International Food Research Journal 21(2): 799-806 (2014)
Journal homepage: *http://www.ifrj.upm.edu.my*

Effect of extraction condition on the chemical and emulsifying properties of pectin from *Cyclea barbata* Miers leaves

Mackaman, P., *Tangsuphoom, N. and Chavasit, V.

Food Science Unit, Institute of Nutrition, Mahidol University, Salaya, Nakhon Pathom 73170, Thailand

Article history

Received: 9 June 2013
Received in revised form: 16 October 2013
Accepted: 17 October 2013

Keywords

Pectin
Extraction
Cyclea barbata Miers
Depolymerization
Emulsifying properties

Abstract

Cyclea barbata Miers is a tropical indigenous plant. The pectin extracted from *Cyclea barbata* Miers leaves (CBMP) has unique gelling characteristic that strongly depends on the extraction condition. This study aims to determine the chemical and emulsifying properties of CBMP extracted under conditions that deteriorate its gelling ability. CBMP extractions were performed using 5 and 50 mM HNO_3 with 40 ppm H_2O_2, and 2 mM NaOH at 80°C for 3 h. Acid increased degrees of methylation (DM) and acetylation (DAc), and protein content of the obtained CBMP; while alkali extraction decreased DM but increased DAc. Depolymerization of CBMP to 20-80 kDa was observed when acid, alkali, and long extracting time were used. CBMP extracted using 5 mM HNO_3 contained 66% DM, 2% DAc and showed better emulsifying properties than other depolymerized CBMP. Emulsion forming and stabilizing abilities of such CBMP resulted from the appreciable amount of protein (6%) to adsorb at the oil-water interface, and the suitable molecular weight (80 kDa) of pectin to provide sufficient thickness of hydrated layer for emulsion stabilization. Extraction under severe conditions resulted in the chemical structure modification which improves the emulsifying properties of CBMP and its potential use as emulsifier.

Introduction

Cyclea barbata Miers (CBM) is a climbing vine growing in Asia, Africa and South America where the gel, prepared by mixing the leaves with water, is used as an ingredient of indigenous foods and medicines (Smitinand and Larsen, 1991). Chemical composition of the gel from CBM leaves has been elucidated and classified as pectin consisting of the partly methylated and acetylated polygalacturonic acid backbone with neutral sugar side chains (Voragen *et al.*, 1995). Pectin from CBM leaves (CBMP) has unique characteristics that its gelation occurs spontaneously in a short period of time at room temperature in the absence of neither sucrose nor calcium, and at the pectin concentration of as low as 0.5% w/v (Arkarapanthu *et al*., 2005). CBMP thus could be a potential gelling agent for low sugar products.

On the other hand, emulsifying and stabilizing abilities of the native CBMP are quite poor since the level of addition is restricted by its high viscosity and tendency to form a gel. Recently, emulsifying properties of pectin have been extensively studied due to the increasing interest in identifying alternative emulsifier from natural sources. Sugar beet pectin, which does not perform neither thickening nor gelling ability, is known to be a good emulsifier due to its naturally high content of acetyl group and the presence of hydrophobic proteinaceous moiety in its structure (Sun and Hughes, 1998; Siew *et al.*, 2008; Funami *et al*., 2011). The emulsifying properties of depolymerized, high methoxyl citrus pectin extracted under highly acidic condition has also been reported to be better than those of the native ones. Such improved emulsifying property results from the lower viscosity and greater exposure of protein moiety associated with the shortened pectin chains (Akhtar *et al*., 2002).

Previous work from our group revealed that the unique gelling properties of the CBMP are hardly preserved during the extraction steps and thus the extraction has to be conducted under very strict condition that even a small alteration of the extraction parameters could deteriorate its gelling ability as well as feasibility of extraction. Extraction parameters that could led to the loss of gelling as well as thickening abilities of CBMP include acid and alkali extractants, high temperature and long time (Arkarapanthu *et al*., 2005). However, the effect of such severe extraction conditions on the chemical properties of CBMP has not yet been reported and we hypothesize that there should be some modifications in the structure and composition of CBMP, which might improve its emulsifying properties. This study aims to investigate the chemical and emulsifying properties of CBMP obtained from severe extraction conditions. The

*Corresponding author.
Email: *nattapol.tng@mahidol.ac.th*
Tel: +66 2800 2380; Fax: +66 2441 9344

results of this study will provide information on the potential of non-thickening and non-gelling CBMP as emulsifying and stabilizing agents which will broaden its application as a food ingredient.

Materials and Methods

Materials

Fresh mature CBM leaves were collected from a farm in Nakhon Pathom, Thailand. After collection, the leaves were trimmed and washed twice with tap water. The cleaned leaves were drained, vacuum-sealed in low-density polyethylene (LDPE) bags and kept in a freezer at -10±3ºC until used for pectin extraction. The fresh CBM leaves consisted of 13.8% protein, 3.3% fat, 8% ash, 0.6% calcium, 10.8% sugar, 31% soluble dietary fiber and 31.5% insoluble dietary fiber on a dry weight basis, as analyzed according to the AOAC official methods (AOAC, 2000). All chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, Missouri, U.S.A.).

Preparation of CBMP

Frozen CBM leaves were chopped in a food processor prior to mixing with the extracting solution, i.e., 5 and 50 mM HNO_3 and 2 mM NaOH, at a weight-to-volume ratio of 1:60. According to the preparation method of depolymerized citrus pectin (Akhtar et al., 2002), 40 ppm H_2O_2 was added to the slurries of CBM leaves and HNO_3. All extractions were performed at 80ºC for 3 h, except that the control sample was extracted using deionized (DI) water at 50ºC for 8 min to obtain native CBMP (Arkarapanthu *et al*., 2005). After extraction, the mixture was vacuum filtered through Whatman No.1 filter paper. Pectin was precipitated by mixing the filtrate with ethanol (95% v/v; Government Pharmaceutical Organization, Bangkok, Thailand) at a volume ratio of 1:2 and left at room temperature (25±2ºC) for 16 h. The precipitate was harvested and washed twice with ethanol, dried in a hot air oven at 60ºC until dry (<10% moisture). Samples were vacuum-sealed in LDPE bags and stored in a desiccator until analyzed.

Chemical properties of CBMP

Galacturonic acid content (GA) was determined by a colorimetric method (Melton and Smith, 2001). One milliliter of 0.005% w/v CBMP solution in DI water was mixed with 6 ml of 12.5 mM sodium tetraborate in H_2SO_4. The mixture was heated in a water bath at 100°C for 10 min, cooled down in an ice bath and mixed with 100 µl of 0.15% w/v 3-hydroxydiphenyl in 0.125 M NaOH prior to measuring the absorbance at 520 nm using a UV-visible spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). GA was determined using a standard curve prepared from galacturonic acid monohydrate (≥97% purity).

Degree of methylation (DM) was determined using Fourier transform infrared (FTIR) spectroscopy according to the method described by Singthong *et al.* (2004). Pectin powder was desiccated in a desiccator prior to being analyzed as is by placing on an attenuated total reflectance sampling accessory (Smart iTR, Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.) of an FTIR spectrometer (Nicolet 6700, Thermo Fisher Scientific) equipped with a single bounce diamond crystal. The spectral values of the sample were obtained by co-adding 64 scans at the resolution of 4 cm^{-1} in mid-infrared region (4000-400 cm^{-1}). The obtained spectra were subtracted with a blank spectra collected from atmosphere. The areas of the peaks at 1760-1745 and 1640-1620 cm^{-1} which represent esterified and free carboxylic groups, respectively, were integrated using a computer software (OMNIC version 8.1, Thermo Electron, Madison, Wisconsin, U.S.A.). DM was calculated using a linear correlation: $DM = 87.609 \times [Area_1/ (Area_1 + Area_2)] + 25.768$, where $Area_1$ and $Area_2$ are areas of the peaks appeared between 1760-1745 and 1640-1620 cm^{-1}, respectively.

Degree of acetylation (DAc) was determined using a colorimetric method (McComb and McCready, 1957). Five milliliters of pectin solution (2% w/v) was mixed with 1 ml of 2.35 M NaOH and 1 ml of 0.5 M hydroxylamine hydrochloride. After 5 min incubation, 5 ml of 0.7 M $HClO_4$ in methanol was added and the volume of the mixture was made to 25 ml by gradually adding the ferric perchlorate solution containing 0.07 M $FeCl_3 \cdot 6H_2O$, 0.5 M H_2SO_4 and 0.5 M $HClO_4$. The sample was left for 5 min until pectin hydroxamic acid-ferric complex was precipitated. The solution was then filtered through a filter paper (Whatman No.1) into a cuvette and measured the absorbance at 520 nm. DAc was determined using a standard curve prepared from glucose pentaacetate (98% purity).

Protein content was determined by Lowry assay (Copeland, 1994). One milliliter of the pectin solution (0.5% w/v) was mixed with 1 ml of Lowry reagent, containing 1 M Na_2CO_3 in 0.5 M NaOH, 0.04 M $CuSO_4 \cdot 5H_2O$ and 0.1 M potassium tartrate at a volume ratio of 20:1:1, vortexed and left in the dark at room temperature for 15 min. Three milliliters of 2 M Folin-Ciocalteu reagent was then added and the mixture was incubated for 30 min prior to measuring the absorbance at 540 nm. Protein concentration was determined using a standard curve prepared from bovine serum albumin (lyophilized powder, ≥96%

protein).

Molecular weight of CBMP was estimated from its intrinsic viscosity. Viscosities of dilute CBMP solutions (0.0125-0.3% w/v) in 0.2 M citrate buffer pH 4.5 containing 0.1 M NaCl were measured using a Cannon-Fenske viscometer (capillary No.100, Schott-Geräte, Hofheim, Germany). The temperature was maintained at 25°C by submerging the viscometer in a temperature-controlled water bath. The intrinsic viscosity ([η]) of pectin was determined by extrapolation of the Huggins equation plot. The viscosity-average molecular weight (Mv) was obtained according to the Mark–Houwink–Sakurada equation: $[\eta] = K(Mv)^{\alpha}$, where K and α are constants for pectins, which are 2.34×10^{-5} and 0.8224, respectively (Kar and Arslan, 1999).

Apparent viscosity of CMBP solutions (1-4% w/v) in 0.2 M citrate buffer pH 4.5 were measured at a shear rate of 122.4 s^{-1} using a Brookfield viscometer (RVT-DVII, Brookfield Engineering Laboratories, Middleboro, Massachusetts, U.S.A.) fitted with a UL adaptor that consists of a cylindrical spindle rotating inside a sample tube. All the measurements were carried out at 25°C in a temperature-controlled water bath.

Preparation of CBMP-stabilized emulsions

CBMP-stabilized oil-in-water emulsions were prepared by mixing soybean oil with CBMP solutions in 0.2 M citrate buffer pH 4.5 to obtain a dispersed phase volume fraction of 0.25 with final polymer concentrations of 0.5-4% w/v. Homogenization was performed using a high performance disperser (Ultra-Turrax T25, IKA-Labortechnik, Staufen, Germany) at 24000 rpm for several minutes. A set of emulsions stabilized by 0.5-6% w/v gum arabic (Instantgum™ BA, Colloïdes Naturels International, Rouen, France; 10% moisture, 4% ash) was also prepared for comparisons. Experiment was repeated three times with freshly prepared emulsions used on each run.

Properties of CBMP-stabilized emulsions

Mean particle size and specific surface area of emulsions were measured using a static laser light-scattering analyzer (Mastersizer 2000, Malvern Instruments, Worcestershire, U.K.) with adsorption parameter value of 0.01. To avoid multiple scattering effects during measurement, samples were diluted in the measuring chamber to obtain approximately 0.001% v/v fat content. The scattering pattern was used by the internal software of the instrument to calculate the particle size and specific surface area (S_v, $m^2.mL^{-1}$) of the droplets using a relative refractive index of 1.10 which is the ratio of the refractive index of olive oil, 1.47, and that of the dispersion medium, 1.33. The mean particle size of the emulsion droplets was characterized as surface-weighed mean diameter, $d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$ and volume-weighed mean diameter, $d_{43} = \sum n_i d_i^4 / \sum n_i d_i^3$, where n_i is the number of droplets of diameter d_i.

Droplet morphology. Twelve microliters of emulsion was placed on a microscope slide and covered with a cover slip. Morphology of oil droplets was observed under 400× magnifications using an inverted microscope equipped with a colour digital video camera (Eclipse Ti-S, Nikon, Tokyo, Japan). Pictures were taken from three different fields on each slide and representative images are presented.

Pectin and protein adsorption. The amounts of pectin and protein adsorbed on the droplet surface of emulsion were inferred from measurements of the concentrations of pectin and protein remaining in the serum phase after centrifugation at 1000 x g for 1 h at 25°C. The unadsorbed protein content in the aqueous phase was determined by Lowry assay as described earlier. The amount of unadsorbed pectin was determined by weighing the dried residue obtained after precipitating the aqueous phase twice in ethanol. The amount of pectin and protein adsorbed at the interface (C_i, $g.L^{-1}$) was calculated by subtracting the concentrations of pectin and protein present in the aqueous phase before emulsification (C_0) with that found in the serum layer after centrifugation (C_s). Proportions of pectin and protein associated with the droplets (F_{ads}) were calculated as: $F_{ads} = C_i/C_0 \times 100$. Surface coverage ($\Gamma$, $mg.m^{-2}$) of pectin and protein were calculated from the specific surface area (S_v, $m^2.mL^{-1}$) and dispersed phase volume fraction (Φ = 0.25) of the emulsion: $\Gamma = C_i/S_v \times \Phi$.

ζ-potential of the droplets was measured by a laser scattering instrument (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, U.K.). Emulsions were diluted to a droplet concentration of about 0.02% v/v in appropriate buffer prior to being loaded in a folded capillary cell at room temperature. Each measurement was the average of 120 (6 sets of 20) measurements and the entire experiment was conducted in triplicate. The ζ-potential was calculated from the electrophoretic mobility using the Smoulokowski model (Hunter, 1981).

Creaming index of emulsion was determined by observing the separation of cream layer. Six milliliters of emulsions were transferred into screw-capped test tubes (10 mm internal diameter, 100 mm height) and stored at 25°C for 7 d. The total height of the emulsion (H_E) and the heights of the serum layer (H_S) were measured. The extent of creaming, expressed as creaming index (CI), was calculated as: $CI = H_S/$

$H_E \times 100$.

Statistical analysis

Data were analyzed using a computer statistics program (SPSS 17.0, SPSS, Chicago, Illinois, U.S.A.). Independent samples t-test or one-way Analysis of Variance together with Tukey's HSD test was used to determine the significance of differences ($p \leq 0.05$) between the samples. Only significantly different results are discussed in the text. Data are presented as the means and standard deviations.

Results and Discussion

Chemical properties of CBMP

Although extraction with water at room or slightly elevated temperature for short period of time is usually employed in the preparation of CBMP, the more severe conditions, i.e., the uses of acid and alkali solutions, high extracting temperature, long extracting time and the addition of H_2O_2, were used in this study in order to achieve chemical modification of the CBMP structure. The pectin yield of all extraction conditions was 4-8% of the CBM leaves on a dry weight basis, which accounts for 13-26% recovery of the soluble dietary fiber presenting in CBM leaves. GA of all CBMP samples was 50-65%, which is similar to that previously reported (Arkarapanthu *et al*., 2005). Extractions using HNO_3 gave CBMP with slightly lower GA than DI water and NaOH (Table 1).

Typical FTIR spectra of control, acid-extracted and alkali-extracted CBMP are presented in Figure 1. The characteristic peaks of polysaccharides from plant cell walls and their major chemical groups could be seen in the spectra of all CBMP samples. The peak occurring in a broad region between 3600-2900 cm^{-1} represents O-H stretching. The peak corresponding $O\text{-}CH_3$ stretching from methyl esters of galacutronic acid, which is expected at 2930 cm^{-1}, was masked by the large O-H stretching peak especially for CBMP extracted with alkali. The peaks at 1760-1745 and 1640-1620 cm^{-1} are from esterified and free carboxylic groups and their intensity ratio was successfully used to determine the DM of the pectin from *Cissampelos pareira* (Singthong *et al*., 2004), another plant that is also belong to the *Meispermaceae* family and has about similar taxonomy to CBM (Arkarapanthu *et al*., 2005). Finally, the peaks observed between 1400 and 950 cm^{-1} correspond to the typical profile of polygalacturonic acid (Kačuráková *et al*., 2000; Černá *et al*., 2003). Acid-extracted CBMP samples were more highly methoxylated (65-75% DM) than the control CBMP (Table 2) although DM of pectin has usually been reported to decrease with the pH of extracting solution (Lavigne *et al*., 2002; Yapo *et al*., 2007; Pinheiro *et al*., 2008; Fissore *et al*., 2010; Wai *et al*., 2010). It is likely that the presence of H_2O_2 in acidic extraction condition inhibits the demethylation of CBMP (Timerbaeva *et al*., 2007). CBMP extracted by alkali solution were low methoxylated (36% DM) due to the alkali-induced demethylation (Brejnholt, 2010). All CBMP were low-acetylated, in which the control CBMP contained <1% of acetylated galacturonic acid molecules. DAc increased with the increasing concentrations of acid and alkali extracting solutions, which accounted for the poor thickening and gelling abilities of acid- and alkali-extracted CBMP (Oosterveld *et al*., 2000; Siew and Williams, 2008). DAc of all CBMP in this study as about similar to that of pectin from apple pomace but higher than that of citrus pectin (Kravtchenko *et al*., 1992; Voragen *et al*., 1995) and much lower than that presents in sugar beet pectin (≥16% DAc) (Axelos and Thibault, 1991).

Table 1. Chemical properties of CBMP

Chemical properties[1]	Extraction condition			
	DI water, 50°C, 8 min (Control)	5 mM HNO_3 + 40 ppm H_2O_2, 80°C, 3 h	50 mM HNO_3 + 40 ppm H_2O_2, 80°C, 3 h	2 mM NaOH, 80°C, 3 h
Galacturonic acid (%)	61.65±0.68^{b}	58.75±1.85^{cd}	56.19±1.21^{d}	64.11±0.82^{a}
Degree of methylation (%)	52.55±1.10^{c}	65.93±1.72^{b}	75.60±2.32^{a}	35.81±0.57^{d}
Degree of acetylation (%)	0.59±0.06^{d}	2.04±0.03^{b}	3.19±0.06^{a}	1.51±0.03^{c}
Protein (%)	2.14±0.12^{c}	6.01±0.39^{a}	4.28±0.22^{b}	0.85±0.17^{c}
Viscosity-average molecular weight (kDa)	741.31±21.78^{a}	80.00±0.79^{b}	25.72±0.26^{c}	23.02±1.05^{c}

[1] Mean ± standard deviation of triplicate analyses
[a,b,c] Means with different superscripts within the same row are significantly different ($p \leq 0.05$).

Table 2. Surface compositions and charge of emulsions (25% v/v oil, pH 4.5) stabilized with CBMP

Surface properties[1]	Concentration and extraction condition of CBMP	
	2% w/v, 5 mM HNO_3 + 40 ppm H_2O_2, 80°C, 3 h	3% w/v, 50 mM HNO_3 + 40 ppm H_2O_2, 80 °C, 3 h
Pectin		
Interfacial concentration ($g.L^{-1}$)	10.26±1.14*	6.53±1.85*
Adsorbed fraction (%)	51.30±5.71*	21.76±6.18*
Surface coverage ($mg.m^{-2}$)	8.55±0.95	7.84±2.23
Protein		
Interfacial concentration ($g.L^{-1}$)	0.81±0.06	0.97±0.02
Adsorbed fraction (%)	67.56±5.07	75.20±1.89
Surface coverage ($mg.m^{-2}$)	1.10±0.08	1.16±0.03
ζ-potential (mV)	-42.77±1.23*	-50.17±0.55*

[1] Mean ± standard deviation of triplicate analyses
* Means with asterisk within the same row are significantly different ($p \leq 0.05$).

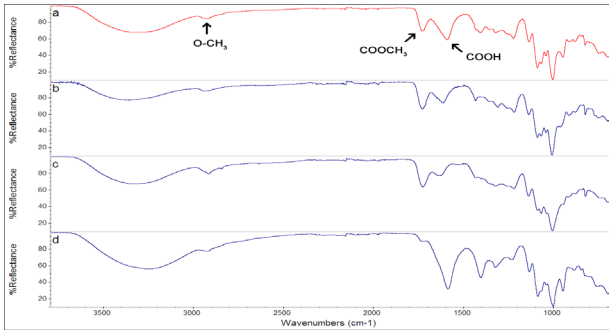

Figure 1. Typical FTIR spectra of control CBMP (a), CBMP extracted using 5 mM HNO_3 + 40 ppm H_2O_2 (b), 50 mM HNO_3 + 40 ppm H_2O_2 (c) and 2 mM NaOH (d) at 80°C for 3 h.

Protein content of CBMP samples ranged between 2-6% which is much lower than that of 9% in sugar beet pectin (Yapo *et al*., 2007). It should be noted that protein associated with pectin includes that covalently linked to the pectin and that co-precipitated with the pectin as free protein. Acid- and alkali-extracted CBMP contained the higher protein contents than the control CBMP due to the better solubility of protein in acid and alkali solutions. Among acid-extracted CBMP, the pectin extracted with the less acidic solution was higher in protein content (Table 1). This could be due to the greater interactions between pectin and protein and/or the larger quantities of co-extracted protein at milder acid pH values (Yapo *et al*., 2007). CBMP extracted with acid and alkali solutions were lower in Mv than the control CBMP due to depolymerization and thus could be considered as depolymerized pectins. The Mv of CBMP extracted using 50 mM HNO_3 and 2 mM NaOH were 20 kDa, while that extracted using 5 mM HNO_3 had a higher Mv of 80 kDa (Table 1). The reduction in Mv of CBMP resulted from acid hydrolysis and β-elimination of the pectin chain in acid and alkali condition (Diaz *et al.*, 2007).

In order to determine the effect of H_2O_2, protein content and Mv of CBMP extracted with 5 mM HNO_3 without H_2O_2 were also analyzed (data not presented). In the absence of H_2O_2, acid-extracted CBMP was higher in protein content, possibly due to the H_2O_2-mediated protein oxidation (Kocha *et al*., 1997), and the Mv was 1.5 times larger than that extracted in the presence of H_2O_2. Oxidizing agents are known to enhance the degradation of carbohydrate polymer chain, e.g., dextran and pectin, through oxidative depolymerization (Moody, 1964).

Solutions at any concentrations up to 4% w/v of CBMP samples extracted with HNO_3 and NaOH did not form gels after being left at room temperature for 2 h; while those of the control CBMP readily gelled within 30 min. Apparent viscosities of the CBMP solutions at any concentrations were <10 mPa.s at 25°C, 122.4 s^{-1} (data not presented). Among the CBMP samples, the highest viscosities were observed in the solutions of CBMP extracted with 5 mM HNO_3 which corresponded to their highest Mv (Table 1). The non-thickening and non-gelling characteristics of CBMP was resulted from the depolymerization and/or modification in the chemical structure of the pectin chain. Acidic conditions can cause hydrolysis while alkali condition leads to β-elimination of the pectin chain (Diaz *et al*., 2007). Both reactions result in a shorter polymer chain which impairs the ability of pectin in increasing the viscosity of the solution. The loss in gelling ability of acid-extracted CBMP could also result from the presence of H_2O_2 which can oxidize the phenolic compounds to quinones. The naturally present phenolic compounds in CBM leaves are responsible for the spontaneous gelling mechanism of CBMP by acting as a bridge between polygalacturonic acid chains (Arkarapanthu *et al*., 2005).

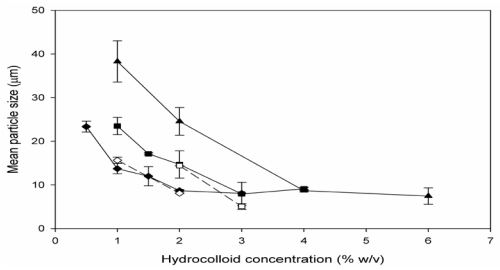

Figure 2. Mean particle diameter of emulsions (25% v/v oil, pH 4.5) stabilized with CBMP extracted using (♦, ◊) 5 mM HNO_3 + 40 ppm H_2O_2, (■, □) 50 mM HNO_3 + 40 ppm H_2O_2 at 80°C for 3 h and (▲) gum arabic. Filled points represent d_{32}; open points represent d_{43}.

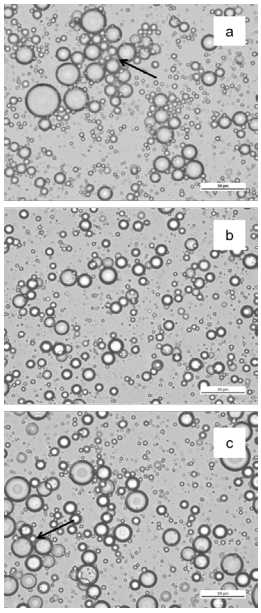

Figure 3. Micrographs taken of emulsions (25% v/v oil, pH 4.5) stabilized with 1 (a) and 2% w/v (b) CBMP extracted using 5 mM HNO_3 + 40 ppm H_2O_2 and 3% w/v CBMP extracted using 50 mM HNO_3 + 40 ppm H_2O_2 (c) at 80°C for 3 h. Scale bar is 50 μm. Arrow indicates the presence of floc.

Properties of CBMP-stabilized emulsions

Abilities of pectin in forming and stabilizing an emulsion depend largely on their chemical composition and structure. The presence of methoxyl and acetyl groups on the polygalacturonic acid chain resulted in the higher hydrophobicity hence the higher surface activity of the pectin. The proteinaceous moiety acts as anchors for pectin to align itself and adsorb on the surface of oil droplets with the pectin part facing off (Williams *et al.*, 2005; Siew and

Williams, 2008). Pectin stabilizes the emulsion by covering the droplet surface and provides electrostatic and steric repulsions among the oil droplets via the negative charge on the pectin chains. Pectin also provides viscosity to the aqueous continuous phase of the emulsion which helps retard the movement of the emulsion droplets and improves creaming stability (Lopes da Silva and Rao, 2006).

Emulsification ability of depolymerized CBMP was determined by preparing emulsions at pH 4.5 with various hydrocolloid concentrations. Emulsion formation was not observed in the oil-water mixtures containing CBMP extracted using 2 mM NaOH at any concentrations up to 4% w/v, probably due to their low DM, DAc and protein content, and too low Mv. The absence of emulsifying properties in 20 kDa pectin has also been reported in other studies (Akhtar *et al.*, 2002; Funami *et al*., 2007). However, emulsifying activity was observed in CBMP extracted using 50 mM HNO_3, of which the Mv was also 20 kDa. This could be due to its higher DM, DAc and protein content than those of NaOH-extracted CBMP (Table 1).

Therefore, only HNO_3-extracted CBMP samples were used to prepare emulsions for further characterizations. Oil-in-water emulsions containing 1-4% w/v CBMP were polydispersed and their particle size distributions were bimodal with span values of ~2 (data not presented). The d_{43}, which is more sensitive to flocculation, was higher than d_{32} in all emulsions containing <2% w/v CBMP (Figure 2). This suggested the presence of flocs, which can also be seen in the micrograph (Figure 3). The particle size of CBMP-stabilized emulsions decreased with increasing pectin concentration until both the d_{32} and d_{43} were $\leq$ 8 μm in emulsions containing 2% w/v of CBMP extracted with 50 mM HNO_3 and 3% w/v CBMP extracted using 5 mM HNO_3 (Figure 2). Such particle sizes were about similar to that of emulsion made with 6% w/v gum arabic. With further increase in the hydrocolloid concentration, the mean particle size of all emulsions remained constant. The depolymerized CBMP thus are able to provide emulsification effect in the same way as gum arabic does even at lower concentration. Pectin is a semi-flexible polymer so it could adsorbs at the oil-water interface better than the random coiled gum arabic (Leroux *et al.*, 2003).

CBMP extracted using 5 mM HNO_3 required lower concentrations than those extracted using 50 mM HNO_3 to form emulsions of similar d_{32}, suggesting its better emulsifying properties. This is well confirmed by the fact that amount of interfacial pectin in the emulsion stabilized with 2% w/v CBMP extracted using 5 mM HNO_3, was twice that, despite the fact they contained less total than the one prepared with 3% w/v CBMP extracted with 50 mM HNO_3 (Table 2). However, the pectin surface coverage of the two emulsions was the same. The likely reason for the lower interfacial adsorption of CBMP extracted using 50 mM HNO_3 than that extracted using 5 mM HNO_3 is their lower protein content (Table 1). Ones might expect that CBMP extracted with 50 mM HNO_3 containing more numbers of methyl and acetyl groups which can be additional anchors for pectin adsorption should be better in surface activity (Nakauma *et al*., 2008; Gavlighi *et al*., 2013). The DM and DAc of CBMP extracted using 50 mM HNO_3 might be too high that the pectins were too highly hydrophobic hence did not adsorb well at the oil-water interface (Table 1). It could also be seen in the micrographs that large droplets and flocculation were more observed in the emulsions stabilized with 3% w/v CMBP extracted using 50 mM HNO_3 (Figure 3c).

The surface coverage of pectin in CBMP-stabilized emulsions were slightly lower that of 10 $mg.m^{-2}$ reported in emulsions made with 2-4% w/v depolymerized citrus pectin (Akhtar *et al*., 2002) but much higher than 1 $mg.m^{-2}$ reported to be monolayer coverage of sugar beet pectin (Nakauma *et al*., 2008; Fissore *et al*., 2010). The high coverage in this study thus suggested the occurrence of multilayer absorption, possibly due to crosslinking of pectin molecules by calcium ions or proteins (Siew and Williams, 2008). More than 70% of the protein presenting in the CBMP-stabilized emulsions adsorbed at the oil-water interface (Table 2). The surface protein coverage of emulsions containing 2-3% w/v CBMP was 1-1.5 $mg.m^{-2}$ (Table 2), which is similar to that of emulsions made with other pectins (Akhtar *et al*., 2002; Leroux *et al.*, 2003; Funami *et al*., 2007; Siew *et al*., 2008; Funami *et al*., 2011), and is known to be the sufficient coverage for protein-stabilized emulsions (Hunt and Dalgleish, 1994; Srinivasan *et al.*, 1996).

Pectin carries a negative charge at pH = 4.5 (i.e., greater than the pK_a of galacturonic acid, 3.5); while the charge of proteinaceous moiety associated with the pectin should be minimally negative (i.e., closes to the pI of plant proteins). Therefore, oil droplets in CBMP-stabilized emulsions in this study were negatively charged with the ζ-potential values of -50 mV for emulsions containing 2% w/v CBMP extracted using 5 mM HNO_3 and 3% w/v of CBMP extracted using 50 mM HNO_3 (Table 2). The similar magnitude of the surface charge is consistent with their equal amount of pectin adsorbed at the interface

(Table 2).

The stability against cream separation during storage of CBMP-stabilized emulsions was determined as CI; a higher CI indicates less creaming stability. Emulsion containing CBMP extracted using 50 mM HNO_3, of which the droplets were more flocculated (data not presented), creamed more quickly and extensively during storage than that containing CBMP extracted using 5 mM HNO_3 although the droplet surface of both emulsions had similar pectin coverage (Table 2). This might be due to the lower Mv of CBMP extracted using 50 mM HNO_3 than that of CBMP extracted using 5 mM HNO_3, which led to the less thick hydrated pectin layer to generate steric repulsion between emulsion droplets (Akhtar *et al*., 2002). The higher viscosity of CBMP extracted using 5 mM HNO_3 also resulted in the more ability of the aqueous phase to retard the upward movement of oil droplets. The emulsion containing 2% w/v of this CBMP contained minimal number of large droplets and floc (Figure 3b), hence more stable to creaming with the CI of 34% after 7 d storage.

From all the data, it is suggested that the emulsifying activity of CBMP is governed by the adsorption on the droplet surface of its proteinaceous moiety or the protein-rich part. The stability of emulsion solely depends on the amount and size of adsorbed pectin since a certain thickness of pectin layer is required to provide sufficient steric repulsion to emulsion droplets.

Conclusion

Extraction under severe conditions resulted in the modifications of chemical composition and substitution of CBMP structure, which made the differences in functional properties between the chemically-modified and the control CBMP. Depolymerized CBMP, which were obtained from extractions using acid or alkali at prolonged duration, did not form gel but exhibited emulsifying properties at different extents depending on their DM, DAc, protein content and Mv. Abilities to form emulsion and provide the stability to emulsion were observed only in certain depolymerized (80 kDa), high methoxy CBMP containing appreciable amounts of DAc and protein. Therefore, it is possible to use severe extraction condition to chemically modify the CBMP in order to improve its emulsifying properties and increase its potential as emulsifier.

Acknowledgements

This study was supported by Mahidol University, Thailand. The authors would like to thank Prof. John Coupland of the Department of Food Science, The Pennsylvania State University, U.S.A. for his constructive comments on the manuscript. Mr. Nawapol Udpuay of the Salaya Central Scientific Instrument Facility, Mahidol University, is acknowledged for his technical assistance on FTIR analysis.

References

Akhtar, M., Dickinson, E., Mazoyer, J. and Langendorff, V. 2002. Emulsion stabilizing properties of depolymerized pectin. Food Hydrocolloids 16: 249-256.

AOAC. 2000. AOAC Official Methods of Analysis. Gaithersburg, Maryland: AOAC International.

Arkarapanthu, A., Chavasit, V., Sungpuag, P. and Phuphathanaphong, L. 2005. Gel extracted from Khruea-ma-noi (*Cyclea barbata* Miers) leaves: chemical composition and gelation properties. Journal of the Science of Food and Agriculture 85: 1741-1749.

Axelos, M. A. V. and Thibault, J. F. 1991. Influence of the substituents of the carboxyl groups and of the rhamnose content on the solution properties and flexibility of pectins. International Journal of Biological Macromolecules 13: 77-82.

Brejnholt, S. M. 2010. Pectin. In Imason, A. (Ed). Food stabilisers, thickeners and gelling agents, p. 237-265. Oxford: Blackwell.

Černá, M., Barros, A. S., Nunes, A., Rocha, S. M., Delgadillo, I., Čopíková, J. and Coimbra, M. A. 2003. Use of FT-IR spectroscopy as a tool for the analysis of polysaccharide food additives. Carbohydrate Polymers 51: 383-389.

Copeland, R. A. 1994. Methods for protein analysis: a practical guide to laboratory. New York: Chapman and Hall.

Diaz, J. V., Anthon, G. E. and Barrett, D. M. 2007. Nonenzymatic degradation of citrus pectin and pectate during prolonged heating: effects of pH, temperature, and degree of methyl esterification. Journal of Agricultural and Food Chemistry 55: 5131-5136.

Fissore, E. N., Ponce, N. M. A., Pla, M. E., Rojas, A. M. and Gerschenson, L. N. 2010. Characterization of acid-extracted pectin-enriched products obtained from red beet (*Beta vulgaris* L. var. conditiva) and butternut (*Cucurbita moschata* Duch ex Poiret). Journal of Agricultural and Food Chemistry 58: 3793-3800.

Funami, T., Zhang, G., Hiroe, M., Noda, S., Nakamura, M., Asai, I., Cowman, M. K., Al-Assaf, S. and Phillips, G. O. 2007. Effects of the proteinaceous moiety on the emulsifying properties of sugar beet pectin. Food Hydrocolloids 21: 1319-1329.

Funami, T., Nakauma, M., Ishihara, S., Tanaka, R., Inoue, T. and Phillips, G. O. 2011. Structural modifications of sugar beet pectin and the relationship of structure to

functionality. Food Hydrocolloids 25: 221-229.

Gavlighi, H. A., Meyer, A. S., Zaidel, D. N. A., Mohammadifar, M. A. and Mikkelsen, J. D. 2013. Stabilization of emulsions by gum tragacanth (*Astragalus* spp.) correlates to the galacturonic acid content and methoxylation degree of the gum. Food Hydrocolloids 31: 5-14.

Hunt, J. A. and Dalgleish, D. G. 1994. Adsorption behavior of whey protein isolate and caseinate in soya oil-in-water emulsions. Food Hydrocolloids 8: 175-187.

Hunter, R. J. 1981. Zeta potential in colloid science: Principles and applications. London: Academic Press.

Kačuráková, M., Capek, P., Sasinková, V., Wellner, N. and Ebringerová, A. 2000. FT-IR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses. Carbohydrate Polymers 43: 195-203.

Kar, F. and Arslan, N. 1999. Effect of temperature and concentration on viscosity of orange peel pectin solutions and intrinsic viscosity-molecular weight relationship. Carbohydrate Polymers 40: 277-284.

Kocha, T., Yamaguchi, M., Ohtaki, H., Fukuda, T. and Aoyagi, T. 1997. Hydrogen peroxide-mediated degradation of protein: different oxidation modes of copper- and iron-dependent hydroxyl radicals on the degradation of albumin. Biochimica et Biophysica Acta 1337: 319-326.

Kravtchenko, T. P., Voragen, A. G. J. and Pilnik, W. 1992. Analytical comparison of three industrial pectin preparations. Carbohydrate Polymers 8: 17-25.

Lavigne, S., Relet, M. C. and Thibault, J. F. 2002. Characterization of pectins extracted from fresh sugar beet under different conditions using and experimental design. Carbohydrate Polymers 49: 145-153.

Leroux, J., Langendorff, V., Schick, G., Vaishnav, V. and Mazoyer, J. 2003. Emulsion stabilizing properties of pectin. Food Hydrocolloids 17: 455-462.

Lopes da Silva, J. A. L. and Rao, M. A. 2006. Pectins: structure, functionality, and uses. In Alistair, M. S., Phillips, G. O. and Williams, P. A. (Eds). Food polysaccharides and their applications, 2nd edn, p. 353-412. Boca Raton, Florida: CRC Press.

McComb, E. A. and McCready, R. M. 1957. Determination of acetyl in pectin and in acetylated carbohydrate polymers. Analytical Chemistry 29: 819-821.

Melton, L. D. and Smith, B. G. 2001. Determination of the uronic acid content of plant cell walls using a colorimetric assay. Current Protocols in Food Analytical Chemistry: E3.3.1-E3.3.4.

Moody, G. J. 1964. The action of hydrogen peroxide on carbohydrate and related compounds. Advances in Carbohydrate Chemistry 19: 149-179.

Nakauma, M., Funami, T., Noda, S., Ishihara, S., Al-Assaf, S., Nishinari, K. and Phillips, G. O. 2008. Comparison of sugar beet pectin, soybean soluble polysaccharide, and gum arabic as food emulsifiers. 1. effect of concentration, pH, and salts on the emulsifying properties. Food Hydrocolloids 22: 1254-1267.

Oosterveld, A., Beldman, G., Searle-van Leeuwen, M. J. F. and Voragen, A. G. J. 2000. Effect of enzymatic deacetylation on gelation of sugar beet pectin in the presence of calcium. Carbohydrate Polymers 43: 249-256.

Pinheiro, E.R., Silva, I. M. D. A., Gonzaga, L. V., Amante, E. R., Teofilo, R. F., Ferreira, M. M. C. and Amboni, R. D. M. C. 2008. Optimization of extraction of high-ester pectin from passion fruit peel (*Passiflora edulis* flavicarpa) with citric acid by using response surface methodology. Bioresource Technology 99: 5561-5566.

Siew, C. K. and Williams, P. A. 2008. Role of protein and ferulic acid in the emulsification properties of sugar beet pectin. Journal of Agricultural and Food Chemistry 56: 4164-4171.

Siew, C. K., Williams, P. A., Cui, S. W. and Wang, Q. 2008. Characterization of the surface-active components of sugar beet pectin and the hydrodynamic thickness of the adsorbed pectin layer. Journal of Agricultural and Food Chemistry 56: 8111-8120.

Singthong, J., Cui, S. W., Ningsanond, S. and Goff, H. D. 2004. Structural characterization, degree of esterification and some gelling properties of Krueo Ma Noy (*Cissampelos pareira*) pectin. Carbohydrate Polymers 58: 391-400.

Smitinand, T. and Larsen, K. 1991. Flora of Thailand. Bangkok, Thailand: The Forest Herbarium, Royal Forest Department.

Srinivasan, M., Singh, H. and Munro, P. A. 1996. Sodium caseinate-stabilized emulsions: factors affecting coverage and composition of surface proteins. Journal of Agricultural and Food Chemistry 44: 3807-3811.

Sun, R. and Hughes, S. 1998. Extraction and physico-chemical characterization of pectin from sugar beet pulp. Polymer Journal 30: 671-677.

Timerbaeva, G. R., Zimin, Y. A., Borisov, I. M., Bondareva, I. A. and Monakov, Y. B. 2007. Radical mechanism of pectin oxidation in aqueous solution. Russian Journal of Applied Chemistry 80: 1914-1917.

Voragen, A. G. J., Pilnik, W., Thibault, J-F., Axelos, M. A. V. and Renard, C. M. G. C. 1995. Pectins. In Stephen, A. M. (Ed) Food polysaccharides and their applications, p. 287-339. New York: Marcel Dekker.

Wai, W. W., Alkarkhi, A. F. M. and Easa, A. M. 2010. Effect of extraction conditions on yield and degree of esterification of durian rind pectin: an experimental design. Food and Bioproducts Processing 88: 209-214.

Williams, P. A., Sayers, C., Viebke, C., Senan, C., Mazoyer, J. and Boulenguer, P. 2005. Elucidation of the emulsification properties of sugar beet pectin. Journal of Agricultural and Food Chemistry 53: 3592-3597.

Yapo, B. M., Robert, C., Etienne, I., Wathelet, B. and Paquot, M. 2007. Effect of extraction conditions on the yield, purity and surface properties of sugar beet pulp pectin extracts. Food Chemistry 100: 1356-1364.