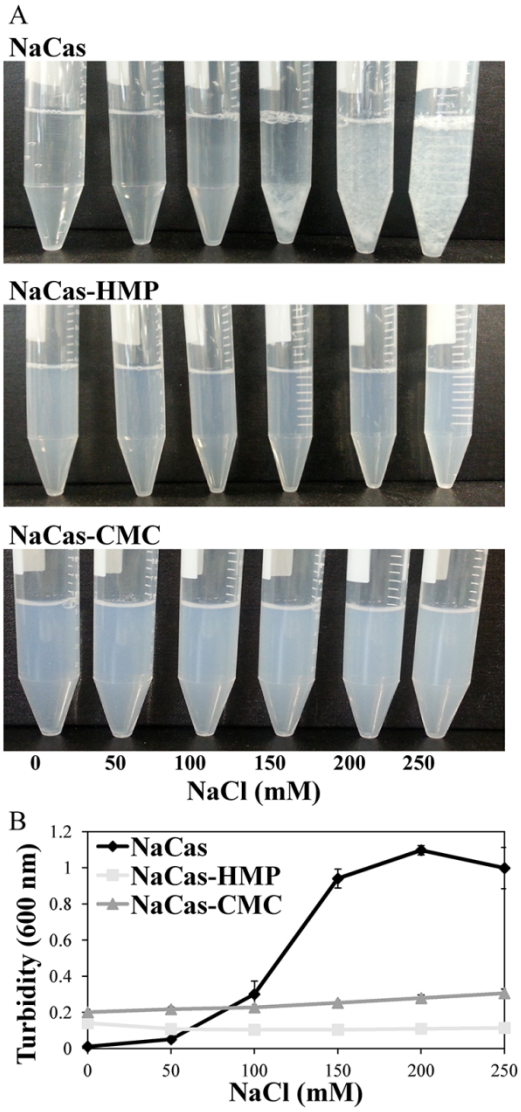

Fig. 2.3 Effect of NaCl concentration on the turbidity of NaCas, NaCas-HMP and NaCas-CMC

Values are means ± standard deviations (n = 3).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

2.3.4 Particle size and zeta potential

The particle size, PDI and zeta potential of NaCas, NaCas-HMP and NaCas-CMC are shown in Table 2.1. The particle size of the NaCas-HMP was larger than those of the NaCas alone and NaCas-CMC. PDI for all the samples were around 0.3, which is indicative of narrow size distribution (Luo et al., 2013). Santipanichwong et al. (2008) also reported that nanoparticles produced with β-lactoglobulin and beet pectin were larger than β-lactoglobulin alone at pH 3.

NaCas was positively charged as pH of the solution was above its pI, while electrostatic complexes were negatively charged due to presence of anionic polysaccharides. Zeta potential measured in this study was similar to those from previous reports (Surh et al., 2006; Du et al., 2009).

Table 2.1 Zeta potential, particle size and PDI of NaCas, NaCas-HMP and NaCas-CMC

	Zeta potential (mV)	Particle size (nm)	PDI
NaCas	23.6 ± 4.0	360.9 ± 25.6	0.24 ± 0.01
NaCas-HMP	-6.6 ± 0.7	400.0 ± 8.6	0.25 ± 0.02
NaCas-CMC	-12.5 ± 0.1	364.8 ± 9.6	0.31 ± 0.004

Values are means ± standard deviations (n = 3).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; CMC, carboxymethyl cellulose; and PDI, polydispersity index

2.3.5 UV-vis absorption and fluorescence spectra of curcumin bound to the complexes

The EE of curcumin for NaCas, NaCas-HMP and NaCas-CMC were 88.1 ± 1.1%, 92.9 ± 1.5% and 94.4 ± 1.7%, respectively. The electrostatic complexes showed higher EE, which was also observed in another study (Li et al., 2015).

Absorption and fluorescence spectra are important tools to study interactions between the compounds and have been used to study the interaction of curcumin with other molecules (Barik et al., 2003; Yang et al., 2013; Li et al., 2015). When curcumin was incubated with NaCas, HMP, CMC, and electrostatic complexes, peak absorbance increased (Fig. 2.4A). A similar increase in intensity was observed in fluorescence spectra (Fig. 2.4B). This indicates that all of the compounds may be interacted with curcumin. Since absorption intensity of the electrostatic complexes was the highest followed by those of NaCas and polysaccharides, the electrostatic complexes may have the highest interaction with curcumin. A similar increase in intensity of curcumin, especially when interacted with electrostatic complex, was observed in other studies (Baglole et al., 2005; Yang et al., 2013; Li et al., 2015). There were red and blue shifts in absorption and fluorescence spectra for curcumin with NaCas and the electrostatic complexes, respectively. These shifts indicate that curcumin moves from aqueous environment to more hydrophobic sites of NaCas (Baglole et al., 2005; Yang et al., 2013; Li et al., 2015).

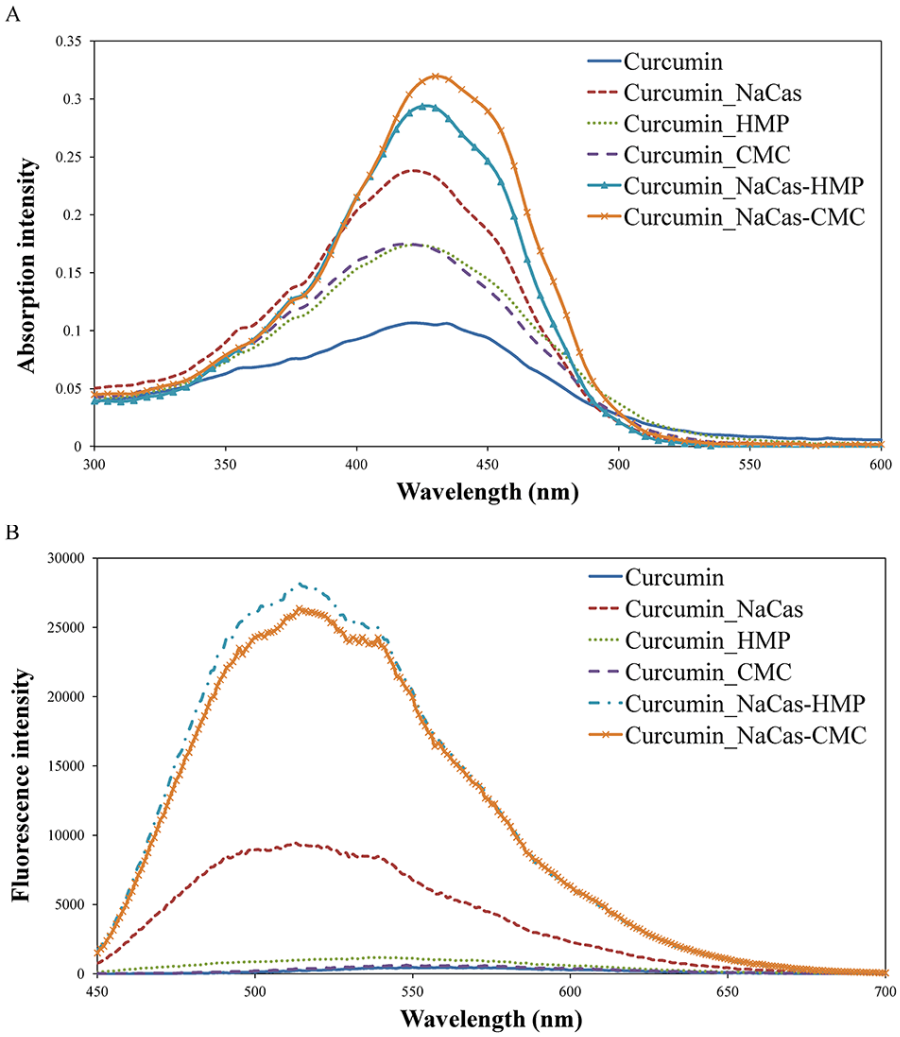

Fig. 2.4 UV-vis absorption spectra (A) and fluorescence spectra (B) of curcumin and curcumin with NaCas, HMP, CMC and electrostatic complexes

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

2.3.6 Fourier transform infrared spectra

FT-IR spectroscopy was used to confirm the binding of curcumin to NaCas, NaCas-HMP and NaCas-CMC (Fig. 2.5). The FT-IR spectrum of curcumin in this study was similar to that of a previous literature (Yallapu et al., 2010). Curcumin showed a characteristic peak around 3,507 cm^{-1} (O-H stretching vibration), which was not found when incubated with the NaCas and electrostatic complexes (Pan et al., 2013). Also, other characteristic peaks were not observed in curcumin with NaCas or the electrostatic complexes. This disappearance of the characteristic peaks indicates that curcumin binds to NaCas or electrostatic complexes (Yallapu et al., 2010).

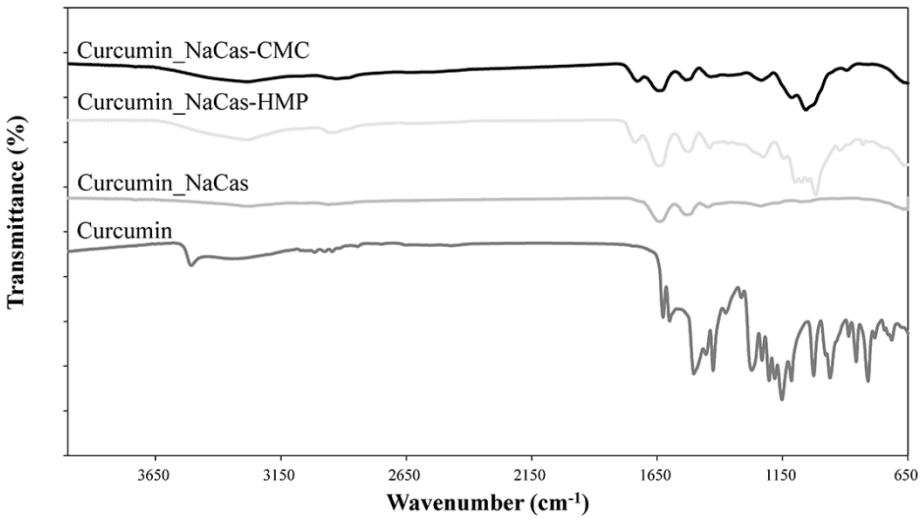

Fig. 2.5 FT-IR spectra of curcumin and curcumin bound to NaCas, NaCas-HMP and NaCas-CMC

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

2.3.7 Binding constant

Casein has tryptophan residue, which is a major contributor of hydrophobicity (Zhang & Zhong, 2013b). Tryptophan has maximum fluorescence emission at 336 nm when excited at 280 nm and this fluorescence is reduced in presence of a quencher. This change in fluorescence could be used to understand interaction between NaCas and curcumin (Cogan et al., 1976). A decrease in fluorescence intensity was observed when curcumin was added more (Fig. 2.6). Two major mechanisms known to be involved in quenching are dynamic and static. Dynamic quenching is caused by collision of a quencher and a fluorophore, while static quenching is caused by formation of non-fluorescent complex between a quencher and a fluorophore (Mohammadi et al., 2009). In this study, Stern-Volmer equation was used to determine whether the quenching is to be dynamic or static quenching using following equation:

$$F_0/F = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q] \quad (1)$$

where F_0 and F are fluorescence intensities without and with the quencher (curcumin), respectively; the k_q is biomolecular quenching rate constant; τ_0 is average lifetime of the biomolecule without quencher and is typically 10^{-8} s; [Q] is concentration of the quencher; and K_{SV} is Stern-Volmer quenching constant (Lakowicz et al., 1980).

From the Stern-Volmer equation, K_{SV} and k_q were calculated and listed in Table

2.2. K_{SV} for NaCas-HMP and NaCas-CMC were significantly higher than that for NaCas ($P < 0.05$). The k_q for NaCas, NaCas-HMP and NaCas-CMC were larger than the maximum collisional quenching constant, 2.0 x 10^{10} $M^{-1}S^{-1}$, indicating that quenching of NaCas by curcumin is due to static quenching (complex formation) (Ware, 1962; Lange et al., 1998). For the static quenching, binding constant of curcumin was calculated using the following equation (Jiang et al., 2004; Guo et al., 2010):

$$\log (F_0 - F) / F = \log K_b + n \log [Q] \quad (2)$$

where F_0, F and Q are the same as in the equation 1; K_b is binding constant; and n is the number of binding sites (Jiang et al., 2004).

Graphs for the equation 2 were shown in the insets of Fig. 2.6, and calculated binding constants and the number of binding sites were listed in Table 2.2. Calculated binding constants for NaCas and curcumin were similar to those for camel β-casein and casein micelle (Sahu et al., 2008; Esmaili et al., 2011). The binding constant increased when NaCas was complexed with polysaccharides. Similar increases were reported for electrostatic complexes between bovine serum albumin and carrageenan (Yang et al., 2013) and between lysozyme and carboxymethyl cellulose (Li et al., 2015; Li & Wang, 2015). Yang et al. (2013) reported that complexation of bovine serum albumin with carrageenan caused a partial denaturation of protein, leading to increased binding of curcumin to the electrostatic complexes. A similar denaturation

might occur in this study. Also, binding constant of the NaCas-CMC was significantly higher than that of the NaCas-HMP. These results, along with spectra data, indicate that the electrostatic complexes have higher affinity toward curcumin than NaCas.

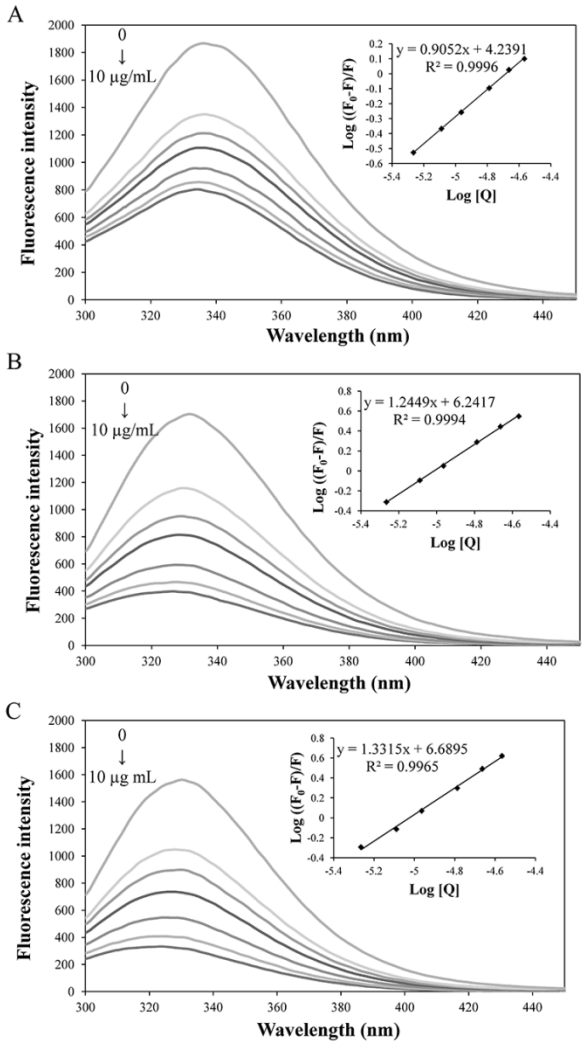

Fig. 2.6 Changes in fluorescence spectra of NaCas (A), NaCas-HMP (B) and NaCas-CMC (C) in response to changes in curcumin concentrations (0, 2, 3, 4, 6, 8 and 10 µg/mL)

Insets show linear plots of Log ($(F_0-F)/F$) vs Log (curcumin) for calculating binding constants.

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

Table 2.2 Stern-Volmer quenching constants (K_{SV}), biomolecular quenching constants (K_q), numbers of binding sites (n) and binding constants (K_b) of curcumin with NaCas, NaCas-HMP and NaCas-CMC

	K_{SV} (x 10^4 M^{-1})	K_q (x 10^{10} $M^{-1}s^{-1}$)	Binding site (n)	K_b (x 10^4 M^{-1})
NaCas	4.87 ± 0.44[a]	60.91 ± 5.49[a]	0.90 ± 0.03[a]	1.75 ± 0.61[a]
NaCas-HMP	13.26 ± 1.33[b]	165.76 ± 16.68[b]	1.21 ± 0.03[b]	131.89 ± 37.16[b]
NaCas-CMC	15.33 ± 1.24[b]	191.58 ± 15.55[b]	1.31 ± 0.02[c]	445.02 ± 69.70[c]

Different letters represent significant difference ($p < 0.05$).

Values are means ± standard deviations ($n = 3$).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

2.3.8 Stability of curcumin

Stability of curcumin at pH 3 is shown in Fig. 2.7. It has been known that curcumin is relatively stable at a lower pH (Wang et al., 1997), but a slow degradation was observed especially at the elevated temperature. However, curcumin showed increased stability when complexed with NaCas, HMP, CMC and the electrostatic complexes in this study. Curcumin incubated with the electrostatic complexes remained almost unchanged while NaCas and polysaccharides showed a lower stabilizing effect. This indicates that curcumin could be stabilized by binding to NaCas and this stabilizing effect could be enhanced by complexation with polysaccharides. These results are in accordance with spectrum and binding constant data. A similar enhanced stability of curcumin was reported for the complexes between BSA and carrageenan and between lysozyme and carboxymethyl cellulose (Yang et al., 2013; Li et al., 2015). Also, the electrostatic complexes showed higher stabilizing effect than their protein counterparts for (−)-epigallocatechin-3-gallate and vitamin D (Ron et al., 2010; Li & Wang, 2015). These results imply that electrostatic complexes have stronger interaction with curcumin resulting in a higher stabilizing effect.

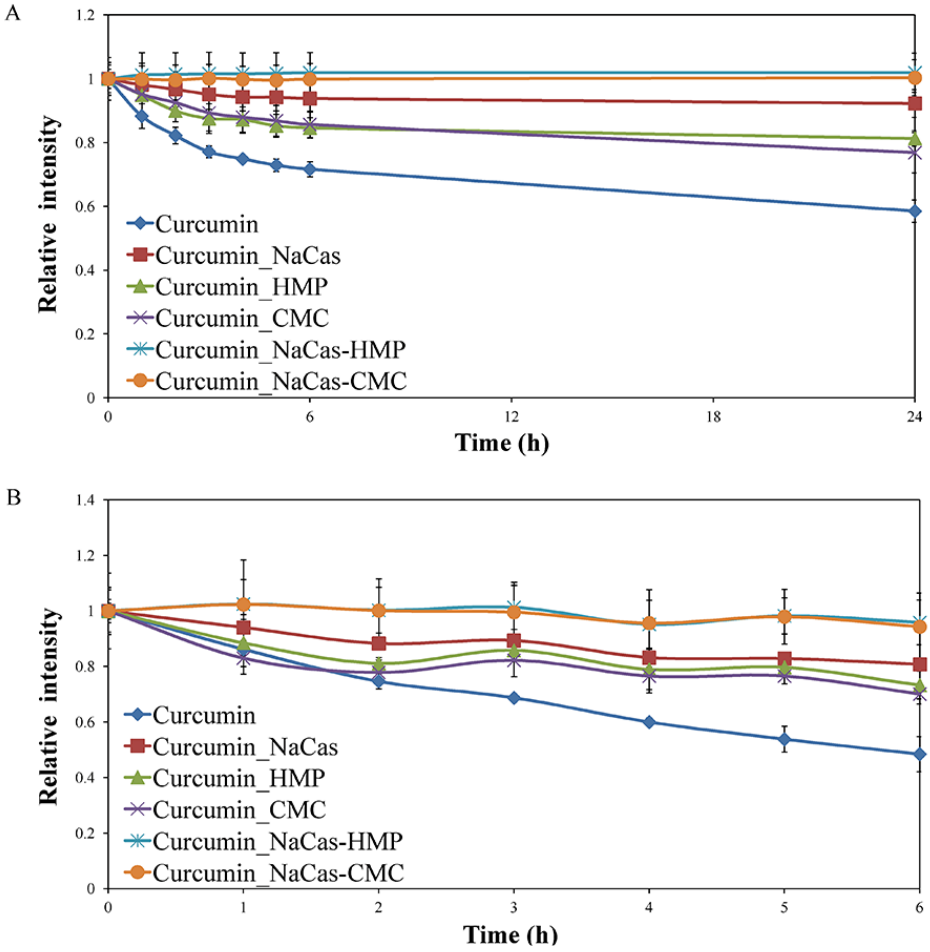

Fig. 2.7 Stability of curcumin in the presence and absence of NaCas, HMP, CMC and electrostatic complexes during storage at room temperature (A) and at 60 °C (B)

Values are means ± standard deviations (n = 3).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

2.4 Conclusion

In this study, electrostatic complexes of NaCas were successfully formed using HMP and CMC. The electrostatic complexes showed higher stability against pH and ionic strength than the native NaCas. Curcumin was successfully bound to NaCas and the electrostatic complexes, which was evident when determined by UV-vis, fluorescence and FT-IR spectra. The electrostatic complexes showed a higher EE and binding affinity toward curcumin than the native NaCas. Curcumin bound to the electrostatic complexes showed the least degree of degradation followed by the native NaCas. HMP and CMC slightly improved stability of curcumin. These results indicate that the electrostatic complex could be used as a carrier material for lipophilic bioactives, especially in acidic beverages, where NaCas is not applicable and transparency is preferred.

Chapter 3

Characterization and Food Application of Curcumin Bound to Electrostatic Complexes of Sodium Caseinate and Polysaccharides (Part 1: Study II)

3.1 Introduction

Curcumin has been extensively studied for its various functionalities including anticancer, antiinflammatory and antioxidant activities (Aggarwal et al., 2003; Rosa et al., 2014). However, efficacy of curcumin is limited due to its low solubility and stability in aqueous media and low bioavailability (Wang et al., 1997; Chen et al., 2015b). Many methods have been used to increase solubility and stability of curcumin including complexation with proteins and electrostatic complexes (Yang et al., 2013; Chen et al., 2015a). Food proteins have advantages as proteins are widely available and cheap (Yang et al., 2013). It was reported in our previous study that sodium caseinate (NaCas) could stabilize curcumin and that the electrostatic complexes between NaCas and high-methoxyl pectin (HMP) or carboxymethyl cellulose (CMC) had higher binding affinity and stabilizing effect than NaCas alone (Cho et al., 2016). However, effect of pH on the size, zeta potential and encapsulation efficiency (EE) was not evaluated in the previous study. Moreover, as binding of curcumin with proteins could affect its functionalities (Pan et al., 2014), bioactivities of curcumin bound to the complexes should be compared to that of native curcumin.

Although encapsulation and stabilization of curcumin have been extensively studied, only a few studies have applied encapsulated curcumin in foods as a functional ingredient or a food colourant. Curcumin bound to the complexes could be a potential food colourant with antimicrobial and antioxidant activities (Prasad et al., 2014). Moreover, electrostatic complexes between proteins and polysaccharides have advantages over proteins in food systems with low pH as the complexes are stable to

aggregation at low pH near isoelectric points of the proteins (Santipanichwong et al., 2008). Also, stability and in vitro bioaccessibility should be determined in food matrixes to evaluate possibility of curcumin bound to the complexes as a food colourant.

In this study, effect of pH on the characteristics of the complexes was evaluated to find optimum pH. Effect of the binding on the antioxidant activity of curcumin was also measured. Finally, potential of the complex-bound curcumin as a food colourant in a model beverage was evaluated.

3.2 Materials and methods

3.2.1 Materials

Food grade NaCas (protein content: 91% wet basis; and moisture content: 5.6%) was kindly provided by Samik Dairy & Food Co. Ltd (Seoul, Korea). Food grade HMP with degree of esterification of 71% and CMC with degree of substitution between 1.0 and 1.2 were obtained from Esfood Co. Ltd. (Pocheon, Korea) and Ashland Chemical Co. (Changzhou, Jiangsu, China), respectively. Ethanol (EtOH), HCl, NaOH, sodium citrate and citric acid were from Samchun Chemicals (Seoul, Korea). Sugar was obtained from local market. Curcumin, sucrose, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, porcine bile extract, 2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulphonic acid) diammonium salt (ABTS) and potassium persulfate were from Sigma Chemical Co. (St. Louis, MO, USA).

3.2.2 Complex formation

The complexes were made according to the method from our previous paper (Cho et al., 2016) with modifications. Briefly, solutions of NaCas, HMP and CMC were separately prepared and adjusted to pH 7 using HCl and NaOH. Then NaCas and polysaccharide solutions were mixed to have final concentrations of 0.1 and 0.2% (w/w), respectively. This ratio was determined to be optimum in our previous paper (Cho et al., 2016). pH was adjusted to a range from 3 to 5, and the mixtures were centrifuged (2,580 × g, 30 min, 4 °C) to remove large insoluble aggregates.

3.2.3 Particle size and zeta potential

Particle size, polydispersity index (PDI) and zeta potential of the complexes were measured using an electrophoretic light scattering spectrophotometer (ELSZ-1000, Otsuka Electronics, Osaka, Japan). All the measurements were performed at 25 ˚C and the angles for particle size and zeta potential analysis were 165˚ and 15˚, respectively. Measurement was made in duplicate.

3.2.4 Measurement of encapsulation efficiency

Effects of solution pH ranging from 3 to 5 and concentrations of curcumin stock solutions from 1 to 3 mg/mL on EE were determined. The complexes were mixed with various concentrations of curcumin (final concentration of curcumin was 10 to 30 μg/mL), vortexed and centrifuged at 2,580 × g for 30 min at 4 ˚C to pellet the free curcumin (Xu et al., 2014). The supernatant was removed and pelleted free curcumin was redissolved in EtOH and quantified by a UV-spectrometer (SpectraMAX, Molecular Devices, Sunnyvale, CA, USA). Curcumin concentration was 10 μg/mL in the rest of the study.

3.2.5 Thermal stability of the complex

The solutions containing the electrostatic complexes were heated for 2 min at 88 ˚C to mimic typical heat treatment condition (Etzel, 2004). Changes in turbidity and curcumin content were evaluated by measuring absorbance at 600 nm and 428 nm, respectively. The results showed that pH 4 was optimal, so this pH was used in the subsequent experiments.

3.2.6 Measurement of antioxidant activity

Effect of binding on the antioxidant activity of curcumin was evaluated using ABTS radical scavenging activity (Brandwilliams et al., 1995). ABTS (7 mM) and potassium persulfate (2.45 mM) stock solutions were prepared in distilled water (DW), mixed at 1:1 ratio and incubated overnight in the dark. The mixture was

diluted with DW (adjusted to pH 4) to have absorbance of 0.700 ± 0.05 at 734 nm. The diluted ABTS solution was mixed with curcumin stock solution in DW or the complexes with and without curcumin at the ratio of 1:1. After 10 min incubation in the dark, absorbance was measured at 734 nm.

3.2.7 Effect of excipient on redispersibility of the freeze-dried complex

To determine optimum ratio of the electrostatic complex to excipient (sucrose), the complexes were mixed with sucrose in the ratio of 1:0 to 1:5 and freeze-dried (Labconco Co., Kansas City, MO, USA). Freeze-dried complexes were redispersed in DW and redispersibility was evaluated by taking digital images and measuring turbidity at 600 nm. Higher concentration of sucrose resulted in lower turbidity, but ratio above 1:4 had no further effect on turbidity. Thus the ratio of 1:4 was used to produce freeze-dried complexes for the application in the food model system. For convenience, the weight of the complex refers to the weight of the complex only, excluding the weight of the excipient.

3.2.8 Application in model beverage

To mimic a commercial beverage, 0.1 M citrate buffer at pH 4 with 13% sucrose and 0.025% sodium benzoate was used (Sari et al., 2012). Freeze-dried complexes were added to the beverage to have final concentration of 0.3% (w/w). Colour of the beverage with the complex-bound curcumin was compared to the beverage with the same concentration of curcumin stock solution in EtOH, using a spectrophotometer.

(CM-5, Konica Minolta, Osaka, Japan). Colours of the above beverages were measured during storage at room temperature under dark for 3 weeks.

3.2.9 *In vitro* bioaccessibility

Bioaccessibility of native curcumin and curcumin bound to the complexes in the beverage was measured according to the modified method from Chen et al. (2015a). The pH of the beverage was adjusted to 3 using 6 N HCl and preincubated at 37 ˚C for 30 min in a shaking incubator (150 rpm; Changshin Scientific Co., Korea). Pepsin stock solution (pH 3) was added and the mixture was incubated at 37 ˚C to initiate digestion process. After 1 h, enzymatic digestion was stopped by increasing the pH to 7. Then pancreatin and bile stock solutions were added, followed by incubation for 2 h at 37 ˚C. The final concentrations of enzymes were 3 mg pepsin, 0.67 mg pancreatin and 4 mg bile extract per g of freeze-dried complex. To quantify bioaccessible curcumin after the digestion process, aliquots were taken and centrifuged at 25,910 × g for 30 min at 4 ˚C. The supernatants were taken, mixed with EtOH and sonicated for 10 min to extract curcumin. The sonicated mixture was centrifuged at 25,910 × g for 30 min at 4 ˚C and curcumin was quantified using curcumin standard curve.

3.2.10 Statistical analysis

Statistical analysis was done using SPSS 22 software (SPSS Inc., Chicago, IL, USA). For one-way analysis of variance (ANOVA), significance between the samples was analysed by Duncan's multiple-range test ($p < 0.05$).

3.3 Results and discussion

3.3.1 Effect of pH on size, zeta potential and polydispersity index of the complex

Particle size of NaCas-HMP was relatively stable to the pH change, while that of NaCas-CMC increased as pH increased (Table 3.1). It is known that NaCas interacts with HMP and CMC at the pH ranging from 3 to 5 (Surh et al., 2006; Liu et al., 2012). However, at pH 5, both NaCas and CMC carry negative charges and thus may have a relatively weaker interaction, which could result in increased particle size (Luo et al., 2015). Moreover, CMC carry a stronger charge than HMP, which could result in stronger repulsion at pH 5. Higher PDI was observed as pH increased from 3 to 5.

NaCas-CMC had higher zeta potential than NaCas-HMP and both of the complexes had higher zeta-potential at higher pH, which is in accordance with previously reported data (Surh et al., 2006; Liu et al., 2012). NaCas-CMC is assumed to be more stable than NaCas-HMP due to a larger zeta potential in all the tested pH range. In general, to attain colloidal stability of electrostatically stabilized dispersion, zeta potential of at least ± 30 mV is recommended and when both electrostatic and steric stabilizations are involved, minimum of ± 20 mV is recommended (Jacobs & Muller, 2002). Since both electrostatic and sterical stabilizations are involved in electrostatic complexes (Santipanichwong et al., 2008), the dispersion is expected to be physically stable at pH 4 and 5.

Table 3.1 Particle size, PDI and zeta potential of the electrostatic complexes at various pH

	pH	Particle size (nm)	PDI	Zeta potential (mV)
NaCas-HMP	3	362.3 ± 4.2^{c}	0.236 ± 0.006^{b}	-8.28 ± 0.49^{a}
	4	457.7 ± 5.1^{b}	0.324 ± 0.013^{a}	-22.19 ± 0.97^{b}
	5	409.9 ± 2.6^{a}	0.323 ± 0.001^{a}	-29.41 ± 1.97^{c}
NaCas-CMC	3	392.1 ± 0.2^{c}	0.311 ± 0.013^{c}	-13.57 ± 0.77^{a}
	4	567.7 ± 4.9^{b}	0.360 ± 0.0003^{b}	-33.59 ± 1.03^{b}
	5	717.5 ± 50.2^{a}	0.409 ± 0.01^{a}	-47.36 ± 1.13^{c}

Different letters represent significant difference ($p < 0.05$).

Values are means ± standard deviations ($n = 3$).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; CMC, carboxymethyl cellulose; and PDI, polydispersity index

3.3.2 Effect of pH and curcumin concentration on encapsulation efficiency

The complexes with higher pH and lower concentration of curcumin showed higher EE (Fig. 3.1). Previous papers also reported that increase in curcumin concentration resulted in reduced EE (Pan et al., 2014; Chen et al., 2015a). In this study, curcumin concentration of 10 μg/mL was used throughout the study as EE was the highest at this concentration.

3.3.3 Thermal stability of curcumin and the complex

The thermal stability of the complex was evaluated by comparing the turbidity before and after the heat treatment (Fig. 3.2A). Initially, the complexes had higher turbidity at lower pH and heat treatment caused increase in turbidity of all the tested samples ($p < 0.05$). NaCas-HMP at pH 4 and CMC at pH 4 and 5 were relatively stable to heat treatment as turbidity of these complexes was lower than the others after the heat treatment ($p < 0.05$).

Also, thermal stability of free curcumin and bound curcumin during the heat treatment was evaluated (Fig. 3.2B). Free curcumin was significantly degraded by the heat treatment, especially at pH 5 ($p < 0.05$). However, curcumin bound to the complexes was stable to the heat treatment. Based on the results of particle size, zeta potential and heat stability, pH 4 was chosen to be optimum.

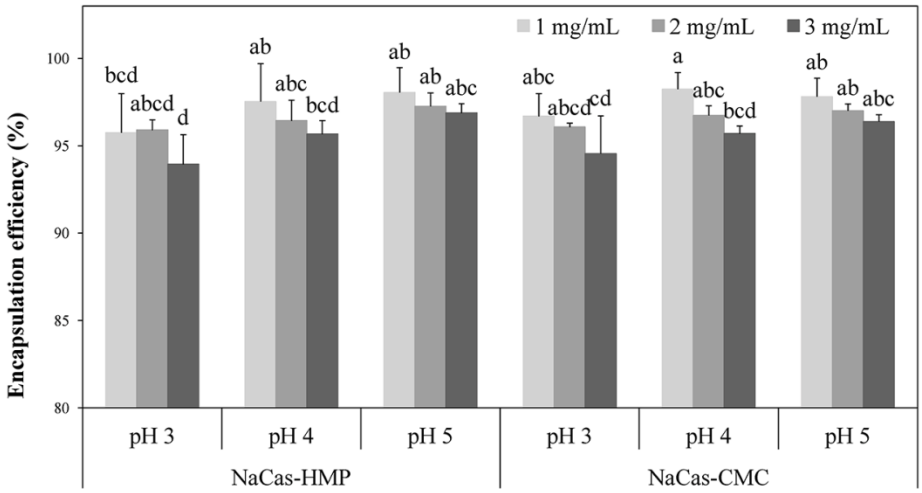

Fig. 3.1 Effect of curcumin concentration and pH on encapsulation efficiency

Different letters represent significant difference ($p < 0.05$).

Values are means ± standard deviations (n = 3).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

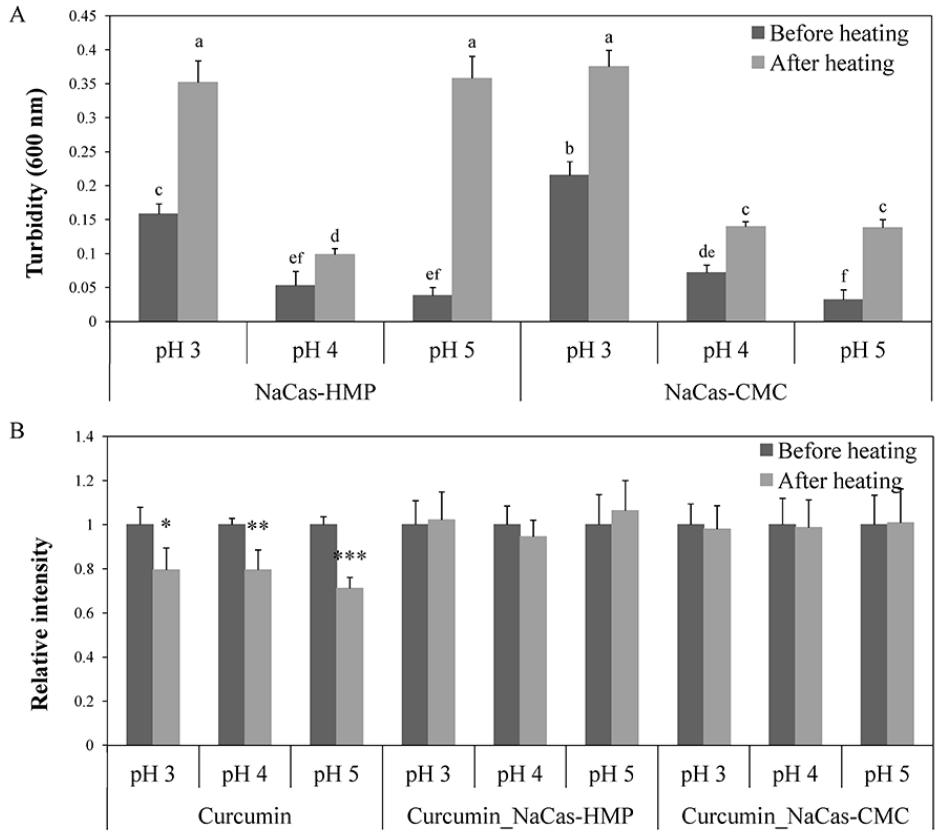

Fig. 3.2 Thermal stability of the complex (A) and curcumin (B) before and after heat treatment

Different letters represent significant difference ($p < 0.05$).

Values are means ± standard deviations (n = 3).

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; t-test

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

3.3.4 Antioxidant activity of native curcumin and complex-bound curcumin

Effect of binding on antioxidant activity of curcumin was evaluated using ABTS radical scavenging assay (Fig. 3.3). The complexes without curcumin showed slight antioxidant activity because NaCas and polysaccharides have an antioxidant capacity (Moseley et al., 2003; Kitts, 2005; Ro et al., 2013). Native curcumin showed slightly lower antioxidant activity than the complex-bound curcumin, which may be due to limited solubility of curcumin in DW ($p < 0.05$). This result indicates that complex-bound curcumin retains its antioxidant activity. Similar retention in antioxidant activity of bound curcumin was reported (Yang et al., 2013; Pan et al., 2014).

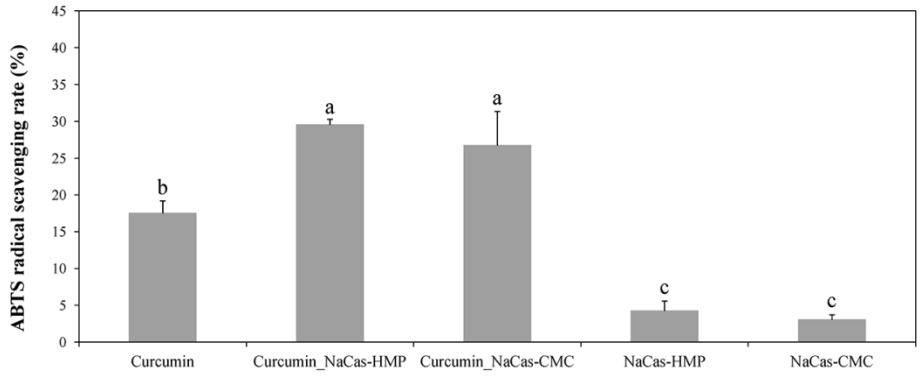

Fig. 3.3 ABTS radical scavenging rate of curcumin in the presence and absence of the electrostatic complex at pH 4

Different letters represent significant difference ($p < 0.05$).

Values are means ± standard deviations ($n = 3$).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

3.3.5 Effect of excipient ratio on redispersion of the freeze-dried complex

Due to long term instability of nanoparticles in aqueous media, freeze-drying is commonly applied (Abdelwahed et al., 2006). Excipients are used to prevent aggregation and destabilization occurring during freeze-drying (Abdelwahed et al., 2006). In this study, sucrose was chosen as an excipient since it is commonly used in foods including beverages and would not undergo Maillard reaction with NaCas (Anhorn et al., 2016). In order to determine optimum ratio of the complex to excipient, ratios of 1:0 to 1:5 were compared for turbidity (Fig. 3.4). Before freeze-drying, addition of sucrose had no effect on turbidity of the solution. Turbidity significantly increased when the freeze-dried complex without the excipient was redispersed in DW ($p < 0.05$). Incorporation of the excipient resulted in lower turbidity upon redispersion. For NaCas-HMP, there was no significant difference in turbidity after redispersion when the ratio was above 1:3 ($p > 0.05$). For NaCas-CMC, the ratio above 1:4 had no further effect on turbidity ($p > 0.05$). Therefore, ratio of 1:4 was used to produce freeze-dried complexes for the model beverage.

Increase in turbidity after redispersion was more predominant in the NaCas-CMC than in the NaCas-HMP. Also, for the NaCas-HMP, the presence of the excipient prevented increase in turbidity upon redispersion ($p > 0.05$); however, for the NaCas-CMC, the increase was only partially prevented ($p < 0.05$). These results indicate that the NaCas-HMP is more stable to freeze-drying-induced aggregation than the NaCas-CMC.

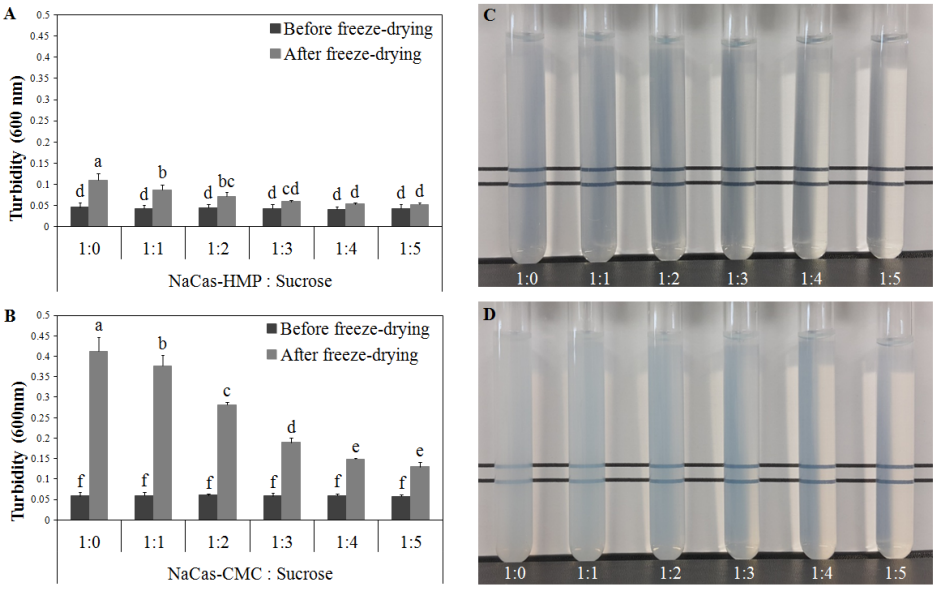

Fig. 3.4 Effect of different ratios of the complex to excipient (sucrose) on the turbidity and visual appearance of NaCas-HMP (A and C) and NaCas-CMC (B and D) before freeze-drying and after redispersion

Different letters represent significant difference ($p < 0.05$).

Values are means ± standard deviations ($n = 3$).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

3.3.6 Application of curcumin bound to the complexes as a food colourant in a model beverage

Hunter Lab values of the complex-bound curcumin in beverages were compared to those of native curcumin (Table 3.2). The beverages with the bound curcumin showed similar colour values compared to those with native curcumin. Incorporation of curcumin bound to the complexes resulted in reduced L value and increased a and b values ($p < 0.05$). Reduction in L value may be due to increased turbidity by the complexes. Our previous study showed that binding of curcumin to the electrostatic complex resulted in increased intensity, which may result in higher b value for the beverages with the bound curcumin (Cho et al., 2016). In accordance with the redispersibility results, the beverage with the curcumin bound to NaCas-CMC was more turbid. The b value was measured for 3 weeks to access stability of the complex-bound curcumin in the beverages (Fig. 3.5). The b value of the bound curcumin was more stable than that of native curcumin.

Binding of curcumin to the complexes enhanced the bioaccessibility of curcumin in the beverage (Fig. 3.6). Free curcumin showed low bioaccessibility due to its low solubility and stability. This result indicates that the binding could enhance bioaccessibility and delivery of curcumin. The bioaccessibility value of curcumin reported in this paper is similar to that of a previous study (Chen et al., 2015a). However, it should be noted that in vitro bioaccessibility assay is a screening tool to evaluate samples which only could provide basic information on bioaccessibility

(Zou et al., 2016). Appropriate in vivo and clinical tests should be followed to confirm the bioaccessibility of curcumin bound to the complexes.

Table 3.2 Colour values of beverages with native curcumin and curcumin bound to the complexes

Colourant	L	a	b
Curcumin	94.96 ± 0.24^{a}	-7.56 ± 0.07^{a}	37.49 ± 1.12^{a}
Curcumin_NaCas-HMP	92.10 ± 0.17^{b}	-12.35 ± 0.07^{b}	49.31 ± 0.54^{b}
Curcumin_NaCas-CMC	90.17 ± 0.34^{c}	-11.19 ± 0.16^{c}	48.42 ± 0.68^{b}

Different letters represent significant difference ($p < 0.05$).

Values are means ± standard deviations (n = 3).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

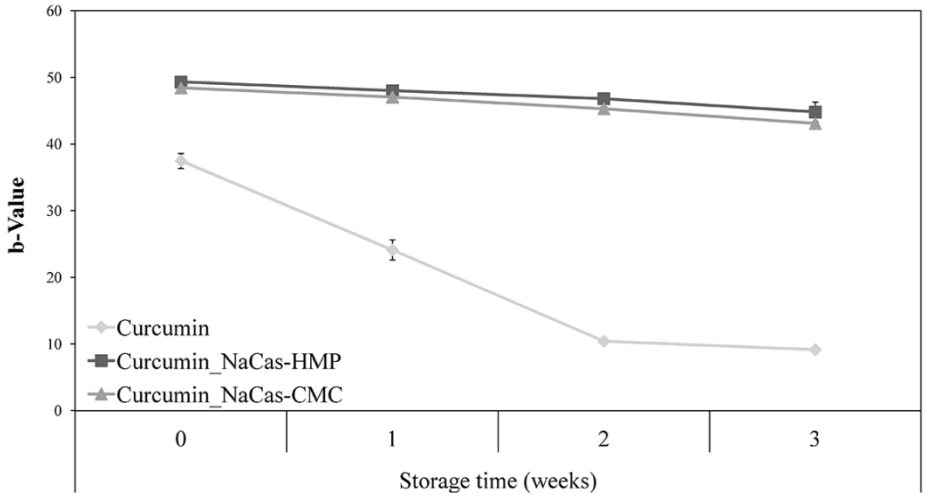

Fig. 3.5 Changes in b values of native curcumin and curcumin bound to the complexes in beverages

Values are means ± standard deviations (n = 3).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

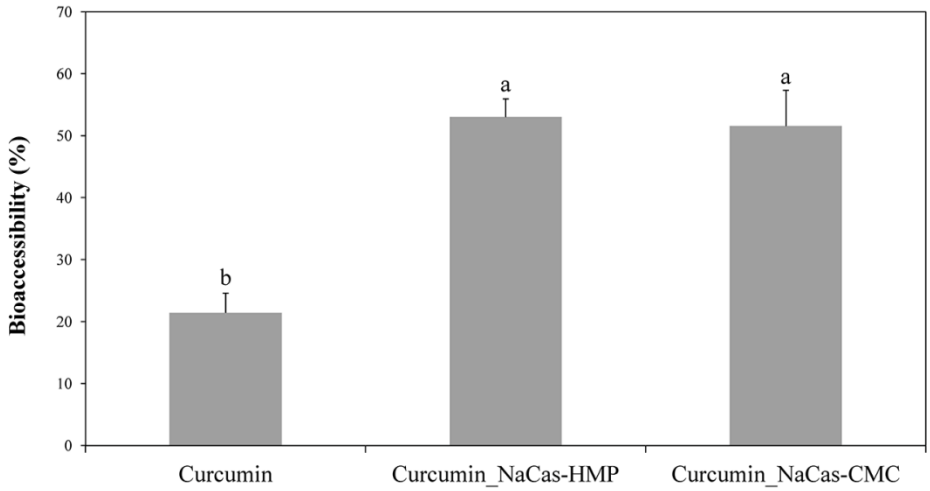

Fig. 3.6 In vitro bioaccessibility of native curcumin and complex-bound curcumin in model beverage after in vitro digestion

Different letters represent significant difference ($p < 0.05$).

Values are means ± standard deviations ($n = 3$).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

3.4 Conclusion

The optimum pH for NaCas-polysaccharide electrostatic complexes was pH 4. Curcumin bound to the electrostatic complexes had similar antioxidant activity to native curcumin, implying that the binding little altered the functionality of curcumin. The aggregation of the complexes during freeze-drying could be partially prevented by the use of an excipient (sucrose). Curcumin bound to the complexes was successfully incorporated into model beverage as a food colourant. The bound curcumin had similar colour values and higher stability and in vitro bioaccessibility than native curcumin. In general, the NaCas-HMP and NaCas-CMC showed similar behaviour except higher freeze-drying stability of the NaCas-HMP. Thus the NaCas-HMP would be a better choice for a product where transparency is needed.

Chapter 4

Effect of Ellagic Acid Incorporation on the Oxidative Stability of Emulsions Stabilized by Sodium Caseinate-Polysaccharide (Part 2: Study III)

4.1 Introduction

Sodium caseinate (NaCas) has been used to encapsulate bioactive compounds (Pan et al., 2014; Luo et al., 2015). NaCas is known to dissociate in alkaline conditions (Vaia et al., 2006). Certain polyphenols including rutin and curcumin are insoluble in aqueous media, but readily soluble in alkaline media due to their deprotonation (Leung et al., 2008; Luo et al., 2015). Dissociation of NaCas and higher solubility of certain polyphenols in alkaline condition could be applied to incorporate polyphenols into NaCas. A pH cycle method (Pan & Zhong, 2016) was used to incorporate rutin and curcumin into NaCas without using organic solvents (Pan et al., 2014; Luo et al., 2015).

Ellagic acid (EA) is a phenolic compound commonly found in berries and nuts (Daniel et al., 1989) with antioxidant and anticancer activities (Bala et al., 2006). EA has low water solubility of about 9.7 μg/mL, but due to its acidic nature, it is more soluble in a basic solvent (Bala et al., 2006). Also, electrostatic complexes between NaCas and an anionic polysaccharide, high-methoxyl pectin (HMP) or carboxymethyl cellulose (CMC), could increase stability of NaCas against changes in pH and ionic strength to expand the application of NaCas in food matrixes (Cho et al., 2016). Thus it was hypothesized that EA could be incorporated into NaCas using a pH cycle method and EA-incorporated NaCas could be used to form electrostatic complexes with HMP or CMC. However, incorporation of EA using a pH cycle method and formation of an electrostatic complex using pH-cycled NaCas have not been tested.

Many health benefits are reported for long chain n-3 polyunsaturated fatty acids (PUFA) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are abundant in fish oil (Abeywardena & Head, 2001). However, PUFA are prone to oxidation during processing and storage, which results in rancidity and loss of nutritional values (Sun et al., 2011). In food systems, lipid usually exists as an emulsion. However, due to increased surface area, oils in emulsion are more susceptible to oxidation (Waraho et al., 2011). Thus antioxidants are commonly added to emulsions to prevent oxidation. Natural antioxidants such as resveratrol, rutin and green tea extract have been used to increase oxidative stability of emulsion (Cui et al., 2014; Wan et al., 2014; von Staszewski et al., 2014). However, these studies were conducted at pH 7; thus study involving pH close to that of general food systems is needed. Also, efficacy of EA-incorporated NaCas on oxidative stability of an emulsion has not been tested. NaCas has been used as a food emulsifier and encapsulant. Thus EA-incorporated NaCas could be used as an emulsifier and incorporated EA will accumulate on oil-water interface with NaCas and improve oxidative stability of oil. Moreover, it was reported that antioxidants in O/W emulsions would be more effective if they are accumulated on oil-water interface (Lucas et al., 2010). Finally, HMP or CMC could be added to increase the stability in a low pH range (Surh et al., 2006; Liu et al., 2012).

In this study, EA was incorporated into NaCas using a pH cycle method, and effect of EA incorporation on the oxidative stability of menhaden oil emulsions stabilized by NaCas-polysaccharide was studied.

4.2 Materials and methods

4.2.1 Materials

Food grade NaCas with 91% protein (wet basis) and 5.6% moisture was a kind gift from Samik Dairy & Food Co. Ltd (Seoul, Korea). Food grade HMP (degree of esterification: 71%) and sodium benzoate were from Esfood Co. Ltd. (Pocheon, Korea). CMC (degree of substitution: 1.0-1.2) was from Ashland Chemical Co. (Changzhou, Jiangsu, China). HCl, NaOH, ethanol (EtOH), hexane, isooctane, 2-propanol, methanol, 1-butanol, ammonium thiocyanate, $BaCl_2$ and $FeSO_4$ were purchased from Samchun Chemicals (Seoul, Korea). EA, 2-pentenal, 2-hexenal, 2,4-heptadienal, fish oil from menhaden, 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid) diammonium salt (ABTS), potassium persulfate, 14% BF_3 and cumene hydroperoxide were from Sigma Chemical Co. (St. Louis, MO, USA). A standard fatty acid methyl ester (FAME) mixture was from Supelco (Bellefonte, PA, USA).

4.2.2 Analysis of fatty acid composition

Fish oil was transesterified using a method from Park and Goins (1994) (AOCS 1980). Fish oil (5 μL), methylene chloride (100 μL) and 0.5 N NaOH in methanol (1 mL) were placed in a test tube, followed by flushing with nitrogen gas and heating at 90 °C for 10 min. The tube was cooled to room temperature, mixed with 14% BF_3 (1 mL), flushed with nitrogen gas and heated at 90 °C for 10 min. After cooling, distilled water (DW; 1 mL) and hexane (2 mL) were added and vigorously mixed. Top hexane

layer with FAME was taken and analyzed by gas chromatography (GC; Agilent 6890, Agilent Technologies, Paolo Alto, CA, USA) using a modified method from Lee et al. (2010). A DB-23 column (30 m × 0.25 mm × 0.25 μm; J & W Scientific, Folsom, CA, USA) was used and the oven was programmed from 50 °C to 160 °C at 25 °C/ min, to 220 °C at 4 °C/min, held for 8 min, and to 250 °C at 25 °C/min, held for 5 min. Temperatures of injector and flame ionization detector were 200 °C and 250 °C, respectively. Carrier gas was helium and split ratio was 30:1 (v/v). Identification and quantification of peaks were done by comparing reference retention times and peak areas as weight percent of the standard FAME mixture. Fatty acid composition of menhaden oil is shown in Table 4.1, and is in accordance with the previously reported data (Stansby, 1981).

4.2.3 Emulsion preparation and stability measurement

To find optimum protein to polysaccharide ratio and pH, various concentrations of polysaccharide and pH were tested. Emulsions were formed from the method of Surh et al. (2006) with some modification. In brief, NaCas and polysaccharides were separately dissolved in DW to have final concentration of 1.11 and 1 % (w/w), respectively. The solutions were stirred for 2 h and then incubated at 4 °C overnight for full hydration. pH was adjusted to 7 using NaOH and HCl. Ten g fish oil was added to 90 g NaCas solution, homogenizing at 10,000 rpm for 2 min to form a crude emulsion (Matsushita Electric Co. Ltd; Osaka, Japan). Then the crude emulsion was homogenized at 500 bar using a nanodispenser (ISA-NLM100; Ilshin autoclave Co.,

Ltd, Daejoen, Korea) 2 times. Sodium benzoate (0.04%) was added as an antimicrobial agent, and the emulsion was mixed with DW, HMP or CMC, and then pH was adjusted from 7 to 3. Aliquot (5 mL) was taken at each pH and stored in a glass test tube (internal diameter 10 mm, height 100 mm) overnight at room temperature before stability measurement. Stability of emulsion was characterized by taking digital images and measuring creaming index (CI) using following equation:

$$CI = 100 \times Hs/He \quad (1)$$

where Hs is height of the serum layer and He is height of the emulsion. Also, emulsions were visualized using an optical microscope (S16C, MICro Scopes, Inc., St. Louis, MO, USA) magnifying 100 times. The result showed that the emulsion with 0.5% NaCas, 0.5% polysaccharide and 5% fish oil at pH 4 was the most stable and this condition was used throughout the study.

4.2.4 Incorporation of ellagic acid into sodium caseinate

EA was incorporated into NaCas using pH cycle method as described by Pan et al. (2014) with some modification. NaCas solution (1 %, w/w) was adjusted to pH 12 using 4 N NaOH and stirred for 30 min. EA powder (0.01%, w/w) was added and the mixture was stirred for 10 min. Previous paper reported that this condition resulted in the highest encapsulation efficiency (Pan et al., 2014). The mixture was adjusted back to pH 7, mixed with HMP or CMC solution and then adjusted to pH 4. The final concentrations of NaCas, polysaccharides and EA were 0.5%, 0.5% and 0.005%, respectively. To produce EA-incorporated emulsion, EA was incorporated into NaCas

using the method mentioned above, but the initial concentrations of NaCas and EA were 1.11% and 0.011%, respectively, to have final concentrations of 0.5% NaCas and 0.005% EA.

4.2.5 Fluorescence spectroscopy

To confirm the binding of EA to NaCas, intrinsic fluorescence of NaCas in the presence and absence of various concentrations of EA was measured (Pan et al., 2014). NaCas (0.01%, w/w) was prepared in DW, adjusting to pH 12. Then the solutions were mixed with various concentrations of EA in EtOH (final concentration of EA was 0-1 μg/mL) and intrinsic fluorescence was measured using FluoroMate FS-2 (Scinco, Seoul, Korea). Excitation wavelength was 280 nm and emission spectrum was recorded from 300 to 500 nm.

4.2.6 Fourier transform infrared spectroscopy

Fourier transform infrared (FT-IR) spectra of electrostatic complexes, complex incorporated with EA, physical mixture of freeze dried electrostatic complexes and EA powder were measured by a Nicolet 6700 FT-IR spectrometer (Thermo Nicolet Corp., Madison, WI, USA). Wavenumber range was from 4000 to 650 cm^{-1} and numbers of scan and resolution were 32 and 8, respectively.

4.2.7 Antioxidant activity

Antioxidant activity of the complexes, complex incorporated with EA and native EA were measured using ABTS radical scavenging method (Brandwilliams et al.,

1995). Briefly, ABTS (7 mM) and potassium persulfate (2.45 mM) stock solutions were prepared in DW, mixed at 1:1 ratio, and incubated overnight in the dark. The solution was diluted with DW at pH 4 to have absorbance of 0.70 ± 0.05 at 734 nm. ABTS solution (0.5 mL) was mixed with the samples (30 μL) and incubated for 5 min in the dark. Absorbance was measured at 734 nm.

4.2.8 Encapsulation efficiency

Encapsulation efficiency (EE) of EA incorporated in the complexes or emulsions was measured using the method from Xu et al. (2014) with some modification. In brief, EA-incorporated complexes or emulsions were centrifuged at 2,580 × *g* for 30 min at 4 °C. The supernatant was removed and the pellet was mixed with EtOH and sonicated for 10 min to extract pelleted free EA. For emulsion, the pellet was washed with DW before the addition of EtOH to completely remove remaining emulsion.

Amount of EA was quantified using high-performance liquid chromatography (HPLC) according to the method of Daniel et al. (1989) and Espin et al. (2007). A Waters Alliance 2695 separations module equipped with a Waters 2996 variable-wavelength diode array detector (Waters, Milford, MA, USA) and an Xbridge C18 column (4 μm, 3.9 mm × 300 mm; Millipore Corp., Milford, MA, USA) was used. Mobile phases were 5% formic acid (A) and acetonitrile (B) and the gradient started from 99% A for 5 min, followed by a linear decrease from 99% A to 40% A over 40 min. The flow rate was fixed to 1 mL/min, injection volume was 20 μL and EA was detected at 254 nm. The samples were filtered with 0.45 μm syringe filter before

HPLC analysis.

4.2.9 Lipid hydroperoxides

The emulsions were stored at room temperature under dark for 2 weeks and aliquots were withdrawn periodically to measure lipid hydroperoxides according to the method from Richards et al., (2002). Emulsion (0.3 mL) was taken, mixed with 1.5 mL isooctane/2-propanol (3:1; v/v) and vortexed 3 times for 10 s each. The mixture was centrifuged at 1,274 × *g* for 1 min and 0.2 mL of clear upper layer was mixed with 2.8 mL methanol/1-butanol (2:1; v/v), 15 µL 3.94 M thiocyanate solution and 15 µL 0.072 M Fe^{2+} solution. After 20 min of incubation at room temperature under dark, absorbance was measured at 510 nm (Optizen 2020UV; Mecasys, Daejeon, Korea) and the amount of lipid hydroperoxides was calculated using a cumene hydroperoxide standard curve.

4.2.10 Volatile lipid oxidation products

Fresh emulsions (1 mL) were placed at 10 mL screw cap vials and sealed with screw caps with PTFE/silicone septa (Supleco, Bellefonte, PA, USA). The vials were stored at room temperature under dark for 2 weeks and headspace volatiles were measured using gas chromatograph mass spectrometer (GCMS; Shimadzu GP2010 Ultra, Shimadzu, Kyoto, Japan), equipped with a DB 5 capillary column (30.00 m × 0.25 mm × 0.25 µm, Agilent J & W Scientific, Folsom, CA, USA) and a divinylbenzene/darboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 µm solid microextraction (SPME) fibre (Supleco, Bellefonte, PA, USA). A SPME fibre was

injected into vial and exposed to the headspace for 10 min at 55 °C. The fibre was desorbed at 250 °C for 10 min in the GC injector using splitless mode. Oven temperature was held at 40 °C for 2 min and then elevated from 40 °C to 160 °C at 6 °C/min and from 160 °C to 280 °C at 10 °C/min. Carrier gas was helium (25.0 cm/s) and injector temperature was 250 °C. Volatile compounds were identified by a mass detector at 70 eV and a 220 °C ionization gauge controller.

4.2.11 Statistical analysis

Statistical analysis was conducted using one-way analysis of variance (ANOVA) (SPSS 22 software, SPSS Inc., Chicago, IL, USA) with Duncan's multiple-range test ($p < 0.05$) to determine significance among the samples.

4.3 Results and discussion

4.3.1 Effect of polysaccharide concentration and pH on the stability of emulsion

In this study, the concentration of NaCas was fixed to 1% when producing crude emulsion as this concentration was reported to be sufficient to cover the oil (10%, w/w) during emulsification (Liu et al., 2012). CI and digital images of the emulsions with various concentrations of polysaccharides and pH are shown in Fig. 4.1 and 4.2.

NaCas-stabilized emulsions were stable at pH 7, but phase separation occurred at pH 3, 4 and 5 since the pH was near the isoelectric point of NaCas. After 2 weeks, severe aggregation occurred in all the emulsions except at pH 7. The emulsions with 0.25% polysaccharides were not stable to aggregation and phase separation, possibly

due to bridging flocculation (Liu et al., 2012). The emulsions with 0.5% polysaccharides were the most stable to aggregation and phase separation at pH 4. In the proceeding experiments, emulsions with 0.5% NaCas, 0.5% polysaccharides and 5% fish oil at pH 4 were used. From the microscopic images, particle size of the emulsions was about 2-4 μm and was not analyzed further as the objective of this study was to examine oxidative stability of the emulsion rather than its physical characterization.

Table 4.1 Fatty acid composition of menhaden fish oil (%, w/w)

Fatty acid	Menhaden fish oil
12:0	0.28 ± 0.07
14:0	8.40 ± 0.05
15:0	0.78 ± 0.01
16:0	18.2 ± 0.12
17:0	0.93 ± 0.01
18:0	3.61 ± 0.14
SFA	32.2 ± 0.32
16:1	11.0 ± 0.08
18:1n-9	5.67 ± 0.03
20:1	0.85 ± 0.25
MUFA	17.5 ± 0.15
18:2n-6	1.57 ± 0.02
18:3n-3	1.42 ± 0.01
20:4n-6	0.62 ± 0.58
20:5n-3	13.9 ± 0.09
22:6n-3	12.6 ± 0.08
PUFA	28.7 ± 0.54
Total identified	79.8 ± 0.20

Values are means ± standard deviations (n=3).

SFA: saturated fatty acids; MUFA: mono unsaturated fatty acids; and PUFA: poly unsaturated fatty acids

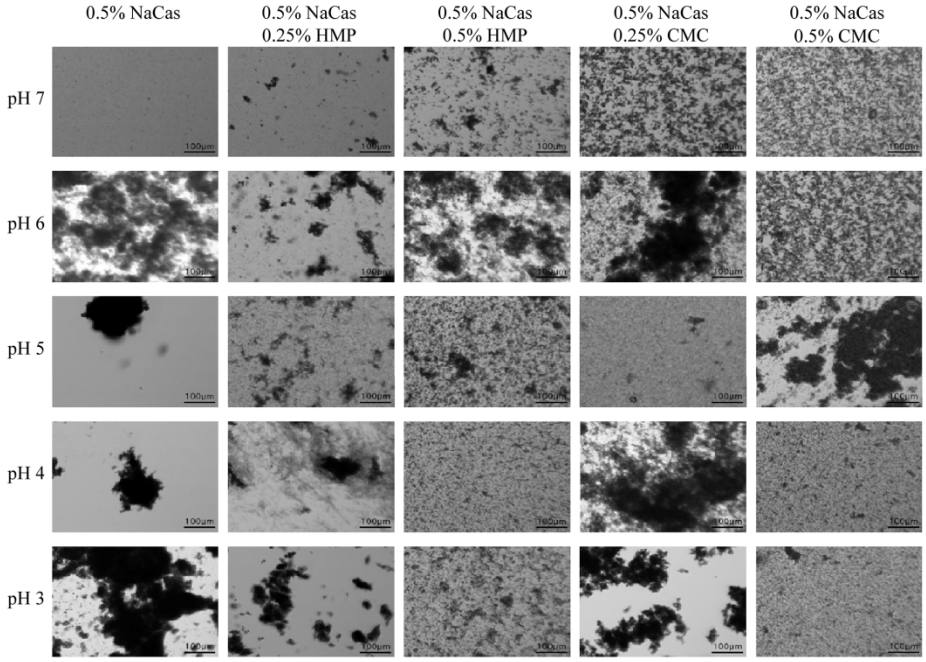

Fig. 4.1 Microscopic images of the emulsions prepared with NaCas, NaCas-HMP and NaCas-CMC at pH 3-7 after 14 days of storage (n = 2)

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

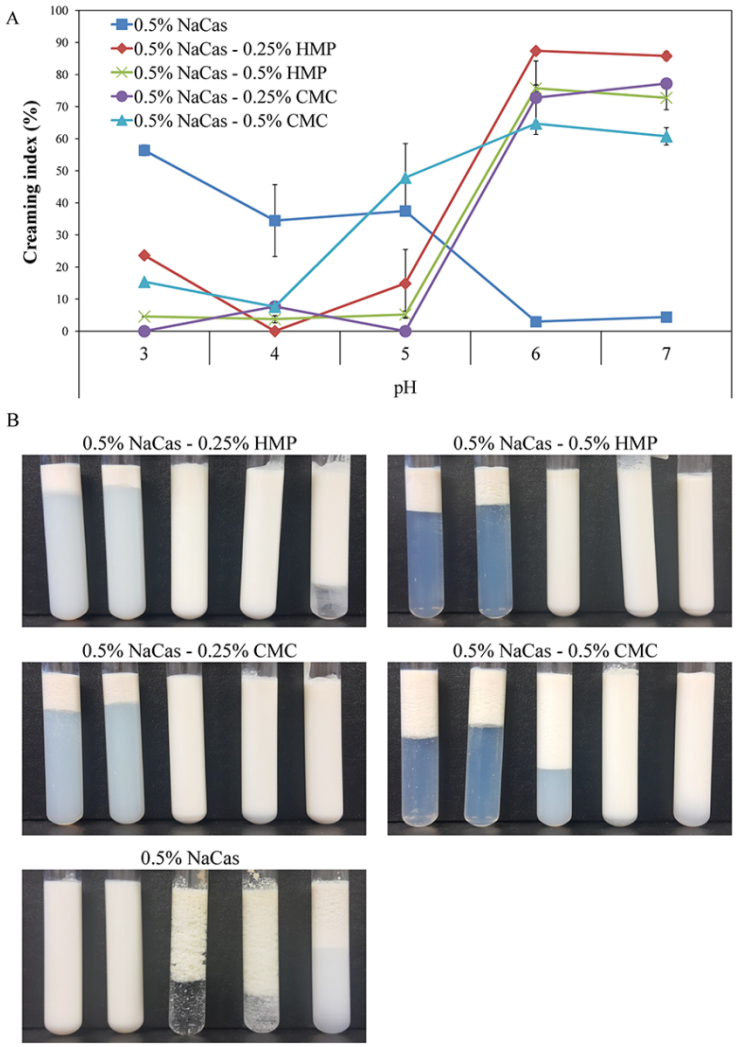

Fig. 4.2 Effect of polysaccharide concentration and pH on creaming index (A) and visual appearance (B) of the emulsions prepared with NaCas, NaCas-HMP and NaCas-CMC at pH 3-7 after 14 days of storage

Values are means ± standard deviations (n = 2).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

4.3.2 Fluorescence spectra of ellagic acid-incorporated sodium caseinate

In order to determine interaction between NaCas and EA, intrinsic fluorescence spectra of NaCas with and without various concentrations of EA were measured (Fig. 4.3). NaCas has maximum intensity at 336 nm when excited at 280 nm and the presence of a quencher resulted in lower intensity (Cogan et al., 1976). Fluorescence intensity of NaCas decreased as the concentration of EA increased, indicating the interaction between NaCas and EA. Decrease in fluorescence intensity could be due to dynamic quenching, the collision of a quencher and a fluorophore or due to static quenching, complex formation between a quencher and fluorophore (Mohammadi et al., 2009). To determine whether the quenching is dynamic or static, Stern-Volmer equation was used:

$$F_0/F = 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q] \quad (2)$$

where F_0 and F are fluorescence intensities in the absence and presence of EA, respectively. K_{SV}, [Q], and k_q are Stern-Volmer quenching constant, concentration of EA and the fluorescence quenching constant, respectively. τ_0 is lifetime of fluorophore fluorescence in the absence of quencher (10^{-8} s; Lakowicz & Weber, 1980). k_q value was 7.85×10^{11} $M^{-1}s^{-1}$, which is higher than the maximum dynamic quenching constant of 2×10^{10} $M^{-1}s^{-1}$ (Lange et al., 1998). This result indicates that the quenching interaction between NaCas and EA was static and NaCas formed complex with EA.

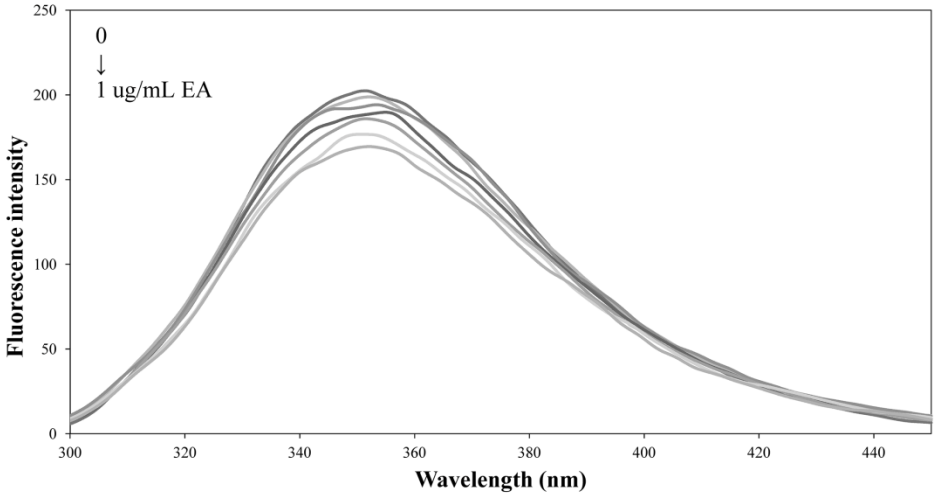

Fig. 4.3 Fluorescence spectra of sodium caseinate (0.01%) in the presence and absence of various concentrations (0-1 µg/mL) of ellagic acid (EA)

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

4.3.3 Fourier transform infrared spectroscopy

FT-IR spectra of EA, EA-incorporated complexes, complexes without EA and physical mixture are shown in Fig. 4.4. The spectrum of EA showed similar characteristic bands as described in previous papers (Bulani et al., 2016; Gopalakrishnan et al., 2014). Most of the characteristic peaks of EA were not visible in physical mixture of EA and the complex as the concentration of EA was much lower than that of the complex. However, some of the characteristic peaks of EA were still visible in the physical mixture. Even in the same concentration of EA and the complex, the spectrum of EA-incorporated complex was almost identical to that of empty complex. These results suggest that EA was incorporated into the complex.

4.3.4 Antioxidant activity of ellagic acid and ellagic acid-incorporated complexes

Antioxidant activity of EA and the electrostatic complexes with and without EA was evaluated using ABTS radical scavenging assay (Fig. 4.5). The complexes showed antioxidant activities as NaCas, HMP and CMC have antioxidant activities (Moseley et al., 2003; Kitts, 2005; Ro et al., 2013). Antioxidant activity of EA-incorporated complexes was similar to that of native EA. This result indicates that binding did not alter the antioxidant activity of EA.

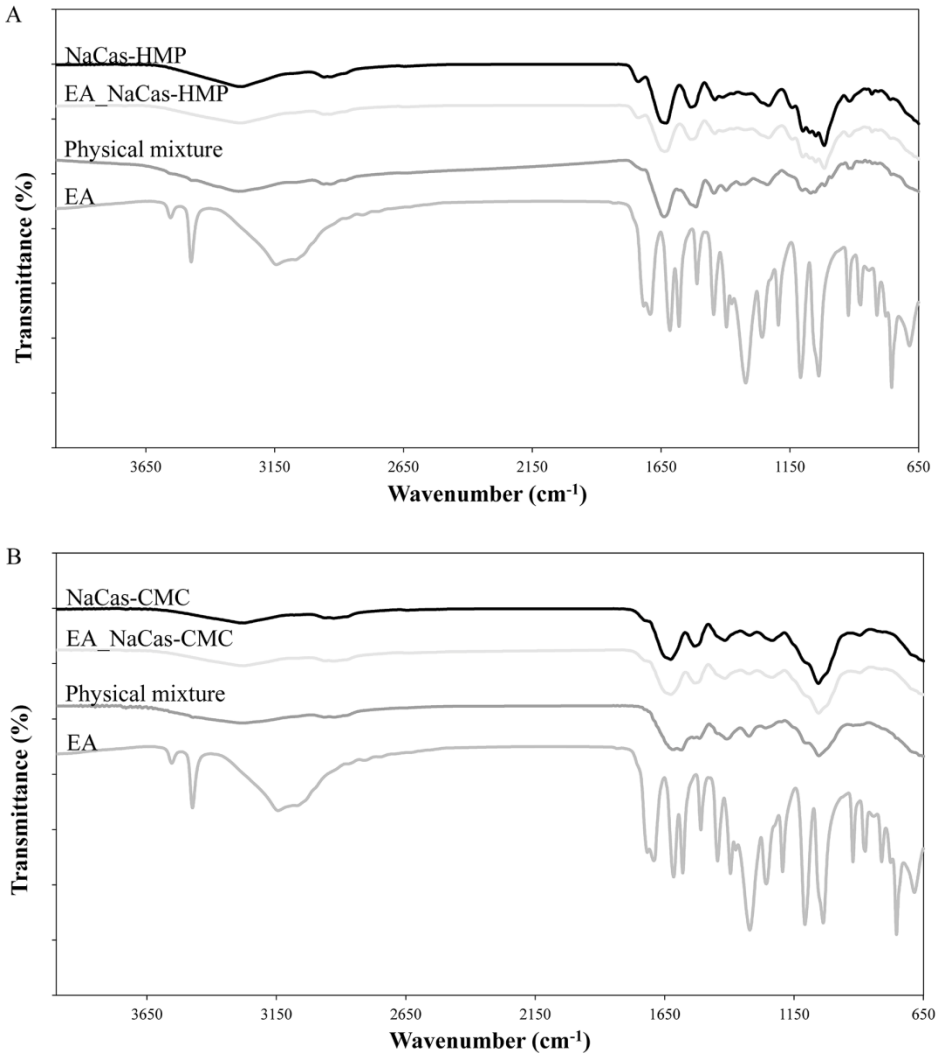

Fig. 4.4 FT-IR spectra of ellagic acid (EA), electrostatic complexes, complex incorporated with EA, physical mixture of freeze dried electrostatic complexes prepared with HMP (A) and CMC (B)

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

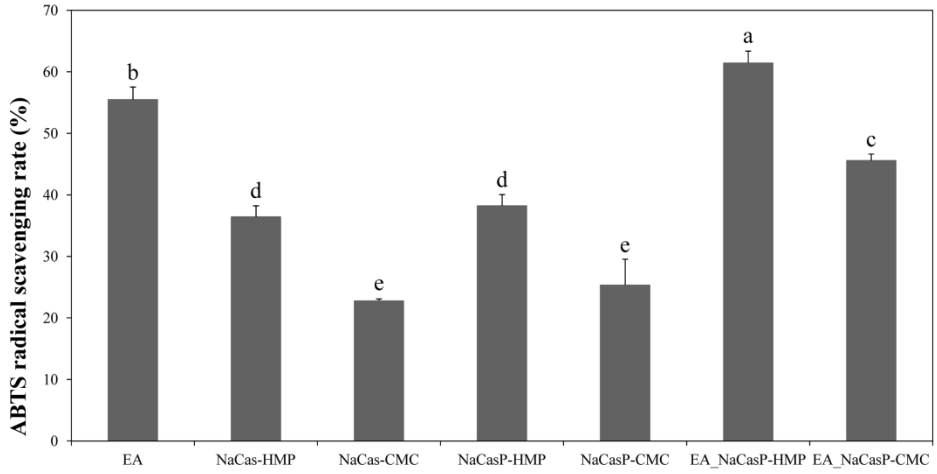

Fig. 4.5 ABTS radical scavenging rate of ellagic acid (EA) and electrostatic complexes with and without incorporated EA

Different letters represent significant difference ($p < 0.05$).

Values are means ± standard deviations (n = 3).

NaCas, sodium caseinate; NaCasP, pH-cycled NaCas; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

4.3.5 Encapsulation efficiency of the complex and emulsion

EE of EA in the complex and emulsion are shown in Fig. 4.6. There was no significant difference between NaCas-HMP and NaCas-CMC. However, EE of the complexes was significantly higher than that of the emulsions ($p < 0.05$). This could be due to the high pressure homogenization process during emulsion formation or presence of oil, which could affect structure of NaCas, consequently binding EA to the protein.

4.3.6 Effect of ellagic acid incorporation on the physical stability of emulsion

To evaluate effect of the pH cycle treatment on the stability of emulsion, CI of the emulsions with native NaCas, pH-cycled NaCas and EA-incorporated NaCas were measured (Fig. 4.7). CI of the emulsions prepared using NaCas-HMP was significantly lower than that of NaCas-CMC ($p < 0.05$), which is in accordance with Fig. 4.2A. However, CI of the emulsions prepared with pH-cycled NaCas or EA-incorporated NaCas were not significantly different from those of the emulsions with native NaCas ($p > 0.05$). Chen et al. (2016) also reported that complexation of curcumin to soy protein isolate did not affect CI of the emulsions.

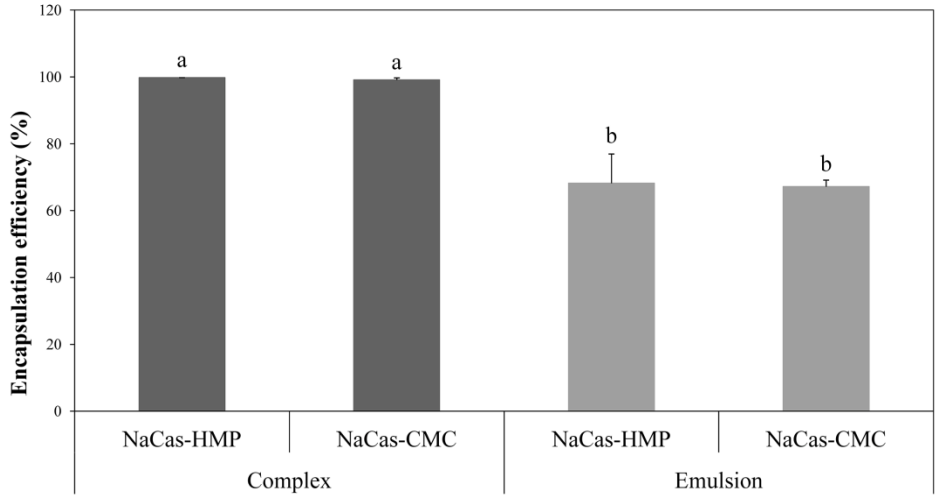

Fig. 4.6 Encapsulation efficiency of ellagic acid (EA) incorporated in the complexes and the emulsions

Different letters represent significant difference ($p < 0.05$).

Values are means ± standard deviations (n = 3).

NaCas, sodium caseinate; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

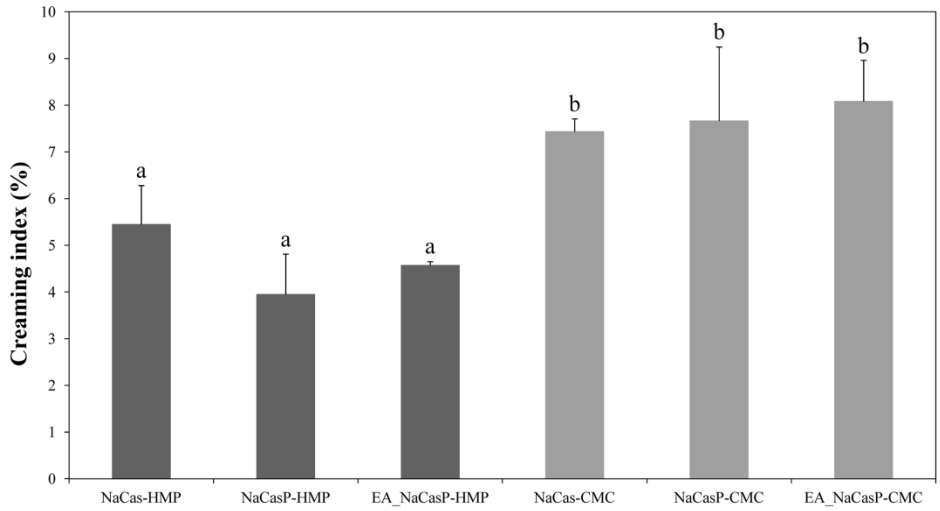

Fig. 4.7 Effect of pH cycle treatment and ellagic acid (EA) incorporation on creaming index of the emulsions after 14 days of storage

Different letters represent significant difference ($p < 0.05$).

Values are means ± standard deviations (n = 3).

NaCas, sodium caseinate; NaCasP, pH-cycled NaCas; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

4.3.7 Effect of ellagic acid incorporation on the oxidative stability of emulsion

Incorporation of EA reduced lipid hydroperoxides in the emulsions compared to the emulsions without EA (Fig. 4.8A). After 14 days of storage, lipid hydroperoxides of NaCas-HMP and NaCas-CMC-stabilized emulsions with EA were 22.5% and 24.0%, respectively, lower compared to the pH-cycled controls. The pH-cycle treatment did not affect formation of lipid hydroperoxides.

Volatile lipid oxidation products in the emulsions stored for 14 days were analyzed by SPME (Fig. 4.8B). Among lipid oxidation products from fish oil or oil rich in n-3 fatty acids, 2-pentenal, 2-hexenal and 2,4-heptadienal were identified in this study (Frankel, 1993; Jimenez-Alvarez et al., 2008). In accordance with the result of lipid hydroperoxides, there was no significant difference between the emulsions with native NaCas and pH-cycled NaCas ($p < 0.05$) except for 2-hexenal in the NaCas-CMC-stabilized emulsion ($p > 0.05$). For the NaCas-HMP-stabilized emulsion, incorporation of EA reduced formation of 2-pentenal, 2-hexenal and 2,4-heptadienal by 70.1%, 39.9% and 34.5%, respectively. For the NaCas-CMC-stabilized emulsion, their reductions were 80.9%, 58.3% and 46.1%, respectively. There was no significant difference between the emulsions with NaCas-HMP and NaCas-CMC ($p > 0.05$). Other papers reported that incorporation of antioxidant resulted in reduced amount of hexanal (Cui et al., 2014, Wan et al., 2014). However, in this study, there was no difference in the concentration of hexanal in all the samples (data not shown). This may be due to difference in the fatty acid composition as previous papers used

soybean or corn oil, while menhaden oil, rich in n-3 PUFA, was used in this study (Table 4.1). Jimenez-Alvarez et al. (2008) reported that 2-propenal, 2-hexenal and 2,4-heptadienal but not hexanal were formed during oxidation of EPA and DHA.

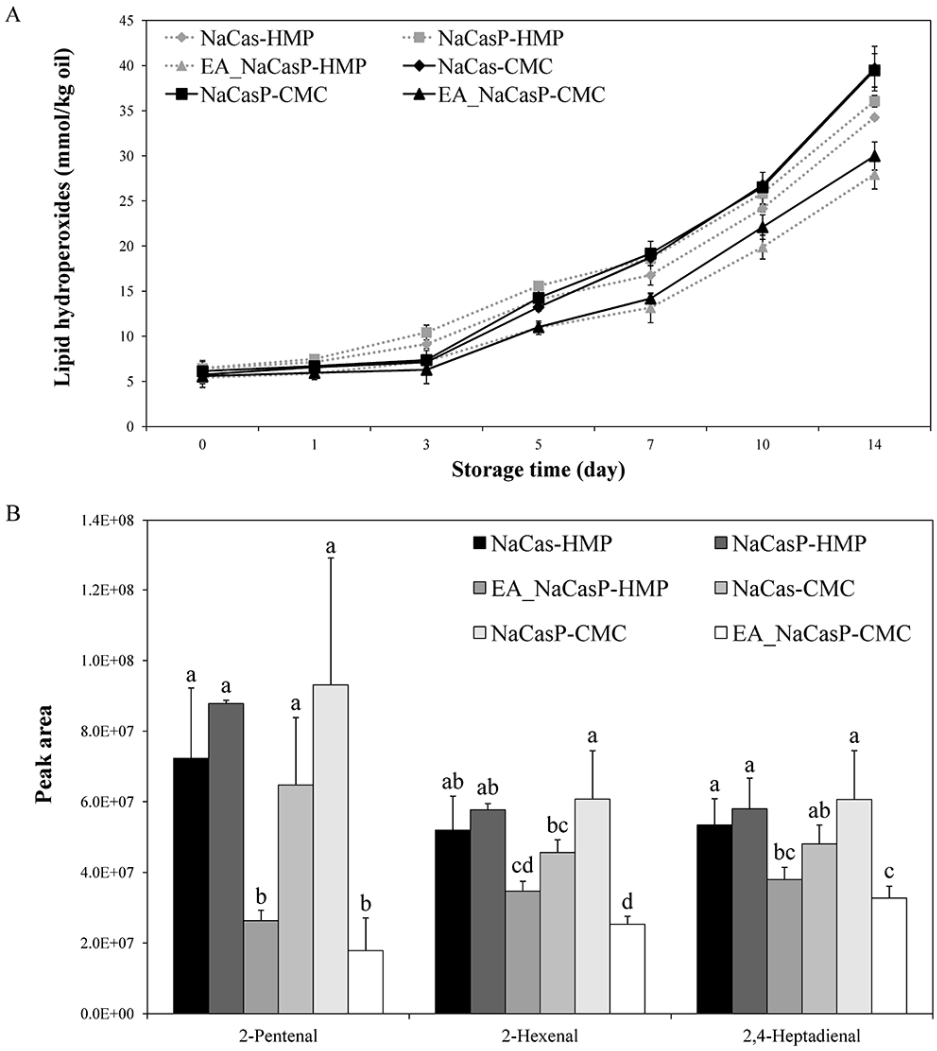

Fig. 4.8 Effect of ellagic acid (EA) on the formation of lipid hydroperoxides (A) and volatile lipid oxidation products (B) of the emulsions during storage for 14 days

Different letters represent significant difference ($p < 0.05$).

Values are means ± standard deviations (n = 3).

NaCas, sodium caseinate; NaCasP, pH-cycled NaCas; HMP, high-methoxyl pectin; and CMC, carboxymethyl cellulose

4.4 Conclusion

A stable NaCas and polysaccharide-stabilized emulsion was formed when 0.5% NaCas, 0.5% polysaccharide and 5% oil was used at pH 4. Incorporation of EA into NaCas using pH-cycle method was confirmed using fluorescence spectra and FT-IR. EA incorporated into NaCas showed similar antioxidant activity to native EA. Incorporation of EA did not affect CI of the emulsions. However, EA increased oxidative stability of the emulsions by reducing lipid hydroperoxides and volatile lipid oxidation products such as 2-pentenal, 2-hexenal and 2,4-heptadienal. There was no significant difference between NaCas-HMP and NaCas-CMC stabilized emulsions. Results of this study suggest that pH cycle method may be used to incorporate EA. Also, NaCas with EA may be used to increase oxidative stability of emulsions. Further study is needed on the effect of EA incorporation on the other properties of the emulsion, including particle size, zeta potential and flocculation.

Chapter 5

Summary and Conclusions

Objective of this study was to find optimum condition to form electrostatic complexes using sodium caseinate (NaCas) and high-methoxyl pectin (HMP) or carboxymethyl cellulose (CMC), and to increase stability, solubility and utilization of curcumin and ellagic acid (EA) using the complexes.

Part 1

Study I: Optimum condition to form electrostatic complexes was evaluated. NaCas and electrostatic complexes were compared for their ability to bind and stabilize curcumin.

1) Electrostatic complexes of NaCas were successfully formed using HMP and CMC.
2) The electrostatic complexes showed higher stability against pH and ionic strength than native NaCas.
3) Curcumin was successfully bound to NaCas and the electrostatic complexes, which was evident when determined by UV-vis, fluorescence and FT-IR spectra.
4) The electrostatic complexes showed a higher encapsulation efficiency and binding affinity toward curcumin than native NaCas.
5) Curcumin bound to the electrostatic complexes showed the least degree of degradation followed by native NaCas. HMP and CMC slightly improved stability of curcumin.

Study II: The optimum pH for the complexes was determined. Curcumin bound to the

complexes were used as a colourant in a model beverage. Stability, colour and in vitro bioaccessibility of curcumin in a model beverage were evaluated.

1) The optimum pH for NaCas-polysaccharide electrostatic complexes was pH 4.

2) Curcumin bound to the electrostatic complexes had similar antioxidant activity to native curcumin.

3) The aggregation of the complexes during freeze-drying could be partially prevented by the use of an excipient (sucrose).

4) Curcumin bound to the complexes was successfully incorporated into model beverage as a food colourant. The bound curcumin had similar colour values and higher stability and in vitro bioaccessibility than native curcumin.

Part 2

Study III: Optimum condition to form stable emulsion using NaCas and polysaccharides was evaluated. EA was incorporated into NaCas using a pH cycle method. Effect of EA incorporation on the oxidative stability of emulsion was determined.

1) A stable NaCas and polysaccharide-stabilized emulsion was formed when 0.5% NaCas, 0.5% polysaccharide and 5% oil was used at pH 4.

2) Incorporation of EA into NaCas using pH cycle method was confirmed using fluorescence spectra and FT-IR.

3) Incorporation of EA did not affect creaming index (CI) of emulsion.

4) Incorporation of EA increased oxidative stability of emulsion by reducing the formation of lipid hydroperoxides and volatile lipid oxidation products, 2-pentenal, 2-hexenal and 2,4-heptadienal.

Results of this study showed that electrostatic complexes could be formed using NaCas and polysaccharides (HMP and CMC). The complexes showed higher stability against changes in pH and ionic strength than NaCas. The optimum pH of the complexes was pH 4. The complexes were able to bind and stabilize curcumin, and curcumin bound to the complexes could be used as a food colourant with higher colour intensity, stability and in vitro bioaccessibility than native curcumin. A pH cycle method could be used to incorporate EA into NaCas. Also, EA-incorporated NaCas could be used as an emulsifier to increase oxidative stability of emulsions without affecting CI. The results of this study suggest that the complexes between NaCas and polysaccharides could be used to deliver water-insoluble bioactives into food products. Moreover, bioactives incorporated into the complexes could provide functional activities including antioxidant activity in food matrixes. Further study is needed to confirm that the complexes could bind and stabilize other hydrophobic bioactives. Moreover, bioaccessibility of curcumin and other hydrophobic bioactives bound to the complexes should be accessed in *in vivo* model. Applicability of the pH cycle method on the other polyphenols should be studied.

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국문 초록

카제인나트륨과 다당류를 이용한 Electrostatic Complex 제조 조건 설정과 Curcumin 또는 Ellagic Acid를 결합시킨 Complex의 이화학 특성

조현노

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폴리페놀 등을 비롯한 생리활성 물질들은 항산화, 항염증능 등을 비롯한 다양한 기능성을 가지는 것으로 보고되었으나 낮은 용해도와 안정성 때문에 식품에 적용은 제한적이었다. 이러한 문제를 해결하기 위한 방법 중에 하나가 단백질-다당류의 complex와 폴리페놀을 결합시키는 것이다. 본 연구의 최종목적은 카제인나트륨(NaCas)과 다당류를 이용하여 electrostatic complex 제조 조건 설정과 이 complex로 curcumin이나 ellagic acid (EA)를 결합시키고 이것의 이화학 특성을 파악하는 것이다.

첫 번째 연구에서는 NaCas와 다당류로 제조한 complex들과 native NaCas의 curcumin과 결합하고 안정화시키는 능력을 비교하였다.

Curcumin은 다양한 기능성을 가지는 것으로 알려졌으나 낮은 생체이용률, 용해도, 안정성 때문에 식품에는 제한적으로 적용되고 있다. 단백질은 curcumin을 안정화시킬 수 있다고 알려져 있으며 단백질-다당류의 complex는 curcumin 안정화 능력이 더 뛰어나다고 알려져 있다. 본 연구에서는 NaCas과 고메톡실펙틴(high-methoxyl pectin; HMP)이나 카복시메틸셀룰로스(carboxymethyl cellulose; CMC)를 이용하여 만든 complex를 사용하였다. 단백질과 다당류의 비율이 1:2일 때 가장 탁도가 낮고 침전물이 적은 complex가 생성되었다. 이 complex들은 NaCas보다 이온농도나 pH의 변화에 더 안정적이었다. Curcumin과 complex 간의 결합은 UV-vis와 형광 스펙트라 및 푸리에 변환 적외분광법(Fourier transform infrared spectroscopy; FT-IR)을 이용하여 확인하였다. 이 complex들은 NaCas보다 curcumin과 더 강하게 결합하였고 curcumin을 안정화시키는 능력 또한 더 뛰어났다. 이러한 결과를 볼 때 낮은 pH에서 NaCas보다 complex들이 더 사용하기 적합하다고 판단된다.

두 번째 연구에서는 complex들의 최적 pH를 찾고 또한 동결건조한 complex들을 음료모델에 식용색소로 적용이 가능할지를 확인하였다. 최적 pH를 찾기 위하여 pH에 따른 complex들의 특성을 비교하였는데, pH 4가 최적인 것으로 판단되었다. 제타 전위 값을 비교한 결과, NaCas-CMC(-33.59)가 NaCas-HMP(-22.19)보다 제타 전위 값이 컸기에 더 안정적

일 것으로 추정된다. Complex와의 결합은 curcumin의 열안정성은 증가시켰으나 항산화능에는 영향을 미치지 않았다. Sucrose를 첨가하면 동결건조 중에 일어나는 complex 간의 응집에 의한 탁도가 감소하였으며 NaCas-HMP가 NaCas-CMC보다 동결건조에 의한 응집이 더 적게 일어났다. 음료 모델에 적용하였을 때 complex와 결합한 curcumin이 색 안정성과 생체이용률이 더 높았다. 이러한 결과를 볼 때 complex, 특히 NaCas-HMP와 결합한 curcumin은 음료 등 투명도가 요구되는 식품에 식용색소로써 적용이 가능할 것으로 생각된다.

세 번째 연구에서는 항산화능이 있는 폴리페놀인 EA와 결합시킨 NaCas를 이용하여 만든 유화물의 산화안정성을 확인하였다. 높은 pH에서 NaCas의 분산과 EA의 용해도 증가를 이용한 pH cycle법을 이용하여 NaCas와 EA를 결합시켰다. NaCas와 EA 간의 결합은 형광 스펙트라와 FT-IR을 이용하여 확인하였다. EA의 첨가가 유화물의 산화안정성에 미치는 영향을 확인하기 위해 유화물 생성을 위한 최적조건을 탐색하였으며 실제 식품과 비슷한 낮은 pH에서의 안정성을 증가시키기 위해 HMP나 CMC를 첨가하였다. 단백질과 다당류의 비율이 1:1일 때 pH 4에서 가장 안정적인 유화물이 생성되었다. EA의 첨가는 유화물의 creaming index에는 영향을 주지 않았으나 과산화물의 생성을 NaCas-HMP와 NaCas-CMC로 안정화시킨 유화물에서 각각 22.5%와 24.0%감소시켰다. 휘발성

지방산화물질의 생성량 또한 EA가 첨가된 유화물에서 더 낮았다. 본 연구를 통해 EA는 pH cycle법을 이용하여 NaCas과 결합시킬 수 있음을 확인하였다. 또한 EA와 결합한 NaCas을 유화제로 사용하여 유화물의 산화안정성을 증가시킬 수 있다는 것을 확인하였다.

본 연구의 결과를 통해 NaCas과 HMP 또는 CMC를 이용하여 electrostatic complex를 만들 수 있으며 이를 이용하여 curcumin이나 EA를 결합시킬 수 있음을 확인하였다. Complex와의 결합을 통해 curcumin의 안정성을 증가시킬 수 있었으며 complex와 결합한 curcumin을 음료 등 투명한 식품에 식용색소로써 적용할 수 있음을 확인하였다. 또한 EA와 결합한 NaCas과 다당류로 안정화시킨 유화물의 산화안정성은 EA가 없는 유화물보다 높았다. 이러한 결과들을 보았을 때 NaCas과 다당류간의 complex를 이용하여 물에 잘 녹지 않는 폴리페놀을 식품에 첨가시킬 수 있을 것으로 생각된다. 또한 이렇게 식품에 첨가된 폴리페놀들은 색소나 항산화능 등과 같은 추가적인 기능성을 제공할 것으로 기대된다.

주요어: 카제인나트륨, 고메톡시펙틴, 카복시메틸셀룰로스, Electrostatic complex, Curcumin, Ellagic acid

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