HYDROLYSIS AND APPLICATION OF PECTINS FROM PASSION FRUIT PEEL OBTAINED BY SUBCRITICAL WATER TREATMENT

By
MISS Khwanjai KLINCHONGKON

A Thesis Submitted in partial Fulfillment of Requirements
for Doctor of Philosophy (FOOD TECHNOLOGY)
Department of FOOD TECHNOLOGY
Graduate School, Silpakorn University
Academic Year 2017

การไฮโดรไลซ์และการใช้เพกตินจากเปลือกเสาวรสที่ได้จากการทรีตด้วยน้ำกึ่งวิกฤต

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Title	Hydrolysis and application of pectins from passion fruit peel obtained by subcritical water treatment
By	Khwanjai KLINCHONGKON
Field of Study	(FOOD TECHNOLOGY)
Advisor	Pramote Khuwijitjaru

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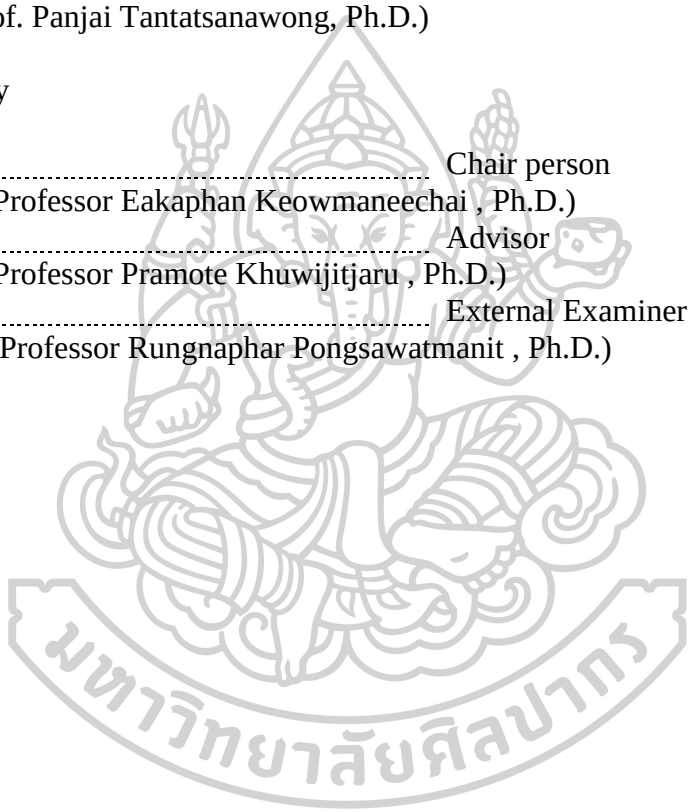

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MISS KHWANJAI KLINCHONGKON : HYDROLYSIS AND APPLICATION OF PECTINS FROM PASSION FRUIT PEEL OBTAINED BY SUBCRITICAL WATER TREATMENT THESIS ADVISOR : ASSISTANT PROFESSOR PRAMOTE KHUWIJITJARU, PH.D.

Passion fruit (*Passiflora edulis*) peel is an underutilized by-product from juice industry. This research investigated the potential of subcritical water for extraction of pectins from the peel and application of the obtained pectins. The effect of ethanol addition on subcritical water extraction of pectin was firstly investigated using a batch-type reactor. The maximum pectin yield of *ca*.12% was obtained from the subcritical water treatment at 140 °C with 10% ethanol. In addition, the ethanol addition also resulted in an increasing of antioxidant activity and total phenolic content in the obtained pectin. However, this extraction technique gave pectins with low molecular weight. To investigate the degradation process of pectin in subcritical water, the degradation of pectin in a continuous flow-type reactor was kinetically evaluated. Reduction of molecular size and formation of reducing end of pectin in subcritical water at 80–160 °C were monitored. The change in degree of polymerization could be modeled by the Emsley equation, whereas the change in reducing end could be modeled by zeroth-order kinetics. The Arrhenius equation was also used to describe the temperature dependency of the reaction rate constants. The activation energies for pectin degradation and reducing end formation were 62.8 and 86.9 kJ/mol, respectively. Subcritical water-hydrolyzed pectins were evaluated for viscosity both in distilled water and buffer (0.05 M of sodium phosphate containing 0.4 M NaCl, pH 7.0) systems, molecular weight, as well as moisture sorption isotherm. The results demonstrated that under more severe hydrolysis conditions the hydrolyzed pectin had lower viscosity. Pectins dissolved in distilled water showed overestimated intrinsic viscosities and molecular weights which caused from electrostatic repulsion effect. Moisture adsorption isotherms of the hydrolyzed pectins could be described well with GAB model. At $a_w \leq 0.75$, the reduction of molecular weight did not affect the water adsorption characteristics of pectin; however, at $a_w > 0.75$, low molecular weight pectins showed higher equilibrium moisture content than high molecular weight pectins. Finally, the application of subcritical water-hydrolyzed pectins (20, 77, and 146 kDa) was demonstrated by conjugation with whey protein isolate using a dry-heating method. SDS-PAGE analysis indicated the formation of conjugates after the reaction. The obtained conjugates were studied for their emulsifying properties in an oil-in-water emulsion with an aqueous phase pH at 5. The smallest emulsion droplet sizes and most stable emulsions were obtained using the conjugates at 6 h of dry-heating. In addition, the conjugates from 146 kDa pectin gave significantly higher emulsion stability than those from 20 kDa pectin. The result indicated that the conjugation could improve the emulsifying properties of whey protein isolate at the pH around its isoelectric point.

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Khwanjai KLINCHONGKON

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CHAPTER 1
General introduction

1.1 Research problem and its significance

In many fruit processing industries, peels are wastes from the production process which their amounts may be nearly half of the raw materials. Conventionally, these wastes were usually eliminated as animal feed. However, the transforming of agricultural by-products into value added products has been receiving much interests recently because their benefits as a good source of bioactive compounds or high value compounds have been revealed (Berardini, Knodler, Schieber, & Carle, 2005; Khuwijitjaru, Pokpong, Klinchongkon, & Adachi, 2014a; Liu, Cao, Huang, Cai, & Yao, 2010; Martínez, Gullón, Schols, Alonso, & Parajó, 2009; Mohan, Banerjee, & Goud, 2015).

The United States Agency for International Development (USAID) reported that in 2013, passion fruit (*Passiflora edulis*) around 1.4 million tons were produced, mostly in Brazil (834,749 tons), Indonesia (141,190 tons), and India (122,630 tons). In addition, around 80,000 tons or 6% of the global production were from other nations include Kenya, Vietnam, Thailand, Venezuela, Malaysia, Zimbabwe, Israel, South Africa, Philippines, Sri Lanka, Pakistan, Suriname, and New Zealand (USAID, 2014). In Thailand, the Department of Agricultural Extension (DOAE) reported that the total production of passion fruit was 8,063 tons in 2016 and mostly produced in Petchaboon (3,415 tons), Loei (2,162 tons), Buriram (1,506 tons), and Chiang Rai (864 tons) provinces (DOAE, 2017). Passion fruit is commonly processed into juice and its peel is discarded as a waste. Passion fruit peel is about 50% of the whole fruit mass. The peel contains approximately 70% of carbohydrate components (Kulkarni & Vijayanand, 2010; Yapo & Koffi, 2008), most of which are cellulose, hemicellulose, and pectin. Pectin content of passion fruit peel ranges from 7.48 to 18% (dry basis) and the peel has a potential to be an alternative raw material for commercial pectin extraction (Kulkarni & Vijayanand, 2010; Oliveira, Giordani, Gurak, Olivera, & Marczak, 2015; Seixas et al., 2014).

The global pectin market increased from 30,000 tons in 2009 to exceed 60,000 tons in 2015 with the average price reaching to $1 billion, resulting from the demand for functional food, as well as cosmetic and pharmaceutical industries (Ciriminna, Fidalgo, Delisi, Ilharco, & Pagliaro, 2016). Pectin is commercially produced from citrus peel (85%), apple pomace (14%), and a minor fraction from sugar beet using hot dilute acid extraction (Ciriminna et al., 2016). As the reasons of corrosion, cost of maintenance, and environmental pollution, using a green technology as a substitution method for extraction is gaining much interests nowadays (Adetunji, Adekunle, Orsat, & Raghavan, 2017).

In subcritical water extraction (SCWE), only water is used as a solvent under high temperature and pressurized conditions. Researchers used this technique to extract pectin from some plant sources such as citrus peel, apple pomace, and sugar beet pulp (Chen, Fu, & Luo, 2015; Wang, Chen, & Lü, 2014; Wang & Lü, 2014). Moreover, it was also combined with other techniques such as ultrasonic pre-treatment to enhance the extraction (Chen et al., 2015). The enhancement of subcritical water extraction can also be done by adding some organic solvents. Ethanol has been used as a modifier in subcritical water extraction to extract bioactive compounds from several sources (Carr, Mammucari, & Foster, 2011; Curren & King, 2001; Jiang et al., 2014; Monrad, Howard, King, Srinivas, & Mauromoustakos, 2010; Tangkhavanich, Oishi, Kobayashi, & Adachi, 2013). However, the extraction of pectin using subcritical water with ethanol modifier has not been reported in the literature. Therefore, this study investigated the effects of ethanol addition in SCWE on pectin extraction from passion fruit peel. Because the molecular weight of the pectin obtained from SCWE was a lower than conventional method, degradation kinetics of pectin in subcritical water were investigated. Finally, properties of subcritical water-hydrolyzed pectin, as well as potential of utilization of these hydrolyzed pectin were also investigated.

1.2 Passion fruit

Passion fruits belongs in genus of *Passiflora* which comprises approximately 500 species. They are mainly distributed in the countries which are in tropical and subtropical regions including America (Ecuador, Brazil, Peru, and Columbia), Asia

(India, Malaysia, Indonesia, Philippines, and Thailand), Africa (Kenya, Uganda, and Rwanda) and Oceania (New Zealand) (Rodriguez-Amaya, 2012). Although there are numerous species, only two varieties are principally cultivated for commercial production, i.e. purple passion fruit (*P. edulis* Sims) and yellow passion fruit (*P. edulis* f. *flavicarpa* Degener) which have been classified by the color of the fruit skin (exocarp). The purple passion fruit has much better taste than the yellow one, but is lower in acid and yield. The rind or peel (pericarp) consists of exocarp and mexocarp. The exocarp is the outer peel where natural pigments as anthocyanin or carotene are contained (Rodriguez-Amaya, 2012), whereas the mexocarp is an inner white soften peel, which is abundant in pectin (Canteri et al., 2010a).

In passion fruit juice processing, the whole fruits are lightly crushed by crushing machine. Then, the juice-filled pulps with seeds are separated from rinds. Afterwards, the pulps and seeds are separated from the juices by a finisher (Bates, Morris, & Crandall, 2001). The juice is then formulated, homogenized, and pasteurized (Fernandes et al., 2011). Therefore, peels, pulps, and seeds are by-products from passion fruit juice production.

Passion fruit is a good source of nutrients especially ascorbic acid (29.8 mg/100 g fruit), niacin (1.460 mg/100 g fruit) and riboflavin (0.131 mg/100 g fruit). Citric acid is the dominating acid found in this fruit (Rodriguez-Amaya, 2012). The peel of passion fruit contained approximately 70% of carbohydrate (Yapo & Koffi, 2008) especially pectin which was found nearby to 20% w/w (Seixas et al., 2014). Canteri et al. (2010a) found that the passion fruit pericarp has total dietary fiber about 65%. Moreover, approximately 13.6% of pectic substances were obtained when the mesocarp was extracted using diluted nitric acid solution. However, not only the peel, but also the seed was a good source of dietary fiber, crude fat (Chau & Huang, 2004), as well as strong antioxidant, piceatannol (Maruki-Uchida et al., 2013). Passion fruit peel is also a source of antioxidants such as vitamin A and C, cyanidin, carotenoids, and polyphenolic compounds (Hernández-Santos et al., 2015).

1.3 Pectin

Pectin is a heteropolysaccharide consists of homogalacturonan (HG) as a main chain and rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) as

the side chains or branch chains as shown in Fig. 1.1. HG is a linear homopolymer of (1→4)-α-linked-D-galacturonic acid and is estimated to contain around 100–200 galacturonic acid units (Brejnholt, 2010). The carboxyl groups of the galacturonic acids are partially methyl esterified (Fig. 1.2) which provides the molecules with a certain degree of hydrophobicity (Trujillo-Ramírez et al., 2018). The degree of esterification (DE) or degree of methylation (DM) of galacturonic acid depends on raw material, extraction, and de-esterification conditions. Pectin with the methylated carboxyl groups more than 50% is called high methoxyl pectin (HM), while pectin with lower than 50% methylated carboxyl groups is called low methoxyl (LM) pectin. In addition to methylation at carboxyl group, acetylation can occur at hydroxyl groups of C-2 and C-3 of galacturonic acid monomer (Brejnholt, 2010). Acetyl groups are abundant in pectins from sugar beet roots, potato tubers, pumpkin, spinach, and easy-to-cook beans (Ngouémazong, Christiaens, Shpigelman, Van Loey, & Hendrickx, 2015), but very low in pectins from apple and citrus (Brejnholt, 2010).

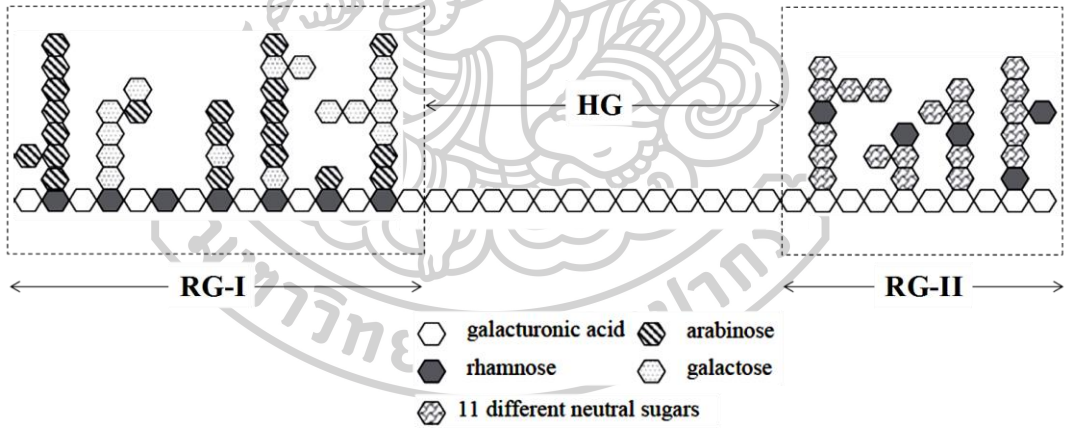

Figure 1.1 Schematic representation of the three major domains in pectin structure: homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II).
Source: Modified from Brejnholt (2010)

The RG-I domain consists of repeating units of the disaccharide (1→2)-α-L-rhamnose-(1→4)-α-D-galacturonic acid as a backbone and 20–80% of rhamnose monomers are branched with side chains of neutral oligosaccharides which mostly are

arabino- and galacto-oligosaccharides, attached at C-3 or C-4 (Schols & Voragen, 2002). The RG-II domain is also branched structure, but different from RG-I since its backbone is composed of HG. This domain is more compact than RG-I and it usually consists of 4 different polymeric side chains and 11 rare sugars including apiose, 3-*O*-methyl-L-fucose, 2-*O*-methyl-D-xylose, 3-*C*-carboxy-5-deoxy-L-xylose (aceric acid), 3-deoxy-D-manno-octulosonic acid, and 3-deoxy-D-lyxoheptulosaric acid (Brejnholt, 2010).

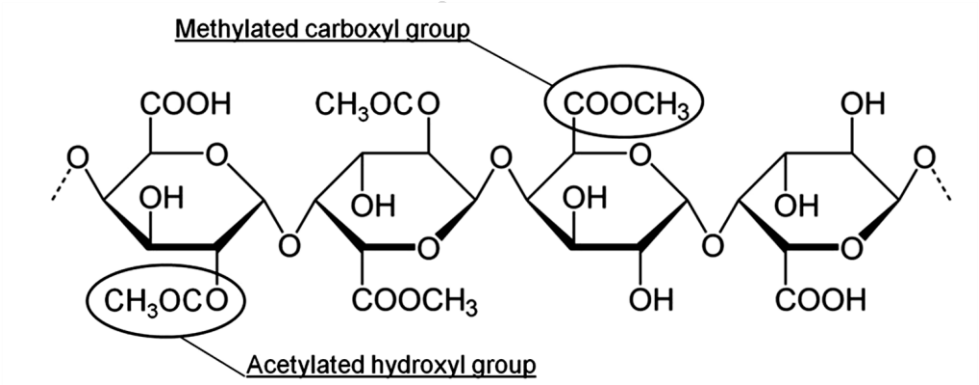

Figure 1.2 Basic molecular structure of pectin with methyl and acetyl groups.
Source: Schmidt et al. (2015)

Pectin is a hydrocolloid which can be used as emulsifying and emulsion-stabilizing agents for food applications. The emulsifying capacity of pectin depends on its protein content, whereas the emulsion-stabilizing relates with the ability of pectin to increase the continuous phase viscosity (Ngouémazong et al., 2015). There were evidences that the emulsification property of low molecular weight sugar beet pectin was influenced by the accessibility of the protein and ferulic acid groups to the surface of the oil droplets (Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003; Williams et al., 2005). The amount of protein reported in sugar beet pectin varied with the extraction conditions (Yapo, Robert, Etienne, Wathelet, & Paquot, 2007). However, the amount could be up to 8.6% w/w (Kirby, Macdougall, & Morris, 2008), which was higher than the protein contents in lemon (3% w/w), apple (1.6% w/w) (Brejnholt, 2010), citrus (1.1% w/w) (Mesbahi, Jamalian, & Farahnaky, 2005), and passion fruit pectins (1.4−5.1% w/w) (Yapo & Koffi, 2006).

Protein has been used as emulsifier in many dairy products, but it is very sensitive to heat or pH change (Euston, 2008). Normally, protein precipitates at isoelectric point resulting in poor emulsifying property. However, conjugation with pectin could improve emulsifying property of protein at the pH closed to isoelectric point (Jiménez-Castaño, Villamiel, & López-Fandiño, 2007; Schmidt et al., 2016). The reducing end of pectin structure can covalently link to amino group of protein via Maillard reaction to form the complex molecule. This resulted in shifting the minimum of protein solubility to more acidic pH (Jiménez-Castaño et al., 2007). Several works indicated that the complex molecule improved the stability of oil-in-water emulsions (Neirynck, Van der Meeren, Gorbe, Dierckx, & Dewettinck, 2004; Qi, Xiao, & Wickham, 2017; Schmidt et al., 2016; Setiowati, Vermeir, Martins, De Meulenaer, & Van der Meeren, 2016).

1.4 Subcritical water

Similar to other substances, water can be existing in 3 phases, solid, liquid, and gas. At 100 °C under atmospheric pressure (0.1 MPa) water changes from liquid phase to gaseous phase and this is a normal boiling point (Fig. 1.3). At the end of the liquid-gas equilibrium line which is at 374 °C and 22.1 MPa, is called the critical point. Above the critical point, the liquid and gaseous phases become indistinguishable, and presents as a supercritical fluid. From the phase diagram of water in Fig. 1.3, subcritical water, also known as pressurized hot water or superheated water, is the liquid water at temperatures between normal boiling point (100 °C) and the critical point (374 °C) under pressure that high enough to keep it in the liquid state. There are two main unique properties of subcritical water relate to the extraction process, which are dielectric constant and ion product of water (Jin, Wang, Zeng, Shen, & Yao, 2014).

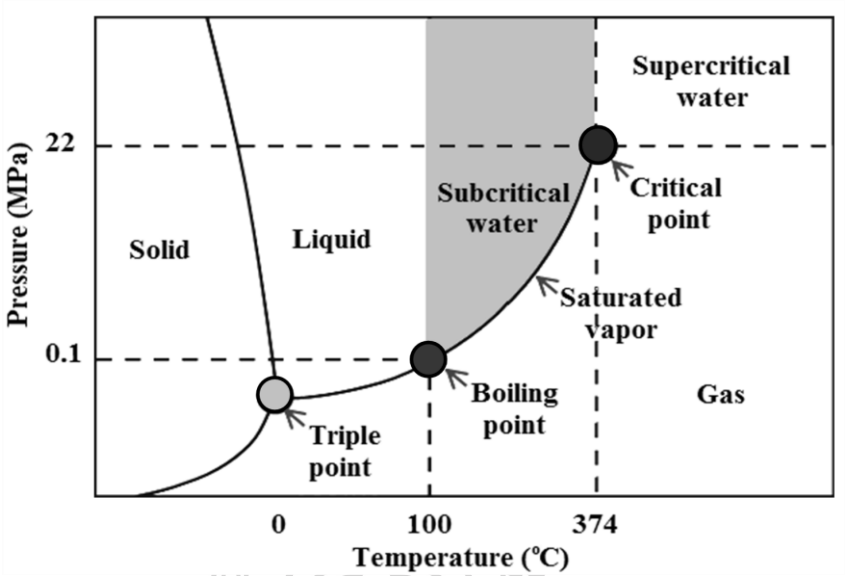

Figure 1.3 Phase diagram of water.

The dielectric constant (ε) is an indication of polarity of solvent. Dielectric constant of water decreases with temperature as shown in Fig. 1.4. At ambient temperature, water is a polar solvent with very high dielectric constant of 79 at 25 °C. But when the temperature increases to 200 °C, the value is lowered down to 35 which is similar to that of methanol (ε = 33 at 25 °C) and it is about 20 at 300 °C which is equal to that of acetone (ε = 21 at 25 °C) (Jin et al., 2014). This makes subcritical water possible to extract both polar and non-polar solutes. Conversely, the ion product (K_w) of water increases with temperature. At room temperature, the concentration of hydronium ions is 10^{-7} M equal to that of hydroxide ions. When the temperature is increased, water molecules dissociate more easily and results in higher hydronium and hydroxide ions concentrations. For this reason, subcritical water can promote a hydrolysis reaction (Khuwijitjaru, 2016).

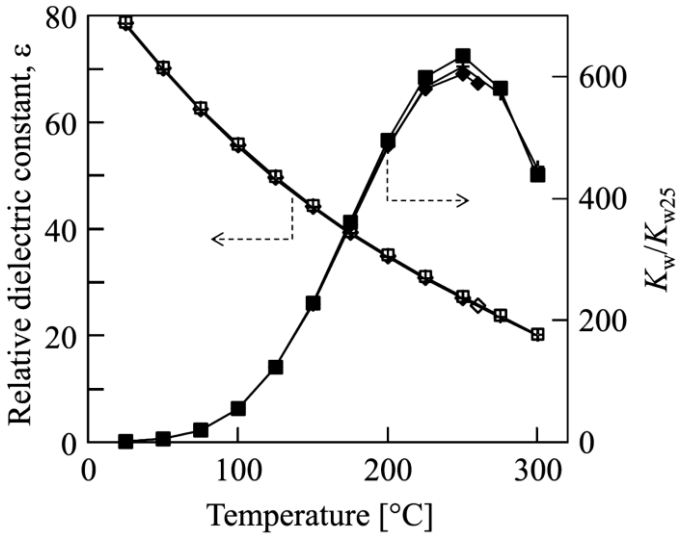

Figure 1.4 Changes in the relative dielectric constant and the ratio of ion product of water at any temperature (25−300 °C) to that at 25 °C (K_w/K_{w25}) under 5 (◇) and 10 (□) MPa of pressure and saturated vapor pressure (×).
Source: Khuwijitjaru (2016)

Several researchers published their works on subcritical water extraction of high valued products from biomass such as the extraction of reducing sugar from ginger bagasse starch (Moreschi, Petenate, & Meireles, 2004) and bamboo (Mohan et al., 2015), oligosaccharides from coconut meal (Khuwijitjaru et al., 2014a), saccharides from Japanese beech (Lü & Saka, 2010), dietary fiber from citrus peel (Tanaka, Takamizu, Hoshino, Sasaki, & Goto, 2012), and bio-oil from wheat straw (Patil, Armbruster, & Martin, 2014). These demonstrates that subcritical water can be used as extraction solvent like organic solvents but it is more safety. Some researchers added ethanol into subcritical water system to enhance extraction capacity, e.g. budesonide (Carr et al., 2011), xanthone from mangosteen pericarp (Yoswathana & Eshtiaghi, 2015), phenolic and methoxy-benzene compounds from lignin (Jiang et al., 2014), and materials with antioxidative activity from defatted rice bran (Chiou, Neoh, Kobayashi, & Adachi, 2012). Ethanol is the harmless solvent, thus it can be used in food production.

Although the extraction of pectic substances is typically accomplished by acid extraction (Brejnholt, 2010), studies on subcritical water extraction of this substance were also published. Martínez, Yáñez, Alons, and Parajó (2010b) employed this method to extract pectic oligosaccharide from citrus peel. They found that the products were oligogalacturonides, arabino-, and galacto-oligosaccharides with the yield about 25% of total waste mass when the extraction temperature at 160 °C was applied. Wang et al. (2014) used subcritical water to extract pectins from citrus peel and apple pomace. They found that the molecular weights of citrus and apple pectin were in range of 56–69 kDa and 28–66 kDa, respectively. Moreover, both of obtained pectins could inhibit more than 60% DPPH radical and 80% ABTS radical scavenging. This indicated the potential of these pectins as functional food ingredients.

1.5 Objectives

1.5.1 To investigate the effect of ethanol addition on the pectin obtained via the SCWE of passion fruit peel.

1.5.2 To investigate the hydrolysis kinetics of passion fruit pectin in subcritical water.

1.5.3 To investigate the properties (viscosity, molecular weight, degree of esterification, and moisture adsorption isotherm) of the subcritical water-hydrolyzed passion fruit pectin.

1.5.4 To investigate the emulsifying properties of the conjugated compound from whey protein isolate and subcritical water-hydrolyzed passion fruit pectin.

An overview of the research is shown in Fig. 1.5.

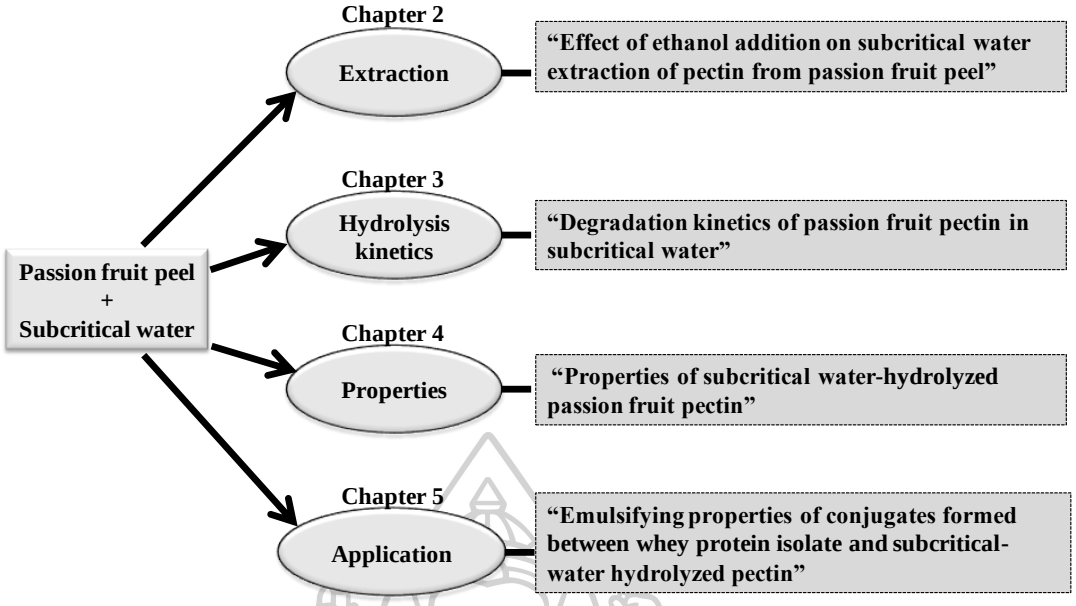

Figure 1.5 Overview of this research.

1.6 Scope of research

In this research, we used the peel from fresh passion fruits which were purchased from a local markets in Thailand. The subcritical water treatments were investigated using a batch-type reactor which its internal pressure changed with the heating temperature and a continuous flow-type reactor which its internal pressure was constantly controlled. The experiments in Chapters 2 and 3 were performed at Department of Food Technology, Faculty of Engineering and Industrial Technology, Silpakorn University, Thailand, while the experiments in Chapters 4 and 5 were performed at Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan and Institute of Process Engineering in Life Sciences, Section I: Food Process Engineering, Karlsruhe Institute of Technology, Karlsruhe, Germany, respectively.

CHAPTER 2

Effect of ethanol addition on subcritical water extraction of pectin from passion fruit peel

The effect of ethanol addition (0−30% v/v) on subcritical water extraction (SCWE) of pectin from passion fruit peel was investigated. The peel was extracted in a batch-type reactor at the treatment temperatures range of 100−160 °C. As a result, low molecular weight pectin in a range of 15.4−38.5 kDa were obtained. Higher treatment temperatures tended to give higher yields while the addition of ethanol gave adverse effect, except for adding small amount at high temperatures. The maximum yield (12.28% dry matter basis) was obtained from the treatment at 140 °C using 10% ethanol. The major composition of extracted pectin was galacturonic acid (72.11−94.76%). Additionally, this study demonstrated that the addition of ethanol resulted in pectin that possessed higher 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and total phenolic content (TPC).

2.1 Introduction

Passion fruit peel is a waste from the juice production and has a potential to be used as an alternative raw material for pectin extraction because it contains pectin in a range of 7.48–18% (dry basis) (Kulkarni & Vijayanand, 2010; Liew, Chin, & Yusof, 2014; Oliveira et al., 2015; Seixas et al., 2014). Pectin is widely used in both food and pharmaceutical industries and it is commercially extracted from apple pomace and citrus peel by hot dilute acid extraction (Brejnholt, 2010). However, the extraction of pectin using subcritical water was reported for citrus peel, apple pomace (Wang et al., 2014), and sugar beet pulp (Martínez et al., 2009). Wang et al. (2014) found that the polysaccharides obtained from this approach were low-molecular weights, which were in range of 56–69 kDa for citrus peel pectin and 28–66 kDa for apple pomace pectin. Wang et al. (2014) also found that those low-molecular weight pectins exhibited antioxidant properties as they showed 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity.

Researchers have studied on adding ethanol into water as a modifier to increase the solubility of low-polarity bioactive compounds such as phenolic compounds in subcritical water (Tangkhavanich et al., 2013). Adding ethanol in subcritical water may adversely affect the extractibility of carbohydrates. However, co-extraction of low-polarity molecules, especially bioactive phytochemicals can be expected and it should add more functionality to obtaining carbohydrates. Recently, we reported that the subcritical water treatment of passion fruit peel resulted in mainly various sizes of pectic substance molecules (Klinchongkon, Khuwijitjaru, Wiboonsirikul, & Adachi, 2017), but the isolation of those carbohydrates has not been performed. In this study, ethanol precipitation of carbohydrates was performed to demonstrate the properties of the obtained pectin. The effect of adding ethanol on SCWE of pectin from passion fruit peel was also investigated.

2.2 Materials and methods

2.2.1 Raw materials and chemicals

Fresh passion fruits were purchased from a local wholesale market (Pathumthani, Thailand). Neutral sugars (D-mannose, L-rhamnose, D-glucose, D-

galactose, D-xylose, D-arabinose, and D-fucose), D-glucuronic acid, D-galacturonic acid, 3-methyl-1-phenyl-2-pyrazoline-5-one (PMP), trifluoroacetic acid (TFA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, and standard dextrans with molecular weights of 5.2, 23.8, 147.6, 409.8, and 667.8 kDa were purchased from Sigma-Aldrich (St. Louis, MA, USA). Absolute ethanol (99.5%) was purchased from Macron (Center Valley, PA, USA). Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). Other reagents and solvents were of analytical grade.

2.2.2 Preparation of passion fruit peel powder

Fresh passion fruit peels were manually separated from the fruits. The peels, which consisted of exocarp and mesocarp, were cut into small pieces *ca*. 1 × 1 cm^2, washed with water to remove residual sugars and acids and blanched with hot water at 95 °C for 1 min to inactivate the enzymes. The peels were dried at 60 °C for 24 h in a hot-air oven and then milled using a food blender into powder that could pass through a sieve with 3.35 mm holes. The final passion fruit peel powder (PFPP) had 8.89% (wet weight basis) moisture content. The PEPP was kept in an aluminum foil bag with silica gel before extraction. Pictures of passion fruit peel powder preparation were shown in appendix.

2.2.3 Subcritical water extraction

SCWE began by adding 5 g of PFPP and 80 mL of water or aqueous ethanol (10, 20, and 30% v/v) into a stainless steel vessel (net volume 125 mL; Taiatsu Techno, Osaka, Japan). The vessel was then closed and heated using a temperature-controlled dry block heater (Nanasiam Intertrade, Bangkok, Thailand) to the desired temperatures (100, 120, 140, and 160 °C). The times required to reach each temperature were 5.64, 6.15, 6.90, and 7.94 min, respectively. After treatment, the vessel was immediately cooled with running tap water to ambient temperature. The hydrolysate was separated by filtering the treated mixture with filter cloth and the pH was determined using a pH meter (S20, Mettler-Toledo, Columbus, OH, USA). The solid residue was dried in a hot air oven at 105 °C for 24 h and the solid loss was calculated as:

$$\text{Solid loss}\,(\%) = \left[1 - \frac{\text{Solid mass after treatment}\,(\text{g})}{\text{Initial solid mass}\,(\text{g})}\right] \times 100 \qquad (2.1)$$

All SCWEs were performed in triplicate. The acid extraction of pectin was also performed using 50 mM HNO_3, 80 °C for 25 min (Canteri et al., 2010b).

2.2.4 Purification of pectin

Purification of pectin was carried out using the modified method from Seixas et al. (2014) and Wang et al. (2014). The hydrolysate obtained from the SCWE was mixed with absolute ethanol at a ratio of 1:2 (v/v). The mixture was stored in a refrigerator at 4 °C for 30 min before filtering through a filter cloth to separate the pectin. The pectin was washed three times using absolute ethanol and dried at 105 °C for 24 h in a hot-air oven. The dried sample was weighed and then used for the yield calculation according to Eq. 3.2. Finally, it was milled with a mortar and pestle into powder for further analyses.

$$\text{Pectin yield}\,(\%) = \left[\frac{\text{Pectin}\,(\text{g})}{\text{Initial sample solid mass}\,(\text{g})}\right] \times 100 \qquad (2.2)$$

2.2.5 Neutral sugars and uronic acid composition

The sugars and uronic acid composition of pectin was determined as described in our previous study (Klinchongkon et al., 2017) with a few modifications. The pectin powder (2 mg) was hydrolyzed with 200 µL of 2 M TFA in a capped vial at 121 °C for 1.5 h to release the neutral monosaccharides and uronic acids. Then, TFA was removed by adding 200 µL of isopropyl alcohol and blowing with nitrogen gas. About 50 µL of distilled water was added to generate a digested pectin solution. Monosaccharides and uronic acids were derivatized with PMP for high performance liquid chromatography (HPLC) analysis. About 50 µL of the digested pectin solution was mixed with 50 µL of 0.3 M NaOH. Subsequently, 50 µL of the mixture was mixed with 50 µL of 0.5 M PMP (in methanol) and then allowed to react at 70 °C for 30 min in a dry block heater (EL-02, Major Science, New Taipei City, Taiwan). The reaction mixture was immediately cooled in an ice bath and neutralized with 50 µL of 0.3 M HCl. The neutralized solution was dried under a stream of nitrogen gas. The residue solid was dissolved in 1 mL of distilled water. To remove the excess PMP

reagent, 1 mL of chloroform was added to the solution and thoroughly mixed using a vortex mixer. The aqueous layer was carefully separated with a Pasteur pipette and filtered through a 0.45 µm nylon membrane filter before HPLC analysis. Two Inertsil ODS 3 columns (150 × 4.6 mm, Ø 5 µm, GL Sciences, Tokyo, Japan) were jointed together and used for chromatographic separation. The columns were maintained at 40 °C in a column heater. The eluent was a mixture of 0.1 M phosphate buffer (pH 6.7) and acetonitrile in a ratio of 84:16 v/v. Elution was carried out at a flow rate of 1.5 mL/min, and 20 µL of the filtered sample was injected into the HPLC. The HPLC system (Shimadzu, Kyoto, Japan) comprised a solvent-delivery module (LC-20AD), a photodiode array UV-Vis detector (SPD-M20A), and a system controller (CBM-20A). The PMP-derivatized monosaccharides and uronic acids were monitored at 245 nm and the area of each peak was used for quantification. The percentage of galacturonic acid or other neutral sugars was calculated according to Eq. 2.3.

$$\text{Galacturonic acid (\%)} = \left[\frac{\text{Galacturonic acid (mg)}}{\text{Pectin (mg)}}\right] \times 100 \qquad (2.3)$$

2.2.6 Degree of esterification

Degree of esterification (DE) of pectin was determined by the titrimetric method (Liu et al., 2010). A dried pectin sample (50 mg) was moistened with 200 µL of absolute ethanol and then dissolved in 15 mL of distilled water by stirring with a magnetic stirrer. After the sample was completely dissolved, the solution was titrated with 0.01 M NaOH in the presence of five drops of phenolphthalein indicator and the volume of the titrant was recorded as V_1. Then, 2 mL of 0.05 M NaOH was added and mixed well. The mixture was allowed to stand for 15 min before 2 mL of 0.05 M HCl was added and stirred until the pink color disappeared. Five drops of phenolphthalein were added, and the solution was titrated again with 0.01 M NaOH to a faint pink color that persisted after stirring. This titration volume was recorded as V_2. The DE was calculated from Eq. 2.4.

$$\text{DE (\%)} = \left[\frac{V_2\,(\text{mL})}{V_1\,(\text{mL}) + V_2\,(\text{mL})}\right] \times 100 \qquad (2.4)$$

2.2.7 Molecular weight analysis

Molecular weight of pectin was determined by size-exclusion chromatography. The sample solution was prepared by mixing 5.0 mg of pectin powder with 2.5 mL of eluent (0.05 M of sodium phosphate buffer containing 0.4 M NaCl and 0.02 % NaN_3, adjusted to pH 7.0) using a magnetic stirrer for 16 h at room temperature. The solution was filtered through a 0.22 µm nylon syringe filter and 20 µL of the filtered sample was separated chromatographically on an Ultrahydrogel Linear column (7.8 × 300 mm, Waters, Milford, MA, USA). A refractive index detector (RID-20A, Shimadzu, Kyoto, Japan) was used for monitoring the eluted compounds. The elution was performed at 0.6 mL/min at ambient temperature.

The molecular weight average values including weight-average molecular weight (M_w) and number-average molecular weight (M_n) were calculated based on a calibration curve obtained by using standard dextrans (see Appendix). The molecular weight at the peak maximum and elution volume of each dextran were used for making a calibration curve and then M_w and M_n were calculated by Eq. 2.5 and 2.6, respectively.

$$M_w = \frac{\sum(H_i \times W_i)}{\sum H_i} \tag{2.5}$$

$$M_n = \frac{\sum H_i}{\sum(H_i / W_i)} \tag{2.6}$$

where H_i is the signal height of each point along the chromatogram measured from the baseline and W_i is the molecular weight of each point along the chromatogram. The mass distribution of sample was expressed in term of polydispersity index (PDI), which is a ratio of M_w to M_n (Gowariker, Viswanathan, & Sreedhar, 1986).

2.2.8 Viscosity measurement

The apparent viscosity of 1% (w/v) pectin solution (in distilled water) was determined at 25 °C using a Brookfield viscometer (DV3T, Brookfield Engineering Laboratories, Middleboro, MA, USA) equipped with an ultra-low adapter. In each test, 16 mL of sample was poured into a sample tube and measured at a shear rate of 70 rpm (85.6 s^{-1}).

2.2.9 DPPH radical scavenging activity

The scavenging activity of DPPH free radical of the obtained pectin was measured using the method reported by Wang et al. (2014). Pectin solution (10 mg/mL in distilled water, 0.5 mL) was mixed with 3.5 mL of DPPH solution (100 µM, in ethanol). The mixture was allowed to react for 30 min in the dark at ambient temperature. The absorbance of the mixture was determined at 517 nm using a UV-vis spectrophotometer (Genesys 10s, Thermo Scientific, Waltham, MA, USA) and the DPPH scavenging activity was calculated according to Eq. 2.7,

$$\text{DPPH radical scavenging activity}\,(\%) = \left[\frac{B - (S - S_c)}{B}\right] \times 100 \qquad (2.7)$$

where B is the absorbance of 3.5 mL of DPPH solution with 0.5 mL of water. S is the absorbance of 3.5 mL of DPPH solution with 0.5 mL of pectin solution and S_c is the absorbance of 3.5 mL of absolute ethanol with 0.5 mL of pectin solution.

2.2.10 Total phenolic content

The total phenolic content (TPC) of the obtained pectin was analyzed using Folin-Ciocalteu assay (Palanisamy et al., 2008). An aliquot of pectin solution (10 mg/mL in distilled water, 1 mL) was added to 5 mL of 10% v/v Folin-Ciocalteu reagent, mixed well and left for 5 min. Then, 4 mL of 7.5% Na_2CO_3 aqueous solution was added to the mixture and thoroughly mixed again. After standing for 2 h in the dark, the absorbance of the mixture was measured at 725 nm by a UV-Vis spectrophotometer. The TPC of the pectin sample was expressed as gallic acid equivalents (GAE).

2.2.11 Statistical analysis

The influence of the treatment temperature and ethanol addition on the measured parameters was evaluated using analyses of variance (ANOVA) performed with SPSS Statistics (version 17, IBM, Armonk, NY, USA). The Tukey's test at $\alpha = 0.05$ was performed for comparison of means.

2.3 Results and discussion

2.3.1 Solid loss, pH, and yield

Subcritical water promotes several decomposition reactions of macromolecules. The decrease in the solid component from the initial passion fruit peel powder during the SCWE was reported as solid loss (Fig. 2.1). It can be seen that the solid loss significantly increased as the treatment temperature increased but significantly decreased as the ethanol addition increased. The highest solid loss (29.81 %) was obtained by the treatment with 0% ethanol (distilled water) at 160 °C. Gao, Kobayashi, and Adachi (2014) showed that increasing the ethanol addition in SCWE slowed the hydrolysis of sucrose. These authors indicated that decreasing the proportion of water might result in milder hydrolysis conditions.

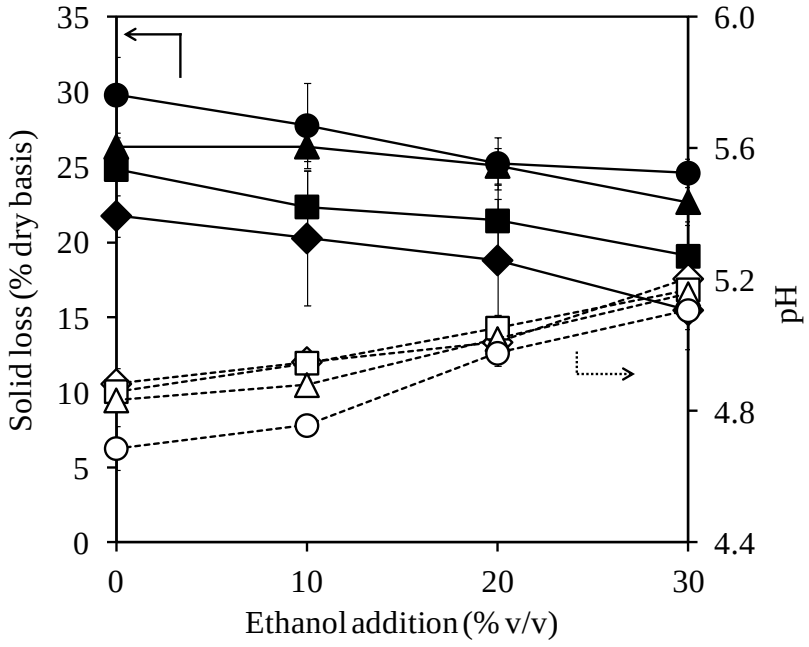

Figure 2.1 The effects of ethanol addition on solid loss (closed symbols) and pH of hydrolysates (open symbols) obtained from subcritical water extraction of passion fruit peel at 100 (◆,◇), 120 (■,□), 140 (▲,△), and 160 °C (●,○). The solid loss and pH from acid extraction of pectin were 28% and 1.9, respectively.

The pH of the passion fruit peel measured by boiling the powder in distilled water for 1 min (Henríquez et al., 2010) was 4.85, which indicated that the peel contained some acids. Figure 2.1 also shows that some acidic components might be formed after SCWE, because the pH values decreased with the treatment temperature for every ethanol addition; the treatment at 160 °C with 0% ethanol gave the lowest pH (4.69), which was lower than the pH of passion fruit peel powder. This observation demonstrated that the degradation of monosaccharides to acids such as acetic acid, formic acid, glycolic acid, or lactic acid (Lü & Saka, 2010; Martínez et al., 2010b) occurred to a slight extent. Martínez et al. (2010b) reported the formation of small amounts of formic acid and acetic acid in the hydrolysate of orange peel waste by SCWE at 140−160 °C. Additionally, as the relative dielectric constant was lowering by adding ethanol, the pH of the aqueous ethanol was significantly higher when the ethanol concentration was higher (Mezdour, Brule, & Korolczuk, 2006).

Pectic substances are complex polymer which are high polar and usually are extracted using relatively acidic system (Brejnholt, 2010). For this reason, the yield of pectin obtained from subcritical water extraction tended to be higher than those obtained using ethanol-added subcritical water extractions at every temperature studied as shown in Fig. 2.2. However, the pectin yield obtained from the condition of 140 °C and 10 % ethanol which was not statistically different from that obtained from subcritical water extraction at the same temperature.

It should be noted that for pectin precipitation we added two volumes of ethanol to all extracted samples, thus ethanol concentrations in the final mixtures during the precipitation might vary between 67 and 77% (v/v). Our preliminary test indicated that there was no significant difference of the pectin yields for precipitation using ethanol at 67 and 77% (v/v). In addition, this ethanol concentration range is not effective for precipitation of oligosaccharides which required at least 85% (v/v) ethanol (Sen et al., 2011).

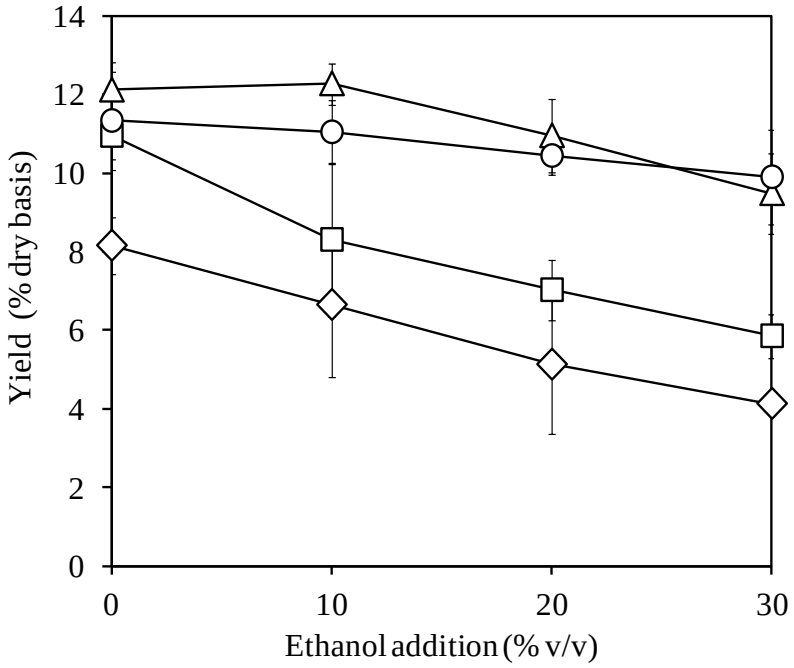

Figure 2.2 The effects of ethanol addition on pectin yield obtained from subcritical water extraction of passion fruit peel at 100 (◇), 120 (□), 140 (△), and 160 °C (○). The pectin yield obtained from acid extraction was 10.37%.

Typically, the pectin yields were approximately 30% of the solid loss values under the same conditions. This might be because other substances such as acid, small saccharides and amino acids that degraded from the peel were not precipitated with ethanol or were removed during the cleaning of pectin. The increase in the solid loss observed at higher temperatures did not parallel with an increased pectin yield. Indeed, the pectin yield at 160 °C seemed to represent the beginning of a downward trend. This decrease might be caused by the high intensity of the extraction conditions at this temperature, which could promote the degradation of the pectin polymer chain into shorter chains or other degradation products that could not be recovered by alcohol precipitation. It should be noted that the pectin yield from the acid extraction in our preliminary study, was 10.37 %, which was lower than that the highest yield obtained from the treatment using SCWE at 140 °C and 10 % ethanol (12.28 %). Additionally, the acid extraction took a longer time and required an acid reagent. The literature

indicates that the yield of pectin obtained from passion fruit ranges from 7.48 to 18.2 % (Kulkarni & Vijayanand, 2010; Oliveira et al., 2015; Seixas et al., 2014).

2.3.2 Uronic acids, neutral sugars, and degree of esterification

In this study, the galacturonic acid content was measured by HPLC and was found to be the main component of pectin. Its amount ranged from 72.11 to 94.76% by weight (Table 2.1), while the other sugars, i.e., mannose, ribose, rhamnose, glucose, galactose, xylose, arabinose and fucose, and glucuronic acid, were also detected at low amounts as shown in Table 2.2 and Fig. 2.3. The treatment temperatures affected on the amount of galacturonic acid at 10% and 20% of ethanol additions while adding ethanol into SCWE tended to decrease galacturonic acid content for the treatments at 120, 140, and 160 °C.

Table 2.1 Galacturonic acid contents (% w/w) of pectin extracted from passion fruit peel using subcritical water added with different ethanol concentrations.

Ethanol addition (% v/v)	Extraction temperature (°C)*			
	100	120	140	160
0	82.5 ± 4.5	90.4 ± 5.8 ^{a}	94.4 ± 2.7 ^{a}	91.7 ± 5.2 ^{a}
10	82.1 ± 6.4 ^{B}	85.6 ± 3.5 ^{abAB}	94.8 ± 1.2 ^{aA}	89.4 ± 4.1 ^{aAB}
20	84.0 ± 5.4 ^{AB}	76.7 ± 1.3 ^{bcB}	75.5 ± 3.1 ^{bB}	91.1 ± 5.2 ^{aA}
30	75.5 ± 6.5	72.1 ± 2.2 ^{c}	73.3 ± 3.9 ^{b}	77.3 ± 2.2 ^{b}

*For each temperature, different small letters mean significant difference. For each ethanol addition, different capital letters mean significant difference (Tukey, $\alpha = 0.05$), n = 3. Galacturonic acid content obtained from acid extraction was 87.5 ± 9.8 %.

Table 2.2 Glucuronic acid and neutral sugars contents of pectin extracted from passion fruit peel using subcritical water added with different ethanol concentrations.

Ethanol addition (% v/v)	Extraction temperature (°C)	Neutral sugar composition (% wt)								
		Mannose	Ribose	Rhamnose	Glucuronic acid	Glucose	Galactose	Xylose	Arabinose	Fucose
0	100	0.65 ± 0.03	0.08 ± 0.01	0.55 ± 0.01	0.25 ± 0.01	1.01 ± 0.02	1.10 ± 0.02	1.24 ± 0.03	0.93 ± 0.02	0.12 ± 0.01
	120	0.62 ± 0.03	0.07 ± 0.00	0.78 ± 0.09	0.26 ± 0.01	0.93 ± 0.13	1.04 ± 0.08	0.95 ± 0.11	0.93 ± 0.05	0.12 ± 0.01
	140	1.01 ± 0.17	0.07 ± 0.00	1.04 ± 0.10	0.20 ± 0.01	1.07 ± 0.13	1.09 ± 0.05	0.91 ± 0.06	1.15 ± 0.11	0.13 ± 0.02
	160	0.71 ± 0.08	0.11 ± 0.01	1.17 ± 0.14	0.25 ± 0.04	1.17 ± 0.15	1.22 ± 0.13	0.84 ± 0.08	1.48 ± 0.17	0.12 ± 0.01
10	100	0.95 ± 0.07	0.09 ± 0.01	0.77 ± 0.11	0.31 ± 0.04	1.67 ± 0.25	1.31 ± 0.05	1.50 ± 0.12	1.09 ± 0.05	0.15 ± 0.01
	120	1.00 ± 0.10	0.08 ± 0.01	0.80 ± 0.01	0.31 ± 0.03	1.72 ± 0.25	1.50 ± 0.18	1.61 ± 0.10	1.24 ± 0.08	0.16 ± 0.01
	140	0.82 ± 0.09	0.10 ± 0.01	1.15 ± 0.13	0.29 ± 0.05	1.18 ± 0.17	1.18 ± 0.09	0.81 ± 0.05	1.22 ± 0.08	0.12 ± 0.00
	160	0.69 ± 0.01	0.10 ± 0.00	1.01 ± 0.13	0.33 ± 0.06	1.29 ± 0.06	1.08 ± 0.06	0.90 ± 0.07	1.37 ± 0.18	0.14 ± 0.01
20	100	0.84 ± 0.04	0.08 ± 0.00	0.83 ± 0.12	0.30 ± 0.01	1.82 ± 0.23	1.37 ± 0.07	1.35 ± 0.05	1.19 ± 0.05	0.15 ± 0.00
	120	0.73 ± 0.05	0.08 ± 0.01	0.63 ± 0.05	0.29 ± 0.02	1.30 ± 0.09	1.12 ± 0.09	1.28 ± 0.11	1.04 ± 0.09	0.12 ± 0.01
	140	0.68 ± 0.06	0.08 ± 0.00	0.73 ± 0.04	0.22 ± 0.01	1.12 ± 0.06	0.87 ± 0.03	0.67 ± 0.06	0.93 ± 0.01	0.10 ± 0.00
	160	0.85 ± 0.03	0.09 ± 0.01	1.27 ± 0.14	0.31 ± 0.03	1.41 ± 0.22	1.41 ± 0.21	0.73 ± 0.09	1.54 ± 0.15	0.12 ± 0.02
30	100	0.81 ± 0.04	0.11 ± 0.02	0.76 ± 0.04	0.30 ± 0.06	1.29 ± 0.13	1.47 ± 0.11	1.63 ± 0.24	1.51 ± 0.15	0.15 ± 0.00
	120	0.46 ± 0.02	0.08 ± 0.01	0.73 ± 0.03	0.25 ± 0.03	0.77 ± 0.03	0.85 ± 0.02	0.62 ± 0.03	0.93 ± 0.05	0.11 ± 0.01
	140	0.44 ± 0.02	0.08 ± 0.00	0.59 ± 0.04	0.20 ± 0.02	0.45 ± 0.05	0.80 ± 0.07	0.68 ± 0.08	0.89 ± 0.06	0.10 ± 0.01
	160	0.65 ± 0.08	0.10 ± 0.01	1.11 ± 0.18	0.33 ± 0.04	1.10 ± 0.12	1.02 ± 0.10	0.51 ± 0.05	1.23 ± 0.12	0.11 ± 0.01
control		0.51 ± 0.02	-	0.90 ± 0.04	0.24 ± 0.01	1.10 ± 0.06	1.19 ± 0.09	0.91 ± 0.05	1.11 ± 0.07	0.12 ± 0.00

n = 3.

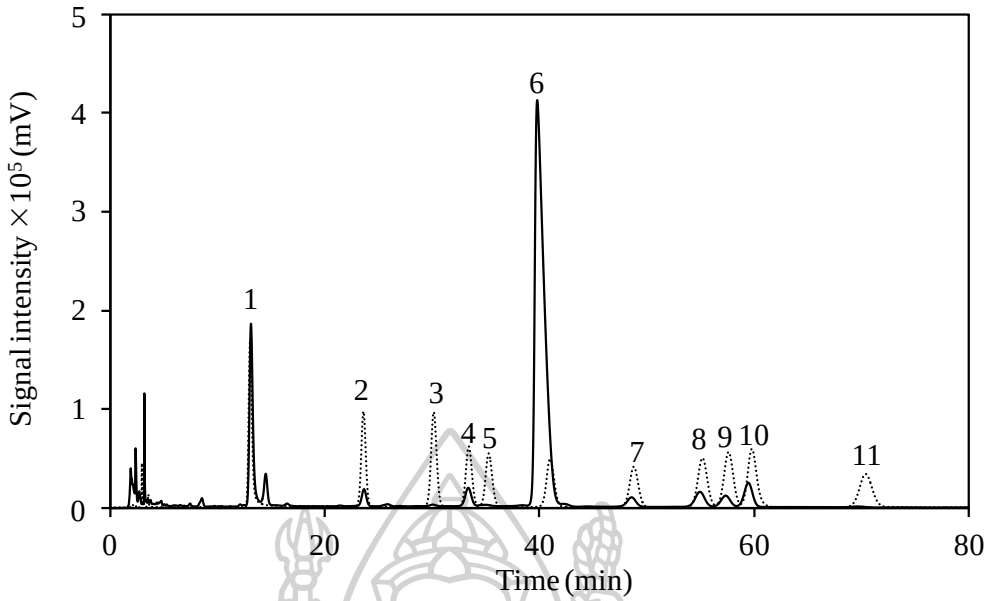

Figure 2.3 HPLC chromatogram of neutral sugars and uronic acids from pectin obtained by subcritical water extraction at 160 °C and 0% of ethanol-added (solid line) comparing with standards (dash line). (1) Excess PMP reagent (13.25 min), (2) mannose (23.81 min), (3) ribose (30.36 min), (4) rhamnose (33.63 min), (5) glucuronic acid (35.48 min), (6) galacturonic acid (41.20 min), (7) glucose (49.00 min), (8) galactose (55.40 min), (9) xylose (57.83 min), (10) arabinose (60.00 min), (11) fucose (70.60 min).

The DE of pectin was determined by the titrimetric method (data not shown). The results indicated that all pectin samples obtained via SCWE with or without ethanol additions could be classified as high methoxy pectin because their DE values were higher than 50%. Literatures show that the pectic substance from passion fruit peel could be classified into both low and high methoxy types depending on extraction conditions (Canteri et al., 2010b; Yapo & Koffi, 2006). Oliveira et al. (2015) demonstrated that the esterified galacturonic acid in passion fruit pectin extracted using diluted nitric acid was de-esterified to a higher extent at lower pH values and longer extraction times. Likewise, Pinheiro et al. (2008), who employed citric acid to extract pectin from passion fruit peel, found that lowering the citric acid concentration from 2.91 to 0.086% increased the DE from 27.69 to 78.59%.

2.3.3 Molecular weight and apparent viscosity

Figure 2.4 shows that adding ethanol into SCWE had no effect on depolymerization of pectin whereas the increment of the treatment temperature significantly affected the pectin chain size. The M_w and M_n of pectin obtained from SCWE were in ranges of 15.4–38.5 kDa and 8.2–17.8 kDa, respectively. These values were low compared to that of the pectin obtained from conventional hot dilute acid extraction reported by Canteri et al. (2010b) (299 kDa) and Seixas et al. (2014) (497 kDa). The result was in agreement with other SCWE studies. Wang et al. (2014) extracted pectin from citrus peel and apple pomace using SCWE (100–170 °C) and found that the M_w of pectin ranged from 56 to 69 kDa and from 28 to 65 kDa, respectively. In addition, Chen et al. (2015) obtained low molecular size pectin (89.4 kDa) from sugar beet pulp by the SCWE (110–130 °C) combined with ultrasonic pre-treatment.

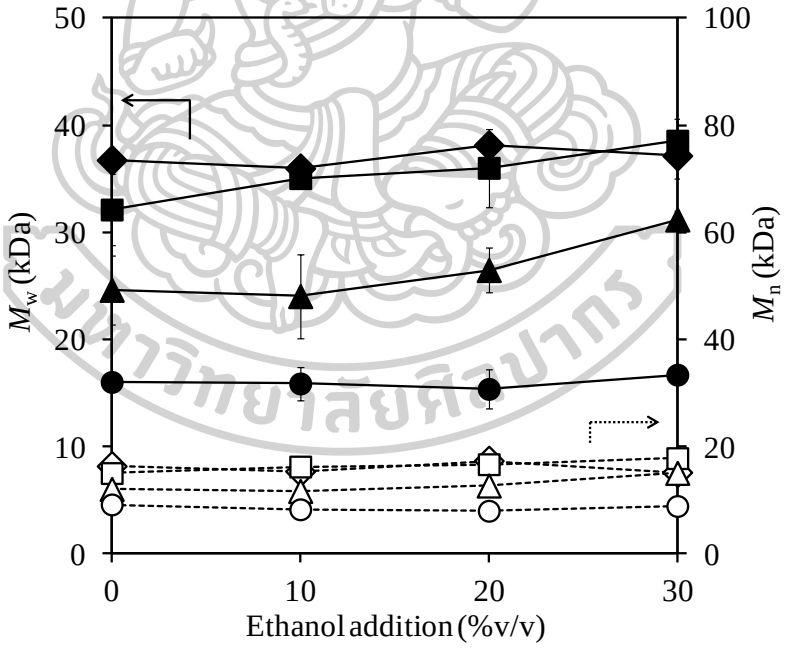

Figure 2.4 The effect of ethanol addition on weight average (M_w) (closed symbols) and number average (M_n) (open symbols) molecular weights of pectin obtained from subcritical water extraction of passion fruit peel at 100 (◆,◇), 120 (■,□), 140 (▲,△), and 160 °C (●,○). M_w and M_n of pectin obtained from acid extraction were 330 and 70 kDa, respectively.

The apparent viscosity of the pectin solutions in Fig. 2.5 tended to decrease as the treatment temperature increased whereas adding ethanol had no effect on it. The viscosities of pectin obtained from SCWE were 10-folds smaller than that obtained from acid extraction (10.64 mPa·s). These results agreed with the decrease of molecular weight as mentioned earlier. Moreover, we also found that the increment of the treatment temperature had effect on lowering the polydispersity index (PDI = M_w/M_n) value while adding ethanol had no effect on the values (data not shown). This indicated that the mass distribution of pectin became narrower with the increase of the treatment temperature. Although the pectin obtained from SCWE could be classified as high methoxy pectin, they were not able to form a gel with 65% sugar. This was because the short chain pectin could not provide enough junction zones for gelling. Nevertheless, low molecular weight pectin can be used as an emulsifier, coating material and prebiotic ingredient (Chen et al., 2015; Gómez, Gullón, Yáñez, Schols, & Alonso, 2016; Leroux et al., 2003).

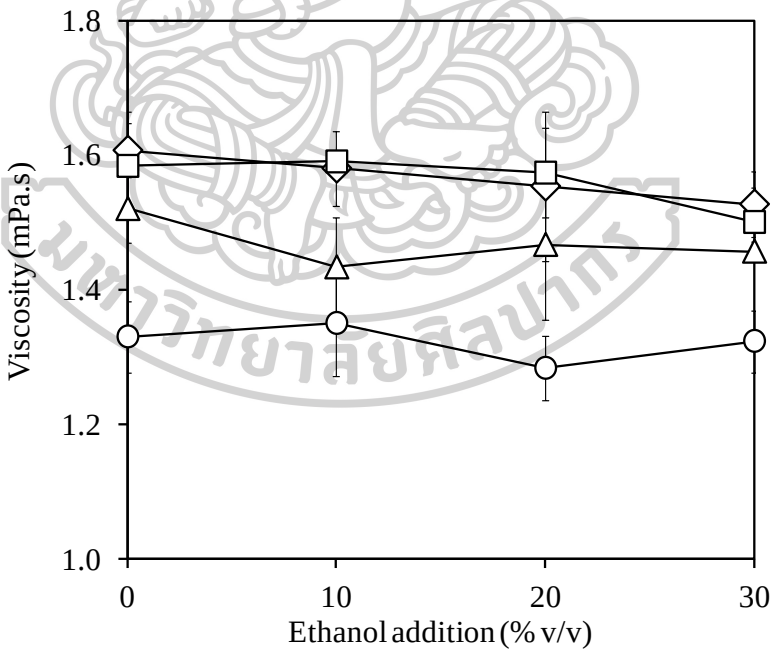

Figure 2.5 The effect of ethanol addition on viscosity (shear rate 85.6 s^{-1}, 25 °C) of 1% pectin obtained from subcritical water extraction of passion fruit peel at 100 (◇), 120 (□), 140 (△), and 160 °C (○). The viscosity of pectin obtained from acid extraction was 10.64 mPa·s.

2.3.4 DPPH radical scavenging activity and total phenolic content

In this study, we found that the obtained pectin samples exhibited DPPH radical scavenging activities (Fig. 2.6). The pectin obtained from SCWE exhibited DPPH radical scavenging activities ranging from 34–45% and the values significantly increased by increasing both the treatment temperature and the ethanol concentration. These results suggested that some antioxidants might also be extracted from the passion fruit peel and precipitated with pectin. It was reported that passion fruit peel contained various antioxidants such as vitamin A and C, carotenoids and phenolic compounds (Hernández-Santos et al., 2015).

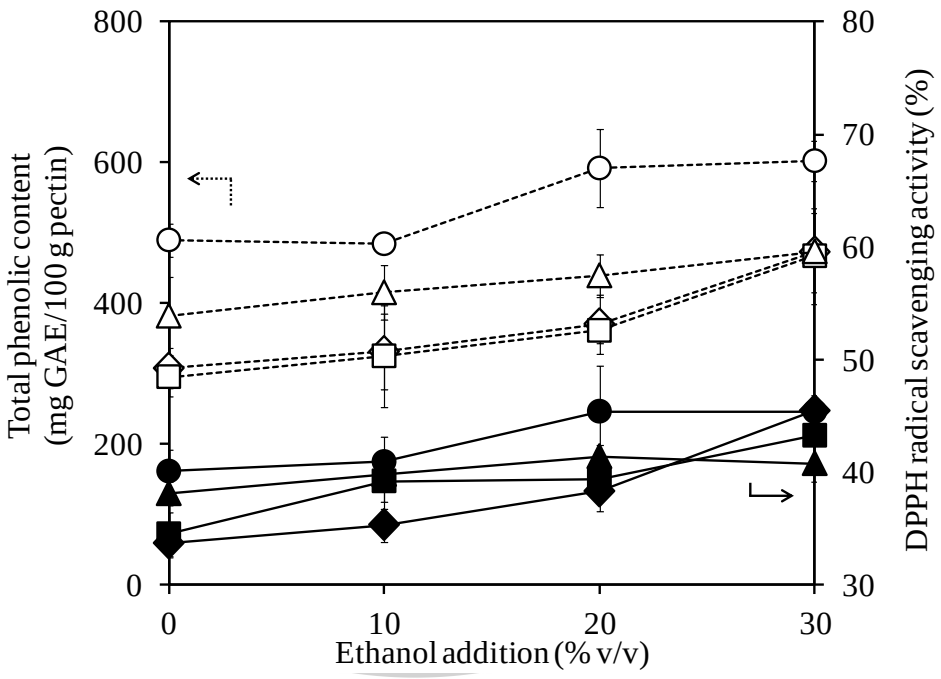

Figure 2.6 The effects of ethanol addition on DPPH radical scavenging activity (closed symbols) and phenolic content (open symbols) of pectin obtained from subcritical water extraction of passion fruit peel at 100 (◆,◇), 120 (■,□), 140 (▲,△), and 160 °C (●,○). DPPH radical scavenging activity and phenolic content of pectin obtained from acid extraction were 13% and 185 mg GAE/100 g pectin, respectively.

In this study, 602 mg GAE/100 g pectin of TPC was obtained at the highest treatment temperature and ethanol concentration (160 °C, 30%) (Fig. 2.6), which was over three times higher than that obtained from acid extraction (185 mg GAE/100 g pectin). Chiou et al. (2012) reported that increasing the ethanol concentration up to 30% v/v and increasing the temperature could increase both the TPC and DPPH radical scavenging activity of the extract from rice bran. This technique should be considered as a novel method for preparing functional pectin, which can be used in food industry.

2.4 Conclusions

This study showed that subcritical water could be used to extract pectin from passion fruit peel with a yield comparable to that obtained using the acid extraction but within a shorter time. The highest pectin yield was 12.28% at the treatment 140 °C using 10% ethanol. The pectin obtained using this method had smaller molecular weights and did not form gel with the addition of sugar. Adding ethanol at a low concentration into subcritical water resulted in milder extraction condition, but increasing the ethanol concentration conferred no further advantages regarding yield, molecular weight, or viscosity. However, using a high ethanol concentration was advantageous for antioxidant activity of the obtained pectin because some phenolic compounds were co-extracted.

CHAPTER 3

Degradation kinetics of passion fruit pectin in subcritical water

The degradation of passion fruit pectin by subcritical water treatment in a continuous flow-type reactor was investigated in the temperature range of 80−160 °C at a constant pressure of 5 MPa. Changes in the degree of polymerization and reducing end formation were monitored and modeled by applying the Emsley equation and zeroth-order kinetics, respectively. The results showed that both the pectin degradation rate constant and the change in the amount of reducing end were enhanced by temperature, and that the temperature dependence of these parameters obeyed the Arrhenius relationship. The activation energies for pectin degradation and reducing end formation were 62.8 and 86.9 kJ/mol, respectively. The non-linear relationship between the number of broken bonds per galacturonic acid unit and the change in the amount of reducing end indicated that pectin cleavage became easier as hydrolysis progressed.

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3.1 Introduction

Passion fruit is a tropical fruit that contains a large amount of pectin in its rind (Canteri et al., 2010b; Kulkarni & Vijayanand, 2010; Yapo & Koffi, 2006). It has been reported that the molecular weight of passion fruit pectin extracted using dilute nitric acid is in the range of 299−497 kDa (Canteri et al., 2010b; Seixas et al., 2014). Pectin is a non-digestible saccharide that cannot be absorbed in the intestine (Gullón et al., 2013); therefore pectin is useful as a dietary fiber. However, because of its high molecular weight, pectin cannot be used effectively by probiotics. In this context, the degradation of pectin into smaller-sized molecules has been fascinating (Chen et al., 2012; Diaz, Anthon, & Barrett, 2007; Zhang et al., 2013).

Degradation of pectin can be achieved by various approaches, especially enzymatic methods (Gwanpua et al., 2014; Schols, Geraeds, Searle-van Leeuwen, Kormelink, & Voragen, 1990). However, it was reported that the degradation of pectin could be accomplished by acid hydrolysis (Diaz et al., 2007). In Chapter 2, we applied subcritical water treatment to the extraction of pectin from passion fruit rind, and found that the molecular weight of the obtained pectin was in the range of 16−37 kDa, which was much smaller than that obtained using conventional acid extraction. Other studies also demonstrated that pectin could be broken into smaller molecules by subcritical water treatment (Chen et al., 2015; Wang et al., 2014).

Recently, we employed subcritical water treatment to hydrolyze polysaccharide from coconut meal to obtain a manno-oligosaccharide (Khuwijitjaru et al., 2014a; Khuwijitjaru, Watsanit, & Adachi, 2012) that may be useful as a prebiotic. Martínez, Gullón, Yáñez, Alonso, and Parajó (2010a) investigated the hydrolysis of sugar beet pectin under subcritical water conditions in the temperature range of 140−200 °C and found that arabino-, galacto-, and galactorunide-oligosaccharides were derived from pectin. In order to gain a deeper understanding of the process of pectin degradation under subcritical water conditions, the kinetics of the molecular size reduction and the increasing of reducing end (monosaccharide residue with hemiacetal functionality) from passion fruit pectin in subcritical water were studied using a flow-type reactor.

3.2 Materials and methods

3.2.1 Raw materials and chemicals

Mature passion fruits were bought from a market in Pathumthani province, Thailand. Galacturonic acid (≥ 97.0%) and dextrans with molecular weights in the range of 5−668 kDa, which were used for preparing a calibration curve using size-exclusion chromatography, were purchased from Sigma-Aldrich (St. Louis, MA, USA). Absolute ethanol (99.5%) was purchased from Macron (Center Valley, PA, USA). Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). Other reagents and solvents were of analytical grade.

3.2.2 Sample preparation

Passion fruit peel was separated, cut, thoroughly washed, blanched, dried, and milled as described in Chapter 2. Passion fruit pectin was prepared using a method described by Canteri et al. (2010b) with some modifications. Briefly, the passion fruit peel powder was extracted using 50 mM nitric acid at 80 °C for 25 min in a capped glass bottle using a solid-to-liquid ratio of 1:50 (w/v). The hydrolysate was immediately separated using fiber cloth and evaporated to reduce the volume four-fold by using a vacuum rotary evaporator (RV 10, IKA, Staufen, Germany). To precipitate the pectin, one part of the evaporated hydrolysate was mixed with two parts of absolute ethanol (v/v) and kept in a refrigerator at 4 °C for 30 min. The precipitated pectin was separated using fiber cloth and then washed with absolute ethanol three times. Finally, the obtained pectin was dried in a hot air oven at 60 °C for 24 h and milled into a fine powder using a mortar and pestle. The degree of esterification of this pectin was 66%.

3.2.3 Degradation of passion fruit pectin in subcritical water

The pectin powder was dissolved in warm distilled water (about 45 °C) under magnetic stirring for 30 min. The solution was then filtered through a Whatman No. 4 paper followed by a No. 1 filter paper to give a final pectin concentration of 0.4 % (w/v). The pectin solution was subjected to degradation at 80, 100, 120, 140, or 160 °C using a continuous flow-type reactor (Fig. 3.1) (Khuwijitjaru, Suaylam, &

Adachi, 2014b). The system was equipped with a high pressure pump (LC-20AD, Shimadzu, Kyoto, Japan) that was used to feed the pectin solution through stainless steel tubings (0.75 mm i.d.) with lengths of 3, 10, and 20 m for respective residence times of 0.5−1, 2−4, and 5−25 min. Each tube was immersed in a silicone oil bath to control the reaction temperature. On both sides of the reactor, a type K thermocouple was connected with a tee connector about 2 cm under the silicone oil surface to monitor the internal temperature. To stop the reaction, the heated solution flowed into another part of the tubing immersed in a cool water bath. A constant pressure of 5 MPa was maintained inside the reactor using a back-pressure valve (P-880, Upchurch Scientific, Oak Harbor, WA, USA). The degraded pectin solution was collected and freeze-dried (Scanvac, Cool Safe, Lynge, Denmark). The dried pectin was stored in a plastic bag at −18 °C until use.

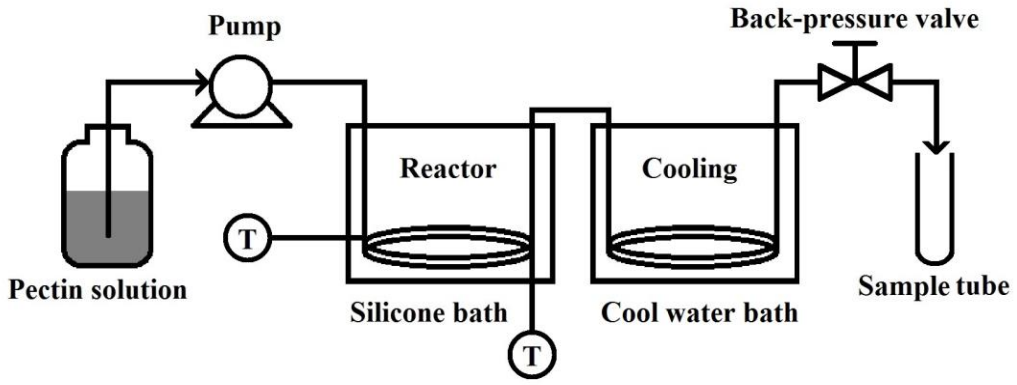

Figure 3.1 Schematic diagram of apparatus for subcritical water treatment.

3.2.4 Degree of polymerization of pectin

To measure the size of the degraded pectin molecules, size-exclusion chromatography (SEC) was carried out using a method described in Chapter 2. Briefly, 2 g/L of pectin solution was prepared by dissolving the freeze-dried pectin in 50 mM of sodium phosphate buffer containing 0.4 M NaCl and 0.02% (w/v) NaN_3 at pH 7 under magnetic stirring for 16 h at ambient temperature. An Ultrahydrogel linear column (7.8 × 300 mm, Waters, Milford, MA, USA) with an Ultrahydrogel guard column and a refractive index detector (RID-20A, Shimadzu, Kyoto, Japan) were

used. Before chromatographic separation, the sample solution was filtered through a 0.22 μm nylon syringe filter and 20 μL of the filtrate was used for characterization. Elution was performed at 0.6 mL/min at ambient temperature using the buffer solution mentioned above. Dextran with various sizes (5, 24, 148, 410, and 668 kDa) was used to prepare a calibration curve. M_w, M_n, and degree of polymerization (DP) were determined using Eq. (2.5), (2.6), and (3.1) respectively. The mass distribution of pectin samples was explained in term of polydispersity index which is a ratio of M_w to M_n.

$$\mathrm{DP} = \frac{M_n}{194.14 - 18.02} \tag{3.1}$$

where 194.14 is the molecular weight of galacturonic acid, and 18.02 is the molecular weight of water.

3.2.5 Reducing end of pectin

The degraded pectin was subjected to reducing end analysis as another indicator of the degree of hydrolysis. The colorimetric methods of Anthon and Barrett (2008) and Diaz et al. (2007) were modified for this measurement. The degraded pectin powder was dissolved in distilled water under magnetic stirring for 30 min at room temperature to give a final pectin concentration of approximately 2−4 g/L. A copper solution consisting of 4 M NaCl, 0.4 M acetate buffer pH 4.8, and 20 mM $CuSO_4$ was prepared. At pH 4.8, uronic acid reduces cupric ions (Cu^{2+}) in the solution more strongly than neutral reducing sugars (Anthon & Barrett, 2008). Therefore, this procedure is a specific measurement of galacturonic acid at the reducing end of the pectin structure. A 0.3 mL aliquot of the sample solution and 0.3 mL of copper solution were mixed together and then heated at 100 °C for 15 min using a dry block heater (EL-02, Major Science, New Taipei City, Taiwan). Subsequently, 2.4 mL of diluted Folin-Ciocalteau reagent (1/40 in distilled water), which was used for determining the cuprous ion (Cu^{+}) in the solution, was immediately added to the mixture and mixed well for 10 s. The absorbance was then measured at 750 nm using a UV-Vis spectrophotometer (Genesys 10s, Thermo Scientific, Waltham, MA, USA). A calibration curve for the reducing groups was prepared using galacturonic acid (GalA) at 0−1,095 μM.

3.3 Results and Discussion

3.3.1 Molecular weight change

The molecular weight of pectin and the products of subcritical water treatment using a flow-type reactor were measured by the SEC method. Standard dextrans were used to develop a calibration curve (log $M = -0.970\ V_e + 13.2$; where M and V_e refer to the molecular weight and elution volume (mL), respectively). The M_w, M_n, and DP of the initial pectin extracted with nitric acid were 259 kDa, 27 kDa, and 154, respectively. Other studies also showed a ten-fold difference between the M_w and M_n for several pectins; for example, the M_w and M_n were respectively 153 and 16 kDa for citrus pectin (Mesbahi et al., 2005), 116 and 13 kDa for beet pectin (Mesbahi et al., 2005), 229 and 15 kDa for tomato pectin (Chou & Kokini, 1987), and 223 and 15 kDa for apple pectin (Chou & Kokini, 1987). Figure 3.2 shows size-exclusion chromatograms of the initial pectin and the products of degradation at 80, 100, 120, 140, and 160 °C after 5 min of reaction time.

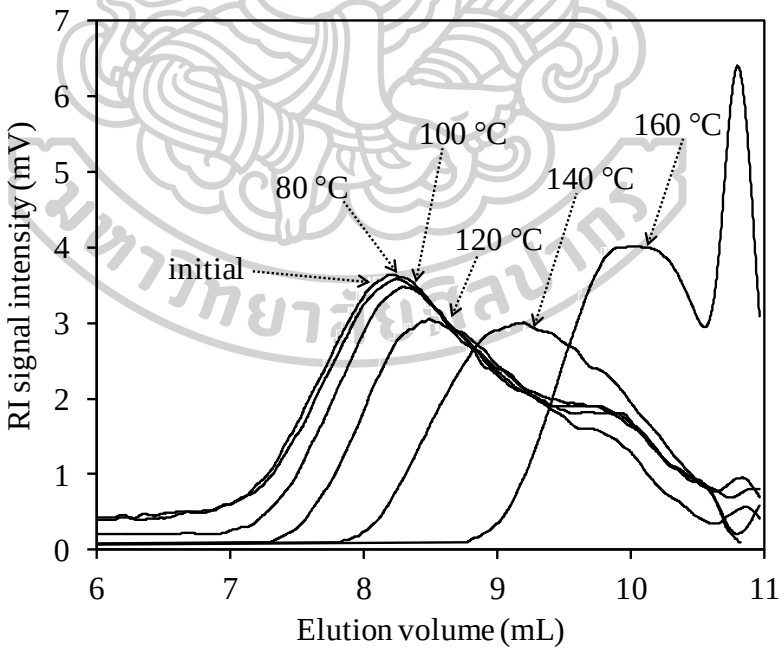

Figure 3.2 Size-exclusion chromatograms of untreated pectin and pectin obtained after five min-subcritical water treatment at 80, 100, 120, 140, and 160 °C.

The chromatograms demonstrate that a higher temperature resulted in a shift to lower molecular weight. The molecular weights of pectin degraded at different treatment temperatures are shown in Table 3.1. The treatment temperature appeared to strongly affect the M_w, since the M_w decreased to means reached 97% of the original at 160 °C within 5 min of reaction time. However, it took 25 min to achieve a decrease of the molecular weight of just 39% of at 80 °C. Moreover, we also calculated the polydispersity index (M_w/M_n) of the pectin samples (Table 3.1), where the polydispersity also decreased with increasing treatment temperature. This fact means that high treatment temperature resulted in a decrease of both the molecular weight and narrowing of the size distribution.

3.3.2 Kinetic analysis of pectin degradation

The Emsley equation has been widely used to explain the decrease in the DP of cellulose during thermal-aging analysis of paper (Ding & Wang, 2008; Emsley, 1994; Gilbert et al., 2010). This model was developed from Ekenstam equation, which is the second order equation as shown in Eq. 3.2:

$$\frac{\mathrm{dDP}}{\mathrm{dt}} = -k_1 \mathrm{DP}^2 \tag{3.2}$$

where DP is the degree of polymerization at time t and k_1 is the degradation rate constant.

However, Emsley et al.(1997) proposed that the degradation rate constant in Ekenstam equation should not be a constant but decrease with time. Thus, the modified model was obtained as:

$$\frac{1}{\mathrm{DP}} - \frac{1}{\mathrm{DP}_0} = \frac{k_{1o}}{k_2}\left(1 - \mathrm{e}^{-k_2 t}\right) \tag{3.3}$$

where k_{1o} is the initial degradation rate constant, and k_2 is the rate of k_1 changing ($k_1 = k_{1o}e^{-k_2 t}$).

In this study, we applied the Emsley equation (Eq. 3.3) to describe the degradation of passion fruit pectin. The parameters were estimated using Microsoft Excel® version 2007 as summarized in Table 3.1. The changes in the number of broken bonds per galacturonic acid unit ($1/\mathrm{DP}–1/\mathrm{DP}_0$) with time at different reaction temperatures are shown in Fig. 3.3. The results indicate that, at any treatment

temperature, $1/DP–1/DP_0$ increased with the residence time. The experimental data fit well to the model. The estimated k_{1o} value increased by about 5, 7, 15, and 82 times with an increase of the treatment temperature from 80 to 100 °C, 120 °C, 140 °C, and 160 °C, respectively. The increase in the k_{1o} value could be ascribed to pectin hydrolysis. Hydrolysis of a biomaterial under hydrothermal conditions may occur via three possible reaction pathways, namely, acid, base, and water catalysis, since the concentration of the ion products (H^+ and OH^- ions) of water increased at higher temperatures. Thus, water acts as an acid or base catalyst (Jin et al., 2014). In Chapter 2, we extracted pectin from passion fruit peel using subcritical water treatment at 100−160 °C, and found that the molecular weight of pectin was more significantly reduced at higher temperatures.

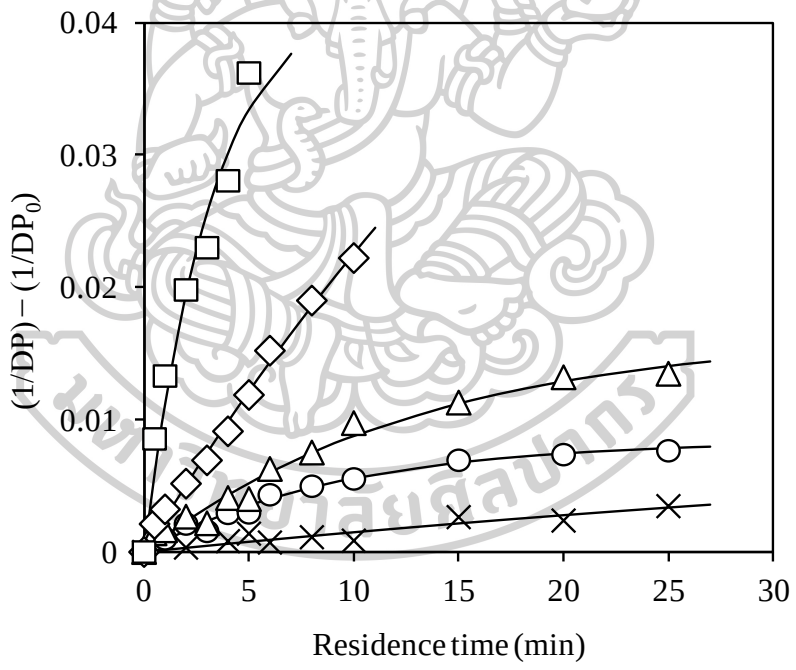

Figure 3.3 Changes in the number of broken bonds per galacturonic acid unit treated at 80 (×), 100 (○), 120 (△), 140 (◇), and 160 °C (□).

Table 3.1 Properties of passion fruit pectin degraded in subcritical water at different temperatures.

Treatment temperature (°C)	Residence time (min)	M_w (kDa)	M_n (kDa)	PDI*	DP	k_{1o} (min^{-1})	k_2 (min^{-1})	Reducing ends (μmol-GalA/g-pectin)	k_{re} (μmol-GalA/g-pectin·min)
80	0−25	158−259	18−27	9−10	100−154	1.59×10^{-4}	1.47×10^{-2}	96−106	3.65×10^{-1}
100	0−25	106−259	12−27	9−10	71−154	9.04×10^{-4}	1.08×10^{-1}	96−122	9.84×10^{-1}
120	0−25	39−259	9−27	4−10	50−154	1.25×10^{-3}	7.54×10^{-2}	96−151	2.44×10^{0}
140	0−10	18−259	6−27	3−10	35−154	2.63×10^{-3}	3.10×10^{-2}	96−259	1.57×10^{1}
160	0−5	7−259	4−27	2−10	23−154	1.31×10^{-2}	3.09×10^{-1}	96−553	9.17×10^{1}

*PDI: polydispersity index, n = 3.

The temperature dependence of k_{1o} could be described by the Arrhenius equation (Eq. 3.4):

$$\ln k_{1o} = \ln A - \frac{E_a}{RT} \tag{3.4}$$

where A is the frequency factor, E_a is the activation energy of the reaction, T is the reaction temperature, and R is the universal gas constant (8.314 J/mol·K).

Figure 3.4 shows the plot of $\ln(k_{1o})$ against $1/T$. The values of E_a and A for pectin degradation were evaluated to be 62.8 kJ/mol and 3.5×10^5 min^{-1}, respectively. A comparison of the E_a obtained in this study with those of obtained in other studies shows that the passion fruit pectin was more easily hydrolyzed than cellulose, since the E_a for pectin hydrolysis was almost two-fold lower than that of cellulose (Emsley, 1994; Gilbert, Jalbert, Tétreault, Morin, & Denos, 2009).

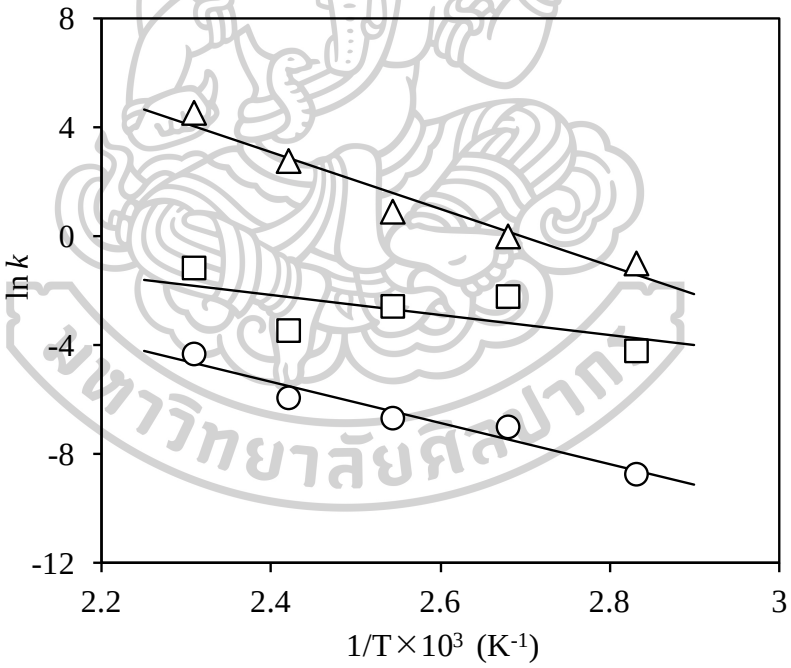

Figure 3.4 Arrhenius plots for initial degradation rate constant, k_{1o} (○), changes in k_{1o} rate constant, k_2 (□), and changes in reducing end rate constant, k_{re} (△).

Moreover, the degradation of citrus pectin was also studied by Diaz et al. (2007), demonstrating that at pH 4.5, in the temperature range of 75–110 °C, the E_a

for acid hydrolysis was 95 kJ/mol. In addition, the plot of ln (k_2) against 1/T, which also shows in Fig. 3.4, demonstrates that the k_2 also tends to increase with temperature. From this plot, the values of E_a and A for k_2 were evaluated to be 30.8 kJ/mol and 8.4 $\times 10^2$ min^{-1}, respectively.

3.3.3 Kinetic model for change in reducing end

The reducing end is an important parameter for analysis of carbohydrate depolymerization (Anthon & Barrett, 2008; Chen et al., 2012; Diaz et al., 2007). In this study, the increase of the reducing end could be described by zeroth-order kinetics as shown in Eq. 3.5:

$$RE - RE_0 = k_{re}t \quad (3.5)$$

where RE is the amount of reducing end at time t and RE_0 is the value at t = 0, and k_{re} is the reaction rate constant.

Figure 3.5 shows that at treatment temperatures of 80, 100, and 120 °C, the amount of reducing end gradually increased with the residence time, and increased rapidly at 140 and 160 °C.

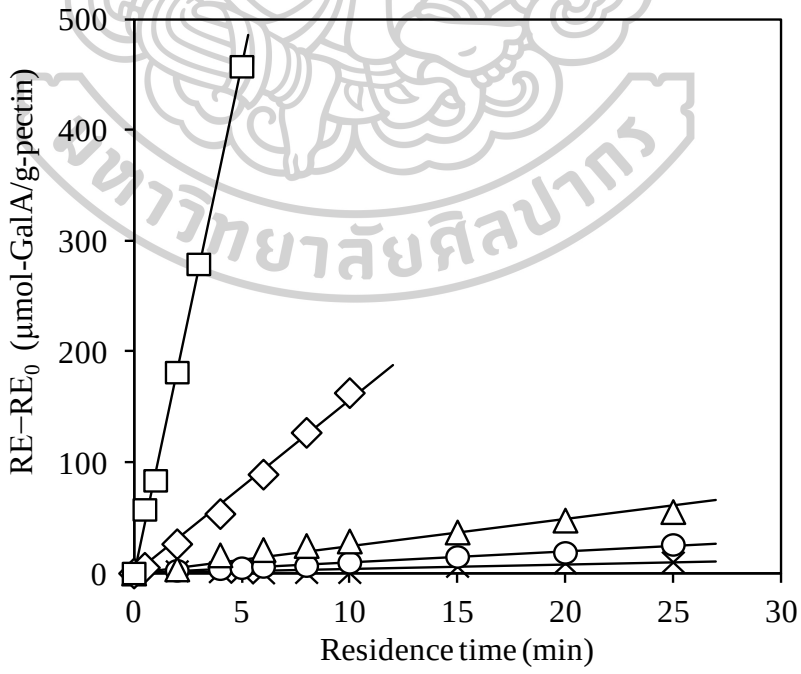

Figure 3.5 Changes in reducing end for pectins treated at 80 (×), 100 (○), 120 (△), 140 (◇), and 160 °C (□).

The estimated k_{re} increased about seven-fold with an increase of the treatment temperature from 80 to 120 °C, and increased about 43- and 251-fold with an increase of the treatment temperature from 80 to 140 °C and 160 °C, respectively. The relationship between ln (k_{re}) and 1/T is also shown in Fig. 3.4. The E_a and A for the change in the reducing end were 86.9 kJ/mol and 1.69×10^{11} μmol-GalA/(g-pectin·min), respectively.

3.3.4 Relationship between $1/DP - 1/DP_0$ and $RE - RE_0$

Chen et al. (2012) found that the relationship between the change in the reducing sugar content and that in $1/M_w$ during degradation of high-methoxyl pectin by dynamic high-pressure microfluidization was linear. However, Fig. 3.6, which shows the relationship between $1/DP - 1/DP_0$ and $RE - RE_0$ for pectins treated under different subcritical water conditions, demonstrates that the mechanism of pectin degradation in the temperature range of 80−160 °C would be the same since all of the data lie on the same line. From the non-linear and rapid increase of the reducing end, it is plausible to conclude that during the depolymerization process, the pectin chains were randomly cleaved; therefore, the amount of reducing end generated increased only moderately at the beginning and then increased drastically. This fact may also indicate that as the hydrolysis progressed, the pectin structure might become less complex making hydrolysis more facile. In addition, it can be expected that when the pectin was completely hydrolyzed ($DP \rightarrow 1$, then $1/DP - 1/DP_0 \approx 1$), the amount of reducing end of the degraded pectin ($RE - RE_0$) may reach a constant value, if further degradation reactions of galacturonic acid and other monosaccharides are assumed negligible under the conditions, or may decrease if other degradation processes occur at a considerable rate.

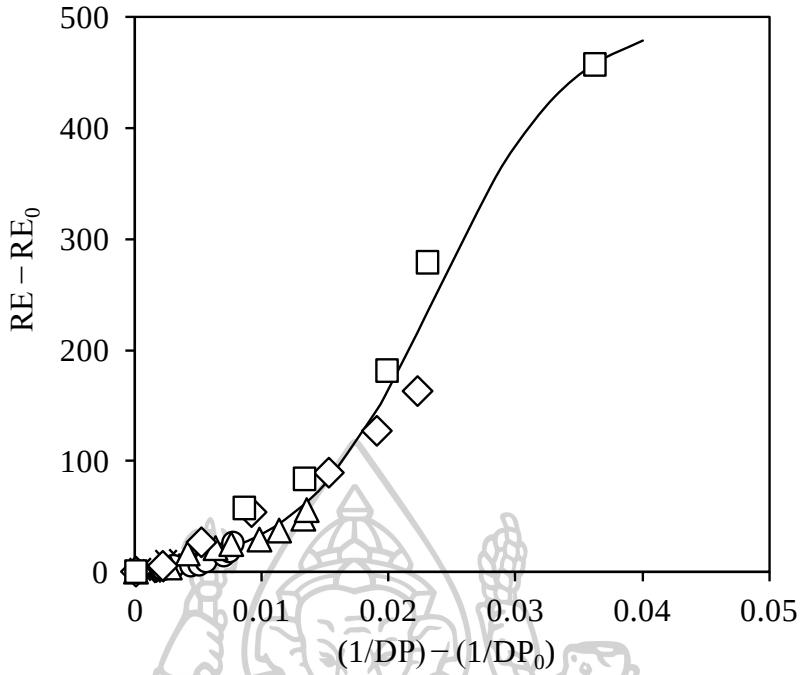

Figure 3.6 Relationship between the number of broken bonds per galacturonic acid unit and reducing end for samples treated at 80 (×), 100 (○), 120 (△), 140 (◇), and 160 °C (□).

3.4 Conclusions

The degradation of passion fruit pectin under subcritical water conditions in a continuous flow-type reactor was kinetically studied. The depolymerization of pectin was modeled using the Emsley equation, whereas the formation of reducing end groups was expressed by zeroth-order kinetics. The degradation reaction was found to be temperature dependent and could be represented by the Arrhenius equation. Understanding of the degradation kinetics is useful for the recovery of pectin with specific molecular weight using subcritical water treatment.

CHAPTER 4

Properties of subcritical water-hydrolyzed passion fruit pectin

Pectin extracted from passion fruit (*Passiflora edulis*) using diluted nitric acid was further hydrolyzed in subcritical water to reduce its molecular weight using a batch-type reactor at different temperatures (100, 120, 140, and 160 °C) and heating rates (3.5 and 7.0 °C/min). The subcritical water-hydrolyzed pectins were investigated in their properties including viscosity, molecular weight, degree of esterification, and water adsorption isotherm. The results demonstrated that under more severe conditions the hydrolyzed pectin had lower viscosity and showed different solution characteristics. The molecular weight of pectin also decreased with the severity of subcritical water hydrolysis. The pectins dissolved in distilled water system had higher intrinsic viscosities and molecular weights than those dissolved in buffer solution. Degrees of esterification of the hydrolyzed pectins only slightly changed in range of 55 to 60%. In addition, the moisture adsorption isotherms of the hydrolyzed pectins were evaluated and could be interpreted well with Guggenheim-Anderson-de Boer (GAB) model. The reduction of molecular weight did not affect the water adsorption characteristics of pectin at $a_w \leq 0.75$, but showed more an equilibrium moisture content at higher a_w

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4.1 Introduction

Pectin is a heterogeneous anionic polysaccharide mostly found in the primary cell wall and middle lamella of higher plant. The physical properties of pectin depend on its structures such as chain length, type of branched chain, amount of esterified group etc. Viscosity is one of the most important properties that must be considered before using pectin. It is affected by the degree of esterification (DE), molecular weight, and ionic strength in the solution (Kar & Arslan, 1999). In Chapter 2, we reported that the molecular weight of passion fruit pectin was significantly decreased after subcritical water treatment. It was reported that the decrease in molecular size of pectin tended to improve an intestinal absorption which might be useful for the cancer and kidney injury treatment (Courts, 2013), as well as promoted the digestion by beneficial bacteria in gastrointestinal tract (Gullón et al., 2013). Pectin is a hygroscopic material that is often used in the food, drug and cosmetic industries as gelling, thickening, water binder, and stabilizing agent (Brejnholt, 2010). Because the initial moisture content of food affects the ingredient mixing step and quality of final product, the information of water sorption characteristics is important. Brejnholt (2010) reported that the equilibrium moisture of high methoxyl (HM) pectin is around 12% at the water activity of 0.7; moreover, most commercial pectin is dried to less than 10% of moisture content and stored in a vapour-tight packaging for good long-term storage. Tsami, Vagenas, and Marinos-Kouris (1992) reported that the water adsorption isotherm of pectin at 25 °C was well fitted with Guggenheim-Anderson-de Boer (GAB) equation. Panchev, Slavov, Nikolova, and Kovacheva (2010) demonstrated that the adsorption of water from pectic substance was influenced by multiple factors. The authors showed that the monolayer moisture content of sunflower pectin was between those of apple and citrus pectins even though the sunflower pectin had the lowest degree of metoxylation and molecular weight.

Subcritical water treatment has been applied for pectin extraction in the recent years (Chen et al., 2015; Klinchongkon et al., 2017; Martínez et al., 2009; Wang et al., 2014). The effects of treatment temperature and reaction time of subcritical water treatment could be combined and expressed in term of “the severity

factor" for the facility of data comparison (Klinchongkon et al., 2017; Koomyart et al., 2016; Martínez et al., 2010b). Recently, we reported that subcritical water extraction is a feasible method for producing low molecular weight pectin (Chapter 2 and 3) or oligosaccharides from passion fruit peel directly (Klinchongkon et al., 2017). However, because the peel contain also cellulose and hemicellulose, therefore, to avoid the contamination of these component, we also investigated the use pectin extracted by conventional method (acid extraction) as the initial raw material for subcritical water hydrolysis. In the previous chapter, only hydrolysis kinetics was studied, thus, the objective of this study was to evaluate the properties (viscosity, molecular weight, DE, and moisture adsorption isotherm) of the subcritical water-hydrolyzed passion fruit pectin. In addition, solvent effect on viscosity and molecular weight measurements was also evaluated.

4.2 Materials and methods

4.2.1 Materials

Ripe passion fruit was purchased from a market in Pathumthani province, Thailand. Absolute ethanol (99.5%) was purchased from Junsei Chemical (Tokyo, Japan). Dextrans T10, T70, and T500 with weight-average molecular weight of 10.5, 71.2, and 507 kDa, respectively, were purchased from Phadia (Uppsala, Sweden). CH_3COOK, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, KI, NaCl, KCl, NaOH, HCl, Na_2HPO_4, NaH_2PO_4, HNO_3, and phenolphthalein were purchased from Wako Pure Chemical Industries (Osaka, Japan). KOH, LiCl, and K_2SO_4 were purchased from Nacalai Tesque (Kyoto, Japan).

4.2.2 Preparation of passion fruit pectin

The passion fruit peel was cut into small pieces, washed with clean water, blanched with hot water at 95 °C for 1 min, dried at 60 °C for 24 h in a hot-air oven, and milled to a smaller size using a food blender as same as the method in Chapter 2. The passion fruit pectin was extracted using diluted acid as reported in Chapter 3. Briefly, the dried passion fruit peel was mixed with a 50 mM HNO_3 solution (1:50 w/v) in a capped glass bottle, and then heated at 80 °C for 25 min in a temperature controlled water bath. The hydrolysate was filtered through a nylon cloth, evaporated

to reduce the volume using a vacuum rotary evaporator (N-1100, Eyela, Tokyo, Japan), and mixed with double volume of absolute ethanol. The mixture was kept in a refrigerator at 4 °C for 30 min. After that, the precipitated pectin was separated by filtering using nylon cloth and washed three times with absolute ethanol. Finally, the pectin was dried at 60 °C for 24 h and milled using a mortar and pestle. This pectin was used as an initial raw material for subcritical water hydrolysis.

4.2.3 Subcritical water hydrolysis

A 30 g/L of passion fruit pectin solution was prepared by gently dissolving the pectin (from 4.2.2) in distilled water under magnetic stirring for 5 h at room temperature. Then, the solution was centrifuged at 8,900 rpm, 4 °C for 10 min (LX-120, Tomy, Tokyo, Japan) to separate the insoluble solid. After that, 80 g of the solution (M_w = 197 kDa, DE = 60%, η = 84.20 mPa·s) was added into a pressure-resistant stainless steel vessel (net volume 125 mL; Taiatsu Techno, Osaka, Japan). The vessel was tightly closed and then heated at 100, 120, 140, or 160 °C using a mantle heater (200 W, Heater Engineer, Tokyo, Japan) connected with a TXN 700B temperature controller (As One, Osaka, Japan). The temperature increasing rate varied at 3.5 and 7.0 °C/min. The vessel was immediately cooled in an ice bath after internal vessel temperature reached to the desired temperature. The temperature profile inside the vessel for each condition during heating and cooling processes is shown in Fig. 4.1.

Since the treatments were operated non-isothermally, the effects of treatment time and temperature were expressed as the severity factor (ln R_o) (Overend, Chornet, & Gascoigne, 1987), which is defined by Eq. 4.1:

$$\ln R_o = \ln\left(\int_0^t \exp\left[\frac{T(t)-100}{14.75}\right]dt\right) \quad (4.1)$$

where t is the treatment time (min), T is the treatment temperature (°C) and 14.75 is an empirical constant (°C).

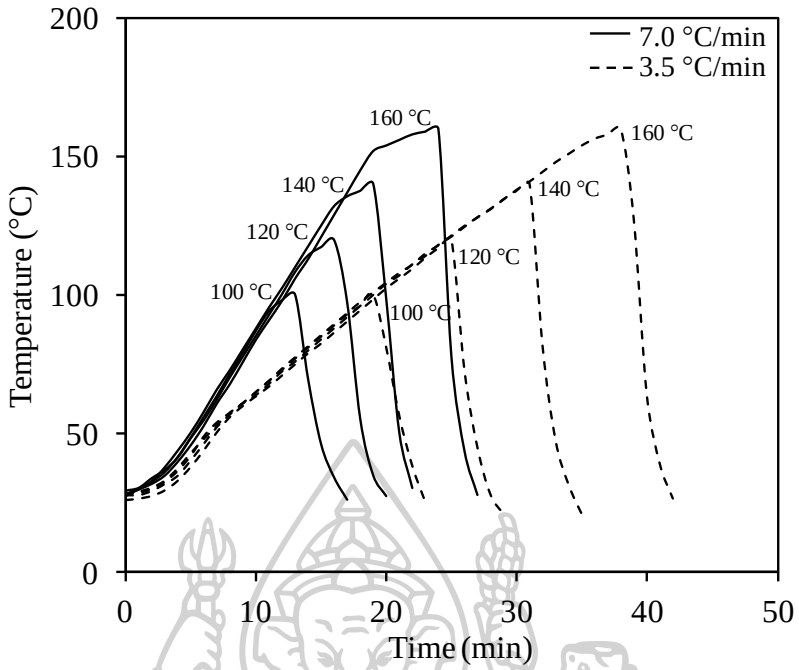

Figure 4.1 Temperature profile of subcritical water hydrolysis at 3.5 and 7.0 °C/min.

The ln R_o of each subcritical water hydrolysis condition is shown in Table 4.1. All experiments were performed in triplicate. An original pectin solution was turbid, brown, and viscous but when it was subcritical water-hydrolyzed, the turbidity and viscosity were reduced. At higher severity factors, the pectin solution became light yellowish-brown and less turbid.

Table 4.1 The severity factor of each subcritical water hydrolysis condition.

Temperature (°C)	ln R_o at different heating rates	
	3.5 °C/min	7 °C/min
100	1.5 ± 0.0	1.3 ± 0.0
120	2.9 ± 0.0	2.7 ± 0.0
140	4.4 ± 0.0	4.1 ± 0.0
160	5.9 ± 0.0	5.8 ± 0.1

n = 3.

4.2.4 Viscosity measurement

A viscosity measurement of pectin was carried out in two different solvent systems, distilled water and buffer systems. For the distilled water system, the hydrolyzed pectin solution was serially diluted with distilled water. Then, the viscosity was measured at 25 °C using a sine-wave vibro-viscometer (SV-10, A&D, Tokyo, Japan). In the case of buffer system, the hydrolyzed pectin was firstly dried using freeze dryer (FDU-1200, Eyela, Tokyo, Japan). Then, the freeze-dried pectin was dissolved in 50 mM phosphate buffer solution pH 7 containing 0.4 M NaCl to prepare 5 g/L solution and then serially diluted with the buffer mentioned above. After that, a viscosity measurement was performed similarly. The specific viscosity (η_{sp}) (dimensionless) and the reduced viscosity (η_{re}) (L/g) of pectin solution were calculated by Eqs. 4.2 and 4.3, respectively:

$$\eta_{sp} = \frac{\eta - \eta_0}{\eta_0} \tag{4.2}$$

$$\eta_{re} = \frac{\eta_{sp}}{c} \tag{4.3}$$

where η and η_0 are apparent viscosities (mPa·s) of pectin solution and pure solvent, respectively and c is the pectin concentration (g/L).

4.2.5 Molecular weight analysis

The freeze-dried pectin was dissolved in distilled water or the 50 mM phosphate buffer solution containing 0.4 M NaCl using a magnetic stirrer for 5 h at room temperature. The solution was filtered through a 0.22 μm syringe filter and 20 μL of the filtered sample was chromatographically separated on YMC-Pack Diol-300 (8.0 × 500 mm, YMC, Kyoto, Japan) at ambient temperature. A refractive index detector (RID-10A, Shimadzu, Kyoto, Japan) was used to monitor the elution profile of pectin. Distilled water or 50 mM phosphate buffer solution containing 0.4 M NaCl at a flow rate of 0.6 mL/min was used as a mobile phase. Dextrans with different molecular sizes were used to prepare a calibration curve (see in appendix). The M_w was determined using Eq. 2.5.

4.2.6 Degree of esterification

DE of pectin sample was measured according to the method described in Chapter 2. A 50 mg of dried pectin was moistened with 400 μL of absolute ethanol and then mixed with 15 mL of water using magnetic stirrer. Five drops of 1% (w/v in ethanol) of phenolphthalein were added into the pectin solution, which was then titrated with 0.01 M NaOH. The volume of first titrated NaOH (V_1 mL) was recorded. After that, the pectin solution was mixed well with 2 mL of 0.05 M NaOH for 15 min and followed by mixing with 0.05 M HCl until the pink color disappeared. Five drops of phenolphthalein were added and the titration with 0.01 M NaOH was performed again. The volume of second titrated NaOH (V_2 mL) was also recorded. The DE was calculated by Eq. 2.4.

4.2.7 Moisture adsorption isotherm

Moisture adsorption isotherms were determined using a static desiccator method. Freeze-dried pectin samples (100 mg each) were placed in separate desiccators (ϕ 15 cm) containing different saturated salts with various water activities (a_w) namely, KOH (a_w = 0.07), LiCl (a_w = 0.11), CH_3COOK (a_w = 0.22), $MgCl_2$ (a_w = 0.32), K_2CO_3 (a_w = 0.43), $Mg(NO_3)_2$ (a_w = 0.51), KI (a_w = 0.68), NaCl (a_w = 0.75), KCl (a_w = 0.84), and K_2SO_4 (a_w = 0.97) at 30 °C in a temperature controlled incubator (IS600, Yamato Scientific, Tokyo, Japan). The pectin samples were weighed every day until the stable weights were gained. Subsequently, the equilibrium moisture content, m_e (g/g dry matter), of each pectin sample was calculated by Eq. 4.4:

$$m_e = \frac{w - w_0}{w_0} \tag{4.4}$$

where w and w_0 are the equilibrium weight and an initial weight of the pectin sample, respectively.

4.3 Results and discussion

4.3.1 Viscosity and molecular weight in distilled water system

After hydrolysis of passion fruit pectin in subcritical water, the hydrolysate was serially diluted and the viscosity of the solution was immediately measured using a vibro-viscometer. The apparent viscosity (η) increased with the pectin

concentration; however, the values were lower for the pectin hydrolyzed at higher severity factors. The plots of reduced viscosity (η_{re}) against the pectin concentration in the distilled water system in Fig. 4.2A demonstrated that, for the hydrolysis at ln R_o in the range 1.3–4.4, the η_{re} values of unhydrolyzed and hydrolyzed pectins decreased with the increasing of pectin concentration at the beginning of the plots. However, at certain concentration, the η_{re} values of those pectins gradually increased with concentration. Polyelectrolyte polymer, such as pectin, usually exhibits anomalous viscosity properties comparing with neutral polymer. The initial decrease of η_{re} in Fig. 4.2A could be described by the prevailing of an electrostatic repulsion over the fragment-fragment interaction (Bromberg, 2001; Contois & Trementozzi, 1955; Fuoss, 1948; Izzo, Cloitre, & Leibler, 2014), whereas the increase of η_{re} at higher concentrations would result from the increase of pectin-pectin interactions among aggregation (Michel, Thibault, & Doublier, 1984; Yoo, Fishman, Hotchkiss, & Lee, 2006).

Fuoss equation has been widely used to explain the viscosity behavior of polyelectrolyte in water system (Fuoss, 1948; Izzo et al., 2014; Lima, Soldi, & Borsali, 2009). In this study, the equation was applied to fit with the η_{re} values at four lowest concentrations (0.2, 0.5, 1 and 2 g/L) for the unhydrolyzed and hydrolyzed pectins at ln R_o in the range 1.3–4.4, which we suspected that the electrostatic effects had occurred. The Fuoss equation (Fuoss, 1948) is shown in Eq. 4.5:

$$\eta_{re} = \frac{a}{1+bc^{1/2}} + d \tag{4.5}$$

where a, b, and d are empirical parameters of the equation, c is the pectin concentration (g/L). Values of a, b, and d were estimated by non-linear regression using Solver in Microsoft Excel 2007. The $a+d$ value represented the intrinsic viscosity ([η]) as described by Fuoss (1948).

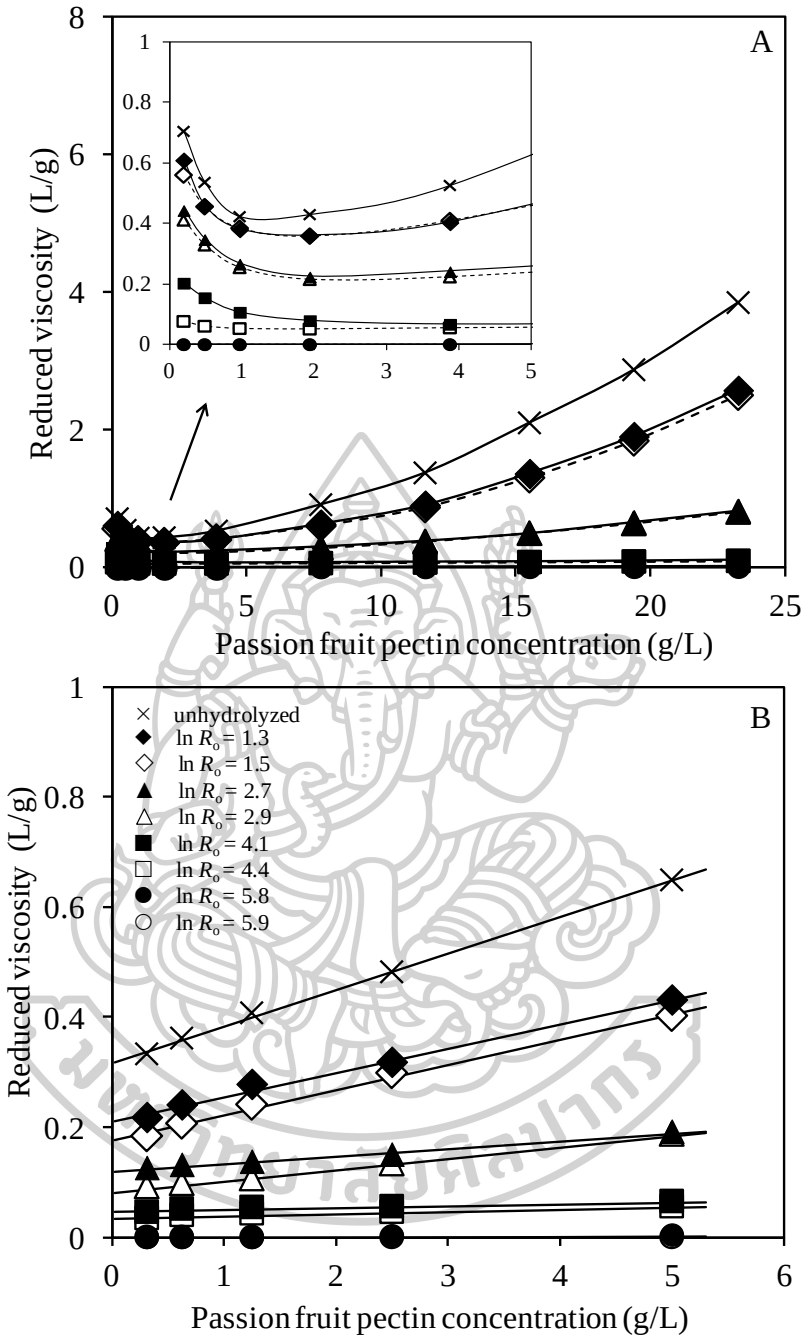

Figure 4.2 Reduced viscosity (η_{re}) as a function of passion fruit pectin concentration: A) in distilled water, and B) in buffer systems. The inset in 4.2A shows the η_{re} in distilled water system at low concentrations.

The intrinsic viscosity ([η]) is the viscosity of pectin solution at the limit of concentration extrapolated to zero. This parameter can also be used to determine the overlap concentration, $c^* = 1/[\eta]$ (Gupta, Elkins, Long, & Wilkes, 2005). Generally, in the dilute solution ($c < c^*$) the individual polymer chains are completely isolated. However, at $c = c^*$, the polymer chain begins to overlap each other leading to intermolecular interactions and becomes closely packed when $c > c^*$. This phenomena results in a sharp increase of viscosity (Bromberg, 2001). The results (Table 4.2) showed that c^* values increased with severity factor. It is also clearly shown by the right-shifting of minimum η_{re} in Fig. 4.2A. However, the concave curve disappeared for the pectin treated under conditions of ln R_o over 4.4. This indicated that the pectin was extensively hydrolyzed and the short-chain pectin, which had low interactions, was obtained. Figure 4.3 shows the [η] value versus ln R_o. The decrement of [η] with ln R_o for the distilled water system shows a non-linear relationship, and the [η] was rapidly dropped at ln R_o about 4.

The aggregation of pectin can occur in the distilled water system and result in an increase of apparent molecular weight (Kar & Arslan, 1999; Lima et al., 2009; Torres et al., 2015; Yoo et al., 2006). As shown in Fig. 4.4, the size-exclusion chromatogram from distilled water system indicates that unhydrolyzed pectin and the pectin hydrolyzed under the most severe condition (ln R_o =5.9) show the large peak at the elution volume before 9 mL which appears before the peak of Dextran T500 (500 kDa); however, these values was not in agreement with literatures and we suspect that they are the peaks of aggregated form of pectins. In Chapter 3, we found that the molecular weight of unhydrolyzed pectin was about 259 kDa and the one hydrolyzed by subcritical water at 160 °C (ln R_o = 4.3) had a molecular weight of only 16 kDa (from Chapter 2).

Table 4.2 Weight-average molecular weight (M_w), degree of esterification (DE), intrinsic viscosity ([η]), overlap concentration (c^*), and the parameters obtained from GAB model.

ln R_o	M_w (kDa)	DE (%)	[η] (L/g)		c^* (g/L)		GAB parameters		
			Distilled water system	Buffer system	Distilled water system	Buffer system	m_0	C	K
1.3	139	59	0.982	0.212	1.02	4.71	0.0565	5.6170	0.9207
1.5	131	59	0.916	0.178	1.09	5.63	0.0591	9.4151	0.9120
2.7	118	58	0.873	0.121	1.15	8.27	0.0438	9.9522	0.9413
2.9	114	57	0.711	0.083	1.41	11.99	0.0609	11.2350	0.9053
4.1	33	56	0.667	0.048	1.50	20.94	0.0466	8.3771	0.9477
4.4	32	56	0.252	0.036	3.96	27.76	0.0401	25.0802	0.9478
5.8	4	56	0.006	0.005	168.15	193.69	0.0449	8.6063	0.9750
5.9	3	55	0.004	0.001	275.81	1748.96	0.0543	9.5984	0.9599
Unhydrolyzed pectin	197	60	13.610	0.318	0.07	3.15	0.0507	5.3952	0.9140

n = 3

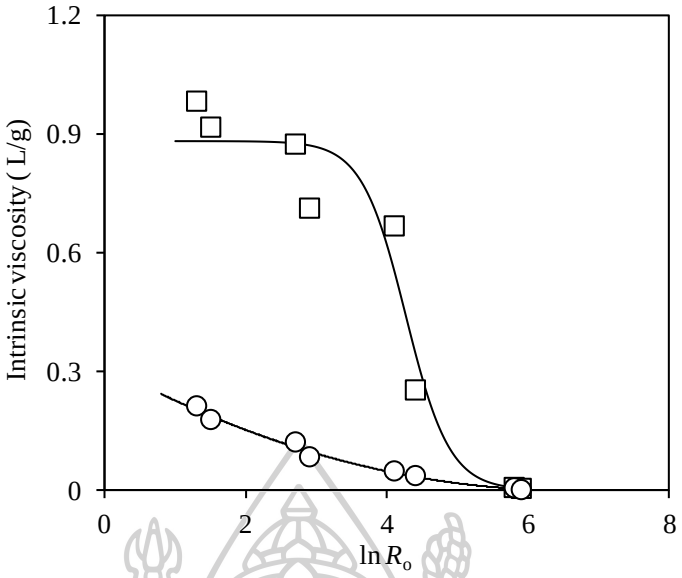

Figure 4.3 The plot of passion fruit pectin intrinsic viscosity [η] as a function of severity factor (ln R_o) in distilled water system given by Fuoss equation (□) and in buffer given by Huggins'equation (○).

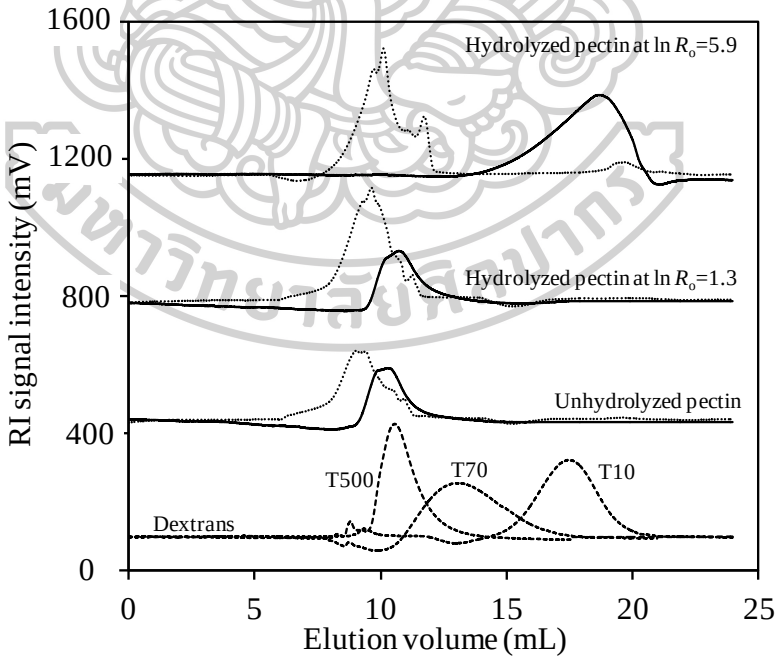

Figure 4.4 Molecular weight distributions of dextrans (dash line), unhydrolyzed and hydrolyzed pectin: dissolved in distilled water (dot line), and in buffer solution (solid line).

4.3.2 Viscosity and molecular weight in buffer system

Literatures have revealed that the addition of salt led to the screening of the electrostatic repulsion and therefore prevented pectin aggregation (Izzo et al., 2014; Lima et al., 2009; Smidsrød & Haug, 1971; Torres et al., 2015; Yoo et al., 2006). However, the study on hydrolyzed pectin is scarcely reported. The results showed that, for the pectin dissolved in buffer solution (ionic strength = 0.5 M), the rising in η_{re} with pectin dilution disappeared (Fig. 4.2B). Thus, the relationship between the η_{re} and pectin concentration could be modeled by Huggins equation as shown in Eq. 4.6 (Kulicke & Clasen, 2004):

$$\eta_{re} = [\eta] + k_H[\eta]^2 c \quad (4.6)$$

where k_H is the parameter called Huggins constant.

Figure 4.3 also indicates that the decrement of $[\eta]$ with ln R_o in buffer system obtained from Huggins equation was different and the values were smaller than those in distilled water system obtained from Fuoss equation, which would be ascribed to the overestimation of $[\eta]$ under electrostatic effect region in case of distilled water system. Yoo et al. (2006) reported that adding of 0.2 M NaCl into pectin solution could reduce the $[\eta]$ 6 times for HM-pectin and 15 times for LM-pectin. In this chapter, we found that, in the buffer solution with the ionic strength of 0.5 M, the $[\eta]$ was reduced about 43 times for unhydrolyzed pectin and about 1 to 14 times for the pectins hydrolyzed at ln R_o = 1.3 to 5.9 (Table 4.2). The c^* values in buffer system also increased with severity factor, but the values were higher than those obtained from distilled water system. This indicates that salt could also prevent the overlapping of hydrolyzed pectin. As shown in Fig. 4.4, in buffer system, a broad peak around elution volume of 19 mL was observed for hydrolyzed pectin at ln R_o = 5.9 which indicated the pectin was extensively hydrolyzed into shorter chains. This could be expected from the fact that under subcritical water conditions, hydrolysis of polysaccharides can be enhanced (Khuwijitjaru, 2016). From buffer system, the molecular weight of the unhydrolyzed pectin was estimated to be 197 kDa; while, the mass decreased from 139 to 3 kDa at ln R_o = 1.3 to 5.9 (Table 4.2).

4.3.3 DE and moisture adsorption isotherm

The DE of pectins was measured by the titrimetic method. Only 8% reduction in the DE value was observed even for the pectin hydrolyzed under the severe condition of ln R_o = 5.9 (Table 4.2) and it was found that all of the investigated pectins could be classified as high methoxyl pectin, since their DE values were higher than 50%. The equilibrium moisture contents (m_e) of samples at different water activities (a_w) were measured at 30 °C and fitted with the GAB model presented in Eq. 4.7:

$$m_e = \frac{m_0 \cdot C \cdot K \cdot a_w}{(1 - K \cdot a_w) \cdot (1 - K \cdot a_w + C \cdot K \cdot a_w)} \quad (4.7)$$

where m_0 is the monolayer moisture content, C is Guggenheim constant, and K is GAB correction factor. Parameters m_0, C, and K were estimated by non-linear regression method using Solver in Microsoft Excel (version 2007).

The GAB model has been widely used to explain the sorption characteristic of hygroscopic materials because it has a few parameters and can be used in wide-range of water activity (0.1–0.9). The experimental results for all samples are presented in Fig. 4.5 and the model fitted well with the data. It was observed that the isotherms obtained from these data were classified as typical sigmoid (type II) characteristic which is the most frequent isotherm for foods (Al-Muhtaseb, McMinn, & Magee, 2002; Basu, Shivhare, & Mujumdar, 2006). The type II isotherm is typical for nonporous and macroporous solid and represents unrestricted monolayer-multilayer adsorption behavior up to water activity values (Lowell, Shields, Thomas, & Thommes, 2004). The intermediate flat region of the isotherm demonstrated that the water was easily adsorbed and formed of monolayer. The equilibrium moisture content of pectins increased with water activity as shown in Fig. 4.5. The estimated GAB parameters are summarized in Table 4.2. The reduction of molecular size did not affect the water adsorption characteristics of pectin at $a_w \leq 0.75$; however, at $a_w >$ 0.75, low molecular weight pectin showed higher m_e value than high molecular weight pectin. Thus, keeping the hydrolyzed pectin under these conditions should be avoided. Commercial pectin is also usually recommended to keep in a vapor-tight package (Brejnholt, 2010). The calculated values for m_0 were in range of 0.04−0.06 g/g dry matter which was comparable with those of apple pectin, sunflower pectin,

and citrus pectin, which were in ranges of 0.04−0.06, 0.03−0.05, and 0.04−0.06 g/g dry matter, respectively, at 25−45 °C (Demertzis, Riganakos, Giannakakos, & Kontominas, 1991; Panchev et al., 2010).

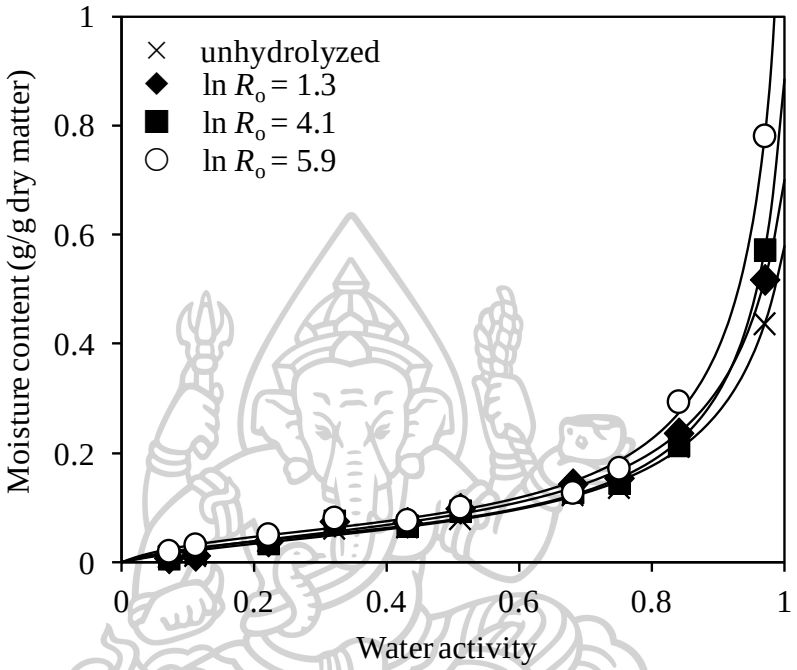

Figure 4.5 Moisture adsorption isotherms of passion fruit pectins at 30 °C with GAB model fitting.

4.4 Conclusions

In this study, we showed that the viscosity and molecular weight of pectin from passion fruit decreased after subcritical water hydrolysis. Also, we demonstrated that the electrostatic effects and the aggregation of pectin occurred in distilled water system which resulted in the overestimation in intrinsic viscosity as well as the molecular size. Additionally, the sorption isotherms of hydrolyzed pectins were unchanged at $a_w \leq 0.75$ and could be well explained by the GAB model. From these results, hydrolysis conditions to obtain certain molecular weight pectin can be predicted using ln R_o.

CHAPTER 5

Emulsifying properties of conjugates formed between whey protein isolate and subcritical-water hydrolyzed pectin

Pectins from passion fruit (*Passiflora edulis*) were hydrolyzed in subcritical water into different molecular weights (20, 77, and 146 kDa) and used to conjugated with whey protein isolate (WPI) by dry-heating method at 80 °C, 79% RH, for 6, 24, and 48 h. Soluble fractions after the conjugation were analyzed by SDS-PAGE. For the conjugation of 6 h, polydispersed bands from 15 kDa to 250 kDa were observed which indicated that WPI-Pectin conjugates were formed. These conjugates were used as emulsifiers (0.1%) in oil-in-water emulsions (pH 5) containing 5% of medium-chain triglyceride. The smallest droplet sizes and most stable emulsions were also obtained using the conjugates at 6 h of the dry-heating. Moreover, comparison of the conjugates from 146 kDa pectin with the ones from 20 kDa pectin revealed that the conjugates from the larger pectin gave significantly higher emulsion stability.

5.1 Introduction

Pectin is a natural complex polymer comprising at least 65% galacturonic acid units with partial methyl esterification and various neutral sugars such as arabinose, galactose, rhamnose, and xylose. It is commercially extracted from citrus peels and apple pomace by acidified water. Normally, the extraction conditions are varied in range of 50–90 °C, for 3–12 h, and at pH 1–3 to give the final product with desired properties and degree of etherification (DE) (Brejnholt, 2010). However, using other novel methods to extract pectin was also reported (Adetunji et al., 2017). Recently, we investigated the extraction of pectin from passion fruit peel using subcritical water which required shorter time for extraction but the obtained pectin were low molecular weight polymers (Chapter 2). Subcritical water could hydrolyze pectin into smaller molecules resulting in the increase of reducing end (Chapter 3), but decrease of viscosity (Chapter 4). Nevertheless, this extraction technique did not affect the DE and water adsorption characteristic of the pectin (Chapter 4).

Pectin is a hydrophilic polysaccharide, which limits its surface activity at oil-water interface. However, the emulsifying of pectin can be improved by the presence of protein moiety, ferulic acid, and acetyl group, which play a major role in the pectin hydrophobic character (Akhtar, Dickinson, Mazoyer, & Langendorff, 2002; Ngouémazong et al., 2015; Williams et al., 2005). Some studies attempted to improve the surface active and emulsifying properties of pectin by conjugation with protein (Schmidt et al., 2016; Trujillo-Ramírez et al., 2018). The reducing end of pectin can react with amino group of protein to form the hybrid molecule. This conjugation can be achieved either in wet (Diftis, Pirzas, & Kiosseoglou, 2005) or dry states (Neirynck et al., 2004; Qi et al., 2017; Schmidt et al., 2016; Setiowati et al., 2016).

Protein has been used as emulsifier in many dairy products such as whipping cream and ice cream which are oil-in-water emulsions. However, protein-based emulsions are very sensitive to destabilize especially when the protein is heated or the pH is changed to isoelectric point (pI) (Euston, 2008). Protein-pectin conjugate could produce smaller oil droplet compared to non-conjugated protein at pH 5.5 (Schmidt et al., 2016) and also showed higher oil-in-water (O/W) emulsion heat stability (Setiowati et al., 2016). Since the obtained pectins from subcritical water treatment

were low molecular weight chains which might be ineffective as an emulsion-stabilizing agent because the short-chain pectin can hardly increase the viscosity of continuous phase. Therefore, in this chapter emulsifying performance of the subcritical water-hydrolyzed pectin conjugated with whey protein isolate in oil-in-water emulsion system at near the pI of whey protein isolate was investigated.

5.2 Materials and methods

5.2.1 Materials

Ripe passion fruit was purchased from Srimuang market in Ratchaburi province, Thailand. Whey protein isolate (WPI), which contains 94 % protein, 5 % moisture, 0.4 % lactose, and 0.3 % fat, was obtained from Fonterra Cooperative Group (Auckland, New Zealand). Medium-chain triglyceride (MCT) was obtained from Symrise AG (Holzminden, Germany)

5.2.2 Subcritical water-hydrolyzed passion fruit pectin preparation

Passion fruit pectin was extracted using hot dilute nitric acid and then hydrolyzed in subcritical water using a batch-type reactor as described in Chapter 4. Three hydrolysis conditions were performed by controlling ln R_o to obtain the hydrolyzed pectin with difference molecular weights as shown in Table 5.1. A total of 5 batches for each condition was performed to obtain *ca*. 12 g of hydrolyzed pectin.

Table 5.1 Hydrolysis conditions and properties of subcritical water-hydrolyzed pectins used for preparation of conjugates.

Name	Hydrolyzed temperature (°C)	Heating rate (°C/min)	ln R_o	DE (%)	M_w (kDa)*	Protein (%)**
LPec	130	8.5	2.9	57	146	
MPec	150	9.0	4.2	57	77	0.66
SPec	170	7.5	5.6	58	20	

***** by size exclusion chromatography method, n = 3.

** by Kjeldahl method (N × 6.25)**,** n = 2.

5.2.3 Preparation of WPI-Pectin conjugates

The preparation of WPI-Pectin conjugates were carried out by the method described by Schmidt et al. (2016) with some modifications. Subcritical water-hydrolyzed passion fruit pectin (10 g) was dissolved in demineralized water (585 g) using a high-speed homogenizer (T-25 Ultra-Turrax, IKA, Staufen, Germany) at 5,000 rpm for 2 min. Then, WPI (5 g) was added into the pectin solution and mixed well at 10,000 rpm until the protein particles were completely dissolved. The mixture was allowed to stand for 3 h at room temperature to equilibrate, and then adjusted to pH 7 using 10% w/w NaOH solution to avoid electrostatic interactions because at this pH WPI and pectin exhibit negative net charge (Jones, Lesmes, Dubin, & McClements, 2010). The protein-pectin mixture was then frozen at -18 °C overnight before freeze-drying at -80 °C, 0.2 mbar (Alpha 2-4 LD plus, Christ, Osterode, Germany). The freeze-dried sample was milled into powder that could pass through a sieve with 0.5 mm-holes using a laboratory grinding mill at 6,000 rpm (POLYMIX® PX-MFC 90D, Kinematica, Luzern, Switzerland). A 2.5 g of each fine-powder sample was spread out in a 10 mm-diameter Petri dish. Then, the conjugation reaction was induced using a dry-heating method by placing the powder in a temperature and humidity controlled chamber (PR-15, Tabai Espec, Osaka, Japan) at 80 °C under a relative humidity of 79% for 6−48 h.

5.2.4 Determination of solubility of WPI-Pectin conjugates

Solubility of the conjugate was analyzed by dispersing 2 g of the heated powder (from 5.2.3) in 100 g of demineralized water using a homogenizer at 5,000 rpm for 2 min. The mixture was measured for pH value using a pH meter, and then adjusted to pH 5 using 10% w/w NaOH or HCl solution. The mixture was centrifuged at 4,600 rpm for 30 min at 20 °C (Rotanta 460 R, Hettich, Kirchlengern, Germany). The supernatant was carefully separated from the insoluble solid, and both fractions were dried using a freeze dryer. All experiments were performed in duplicate. Solubility was calculated by Eq. 5.1:

$$\text{Solubility } (\%) = \frac{(w_t - w_i)}{w_t} \times 100 \qquad (5.1)$$

where w_t is the weight of conjugate powder (g), and w_i is the weight of dried insoluble solid (g). The freeze-dried soluble fraction was used as conjugate in the next experiments.

5.2.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) experiment was performed according to the method described by Schmidt et al. (2016) with some modifications. Both 4 mg/mL of the conjugate samples and 2 mg/mL of WPI were prepared by dissolving each sample in demineralized water. A 60 μL of each solution was mixed with 20 μL of reducing buffer (Roti®-Load 1, Carl Roth, Karlsruhe, Germany), and then heated at 99 °C for 5 min to denature protein complex. After that, 20 μL of this solution, as well as, protein standard solution with molecular sizes in range of 10−250 kDa (Precision Plus Protein™ Dual Color Standards, Bio-Rad Laboratories, CA, USA) were loaded onto a precast 4–20 % gradient polyacrylamide gel (Mini-PROTEAN® TGX™, Bio-Rad Laboratories, CA, USA). The electrophoresis was conducted at 90 V for 90 min. A Tris-glycine solution (25 mM Tris, 192 mM glycine, 0.1% SDS, pH ~8.3) was used as running buffer. After electrophoresis separation, the gel was placed in glacial acetic acid for 5 min for fixing proteins on the gel. Then, proteins on the gel were stained using Coomassie brilliant blue solution containing 10% acetic acid for 20 min, and destained using 0.5% $CuSO_4 \cdot 5H_2O$ in 10% acetic acid and 25% ethanol solution until the protein bands were visible.

5.2.6 Emulsion preparation

The freeze dried conjugate from 5.2.4 was used as an emulsifier for emulsion preparation. Emulsion which composed of 5% of MCT and 0.1% of emulsifier was prepared in duplicate. A continuous phase was prepared by dissolving 0.3 g of the emulsifier in 284.7 g of demineralized water at 30 °C, and adjusted to pH 5. While the continuous phase was homogenized at 5,000 rpm, 15 g of MCT was slowly dropped into the continuous phase. After adding all MCT, the emulsion was further homogenized at 5,000 rpm for 2 min. Then, the emulsion was transferred to a high pressure homogenizer (Microfluidizer® MF 110 EH, Microfluidics Corporation,

Newton, MA, USA) for preparing fine emulsion. The emulsion was emulsified at 40, 60, 80, and 100 MPa.

5.2.7 Droplet size measurement

Droplet size of the emulsion was measured by a laser diffraction particle size analyzer (LA-950, Horiba, Kyoto, Japan). The refractive indexes of 1.333 and 1.473 were used for the continuous and oil phases, respectively. The emulsion droplet size was expressed in term of the Sauter mean diameter, $d_{3,2}$ (μm).

5.3 Results and discussion

5.3.1 Color, pH, and solubility of the sample powder

Generally, when proteins and polysaccharides are mixed and heated, Maillard reaction can spontaneously occurred. In this work, subcritical water-hydrolyzed passion fruit pectins with difference molecular sizes were conjugated with WPI by dry-heating method. The appearance of powders changed from light brown to dark brown after heating for 6 to 48 h. This indicated that Maillard reaction proceeded at higher extents at longer heating time since brown color intensity is the easiest measurable consequence of the Maillard reaction (Spotti et al., 2013; Wang & Zhong, 2014; Wang, Bao, & Chen, 2013). As can be seen from Fig. 5.1A, distinctive increase in browning intensities of whey protein conjugated with lower molecular weight pectin (SPec, 20 kDa) was also observed. In Chapter 3, we reported that the lower molecular weight of pectin, the higher reducing end group. This reactive carbonyl group can accelerate the rate of browning reaction (Spotti et al., 2013). After the dry-heating treatment, the samples were dissolved in water to estimate their pH values. The results showed that the pH value decreased with the heating time (Fig. 5.2A). The decrease of pH during glycation of WPI and low methoxyl pectin by dry-heating at 60 °C and 74% RH was also reported by Setiowati et al. (2016), and the authors suggested that the intermediate acidic compounds generated during Maillard reaction resulted in pH decreasing.

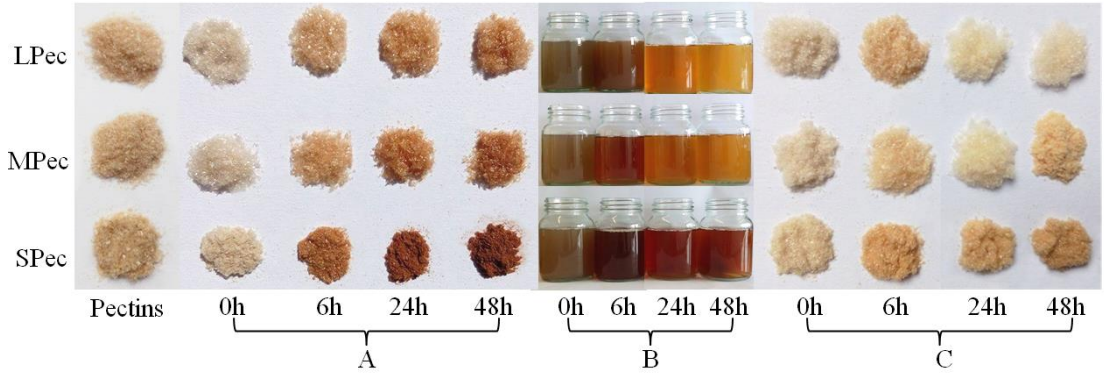

Figure 5.1 Color changing of samples: A) samples with conjugation under dry-heating condition at 0, 6, 24, and 48 h, B) soluble fractions at pH 5, and C) freeze-dried soluble fractions at pH 5.

After pH measurement, the sample suspensions were adjusted to pH 5 and then centrifuged to separate the insoluble solids. The soluble fractions and powders obtained after freeze-drying were shown in Fig. 5.1B and Fig. 5.1C, respectively. It can be seen that the brown color intensities of freeze-dried soluble fractions of the samples decreased. This may be because the brown pigments were removed after centrifugation. At the final stage of the Maillard reaction, melanoidins which are high molecular weight brown pigments are produced via polymerization. Some of these pigments are completely insoluble in water (Cömert & Gökmen, 2017; Nunes & Coimbra, 2007).

It is well known that the solubility of a protein decreases at the pH around its isoelectric point (pI) where the net charge of the protein is about zero. The pI of WPI was around pH 5.2 (Ju & Kilara, 1998). Martinez-Alvarenga et al. (2014) reported that the solubilities of unheated WPI and dry-heated WPI at pH 5 were 85 and 27%, respectively which indicated that after heat-drying, WPI losses their hydrophilicity. In this study, it was found that the unheated WPI-Pectin mixtures (0 h of heating) showed their solubilities about 97% for all types of pectin as shown in Fig. 5.2B. This suggested that the Maillard reaction between WPI and pectins did not occur. High solubility (93%) of unheated WPI-Pectin mixture at pH 5.5 was also reported by Neirynck et al. (2004).

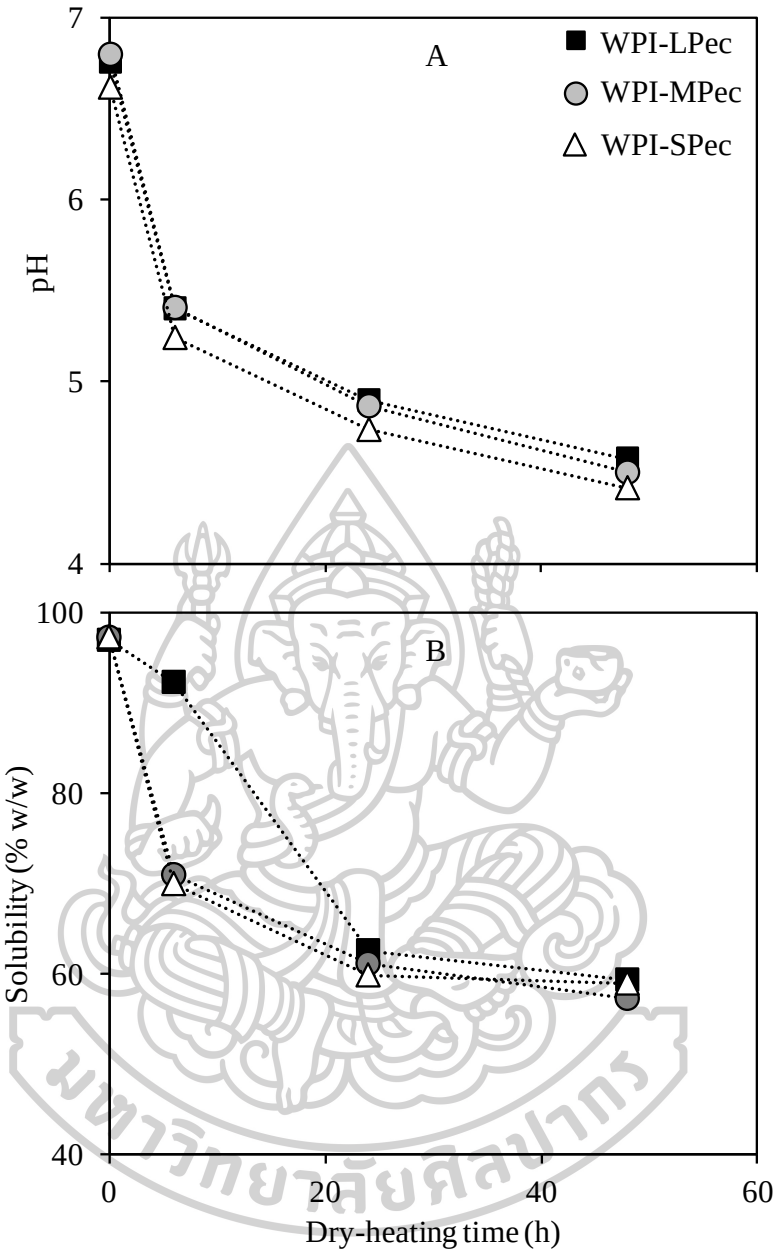

Figure 5.2 pH (A) and solubility (B) of WPI-Pectin mixtures treated under dry-heating condition at 0, 6, 24, and 48 h.

After 6 h of heating, the solubility of WPI-LPec slightly decreased from 97 to 92%, whereas the solubilities of WPI-MPec and WPI-SPec sharply decreased from 97 to 71 and 70%, respectively. This possibly due to the fact that the longer-chain pectin possesses higher number of hydrophilic groups than the shorter-chain ones, resulting in higher soluble fractions. Nevertheless, the solubility of WPI-LPec

dramatically decreased at 24 h of heating. This might be because not only Maillard reaction, but also pectin degradation were occurred in the systems, resulting in an increasing of shorter-chain pectin which could promote the Maillard reaction. The slight decreases of solubilities were observed with longer heating time regardless of the pectin molecular size, the solubilities were approximately 60% for all WPI-Pectin mixtures at 48 h of heating, which indicated that the effect of pH dominated the effect of pectin size. Compared to dry-heated WPI alone (Martinez-Alvarenga et al., 2014), our results indicated that the conjugation of WPI with subcritical water-hydrolyzed pectins gave the compounds with higher water solubility which might cause by the increasing of hydroxyl groups from pectin structure.

5.3.2 SDS-PAGE

The soluble solid fractions were also characterized by SDS-PAGE analysis comparing with native WPI and protein standards as shown in Fig. 5.3. The SDS-PAGE gel indicated that all unheated samples (0 h) showed the molecular size distribution patterns similar to native WPI with four noticeable bands, corresponding to α-lactalbumin (14 kDa), β-lactoglobulin (18 kDa), β-lactoglobulin dimer (37 kDa), and bovine serum albumin (67 kDa) (Schmidt et al., 2016; Setiowati et al., 2016). This indicated that no electrostatic complexes between WPI and pectin were formed (Neirynck et al., 2004). However, for 6 h of heating, the gel showed polydisperse uniform band of molecular size distribution for all samples from 14 kDa to higher than 150 kDa, but the polydispersed-band seemed to be fading as the pectin molecular size was lowered. Generally, disulfide polymerization of protein can occur by heat-treatment and resulted in protein with higher molecular weight (Belitz, Grosch, & Schieberle, 2009). However, because the reducing condition was conducted in this SDS-PAGE, disulfide bonds in protein structure were eliminated. In addition, pectin molecule was not detected by SDS-PAGE analysis (Neirynck et al., 2004; Schmidt et al., 2016). Therefore, it could be concluded that the high molecular weight compounds found in this study were from conjugation between WPI and subcritical water-hydrolyzed pectin. However, longer heat treatment resulted in higher protein precipitation, therefore the fading of protein bands were observed at 24 and 48 h of heating.

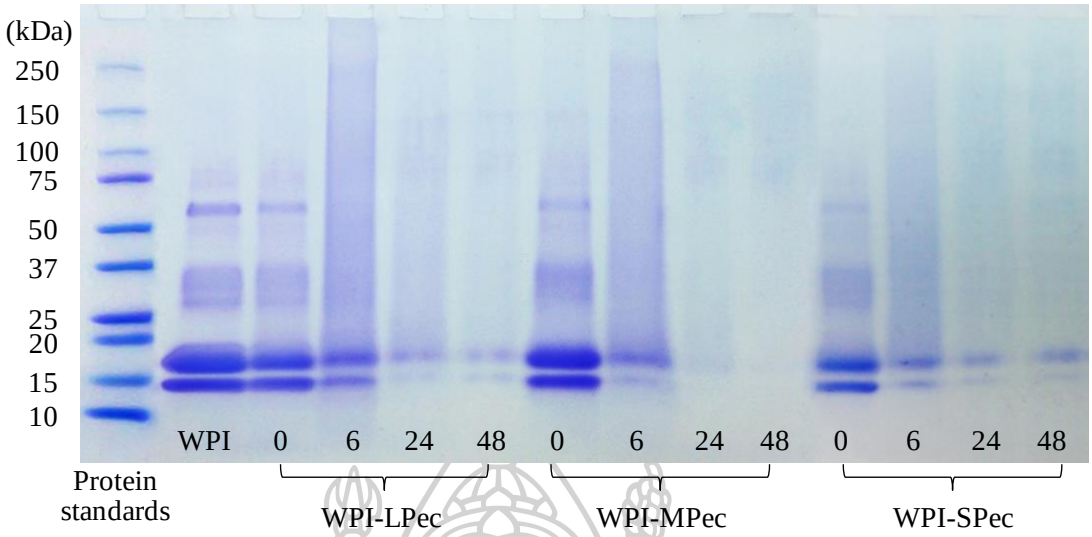

Figure 5.3 SDS-PAGE gel of the native WPI, WPI-Pectin mixtures (0 h), and WPI-Pectin conjugates treated at 80 °C, 79% RH for 6, 24, and 48 h.

5.3.3 Emulsifying performance

To produce emulsion with smaller droplet size, high-energy emulsification method, microfluidization was applied. Emulsification were performed at 40, 60, 80, and 100 MPa which correspond to the energy density (E_v) of 40, 60, 80, and 100 $MJ \cdot m^{-3}$, respectively (Jafari, He, & Bhandari, 2007). The results in Fig. 5.4 shows that the Sauter mean diameters ($d_{3,2}$) of the emulsions added with all conjugates obtained from 6 h of heating gave emulsions with small droplet size lower than 1.0 μm and the droplet sizes tended to decrease when the emulsification pressure was increased. In contrast, the conjugates obtained from 48 h gave the emulsion droplet sizes higher than a micrometer except for the WPI-LPec. This might cause by the depletion of whey protein when conjugating with lower molecular weight pectin and longer heating time as indicated by SDS-PAGE analysis. In addition, all types of conjugates gave emulsion droplet sizes lower than using only hydrolyzed pectin ($d_{3,2}$ = 3.7−6.2 μm) or WPI ($d_{3,2}$ > 10 μm) as emulsifiers.

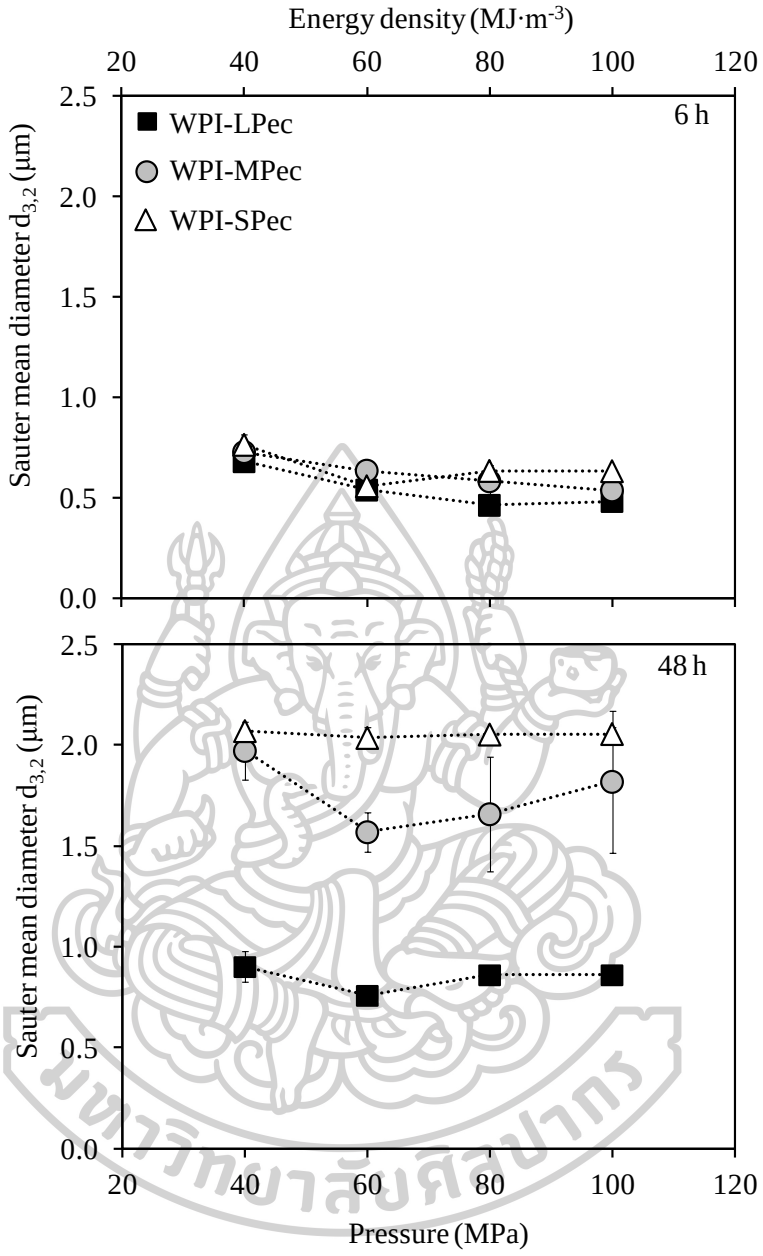

Figure 5.4 Sauter mean diameter ($d_{3,2}$) of emulsion droplet produced from WPI-Pectin conjugates obtained from dry-heating of WPI with 20 (SPec), 77 (MPec), and 146 (LPec) kDa subcritical water hydrolyzed pectin at 6 and 48 h and different emulsification pressures.

Figure 5.5 clearly showed that the conjugation at 6 h resulted in high surface activity conjugates and therefore gave the smallest emulsion droplet sizes. The increases of $d_{3,2}$ were observed in the emulsions added with the conjugates obtained

from 24 and 48 h of heating. These may due to the flocculation or coalescence of disrupted droplets were occurred, resulting from the absorption rates of emulsifiers onto the created interface were slower than the collision rates of droplets which particularly increased during homogenization (McClements, 2016). Long-time heat treatment (48 h) resulted in the conjugates with lower surface activity which corresponding to protein denaturation (Belitz et al., 2009; McClements, 2016).The larger increase of $d_{3,2}$ also occurred when using smaller pectin conjugates. The smaller size of polysaccharide can easily conjugate with protein and therefore long time reaction lead to losing of surface activity (Jiménez-Castaño et al., 2007).

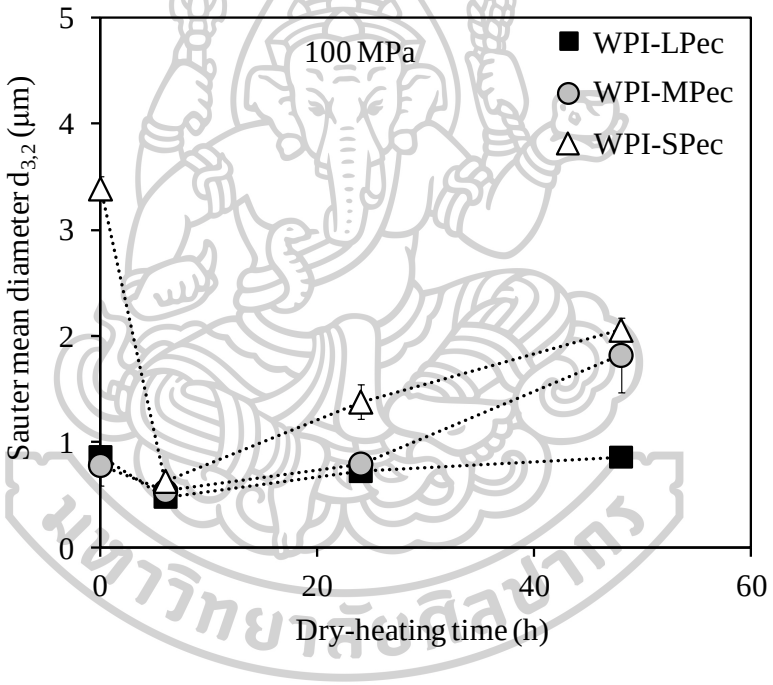

Figure 5.5 Sauter mean diameter ($d_{3,2}$) of emulsion droplet produced from WPI-Pectin conjugates obtained from dry-heating of WPI with 20 (SPec), 77 (MPec), and 146 (LPec) kDa subcritical water hydrolyzed pectin at 100 MPa and different conjugation times.

Because antibacterial chemical was not added, the emulsions were stored in a refrigerator at 4 °C, and then the stability of emulsions was evaluated by comparison of the emulsion droplet sizes on the first day (day 1) and 28 days after emulsification (day 28) as shown in Fig. 5.6. WPI-LPec and WPI-SPec conjugates obtained from 6 h

of heating gave obviously high emulsion stability. On the other hand, conjugates at 48 h showed significant increase in emulsion droplet sizes, especially the emulsion with WPI-SPec conjugate. Interestingly, the droplet size of emulsion added with unheated WPI-LPec mixture did not significantly change at day 28. This may because the steric hindrance of pectin portion helped stabilizing the emulsion. The smaller change in droplet size of emulsion stabilized by high molecular size citrus pectin (146.3 kDa) compared with the one stabilized by low molecular size (47.8 kDa) were observed after a storage period of 50 days (Akhtar et al., 2002); moreover the authors also reported that too much depolymerized pectin produced very thin adsorbed layers which unable to prevent coalescence. Therefore, it could be concluded the WPI-Pectin conjugates obtained from too small size pectin as well as too long dry-heat treatment durations resulted in poor emulsifying performance.

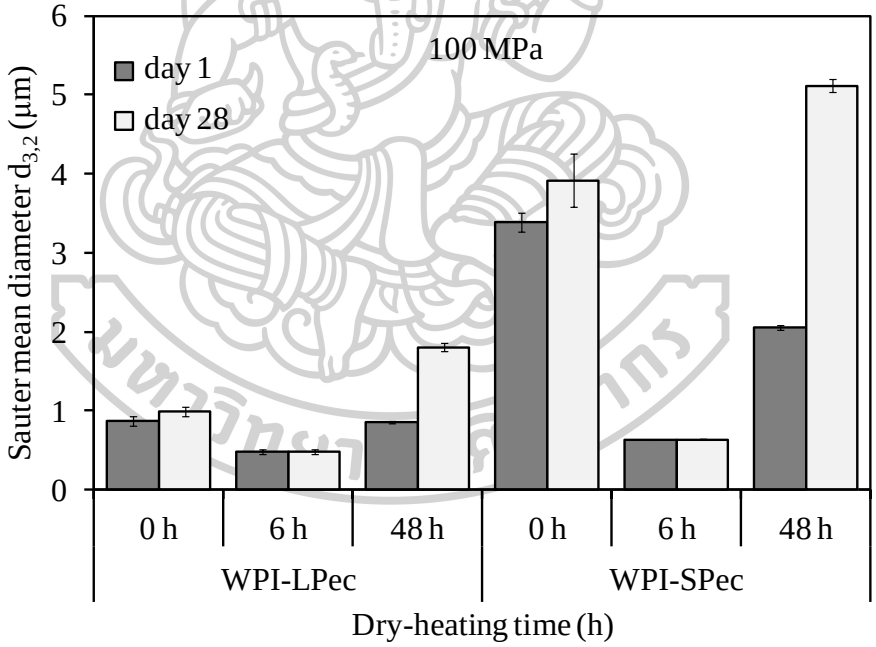

Figure 5.6 Sauter mean diameter ($d_{3,2}$) of emulsion droplet produced from WPI-Pectin conjugates obtained from dry-heating of WPI with 20 (SPec) and 146 (LPec) kDa subcritical water hydrolyzed pectin on the first day (day 1) and 28 days (day 28) after emulsification.

5.4 Conclusions

The study in this chapter investigated, for the first time, emulsifying performance of subcritical water-hydrolyzed pectins conjugated with WPI via Maillard reaction. The results demonstrated that the conjugates obtained by the dry-heating treatment at 80 °C, 79% RH, 6 h gave the small emulsion droplet sizes lower than 1.0 μm with good stability during 28 days of storage even though the emulsion was performed at pH 5 which close to pI of WPI. This suggested that subcritical water-hydrolyzed pectin can be used for improving the emulsifying property of WPI in emulsion with pH close to its pI.

CHAPTER 6

Summary of findings, conclusions, and recommendations

This study showed that subcritical water can be used for the extraction of pectin from passion fruit peel with a production yield comparable to that obtained using conventional method but within a shorter time. Adding ethanol into subcritical water resulted in decreasing of water polarity as well as water acidity and therefore lowering the pectin yield. However, adding ethanol was advantage for antioxidant activity of the obtained pectin because some phenolic compounds were co-extracted. In addition, subcritical water extraction resulted in low-molecular weight pectin which was not suitable for using as gelling or thickening agents.

In order to understand the degradation process of pectin in subcritical water, the degradation of passion fruit pectin in a continuous flow-type reactor was kinetically studied. The results showed that depolymerization of pectin could be modeled by the Emsley equation, whereas the formation of reducing end groups could be expressed by zeroth-order kinetics. The temperature dependency of the degradation rate constants could be expressed by the Arrhenius equation. Kinetics study provided an important information on controlling the degradation extent of pectin in subcritical water.

Even though subcritical water treatment reduced the size of pectins, the DE of all hydrolyzed pectins were higher than 50%. Water absorption isotherms of hydrolyzed pectins were unchanged at $a_w \leq 0.75$ and could be well described by the GAB model. Additionally, this study also showed that pectin could aggregate in distilled water which resulted in the overestimation in intrinsic viscosity as well as the molecular size by size exclusion chromatography but this effect could be prevent by adding salts to the solution.

Finally, application of subcritical water-hydrolyzed pectin was demonstrated. The present study showed that subcritical water-hydrolyzed pectins conjugated with WPI via Maillard reaction had a potential for improving the emulsifying property of WPI at the pH around its pI.

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Klinchongkon, K., Chanthong, N., Ruchain, K., Khuwijitjaru, P., & Adachi, S. (2017). Effect of ethanol addition on subcritical water extraction of pectic polysaccharides from passion fruit peel. *Journal of Food Processing and Preservation, 41*(5), e13138. doi:10.1111/jfpp.13138

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Emulsifying properties of conjugates formed between whey protein isolate and subcritical-water hydrolyzed pectin (to be submitted to *Food Hydrocolloids*)

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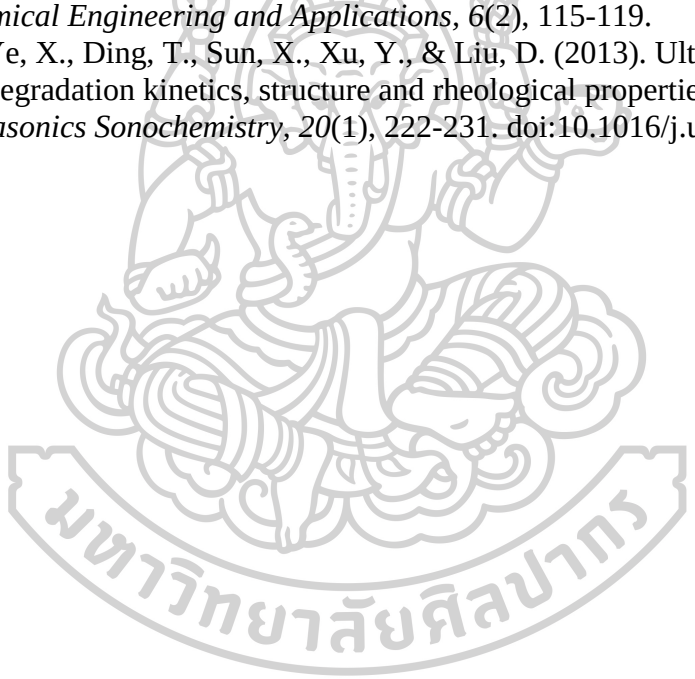

APPENDIX

Figure A1 Preparation of passion fruit peel powder: A) fresh passion fruits, B) peels without pulps and seeds, and C) dried-peel powder.

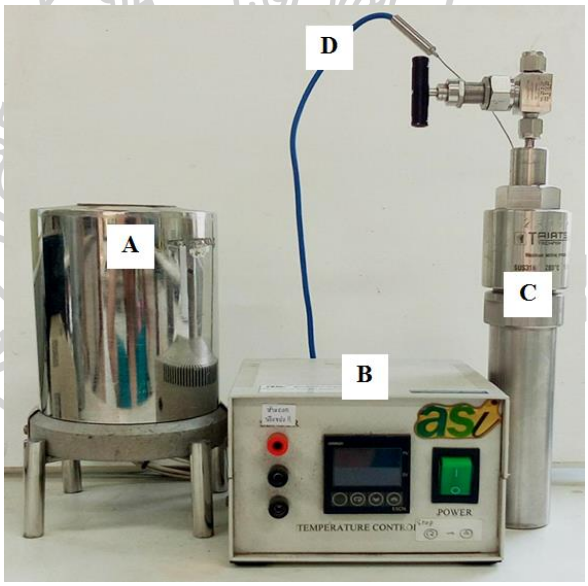

Figure A2 Subcritical water instrument for experiment in Chapter 2: A) block heater, B) temperature controller, C) stainless steel vessel, and D) type K thermocouple.

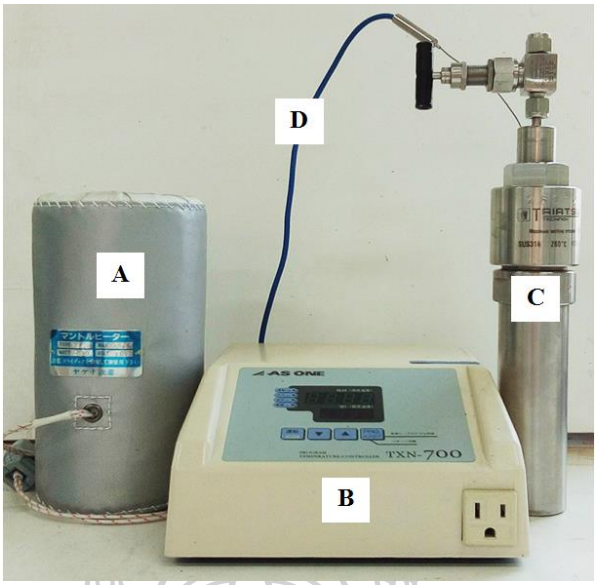

Figure A3 Subcritical water instrument for experiments in Chapters 4 and 5: A) mantle heater, B) temperature controller, C) stainless steel vessel, and D) type K thermocouple.

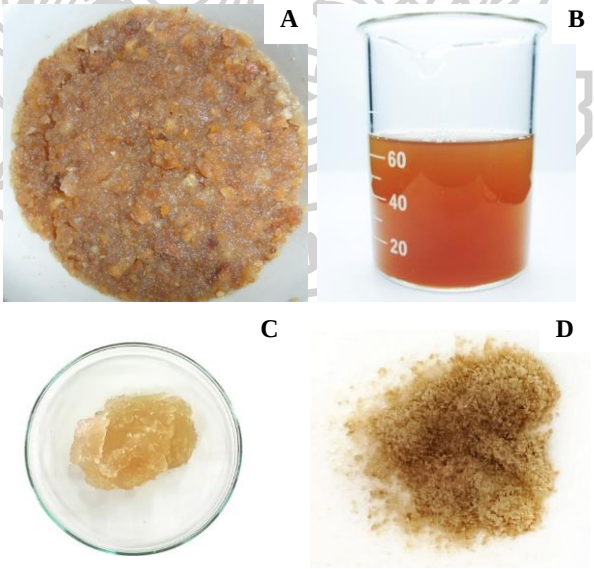

Figure A4 Subcritical water extraction of pectin from passion fruit peel performed Chapter 2: A) solid residues after filtration, B) hydrolysate, C) pectin after ethanol precipitation, and D) dried-pectin powder.

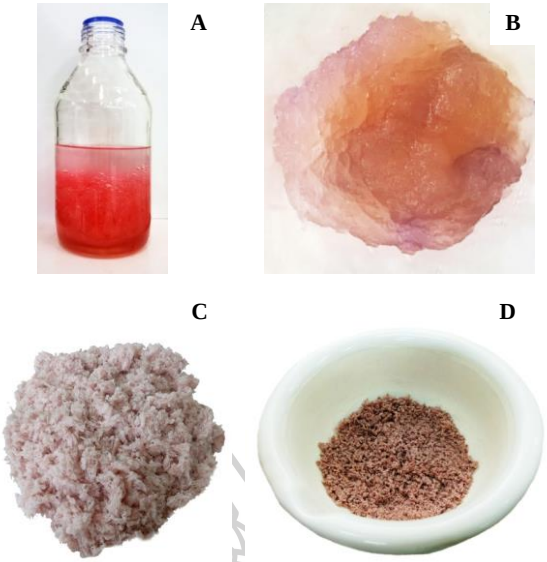

Figure A5 Extraction of pectin from passion fruit peel using hot dilute nitric acid: A) pectin precipitation using absolute ethanol, B) pectin after ethanol precipitation, C) pectin after washing with absolute ethanol, and D) dried-pectin powder.

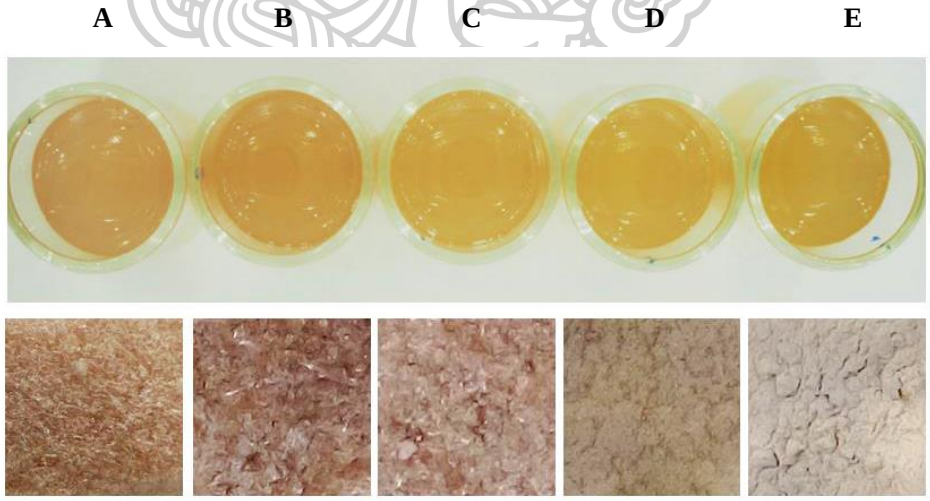

Figure A6 Subcritical water-hydrolyzed passion fruit pectin from Chapter 4. Initial pectin or non-hydrolyzed pectin (A) and pectin hydrolyzed at ln R_o=1.3 (B), ln R_o=2.7 (C), ln R_o=4.1 (D), and ln R_o=5.8 (E). Pectin hydrolysate (upper row) and freeze-dried pectin hydrolysate powder (lower row).

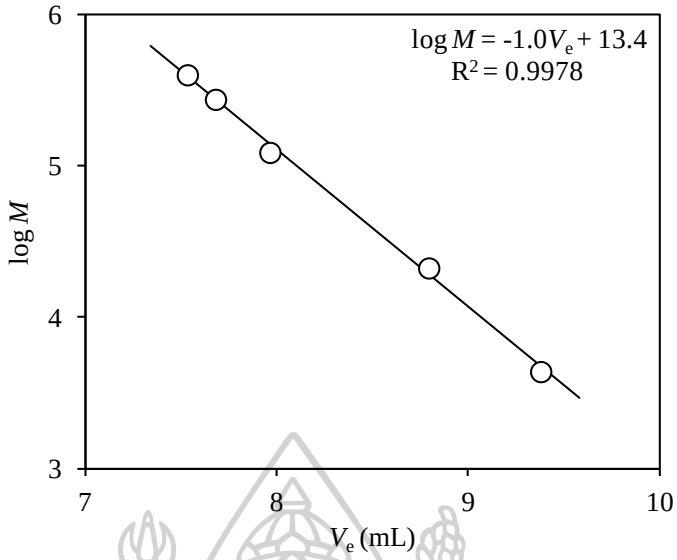

Figure A7 Dextran standards curve used in Chapter 2. The curve shows relationship between logarithm of molecular weight (log *M*) and elution volume (V_e) corresponding to each dextran chromatographic peak.

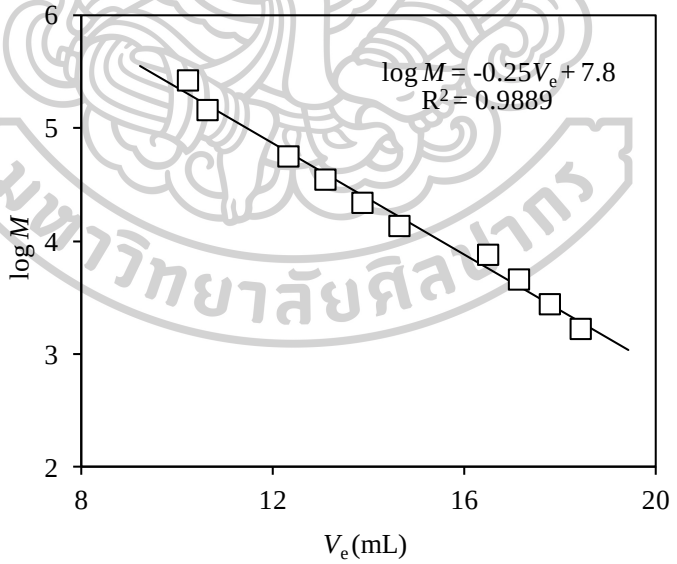

Figure A8 Dextran standards curve used in Chapter 4. Because the dextrans used in this study was not the narrow molecular weight standards, a log-normal model was used to estimate the molar frequency distribution of those dextrans, as described in Cabaniss, Zhou, Maurice, Chin, and Aiken (2000). The relationship between log *M* and V_e was achieved via cumulative frequency calculation.

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Conference

1) Khuwijitjaru, P., Khuhaprema, S., Thongphu, A., Klinchongkon, K., & Adachi, S. (2017). Extraction of pectins from mango, pomelo, lime, and passion fruit peels by subcritical water treatment. 18th Annual Meeting of Japan Society for Food Engineering, Kansai University, Osaka, Japan.

2) Klinchongkon, K., Khuwijitjaru, P., & Adachi, S. (2016). Depolymerization kinetics of passion fruit pectin in subcritical water. JSBBA Kansai 3rd Student Forum, Takigawa-kaikan, Kobe University, Kobe, Japan.

3) Klinchongkon, K., Khuwijitjaru, P., & Adachi, S. (2016). Kinetics on hydrolysis of passion fruit pectin in subcritical water. Wageningen University – Kyoto University International Exchange Program − Seminar on Food Science by Young Researchers, Kyoto University, Kyoto, Japan.

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8) Klinchongkon, K., & Khuwijitjaru, P. (2013). Carbohydrate composition of product from subcritical water treatment of passion fruit peel. The 27th National Graduate Research Conference, Naresuan University, Phitsanulok, Thailand.

9) Klinchongkon, K., Khuwijitjaru, P., Wiboonsirikul, J., & Adachi, S. (2012). Subcritical water extraction of carbohydrate from passion fruit peels. 13th Japan Society

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10) Klinchongkon, K., Khuwijitjaru, P., Wiboonsirikul, J., & Adachi, S. (2012). Subcritical water treatment of passion fruit peel. Food Innovation Asia Conference 2012, BITEC, Bangkok, Thailand.

AWARD RECEIVED

1) "The Most Influential Presentation" in the JSBBA (Japan Society for Bioscience, Biotechnology and Agrochemistry) Kansai 3rd Student Forum, Kobe, Japan. November 5th, 2016.
2) "Poster Award" (3rd place) in the 13th National Postharvest Technology Conference, Nakhon Ratchasima, Thailand. June 18th-19th, 2015.