A Thesis for the Degree of Master of Science

Effects of pectin molecular structure on *in vitro* curcumin release of calcium pectinate beads

펙틴 분자 구조에 따른 커큐민-칼슘 펙틴 비드의 소화 특성 분석

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농학석사학위논문

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Effects of pectin molecular structure on *in vitro* curcumin release of calcium pectinate beads

by

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ABSTRACT

In an attempt to investigate the functional properties of pectin, degree of esterification (DE) of commercial high methoxyl pectin (HMP) was modified with a pectin methyl esterase (PME) isolated from papaya fruit, and modified pectin (DE 50) and commercial pectin including low methoxyl (LM) and low methoxyl amidated (LMA) pectin were prepared as encapsulating curcumin in bead. Commercial LM pectin with DE 50 and DE 5, and LMA pectin with DE 35, degree of amidation (DA) 15 and DE27, DA20 were used for experiment. Curcumin, polyphenol compound, is well-known its diverse functionalities, but its poor solubility and vulnerability to alkali, light, and oxidation prohibit its wide application in food industry. In this study, depending on different molecular structure of pectin, efficient encapsulation of curcumin was pursued, and the physicochemical properties and *in vitro* release of curcumin in simulated gastrointestinal tract (GIT) model were elucidated.

Curcumin-loaded calcium pectinate beads were prepared by dropping 2% of pectin solution containing curcumin (250 mg) and sodium caseinate as a surfactant into 8% of $CaCl_2$ solution. To determine a potential use as a site specific nutraceutical delivery, modified pectin (1-2%) with various levels of Ca^{2+} concentration (2-8%) was also used for bead

preparation.

After formation of the curcumin-loaded calcium pectinate beads, all beads were spheres when 2% of pectin and 8% of Ca^{2+} introduced except the LMP with DE 7, which had a cone aside of spherical bead surface. The beads produced from the modified pectin, the size was notably small compared to the others by entrapping at the highest dose of curcumin. Deesterification in block-wise increased Ca^{2+} sensitivity then induced strong gelation prohibiting the degradation of pectinate beads during digestion with the lease release of curcumin. In contrast, pectin with same DE and Mw as to PME-modified pectin has random charge distribution developing the weak gel which entrapped less curcumin and showed more than 60-fold release rate of curcumin. Generally, commercial pectin with low DE have shown the high Ca^{2+} sensitivity, but its small Mw due to chemical process for demethylesterification limited the functional properties. LMA pectins provided rather strong gel networks enhanced by hydrogen bonds between their amide groups. However, these amide groups might give an opened structure at high DA by distributing in block-wise manner disrupting the calcium-pectin crosslinking, which resulted in low EE and intermediate release rate of curcumin. Different matrix networks depending on molecular structure of pectins were also depicted by SEM, which also supported the explanations.

This difference in entrapment ability and release rate induced by specific structure of pectin suggested a great potential for using pectin to control the release of curcumin and other lipophilic nutraceuticals in the human body.

Keywords: pectin, pectin methyl esterase, molecular structure, curcumin-loaded calcium pectinate bead, physicochemical properties, simulated gastrointestinal tract model

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INTRODUCTION

Pectin, a hydrocolloid found in plant cells, is a heteropolysaccharide composed of neutral and acidic sugars. It is widely used as a functional ingredient for foods and cosmetics such as jelling agent, thickener, emulsifier, and viscosity modifier, etc., and also as a drug releaser for medical products. It is consisted of homogalacturonan (HG) which is a linear chain of 1,4-linked α-D-galacturonic acid. Its rheological and chemical properties are highly dependent on molecular weight (Mw) and structure of HG including the degree of methylesterification (DE) and pattern of demethylation (Hills, Mottern, Nutting, & Speiser, 1949; Hotchkiss et al., 2002; Y. Kim et al., 2013; Limberg et al., 2000; Ralet, Dronnet, Buchholt, & Thibault, 2001).

Pectins, normally extracted from plant cell wall with DM 70, are commercially processed using chemicals accompanying decrease of Mw to demethylesterify or to obtain amidated pectin which has thermal reversibility. In addition, pectins are conventionally classified based on their DE into high (>50% DE) or low (<50% DE) methoxyl pectin. High methoxyl pectin (HMP) has hydrophobic interactions and hydrogen bonds between methoxyl groups, and its gelation is promoted by high sugar concentration and acidic medium (pH 3.5) (Oakenfull, 1984). On the other hand, low methoxyl pectin (LMP) forms a rigid gel by cross-linking through ionic interactions between

divalent calcium ions (Ca^{2+}) and the negatively charged carboxyl groups in the pectin, referred to as 'egg-box' conformation (Grant, 1973; Powell, 1982). According to the recent studies, charge density and charge distribution also play a key role in gelling properties of pectin as well as Mw and DE. In other words, although DE values were same, the functional properties of pectin are dependent on the pattern of methylesterification (Kim et al., 2013; Savary, 2003; Thibault, 1993; Van Buggenhout et al., 2006)

Plant pectin methylesterases (PME; E.C.3.1.1.11) de-esterify the methylesters sequentially in block-wise manner, which is more likely to produce pectins having enhanced sensitivity against calcium for cross-linking than randomly demethylesterified pectins prepared by using chemical saponification or fungal PMEs. It can also maintain Mw during demethylesterification, an important factor of functionality (Jung, Arnold, & Wicker, 2013; Ngouemazong et al., 2012). The PME isolated from papaya fruit (*Carica papaya L.*) has been characterized by Vasu, Savary, and Cameron (2012). This PME (CpL-PME) has a broad optimum pH range from ~6 to 9 and is also stable up to 70°C, of which properties would give a great chance to modify pectin nanostructure in industrial applications that are not possible with the current method using fungal PMEs. However, a more comprehensive understanding of functional properties of pectin based on

nanostructure is needed, especially on methyl ester patterns.

Manipulation of beads by ionotropic gelation of pectin, which is almost totally degraded by pectinolytic enzymes produced by colonic microflora and not digested by gastric or intestinal enzymes, is an ideal method to achieve site specific nutraceutical release. The beads can be fabricated using an injection method, which is one of the simplest and most widely used approaches for bioactive compounds encapsulation, protection, and delivery (Matalanis, Jones, & McClements, 2011; Zhang et al., 2016). In addition, pectin beads have a better ability to protect nutraceutical from degradation under the upper gastrointestinal tract (GIT), as compared with other forms such as emulsions or hydrogels (Bourgeois, Laham, Besnard, Andremont, & Fattal, 2005; T. H. Kim, Park, Kim, & Cho, 2003; Thakur, Singh, & Handa, 1997). Furthermore, pectin beads are efficiently able to deliver nutraceuticals to human organs by encapsulating nutraceuticals at high doses when administered orally (El-Gibaly, 2002; Musabayane, Munjeri, Bwititi, & Osim, 2000; Sriamornsak, 1998). Several previous attempts have been made to deliver the nutraceutical and control of its release using pectin beads and gel matrixes. However, there is currently no information of nutraceutical release from pectin beads related to the nanostructural changes or differences.

Curcumin is a yellow-orange polyphenolic compound isolated from

the rhizomes of *Curcuma longa* (turmeric) (Hu, Kong, Hu, Gao, & Pang, 2015), which has been used as spice and food coloring agent, and in traditional Indian medicine (Aggarwal, Surh, & Shishodia, 2007). Curcumin is well-known for its diverse functionalities such as anti-oxidant, anti-inflammatory, antimicrobial, anti-amyloid and anti-tumor, as reported recently (Wang et al., 2015). Additionally, it also has potential nutraceutical activities and has an ability to inhibit carcinogenesis in a variety of cell lines including colon, breast, gastric, liver, leukaemia, oral epithelial, ovarian, pancreatic, and prostate cancers (Maheshwari, Singh, Gaddipati, & Srimal, 2006; Sookkasem, Chatpun, Yuenyongsawad, & Wiwattanapatapee, 2015). Furthermore, curcumin has been noticed to have properties to inhibit a growth of colon cancer, and induce apoptosis in colon cancer cells, of which use could be a potentially promising approach for cancer therapy (Sookkasem et al., 2015). Another benefit of curcumin is its low toxicity even when ingested at relatively high doses, which has drawn a particular interest as a natural nutraceutical ingredient in health-promoting foods what consumers demand to take (Anand, Kunnumakkara, Newman, & Aggarwal, 2007).

However, several physicochemical characteristics of curcumin limit its application in functional food products, such as its intense color, strong flavor, insolubility, sensitivity to alkaline conditions, photodegradation, and

instability against oxidation or heat (Kurien, Singh, Matsumoto, & Scofield, 2007; Letchford, Liggins, & Burt, 2008; Tonnesen, Masson, & Loftsson, 2002). These problems can be overcome by encapsulating curcumin within food-grade colloidal system which can improve its chemical or biochemical stability and control its fate in gastrointestinal tract (Zhang et al., 2016).

In this study, curcumin was encapsulated by commercial and PME-modified pectins using ionotropic gelation by dropping the pectin solution into calcium chloride solution. The factors such as DE, Mw, size and distribution of unmethylesterified block, which have a significant effect on the physicochemical properties and the release behavior of curcumin from the beads in GIT, were investigated in this study. The hypothesis of this study was that the beads formed by pectins having different molecular structure would have different structural and physicochemical properties, which would result in differences in the gastrointestinal fate and bioaccessibility of the encapsulated curcumin. A simulated GIT (mouth, stomach, and small intestine) model was introduced to study the potential gastrointestinal fate of the different curcumin delivery systems. This study may provide the information that modulation of the bioavailability of encapsulated lipophilic nutraceuticals on specific site would be facilitated by the rational design of pectin beads.

The objectives of this study were to elucidate the effects of pectin

molecular structure and charge distribution on bioaccessibility of curcumin, and to investigate the physicochemical properties of curcumin-calcium pectinate beads with different molecular structure of pectins. In addition, the potential use of pectin as site specific nutraceutical delivery in gastrointestinal (GI) system was also examined.

MATERIALS AND METHODS

1. Materials

GENU pectins were provided by CP Kelco (Atlanta, GA, USA). The low-methoxyl pectin was type Explorer-65CS with DE 50 (CP50) and LM-5CS with DE 7 (CP7). The low-methoxyl amidated pectins were type LM-101AS with DE 35 and DA 15 (AP35) and type LM-104AS with DE 27 and DA 20 (AP27). For modification, the high-methoxyl pectin, type YM-100H with DE 72 (H72), was used as pectin source, and Liquipanol® T-200 from Enzyme Development Corporation (New York, NY, USA) was used as PME source driven from the dried latex of the fruit of *Carica papaya L* (CpL-PME, GRAS – Generally Recognized As Safe – 21 CFR 184.1585). Calcium chloride dihydrate was purchased from Showa Corporation (Gyoda, Saitama, Japan). Curcumin and sodium caseinate were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2. Methods

2.1. Enzymatic modification of pectin

Pectin with defined DE 72 was used for the enzymatic treatment adopted from the study of Hotchkiss et al. (2002) with some modifications. Pectin was dissolved at 2% (w/v) in distilled water with stirring overnight. Pectin solution (250 mL) was put into a 500 mL of mass cylinder, and 50 mL of 2 M LiCl was added with a plastic pipette. Distilled water was added up to 500 mL, and then the mixture was placed in a water-jacketed reaction beaker at 30℃. The pH of the mixture was adjusted to 7.0 with addition of 0.1 M LiOH. The CpL-PME (175 U/L) was added to the reaction mixture (Randall G. Cameron, Luzio, Goodner, & Williams, 2008; R. G. Cameron, Luzio, Vasu, Savary, & Williams, 2011), and pH was kept at 7.0 with 0.1M LiOH as the titrant controlled by the electriode of an autotitration system (842 Titrando, Metrohm, Switzerland). The PME-treatment was terminated at the point of DE 50. After deesterification, 3 volumes of ethanol (99.9%) was added to the reaction mixture and adjusted to pH 3.8 with 1 M HCl to inactivate the enzyme and precipitate the pectin. Then, the mixture was chilled to 4℃ and centrifuged (25,000 ×g, 30 min, 4℃), and the supernatant was discarded. The pellet was placed in liquid nitrogen, lyophilized and homogenized in a blender (BL142, TEFAL, France). The pectin was frozen at -80℃ until future analysis.

2.2. Measurement of sucrose contents

Sucrose is blended with commercial pectins for standardization purposes during manufacturing (Nussinovitch, 1997). The residual sucrose was measured using high performance liquid chromatography. The determined sucrose content was considered for the preparation of curcumin-calcium pectinate beads. The amount of pectin and sucrose were equalized by addition of sucrose and adjustment of total weight of pectin samples to compare properties under the accurate pectin concentration.

Pectin was dissolved at 1% (w/v) in distilled water and put into Amicon® Ultra filter (0.5 PL 10K, Merk) placed in Eppendorf tube and then centrifuged (Labogene 1248R; Labogene, Gangnam, Seoul) at 8,000 ×g for 30 min. Five-hundred microliters of the filtrates were filtered again using syringe filters (0.2 μm PVDF Acrodisc LC 13, PALL Life Science, NY, USA). Fifty microliters were injected into a high-performance anion-exchange chromatography (HPAEC) system equipped with a Carbo-pak PA1 anion-exchange column (4×250 mm, Dionex, Sunnyvale, CA, USA) and a pulsed amperometric detector (PAD). This analysis was performed using 200 mM NaOH for sample elution with isocratic flow rate of 1 mL/min for 15 min. Sucrose was quantified by comparing the peak areas with peak areas of the standard solutions of known concentration.

2.3. Determination of molecular weight

Molecular weight (Mw) of commercial and PME-treated pectins were determined using high performance gel permeation chromatography (HPGPC) combined with refractive index detector (Thermo Dionex HPLC Ultimate3000 RI system, USA). Pectin samples (0.25% w/v) were prepared in distilled water and then filtered with syringe filters (0.45 μm, DISMIC 13 CP, ADVANTEC, Japan) prior to injection (10-100 μL). The HPGPC system used running columns (Waters Ultrahydrogel 120, 500, 1000 serial) at 40°C. The flow rate of mobile phase (Sodium azide 0.1M in water) was 1 mL/min. The molecular weight was measured with the pullulans standards. The results were analyzed by Chromeleon 6.8 Extention-pak software.

2.4. Preparation of curcumin-loaded calcium pectinate beads preparation

The culcumin-calcium pectinate beads were prepared using slightly modified method described by Nguyen, Winckler, Loison, Wache, and Chambin (2014). Pectin solution was prepared by dissolving 0.4 g of pectin in 10 mL of distilled water with overnight stirring at 300 rpm. Then 1 g of sodium caseinate was added and dissolved in the pectin solution with the volume of mixture adjusted to 20 mL with distilled water to obtain 2% pectin

solution (w/v) by stirring with high speed (at 700 rpm) for 1 h. After 250 mg of curcumin was added to the solution and dispersed, the mixture was uniformly mixed by stirring for 30 min. The mixture was pumped through a tube (F1825103, Gilson, Middleton, WI, USA) using a peristaltic pump (Miniplus 3, Gilson, 95400 Villiers le Bel, France), with 6 cm of height dropping into a 8% (w/v) $CaCl_2$ solution under continuous stirring at 150 rpm. The lost amount of curcumin at this point was considered for calculation in the following studies, of which the quantification methods were described in section 2.7. The beads were cured for 10 min by placing in the same $CaCl_2$ solution before washing with 20 mL of distilled water. They were filtered and collected to weigh the total and fifty randomly selected beads. The obtained beads were lyophilized and stored at 4°C prior to future analysis.

2.5. Morphology of curcumin-calcium pectinate beads

2.5.1. Stereomicroscopy

A stereomicroscope (stemi-DV4, Carl Zeiss, Oberkochen, Germany) was used to examine the size and appearance of solid beads. Curcumin-entrapped calcium pectinate beads were prepared following the method described above.

2.5.2. Colorimetric properties

Colorimetric properties of curcumin-calcium pectinate beads were determined with a colorimeter (CR-400 Chroma Meter, Konica Minolta Sensing Inc., Osaka, Japan) and were expressed using the Hunter system, which identifies color in three attributes: L (white = 100, black = 0); a (red = positive, green = negative) and b (yellow = positive, blue = negative).

2.5.3. Field emission scanning electron microscopy (FESEM)

The curcumin-calcium pectinate beads were examined using a field emission scanning electron microscope (FESEM SUPRA 55VP, Carl Zeiss, Oberkochen, Germany). Prior to the SEM analysis, a bead was mounted on a SEM stub with carbon double-stick tape, and then coated with platinum using a sputter coater.

2.6. Physicochemical properties

In order to examine the effect of pectin molecular structure on the formation of beads and gelling properties, weight loss, water holding capacity, and rheological properties were measured for commercial and PME-modified pectin gels.

2.6.1. Weight loss during beads preparation

The gravimetric analysis was used to determine the weight loss of the beads during lyophilization (Das & Ng, 2010). Fifty randomly selected beads from each batch of samples were weighed with an analytical balance with readability of 0.0001 g before and after lyophilization. The weight loss (WL) was calculated using the following equation:

$$\mathrm{WL}(\%) = \frac{W_W - W_D}{W_W} \times 100\%$$

where W_W is the weight of the beads measured just after washing, and W_D is the weight of the beads after lyophilization.

2.6.2. Water holding capacity (WHC)

WHC was measured by a centrifugal method as described by Kocher (1993) with slight modification. The PD-10 column (GE Healthcare, Freiburg, Germany) was used without packing but with column frit (pore size: 20-85 μm) to support the beads. Beads were weighed ~25 mg and placed in a PD-10 column with a PD-10 column adaptor. They were put into a 50 ml polyethylene tube, and then centrifuged at 3,134 ×g and 4℃ for 30 min. WHC was calculated using the following equation:

$$\mathrm{WHC}(\%) = \left(1 - \frac{W_R}{B_H}\right) \times 100\%$$

where B_H is the weight of the rehydrated beads, and W_R is the

weight of the released water after centrifugation.

2.6.3. Fourier transform infrared spectroscopy (FTIR)

IR spectra were obtained using FTIR (Nicolet 6700, Thermo Scientific, CA, USA). The spectra were recorded in the transmission mode from 4000 to 400 cm^{-1} (mid infrared region) at the resolution of 4 cm^{-1}. Samples were diluted 1:100 with KBr before acquisition. The background of pure KBr was acquired before the sample was scanned. The spectra were analyzed using the data analysis software, OMNIC (Thermo Scientific, CA, USA).

2.7. Total curcumin content and entrapment efficiency (EE)

EE of curcumin was determined using the method described by Das and Ng (2010) with slight modification. Briefly, beads (~25 mg) were immersed in 10 mL of 50 mM phosphate buffer (PBS), and 1μL of Pectinex® Ultra SP-L (Novozymes, Denmark) was added to completely break down the beads by cleaving pectin into monosaccharide units. The enzyme reaction was conducted at 37°C for 24 h by continuous stirring on a magnetic stirrer. Then, 10 mL of methanol was added to the system to ensure

solubilization of poorly soluble curcumin and terminate the enzyme reaction, and mixed well by stirring. The mixture was centrifuged at 3,134 ×g and 4℃ for 30 min to remove the pectin. The supernatant containing the curcumin was diluted to the calibration range (0.1-20 μg mL^{-1}) with methanol-PBS mixture (1:1). Curcumin content in the beads was determined spectrophotometrically (UV–Visible Spectrophotometer V-530, Jasco, Japan) at a wavelength of 426 nm. EE of curcumin was calculated using the following equation:

$$\mathrm{EE}(\%) = \frac{AQ}{TQ} \times 100\%$$

where AQ is the actual quantity of curcumin entrapped in the beads, and TQ is the theoretical quantity of curcumin (initial curcumin loading dose during the preparation of the beads considering the lost amount of curcumin).

The loss of curcumin during the beads preparation was also determined by collecting the residue of the mixture from the manufacture of beads and washing the pump line. The residue was immersed in 10 mL of PBS and the pump line was washed with 10 mL of PBS, respectively. The following steps were the same as described above. The mean of TQ was calculated by subtracting the lost amount of curcumin from 250 mg of curcumin which was initial quantity for bead preparation.

2.8. *In vitro* release of curcumin-calcium pectinate beads: Simulated gastrointestinal tract model

The *in vitro* release of curcumin from the curcumin-calcium pectinate beads was determined based on a slightly modified method of Mun, Kim, and McClements (2015). A relatively simple simulated gastrointestinal tract (GIT) model was introduced to simulate the biological fate of ingested samples, comprising oral, gastric, and intestinal phases. Curcumin-entrapped pectin bead samples (~25 mg) were placed in each of 50 mL conical tubes with a glass bead before the samples were exposed to a simulated mouth system.

2.8.1. Oral phase

Simulated saliva fluid (SSF) was prepared using a method reported previously (Sarkar, Goh, & Singh, 2009). Mucin (30 g/L) was dissolved in SSF on a magnetic stirrer for overnight. The mixture was adjusted to pH 6.8 with 3 M NaOH and equilibrated in a water bath at 37℃ prior to use. A 5 mL aliquot of SSF was added to each of conical tubes, and the samples were incubated and orbitally shaken in a shaking incubator (240 rpm, 37℃) for 10 min. The mouth system was terminated by filtering the beads through 100-mesh sieve. The sampling was conducted at 3, 5, and 10 min to determine the curcumin released from the beads as a function of time. These were

diluted 100-fold with methanol-PBS mixture (1:1), and the absorbance of the released curcumin was measured at 426 nm. The diluted SSF was used as blank.

2.8.2. Gastric phase

Simulated gastric fluid (SGF) was prepared by dissolving 2 g of NaCl and adding 7 mL of HCl (37%) in 1 L of water according to a previous study (Sarkar, Goh, Singh, & Singh, 2009). Then, pepsin (3.2 g/L) was added to SGF prior to the *in vitro* digestion. Afterwards, the SGF was adjusted to pH 2.5 using 3 M NaOH and placed in a 37°C water bath to equilibrate the temperature. A 5 mL of SGF was added to each of conical tubes containing the filtered beads from mouth system. The samples were incubated at 37°C for 2 h under the same conditions for the mouth system. After 2h reaction, the digestive fluid was discarded by filtering the beads through 100-mesh sieve. The sampling was conducted at 40, 70, 100, and 130 min, and the measurement was followed as described above.

2.8.3. Small intestinal phase

The conditions in the small intestinal phase of the GIT were simulated following the method described previously (Salvia-Trujillo, Qian,

Martin-Belloso, & McClements, 2013). Bile extract (187.5 mg/3.5 mL) was dissolved in 10 mM phosphate buffer (pH 7.0) with continuous stirring for overnight. Completely dissolved bile extract solution was adjusted to pH 7.0, then 1.5 mL of salt solution (10 mM of calcium chloride and 150 mM of sodium chloride) per batch of samples were added to the solution, and the mixture was adjusted to pH 7.0 again. Afterwards, freshly prepared pancreatin (187.5 mg/2.5 mL) was dissolved in phosphate buffer, and the suspension (2.5 mL/batch of samples) was added into the mixture. An aliquot of 5 mL mixture equilibrated in a 37℃ water bath was added to each of conical tubes containing the filtered beads from gastric system. The samples were incubated under the same conditions for gastric system. The sampling was conducted at 160, 190, 220, and 250 min, and the measurement followed the same procedure. At the end of the reaction, the filtered beads were stored at 4℃ for stereomicroscopy.

2.9. Statistical analysis

All experimental data were analyzed using analysis of variance (ANOVA) and expressed as mean ± standard deviation of replicate measurement. Significant differences ($p<0.05$) among mean values were compared using the Duncan's multiple range test. These statistical analyses

were conducted using IBM SPSS statistics version 21.0 (IBM, Armonk, NY, USA).

RESULTS AND DISCUSSION

1. Sucrose contents of commercial and PME-modified pectins

In general, sucrose was added to pectin for standardization purpose (density increase, dust particle reduction) during the manufacturing process of commercial (Nussinovitch, 1997). The sucrose contents of pectins were quantified using HPLC to determine the exact pectin and sucrose content in commercial and PME-modified pectins. The results are shown in Table 1. The sucrose content in H72 was reduced due to alcohol precipitation during the CpL-PME treatment, and approximately less than 1% remained in MP50. In commercial pectins, approximately 28.4-39.3% of sucrose was quantified. Thus, sucrose was added to several samples to equilibrate the final amount of sucrose with fixed pectin concentration at 2% during the bead preparation.

Table 1. Sucrose contents of commercial and PME-modified pectins

Samples[1)]	H72	MP50	CP50	CP7	AP35	AP27
Sucrose[2)](%)	28.22±0.19	0.83±0.14	34.94±0.35	28.44±0.25	39.33±0.12	32.62±0.44

[1)] H72 = commercial high methoxyl pectin (DE72); MP50 = PME-modified pectin (DE50); CP50 = commercial low methoxyl pectin (DE50); CP7 = commercial low methoxyl pectin (DE7); AP35 = commercial low methoxyl amidated pectin (DE35, DA15); AP27 = commercial low methoxyl amidated pectin (DE27, DA20)

[2)] Mean± SD; Means of triplicate.

2. Molecular weights of commercial and PME-modified pectins

The molecular weight (Mw) of pectin is one of the important key to determine its functionality (Hills et al., 1949). Therefore, Mws of commercial and PME-modified pectins were analyzed using high performance size exclusion chromatography with refractive index detector. The differential molecular weights are shown in Table 2. Demethyl esterification using CpL-PME modified only DE of the pectin without decreasing in Mw (Kim et al., 2013; Vasu et al., 2012). MP50 showed only slight decrease in Mw when compared to H72 but still maintained large molecular weight similar to that of CP50. The commercial pectin with the lowest DE, CP7, showed the smallest Mw which might be due to the possibility of chemical demethyesterification which usually accompanies significant decrease in Mw by cleaving homogalacturonan backbone of pectin (Jung et al., 2013; Ngouemazong et al., 2012). For the same reason, Mw of the amidated pectins also low, and AP27 with lower DE resulted in even smaller Mw but larger than that of CP7.

Table 2. Molecular weight (Mw) of commercial and PME-modified pectins

Samples[1)]	H72	MP50	CP50	CP7	AP35	AP27
Mw	6.3×10^5	5.9×10^5	5.9×10^5	2.5×10^5	4.8×10^5	4.1×10^5

[1)] H72 = commercial high methoxyl pectin (DE72); MP50 = PME-modified pectin (DE50); CP50 = commercial low methoxyl pectin (DE50); CP7 = commercial low methoxyl pectin (DE7); AP35 = commercial low methoxyl amidated pectin (DE35, DA15); AP27 = commercial low methoxyl amidated pectin (DE27, DA20)

3. Morphology of curcumin-loaded calcium pectinate beads

3.1. Shape and size of beads

Photographs and diameter of the undried and dried beads developed by calcium mediated ionotropic gelation are presented in Figure 1 and Table 3, respectively. The proper concentration of pectin (2% w/v) and calcium chloride (8% w/v) could provide uniform bead formation throughout the experiment. For undried beads, all beads displayed spherical shape, except for the CP 7, which had cone with diameter ranged from 1.18-1.95. In general, low methoxyl pectins (LMP) which has more demethylesterified group formed more rigid gel structure. Furthermore, low methoxyl amidated pectins (LMA) more react with calcium ion than nonamidated pectins because the presence of amide groups reduces hydrophilicity. In addition, hydrogen bonding between the amide groups also develops stronger gels (Liu, Fishman, Kost, & Hicks, 2003; Thakur et al., 1997). In accordance with the previous studies, the beads manufactured with LMA (AP35, AP27) were smaller in size than LMP (CP50) because the stronger chemical interactions inside of the beads drew inward and formed structures during the beads preparation. The beads formed with CP7 showed the biggest size due to its cone shape, but the spherical part was apparently smaller than LMA beads.

The gelling properties are highly dependent on DE and Mw. Hence, CP7 which has much lower DE formed smaller beads in spherical part, but it was not enough to develop the perfect spherical bead due to its low Mw. However, PME-modified pectin (MP50) evidently formed the smallest beads with diameter ranged from 1.18 to 1.25 mm. MP50 which has demethylesterified in block-wise manner and maintained Mw showed higher sensitivity to calcium divalent, which strongly induced to pull structures inward during the formation of beads resulting in small size.

Lyophilized beads showed a slight decrease in diameter and they were ranged from 1 to 1.85 mm. The size of beads is an important feature of stability and reactivity of food in solutions (Gbassi, 2013) since Food and Drug Administration (FDA) published the appropriate maximal bead size as 2.8 mm (10 % variation of the target, ~2.5 mm) based on chewing and swallowing particle size, accordingly, the resulted dried beads in this study were well established ("Guidance for Industry Size of Beads in Drug Products Labeled for Sprinkle," 2012). Unlike the wet beads sample, some lyophilized bead samples showed irregular shape. While the size of spherical shaped bead was easily defined by their diameter, that of non-spherical shaped bead was hard to describe using their diameter. Non-spherical beads showed a cone shape (Figure 1c), or irregular spherical shape (Figure 1a), but a more spherical and round shape with similar sizes were observed from

the others.

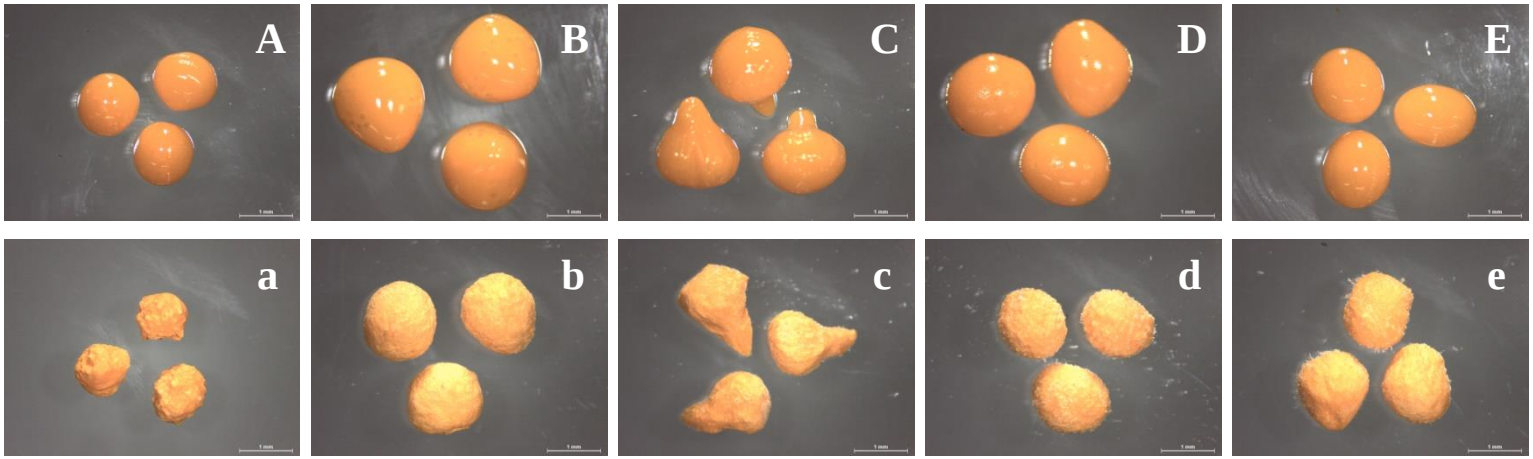


Figure 1. Photographs of curcumin-loaded calcium pectinate beads at the magnification of ×8. Undried beads (A-E) and lyophilized beads (a-e). (A,a) MP50 = PME-modified pectin (DE50); (B,b) CP50 = commercial low methoxyl pectin (DE50); (C,c) CP7 = commercial low methoxyl pectin (DE7); (D,d) AP35 = commercial low methoxyl amidated pectin (DE35, DA15); (E,e) AP27 = commercial low methoxyl amidated pectin (DE27, DA20)

Table 3. Size[1)] of curcumin-loaded calcium pectinate beads

Samples[2)]	MP50	CP50	CP7	AP35	AP27
Before drying	1.18-1.25	1.65-1.9	1.9-1.95	1.6-1.9	1.4-1.55
After drying	1-1.2	1.4-1.6	1.75-1.85	1.35-1.5	1.35-1.6

[1)] Measured diameter (mm)

[2)] MP50 = PME-modified pectin (DE50); CP50 = commercial low methoxyl pectin (DE50); CP7 = commercial low methoxyl pectin (DE7); AP35 = commercial low methoxyl amidated pectin (DE35, DA15); AP27 = commercial low methoxyl amidated pectin (DE27, DA20)

3.2. Colorimetric properties of beads

Colorimetric properties were determined using a Hunter system colorimeter. L-value indicates lightness, and positive a- and b-values indicate red and yellow, respectively. Lightness was highly correlated to particle size observed from Table 3. Higher L-value indicates that the light is more reflected from the surfaces of particles, due to back scattering (Chung, Degner, & McClements, 2013). On the other hand, a- and b-values were more likely to be related to entrapment efficiency (EE) because higher a- and b-values implied that more orange-colored curcumin was encapsulated within the networks of beads. This is obvious from MP50 which showed the highest a-value of 27.54, ($p<0.05$, Table 4), also displayed the highest EE (Table 7). PME-modified pectin prepared by using CpL-PME has free carboxyl groups distributed in block-wise manner due to successive demethyesterification action of this PME (Vasu et al., 2012). Then, enhanced calcium sensitivity could encourage the pectin to have more chance to entrap curcumin during instantaneous bead formation when dropped into $CaCl_2$ solution. However, amidated pectin beads showed not much different a- and b-values from other samples even though they had much lower EE, suggesting that curcumin was distributed to surfaces rather than inside of beads. EE would be discussed more specifically in section 5.

Table 4. Hunter L-, a-, b-values[1)] of curcumin-loaded calcium pectinate beads

Sample[2)]	After drying		
	L	a	b
MP50	67.59±0.85[d3)]	27.54±1.05[a]	62.96±1.19[b]
CP50	70.95±0.93[b]	21.36±0.90[d]	61.78±0.36[c]
CP7	71.78±0.86[a]	23.67±1.04[b]	64.06±0.54[a]
AP35	70.57±0.21[b]	23.58±0.74[b]	60.65±1.15[d]
AP27	69.06±0.11[c]	22.32±0.27[c]	62.83±0.46[b]

[1)] Mean± SD; Means of triplicate per batch (2 batches).

[2)] MP50 = PME-modified pectin (DE50); CP50 = commercial low methoxyl pectin (DE50); CP7 = commercial low methoxyl pectin (DE7); AP35 = commercial low methoxyl amidated pectin (DE35, DA15); AP27 = commercial low methoxyl amidated pectin (DE27, DA20)

[3)] The values with different superscripts in the same column are significantly different ($p<0.05$).

3.3. Scanning electron microscopical analysis

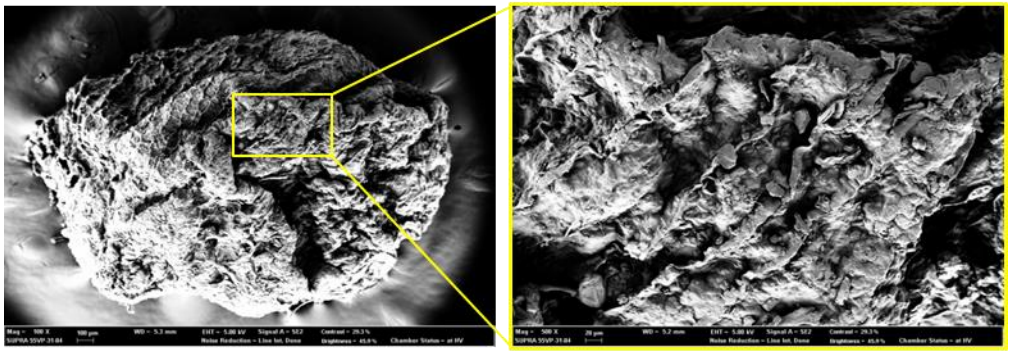
The surface and cross-section morphologies of curcumin-loaded calcium pectinate beads were observed with SEM at different magnifications and are illustrated in Figure 2. It is known that the water content before freeze-drying is the key contribution factor to their irregular shape after freeze-drying (Zohar-Perez, Chet, & Nussinovitch, 2004). Water evaporation occurres quickly during freeze-drying process, accordingly, spherical shaped beads would be shrunken and resulted in squeezed shape. MP 50 bead showed a rough and rugged surface, thick inner structures and shells embedded with the curcumin crystals (Figure 2(A)). It is because that its high Mw and Ca^{2+} sensitivity due to the distribution of negative charge in block-wise manner strongly formed the thicker structure. In addition, its block-wise charge distribution would be crosslinked lopsidedly, which resulted in a huge cavity against the complexes. It is assumed that an amount of curcumin might exist in that place by encapsulating with pectin in an instant. Similar finding was depicted in Figure 2(E), and the pectin with low DE and high DA might develop the lopsided structure as well because of its amidated group prefers to be distributed in block-wise manner which might hinder the crosslink between negatively charged carboxyl group and Ca^{2+} (Racape, 1989). However, the cross-section of the other amidated pectin

bead, AP35, showed fine structures and no empty spaces. Munjeri (1997) explained that the site of cross-linking would not be disrupted by smaller amide groups, but by bulky methoxyl groups. Small and fine structure also resulted in the lowest EE (Table 7).

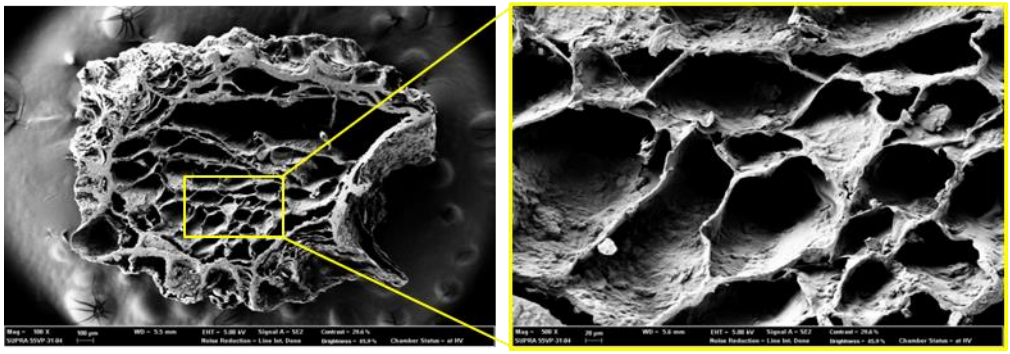
In the case of CP50, the rough surface but rather smoother inner structures were found in Figure 2(B). This pectin had the same DM and Mw with MP50 but different charge distribution. Unlike the lopsided structure in MP50, more regular structures were shown in CP50 due to its randomized demethylesterification. Besides, the bead was larger with less shrinkage and thinner structure compared with MP50. Figure 2(C) also presented clear and smoother surface with little winkles, and thin and ragged inner structures were found. Regularl inner structures were developed with rather bigger sizes of holes. CP7 had low DE involved in better interaction with Ca^{2+}, but low Mw was presumed to be not enough to build a strong structure.

(A)

a-1 **b-1**

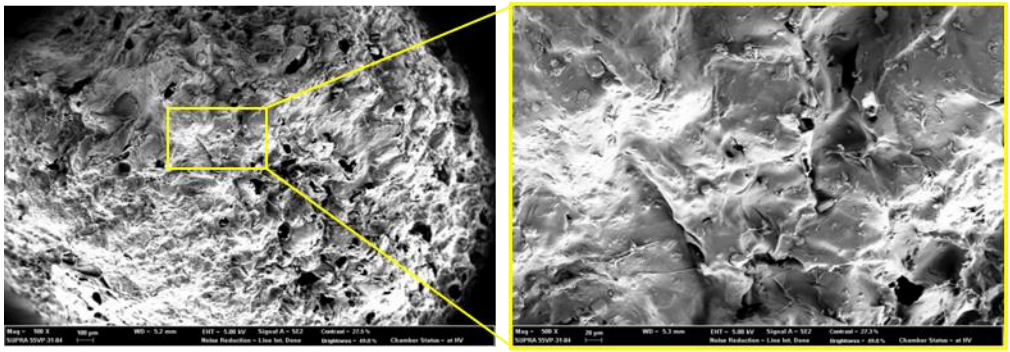


a-2 **b-2**

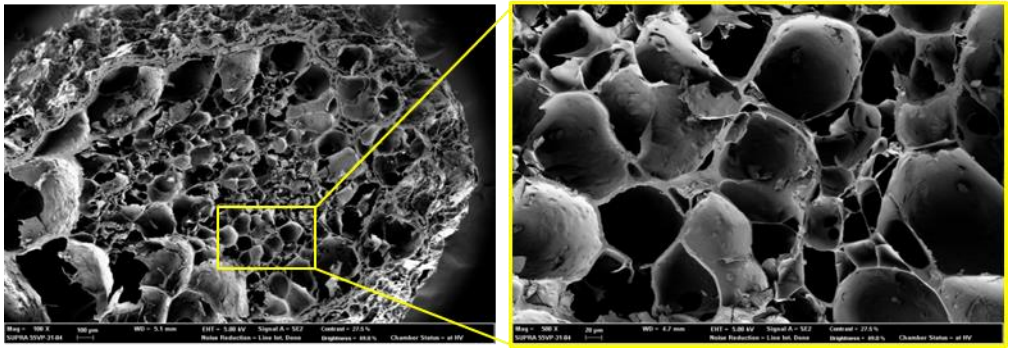


(B)

a-1 **b-1**

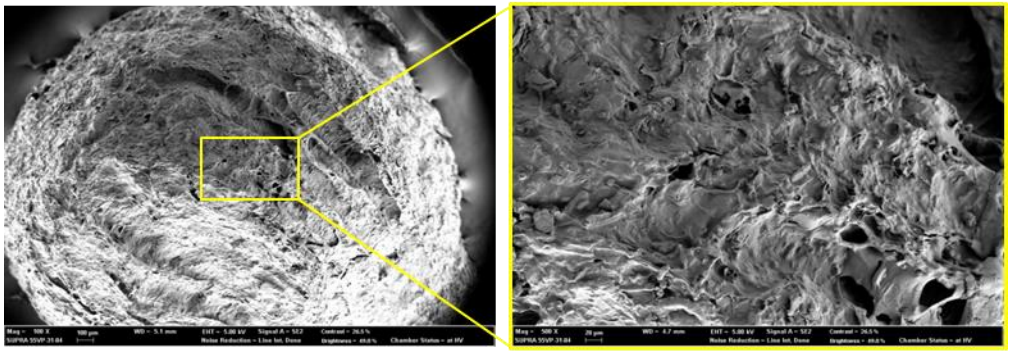


a-2 **b-2**

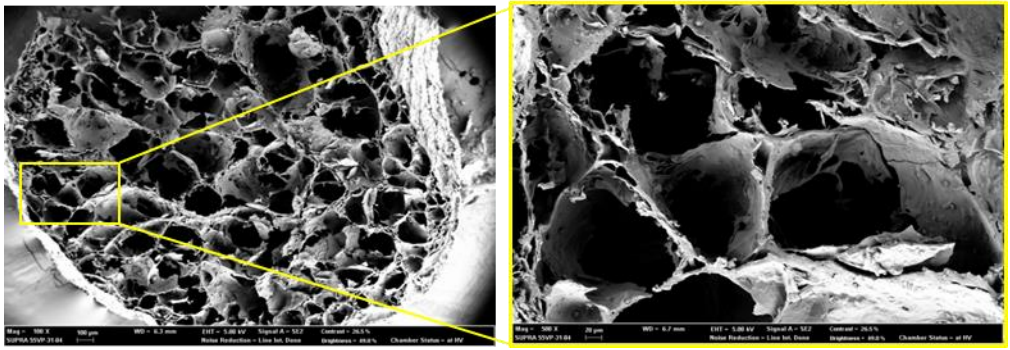


(C)

a-1 **b-1**

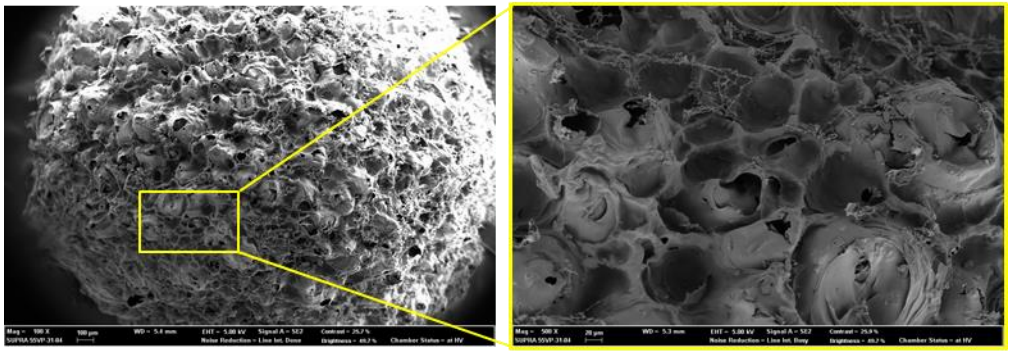


a-2 **b-2**

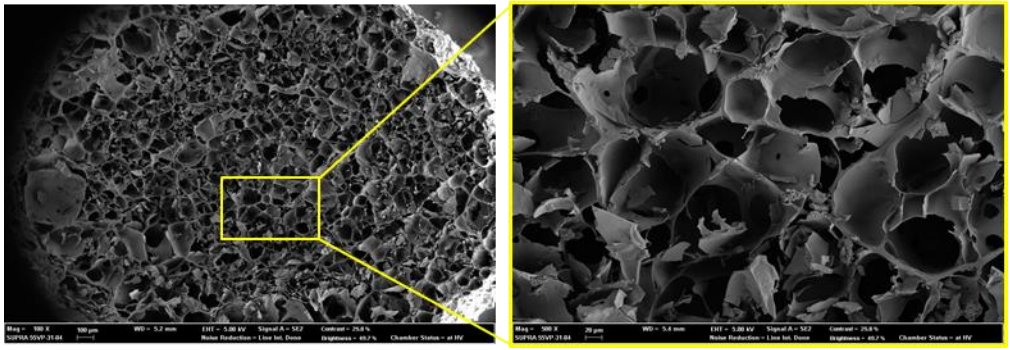


(D)

a-1 **b-1**



a-2 **b-2**



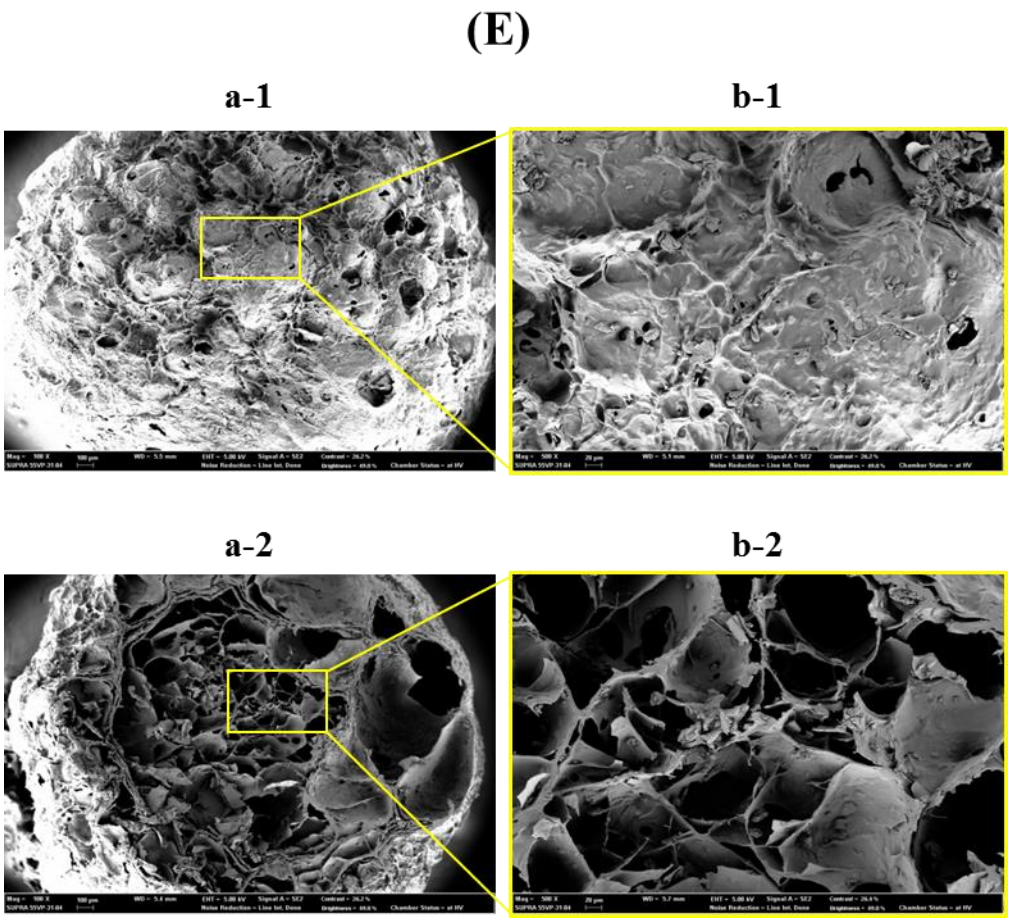


Figure 2. Scanning electron micrographs (a = 100×, b = 500× magnifications) of curcumin-loaded calcium pectinate beads (1 = surface, 2 = cross-section); (A) MP50 = PME-modified pectin (DE50); (B) CP50 = commercial low methoxyl pectin (DE50); (C) CP7 = commercial low methoxyl pectin (DE7); (D) AP35 = commercial low methoxyl amidated pectin (DE35, DA15); (E) AP27 = commercial low methoxyl amidated pectin (DE27, DA20).

4. Physicochemical properties of curcumin-loaded calcium pectinate beads

4.1. Weight loss during beads preparation

Weight of 50 undried as well as dried beads was measured to determine weight loss during lyophilization. Beads lost 88-92% of their initial weight depending on the type of pectins after lyophilazation (Table 5). The weight of undried beads should be the sum of the weights of water and calcium associated pectin. Therefore, the amounts of water squeezed and free calcium leached out of the beads were assumed to be the weight loss. According to Yotsutanagi (1987), the interior water would be squeezed out during the curing process concentrically progressed from the droplet surface to the center, and the third power of the radius would be involved to the water release as the tightening proceeds. In other words, the sphere with more concentric segment would leach out more volume of water, resulting in a more weight loss. Hence, MP50 having free carboxyl groups in block-wise, lost 91.7% of the initial weight, which was significantly higher from others ($p<0.05$), because it was strongly crosslinked with Ca^{2+} by constricting (Figure 2) and resulting in the smallest size (Figure 1). On the other hand, there were no significant differences ($p>0.05$) among others except for the

amidated pectins. Amidated groups in pectin molecules are distributed randomly or semi-randomly, which tends to be in blocks with hydrogen interactions (Munjeri, 1997; Racape, 1989). These structures might have a relatively open structure, so the sample with less DA (AP35; 88.3% WL) would lose less water than the one with more DA (AP27; 89.7% WL).

Table 5. Weight[1)] changes and weight loss (WL) of curcumin-loaded calcium pectinate beads

Sample[2)]	Undried beads	Dried beads	WL (%)
MP50	1.10±0.12	0.09±0.01	91.7±0.2[a3)]
CP50	1.49±0.21	0.16±0.03	89.0±0.5[bc]
CP7	1.35±0.04	0.15±0.00	89.1±0.3[bc]
AP35	1.46±0.01	0.17±0.01	88.3±0.4[c]
AP27	1.93±0.28	0.16±0.02	89.7±0.8[b]

[1)] Mean± SD; Means of duplicate (50 ea. beads).

[2)] MP50 = PME-modified pectin (DE50); CP50 = commercial low methoxyl pectin (DE50); CP7 = commercial low methoxyl pectin (DE7); AP35 = commercial low methoxyl amidated pectin (DE35, DA15); AP27 = commercial low methoxyl amidated pectin (DE27, DA20)

[3)] The values with different superscripts are significantly different ($p<0.05$).

4.2. Water holding capacity (WHC) of beads

WHC is related with the network of beads. Pectin bead networks vary with its factors influencing gelling properties such as DE, Mw, and charge distribution (Y. Kim et al., 2013). In Table 6, MP50, which formed thick and strong structure able to hold more water and prevent the water release from the bead (Figure 2), showed the highest WHC (84.5%). The WHC of amidated pectins, furthermore, were 83.7% and 77.6% for AP27 and AP35, respectively. The amide groups distributed block-wise or in a semi-random manner are known to facilitate the formation of hydrogen bonds, which suggested that they could have a chance to entrap more water (Guillotin, Van Kampen, Boulenguer, Schols, & Voragen, 2006; Racape, 1989). However, CP50 resulted in the lowest WHC (18.7%) although the DE and Mw were the same as these of MP50. When dried CP50 beads were rehydrated, it absorbed a plenty of water showing the highest weight (data not shown), but it lost most of water during centrifugation. Because CP50 is randomly demethylesterified and has rather high DE meaning its low calcium sensitivity, strong structure could not be formed and lost the water more easily. This result implied that the charge distribution in pectin is an important factor to determine the properties of pectin beads. In the case of CP7, it was the second lowest result as 58.3% even though its DE was the

lowest. Generally, the process of lowering DE of commercial pectins using chemicals accompanies a decrease of Mw by collapsing the galacturonan backbone (Hills et al., 1949; Rexova-Benkova & Markovic, 1976). Therefore, the beads with decreased Mw could not hold the water during centrifugation due to its thin and rough inner structure (Figure 2C).

Table 6. Water holding capacity (WHC) of beads[1)]

Samples[2)]	MP50	CP50	CP7	AP35	AP27
WHC (%)	84.5±0.8[a3)]	18.7±0.4[d]	58.3±1.7[c]	77.6±1.0[b]	83.7±1.8[a]

[1)] Mean± SD; Means of triplicate.

[2)] MP50 = PME-modified pectin (DE50); CP50 = commercial low methoxyl pectin (DE50); CP7 = commercial low methoxyl pectin (DE7); AP35 = commercial low methoxyl amidated pectin (DE35, DA15); AP27 = commercial low methoxyl amidated pectin (DE27, DA20)

[3)] The values with different superscripts are significantly different ($p<0.05$).

4.3. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of pure curcumin, pectins, and curcumin-loaded calcium pectinate beads are depicted in Figure 3. In the case of powdered pectins, a broad peak appeared in the range 3200-3600 cm^{-1} which indicates hydroxyl groups. In that range, a lower peak was presented from MP50 because sucrose content added to commercial pectins for standardization was lower than the others due to alcohol precipitation during the pectin modification. The spectra depict C-H stretching at 2944 cm^{-1} and a COOH C-H stretching at 1741 cm^{-1}. A prominent COO- stretching at 1668 cm^{-1}, 1594 cm^{-1} and 1415 cm^{-1} was observed, and especially CP7, which has the lowest DE indicating the most free carboxyl groups, showed noticeably strong peaks at those wavenumber ranges. In addition, a characteristic intense band corresponding to CH-O-CH was shown at 1017 cm^{-1} for all type of pectins except for MP50 which contained low sucrose content (Aburto, Moran, Galano, & Torres-García, 2015; Maciel, Yoshida, & Franco, 2015; Umoren, Obot, Madhankumar, & Gasem, 2015). After manufacturing of curcumin-loaded calcium pectinate beads, the broad band at 3200-3600 cm^{-1} were shown from the all samples with similar intensity because the sucrose and pectin amounts were adjusted equally during bead preparation. The major peaks of carboxyl group, furthermore, were vanished or shifted due to

crosslinking with Ca^{2+}.

Kolev et al. have reported the vibrational spectra of curcumin. The sharp peak at 3508 cm^{-1} and a broad peak at 3293 cm^{-1} indicate the presence of OH. The strong band at 1626 cm^{-1} is attributed to a predominantly mixed C=C and C=O. Another strong peak at 1601 cm^{-1} has the symmetric aromatic ring stretching vibrations. The C=O peak was obtained at 1508 cm^{-1}, while the 1272 cm^{-1} peak was assigned to the enol C-O. C-O-C peak at 1023 cm^{-1}, benzoate trans-CH vibration at 959 cm^{-1}, and cis-CH vibration of aromatic ring at 713 cm^{-1} were also found (Kolev et al., 2005; Mohan, Sreelakshmi, Muraleedharan, & Joseph, 2012). All these major peaks appeared in the curcumin-loaded calcium pectinate beads, suggesting that curcumin was not chemically modified when encapsulated by pectins (Das & Ng, 2010).

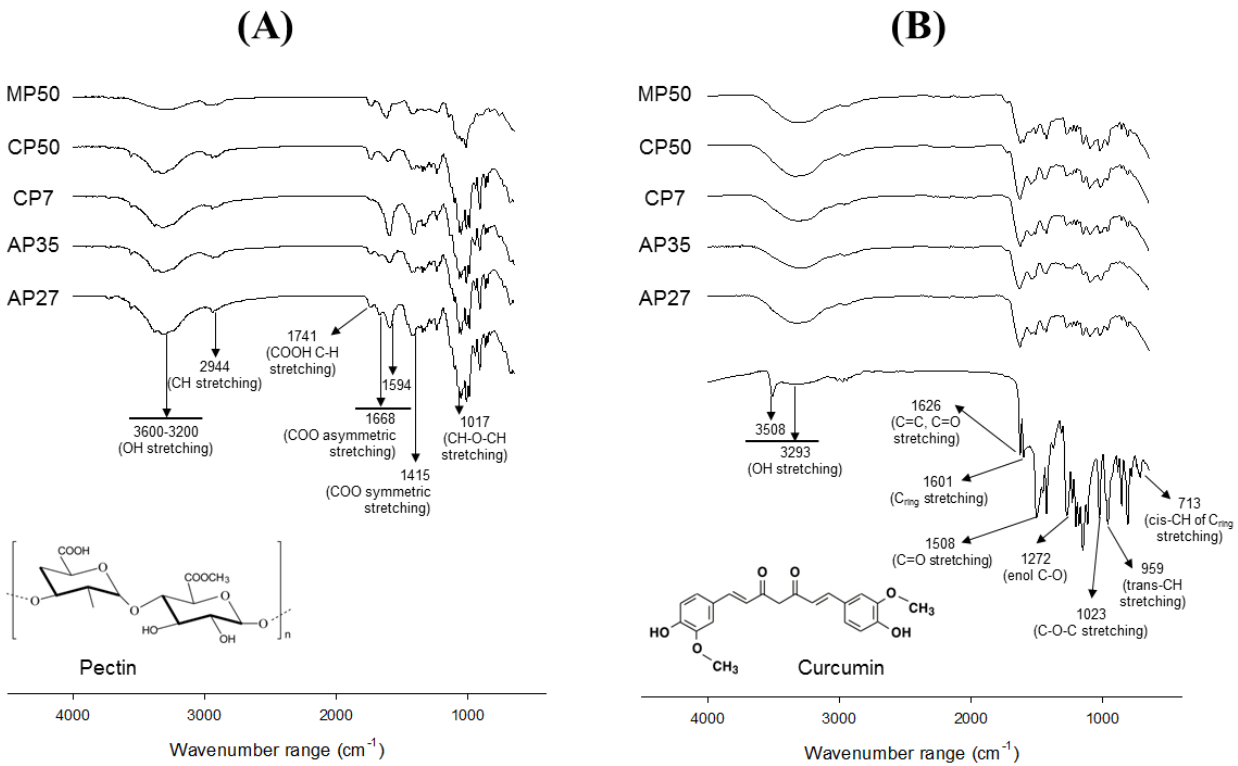


Figure 3. FTIR spectra of pectin and curcumin powder (A), and curcumin-loaded calcium pectinate beads (B)

5. Entrapment efficiency (EE)

EE of each pectin type was presented in Table 7. Instantaneous gelation of pectin when dropping into the $CaCl_2$ allowed easy entrapment of curcumin in calcium pectinate beads (Das & Ng, 2010). However, significant differences ($p<0.05$) of curcumin entrapment were found in all cases. The highest EE was 80.7% obtained from MP50 which had been modified with CpL-PME demethylesterifying in block-wise manner without a decrease of Mw. The retained Mw and increased calcium sensitivity due to blocks by PME modification would contribute to entrapment of an amount of curcumin by forming the beads into strong structure which can restrict the curcumin from coming out of the beads. Indeed, according to Figure 2(A), MP50 had a huge cavity inside the beads due to block-wise demethylesterification developing structure lopsidedly, and much curcumin was presumably embedded in there. For CP50, on the other hand, there was EE more than 20% lower compared with MP50 although they had the same DE and Mw. This study reasoned that random demethylesterification would form a weak structure more regularly, which increases the loss of curcumin and decreases the chance to trap more curcumin than the structure strongly formed with empty space.

The beads made with CP7 also showed not so high EE (68.1%). As

described in section 3.3, its low Mw in spite of the lowest DE played a role in developing the structure roughly and not strongly enough to lead to more loss of curcumin and less entrapping. Furthermore, EEs of beads manipulated with amidated pectins were much lower than others, i.e. 37% and 48.1% from AP35 and AP27, respectively. The sites for cross-linking induced by calcium between pectin chains may be further limited because the amide groups tend to be distributed in blocks, with a random distribution of carboxyl and methoxyl groups (Munjeri, 1997; Racape, 1989). These beads may, therefore, be relatively open allowing free nutraceutical diffusion.

Table 7. Entrapment efficiency (EE) of curcumin in calcium pectinate beads

Samples[1)]	MP50	CP50	CP7	AP35	AP27
EE[2)] (%)	80.7±1.0[a3)]	60.0±0.8[c]	68.1±2.4[b]	37.0±5.0[e]	48.1±1.9[d]

[1)] MP50 = PME-modified pectin (DE50); CP50 = commercial low methoxyl pectin (DE50); CP7 = commercial low methoxyl pectin (DE7); AP35 = commercial low methoxyl amidated pectin (DE35, DA15); AP27 = commercial low methoxyl amidated pectin (DE27, DA20)

[2)] Mean± SD; Means of triplicates per batch (2 batches)

[3)] The values with different superscripts are significantly different ($p<0.05$).

6. *In vitro* release of curcumin-calcium pectinate beads: Simulated gastrointestinal tract (GIT) model

6.1. Influence of pectin molecular structure on curcumin bioaccessibility

The bioaccessibility of encapsulated curcumin in the beads was investigated by introducing simulated GIT model and the results are presented in Firgure 4. The release of curcumin was dependant on the molecular structure of pectin. Almost no release of curcumin was shown for all samples at oral phase. In the gastric phase (pH 2.5), the pectinate bead surfaces were exposed to acidic environment and consequently few curcumin release occurred, which corresponded to the reported by Nguyen et al. (2014). According to Norziah (2001), pectin is depolymerized and split spontaneously by deesterification. However, there was no damage of the bead matrix despite the hydrolytic process leading to a slight attack on the surface. Indeed, bead form is not so degradable in the upper GIT compared to other forms such as emulsions and hydrogels (Bourgeois et al., 2005). Moreover, it has been suggested that the interaction between sodium caseinate and pectin might be able to delay the erosion of polymer matrix, and a part of curcumin was entrapped inside of the casein micelles which

retarded the release of curcumin by enhancing the encapsulation matrix (Nguyen et al., 2014).

When the medium changed from gastric phase to intestinal phase, several pectinate beads were damaged inducing the release of curcumin. The CP50 beads rapidly released curcumin in intestinal medium, and the bead was completely disintegrated and consequently released about 60% of the curcumin. The beads were fully digested and disappeared after 190 min with quite constant release rate, but the release did not reach 100%. It was assumed that polyphenol compounds interact with proteins such as caseinatae or digestive enzymes in this case, and then they might aggregate and precipitate (Ozdal, Capanoglu, & Altay, 2013). The other case was also presumed that the released curcumin was exposed to simulated intestinal fluid, and so it might be destroyed due to its vulnerability to harsh conditions resulting in poor bioavailability (Letchford et al., 2008; Liu et al., 2003). While about 40% and 12% of curcumin was released from AP35 and AP27, respectively, these beads seemed to be damaged after digestion as shown in figure 5. AP35 had rather higher DE than AP27 but lower than CP50, resulting in the release value between those of pectins. The other amidated pectin, AP27, showed much lower release of ~12% compared with AP35, because it had more amide groups enhancing the bead structure by their hydrogen bonds, which led to less release of curcumin from the bead.

The beads of MP50 and CP7 resulted in very low release of 0.98% and 1.25%, respectively. The molecules of MP50 and CP7 consisted of blocks with very low DE were involved in developing hard structure of bead due to their high Ca^{2+} sensitivity which led to strong cross-links. Therefore, the digestive fluids could not depolymerize the pectinate matrix, and thus the curcumin would be delivered to colon without any damage. It has been reported that in aqueous solution of pH 3-4 pectin has a good stability, but the depolymerization of pectin occurs at above this pH (Pillay & Fassihi, 1999). On the contrary, despite the change in pH from gastric phase to intestinal phase, the release of curcumin was constant in these beads. Furthermore, especially MP50 showed intact beads even after digestion.

In human digestion, the released curcumin in intestine will be absorbed with oil and play a role via its functionalities. The remained curcumin in the beads will be delivered to colon and then released from the pectinate beads decomposed by colonic microflora producing pectinolytic enzymes (Matalanis et al., 2011; Zhang et al., 2016). In this study, all samples have shown significantly different results but there was no significant difference ($p>0.05$) between MP50 and CP7. Among the samples, MP50 which entrapped the most curcumin (>80% EE) and showed the least curcumin release during the digestion has been more investigated to assess the potential use of pectin as efficient site-specific nutraceutical delivery in

gastrointestinal system by regulating pectin and calcium concentrations in the section 6.2. It is assumed other lipophilic nutraceuticals having partition coefficient analogous to curcumin may give similar results.

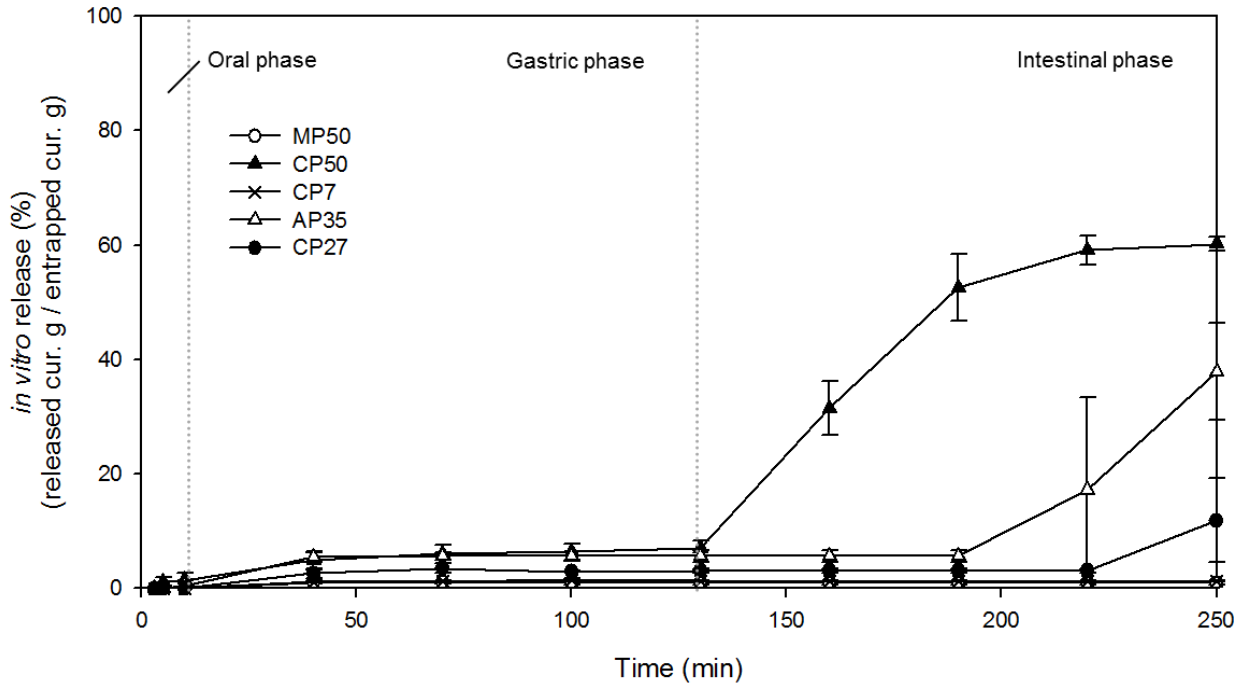


Figure 4. Comparison of curcumin *in vitro* release from pectin beads in GIT system. MP50 = PME-modified pectin (DE50); CP50 = commercial low methoxyl pectin (DE50); CP7 = commercial low methoxyl pectin (DE7); AP35 = commercial low methoxyl amidated pectin (DE35, DA15); AP27 = commercial low methoxyl amidated pectin (DE27, DA20)

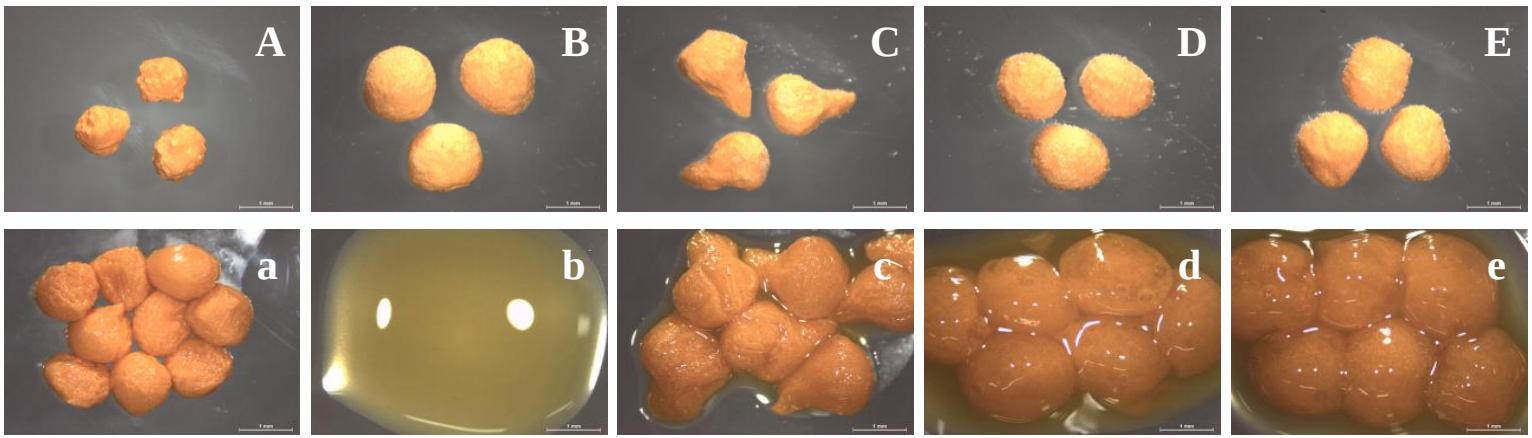


Figure 5. Photographs of curcumin-loaded calcium pectinate beads at the magnification of ×8. Before digestion (A-E) and after digestion (a-e). (A,a) MP50 = PME-modified pectin (DE50); (B,b) CP50 = commercial low methoxyl pectin (DE50); (C,c) CP7 = commercial low methoxyl pectin (DE7); (D,d) AP35 = commercial low methoxyl amidated pectin (DE35, DA15); (E,e) AP27 = commercial low methoxyl amidated pectin (DE27, DA20)

6.2. Influence of pectin and calcium concentrations on curcumin bioaccessibility

For the present investigation, to determine it is desirable to design the formulation such that it controls curcumin release in GIT, *in vitro* release study was carried out with various pectin and calcium concentrations. The method for preparation of curcumin-loaded calcium pectinate bead was followed in the same way as explained above, but the pectin, MP50, was used at 1 and 2% (w/v) as well as aqueous of 2, 4, 6, 8% (w/v) calcium chloride was used. The samples were named as 8C2P if 8% of calcium and 2% of pectin were used for bead manufacturing. The bead, 2C1P, was not formed. The release of curcumin results were depicted in Figure 6.

Depending on pectin concentration, the different amount of curcumin was released. The beads prepared with 1% pectin released about 16-18% of curcumin during digestion. The curcumin encapsulated by samples, 8C1P and 6C1P, was release quickly in oral phase and maintained constantly with slightly increased release from 6C1M in intestinal phase, but there was no significant difference between those samples (8C1M = 18.12%, 6C1M = 17.81%). The beads of 4C1P showed 15.82% of final release which was significantly different with other 1% of pectin samples. This sample released less curcumin under oral and gastric medium but quickly release it

when the medium changed into intestinal condition. The acidic and basic damages due to the simulated gastrointestinal fluids induced disintegration of the beads by chemical erosion of the pectinate matrix of surfaces. In this case, it was suggested 8C1P and 6C1P had more EE as 69% and 52%, respectively, which resulted higher release of curcumin distributed more on surfaces than 4C1P with less EE (47%) entrapping the curcumin inside rather than the surfaces.

On the other hand, 2% of pectin samples presented much lower release than the beads made with 1% of pectin. The increase of calcium concentration resulted in the decrease of curcumin release. There was no significant difference between 2-6% of calcium concentration with 2% of pectin (3.43, 3.05, 2.47%, respectively), and only 8C2P (0.98%) was significantly different with others.

In all experiments with simulated intestinal fluids, phosphate buffer was used in the medium composition. Some reports explained the phosphate ions in the medium captures the calcium ions inducing a fast disintegration of calcium pectinate bead structure (Dhalleine et al., 2011). Here, no disintegration was observed and the ion exchange leaded only the gel swelling of beads as shown in Figure 7. It is because the network of beads was strengthened by the interaction between calcium and pectin blocks. In addition, the lower concentration of pectin developed irregular shape of

beads compared to 2% of pectin beads in spherical shape whatever the calcium concentration.

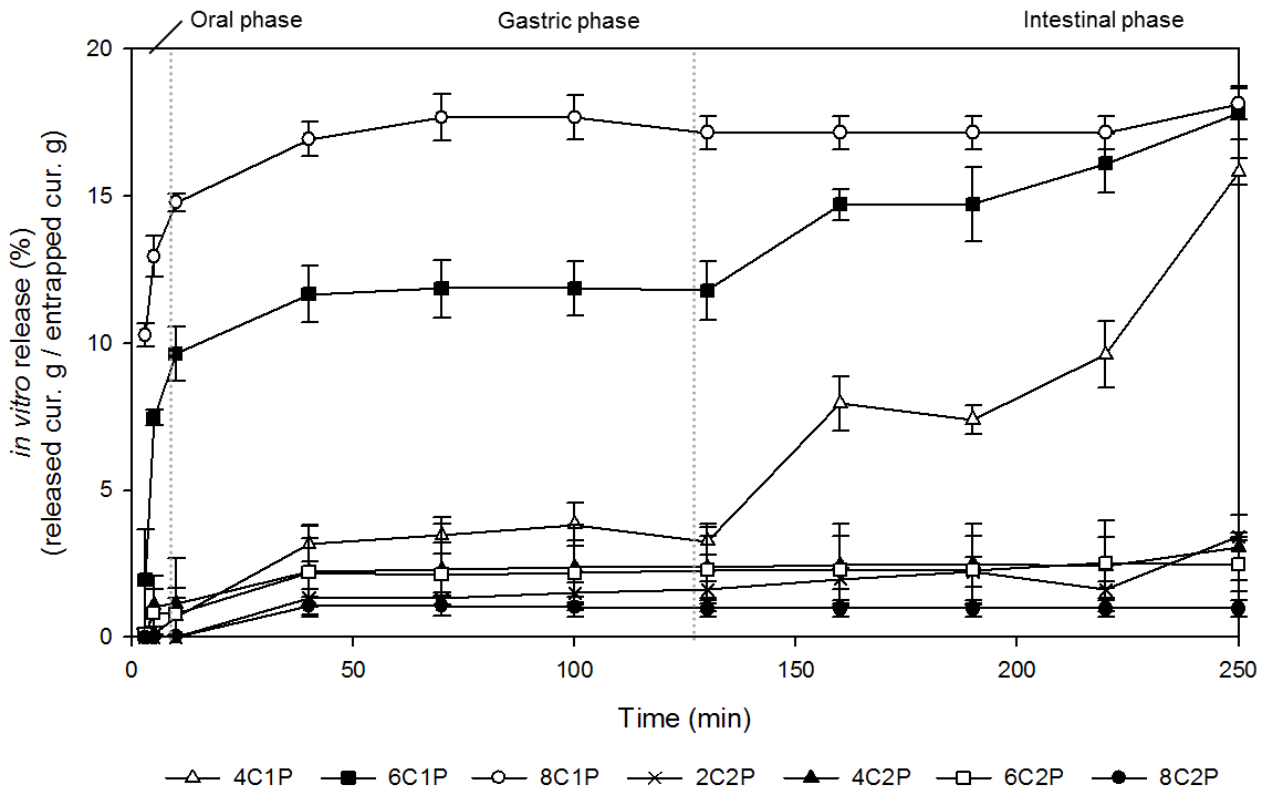


Figure 6. Comparison of curcumin *in vitro* release from pectin beads in GIT system. Sample named aCbP means a% of calcium, b% of pectin (MP50). 2P1C was not formed.

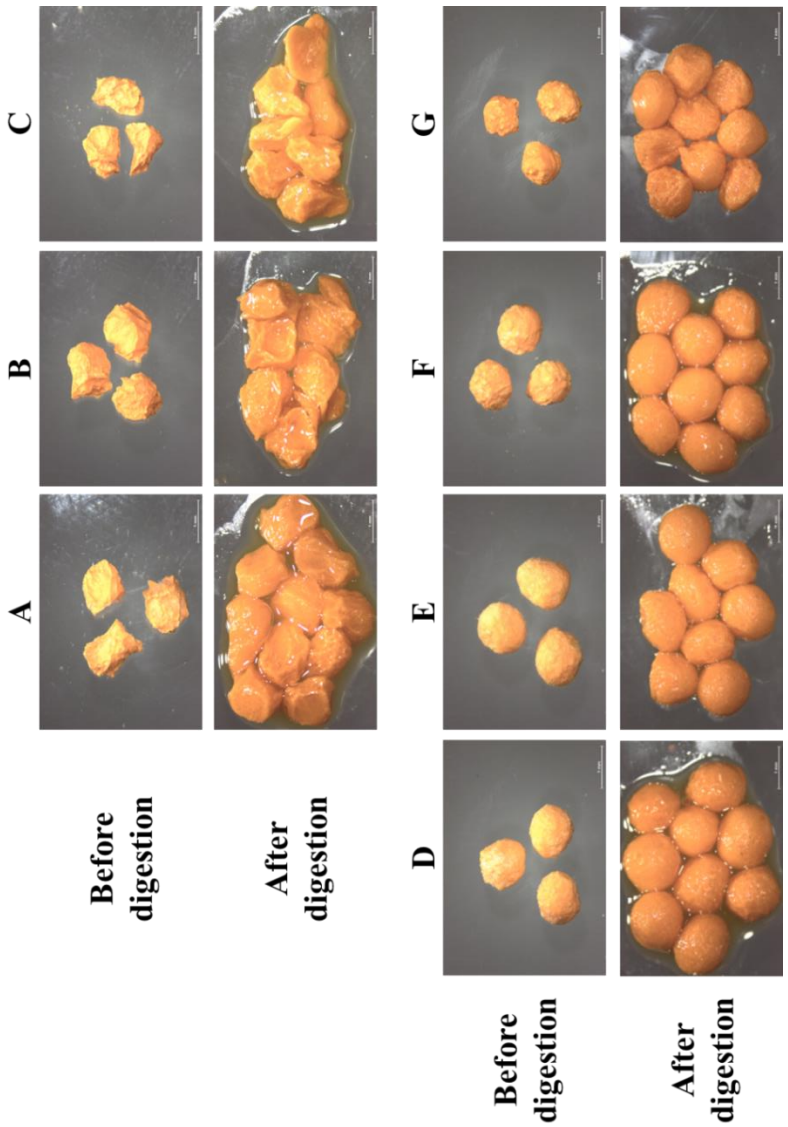


Figure 7. Photographs of curcumin-loaded calcium pectinate beads before and after digestion at the magnification of ×8. Sample named aCbP means a% of calcium, b% of pectin (MP50). 2P1C was not formed. (A) 4C1P; (B) 6C1P; (C) 8C1P; (D) 2C2P; (E) 4C2P; (F) 6C2P; (G) 8C2P.

CONCLUSION

Pectin molecular structure including degree of esterification (DE), molecular weight (Mw), and charge distribution plays a key role in gelling properties. In recent study, the functional properties of pectin are dependent on the pattern of methylesterification although DE values were same. Therefore, effect of different pectin molecular structures on physicochemical and decomposition properties in simulated gastrointestinal tract (GIT) model by encapsulating curcumin in bead form was investigated.

Commercial and PME-modified pectin beads were manufactured entrapping the curcumin to elucidate the effects of pectin molecular structure on physicochemical properties of beads and *in vitro* release of curcumin. After PME modification, the pectin maintained high Mw showed much enhanced gel properties at comparable DE due to the block increasing Ca^{2+} sensitivity for crosslinking. This modified pectin resulted in the smallest bead size, the highest entrapment efficiency (EE), and the lowest release of curcumin in simulated GIT model because its high Mw and the presence of block strongly induced to pull structures inward during the formation of beads. Regulation of pectin and Ca^{2+} concentration also showed different results in GI system. However, at comparable Mw and DE, randomly demethylesterified pectin formed weak gel leading low EE and disintegration

of beads during digestion. In case of the commercial pectin with the lowest DE, the matrix of pectinate beads was strongly formed but it was not as dense as the pectin with block even if with higher DE, due to its small Mw decreased during deesterification using chemicals. Low methoxyl amidated pectin (LMA) also developed stronger gels compared to the low methoxyl pectin (LMP) when they had same percentage of deesterified group because of their hydrogen bonds between the amide groups. Indeed, the opened structure might be formed which leaded low EE and intermediate release rate because the amide group might inhibit the crosslink between free carboxyl group and Ca^{2+} by placing in block-wise manner, but smaller amide group would not disrupt it.

This study provided depending on different molecular structure of pectin the ability of entrapping the nutraceuticals would be changed. Therefore, it can be a potential use as site specific drug delivery in GI system by modifying and regulating the molecular structure of pectin or concentrations ideally resulting in controlling the release of bioactive materials.

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국문초록

본 연구는 펙틴의 분자 구조에 따른 펙틴으로 캡슐화된 커큐민의 물리화학적 특성과 *in vitro* 소화특성을 알아보기 위해 수행되었다. 펙틴의 기능적 특성은 펙틴의 메틸화 수준(degree of methyl esterification; DE)과 분자량, 그리고 탈 메틸화된 부분의 크기와 분포에 따라 달라진다. 화학적 방법으로 DE 를 변경시킨 상업용 펙틴과 파파야 유래의 pectin methyl esterase (PME)를 이용하여 펙틴의 메틸화 수준을 변경한 펙틴을 이용하여 커큐민-칼슘 펙틴 비드를 제조하였다.

커큐민은 강황으로부터 얻어지는 폴리페놀 성분으로 건강에 유익한 기능성 식품 소재로 이용될 수 있으나, 커큐민의 불용성, 알칼리와 빛에 의한 분해 등과 같은 한계점이 있어 식품산업에서 이용되는데에는 어려움이 있다. 이러한 한계점들은 캡슐화를 통해서 극복할 수 있으며, 생체이용율 또한 향상시킬 수 있다.

이 연구에서는 250 mg 의 커큐민을 함유하는 2% 농도의 다양한 DE 를 가진 상업용 저 메톡실 (low methoxyl; LM) 및

아마이드화 저 메톡실 (LM amidated; LMA) 펙틴과 PME 를 이용하여 변형시킨 펙틴 용액을 8% 칼슘클로라이드 용액에 떨어뜨림으로써 커큐민 비드를 형성한 뒤, *in vitro* 소화 특성을 조사하였다.

모든 비드는 1.5-2.0 mm 의 직경을 가졌으나, PME 를 처리한 펙틴에서는 그 크기가 보다 작았다. 이는 PME 가 펙틴의 탈 메틸화를 연속적으로 진행하여 펙틴 분자 구조에 탈 메틸화가 연속적으로 일어난 블록을 형성함으로써 칼슘 민감도가 증가하였기 때문이다. 뿐만 아니라, 본래의 분자량을 유지함으로써 PME 변형 펙틴이 젤을 형성할 때에 더욱 많은 양의 커큐민을 포집하고 단단한 비드를 형성하였다. 무작위로 탈 메틸화된 상업용 LM 펙틴의 경우, 소장을 모방한 소화 과정 중에서 완전히 분해되어, 본 모습을 그대로 유지했던 PME 변형 펙틴 비드와는 상반된 결과를 보였다. 따라서, 블록 형태의 탈 메틸화 유무가 펙틴 젤의 특성을 결정짓는 중요한 요인임을 확인하였다. 또한 보다 낮은 DE 로 높은 칼슘 민감도를 가지더라도, 화학적으로 탈 메틸화 되어 감소한 분자량이 젤 네트워크 형성에 영향을 미쳐 PME 변형 펙틴보다 다소 약한 젤을 형성하였음을 커큐민의 포집

능력과 소화기관에서의 방출 결과로 확인하였다. LMA 펙틴의 경우, 분자 구조 내에 존재하는 아마이드기의 수소결합으로 인해 보다 단단한 젤을 형성할 수 있으나, 다소 열린 구조를 형성한다. 이러한 구조적 특성으로 말미암아 적은 양의 커큐민을 포집하였고, 소화 과정 중에서 아마이드기의 수준에 따라 12-40% 수준의 커큐민을 방출하였다. 주사전자현미경으로 비드의 표면과 단면을 관찰한 결과, 펙틴의 분자 구조의 차이에 따른 결과를 보였다.

펙틴의 분자 구조 차이에 따른 커큐민 포집력과 방출의 차이는 커큐민과 같은 다양한 생체 유용 물질을 캡슐화하여 특정 장기 위치에서의 방출을 조절할 수 있는 가능성을 제공하여 그 활용성이 클 것으로 예상된다.

주요어: 펙틴, 펙틴 메틸에스테라제, 펙틴 분자 구조, 커큐민-칼슘 펙틴 비드, 물리화학적 특성, 소화 방출 양상

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