ENZYME-ASSISTED EXTRACTION AND PHYSICO-CHEMICAL CHARACTERIZATION OF PECTIN-LIKE POLYSACCHARIDES FROM *THEOBROMA* COCOA POD HUSKS

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by

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
CDTA	cyclohexane diamino tetraacetic acid
CP	citrus pectin
CPH	cocoa pod husks
CPHF	cocoa pod husks flour
DE	degree of esterification
DPPH	1,1-diphenyl-2-picryhydrazyl
DPPHsc	1,1-diphenyl-2-picryhydrazyl scavenging
EDTA	ethylene diamino tetraacetic acid
Fig.	figure
FL	fibre layer
FRAP	ferric reducing antioxidant power
FTIR	Fourier transform infrared spectroscopy
GAE	gallic acid equivalent
GalA	galacturonic acid
g	gram
h	hour
HCl	hydrochloric acid
HG	homogalacturonan
HMP	high methoxyl pectin
IDF	insoluble dietary fibre
IP	inner parenchyma
IPPA	International Pectin Producers Association
Kcal	Kilocalories
LMP	low methoxyl pectin
MAE	microwave-assisted extraction
min	minute
mg	miligram
OHC	oil holding capacity
OP	outer parenchyma
p	probability value
PP	Pectin-like polysaccharide
PPs	Pectin-like polysaccharides
r	Pearson correlation coefficient

RG I	type I Rhamnogalacturonan
RG II	type II Rhamnogalacturonan
SD	standard deviation
SDF	soluble dietary fibre
SDS	sodium dodecyl sulphate
SEM	scanning electron microscope
TPC	total phenolic content
TPTZ	2,4,6-tris(2-pyridyl)-s-triazine
UAE	ultrasound-assisted extraction
US	United States
UK	United Kingdom
U	unit
WSP	water soluble polysaccharide

LIST OF SYMBOLS

~	approximately
%	percentage
µ	micro
ºC	degree of Celsius
&	and
α	alpha
β	beta
=	equal to
<	less than
>	greater than
±	plus and minus
-	not available

PENGEKSTRAKAN BANTUAN ENZIM DAN PENCIRIAN FIZIKAL-KIMIA POLISAKARIDA BERSIFAT PEKTIN DARIPADA KULIT KOKO *THEOBROMA*

ABSTRAK

Polisakarida bersifat pektin telah diekstrak daripada sabut kulit koko. Kajian ini bertujuan untuk membangunkan satu pendekatan pengekstrakan bantuan enzim yang lestari untuk meningkatkan hasil pengekstrakan. Beberapa keadaan pengekstrakan (jenis enzim, kepekatan enzim, pH dan suhu) telah dikaji dan diikuti dengan pencirian polisakarida bersifat pektin berdasarkan ciri-ciri antioksidan dan fiziko-kimia. Hasil kajian menunjukkan bahawa hasil pengekstrakan yang tinggi sehingga 41.5% telah dicapai dengan kandungan asid galakturonik dalam lingkungan 5.14 hingga 20.91% dan kandungan polisakarida dari 6.12 hingga 61.18%. Darjah esterifikasi yang berbeza iaitu dari 35 hingga 58% telah diperhatikan dalam polisakarida bersifat pektin. Selain itu, polisakarida bersifat pektin yang telah diekstrak didapati mempunyai penampung kapasiti minyak yang tinggi di antara 6.26 hingga 29.57 g minyak /g ekstrak. Ekstrak ini didapati mempunyai keupayaan untuk membentuk gel dan mempamerkan kelakuan ricih menipis bukan-Newtonian. Selain itu, didapati bahawa ekstrak mengandungi komponen fenolik dengan kandungan yang terdiri daripada 7.90 hingga 47.29 μg GAE/mg, peratusan 1,1-diphenyl-2-picryhydrazyl (DPPH) daripada 0.95 hingga 51.93% dan nilai FRAP adalah di antara 0.43 hingga 3.07 μmol/ mg ekstrak. Keputusan FTIR menunjukkan bahawa polisakarida bersifat pektin berbeza dengan piawai pektin limau komersial. Secara umum, pengekstrakan menggunakan hemisellulase dengan kepekatan 0.1% w/v pada 50°C dan pH 4.5 memberikan ciri-ciri yang lebih baik untuk polisakarida bersifat pektin dari segi hasil pengektrakan, jumlah kandungan polisakarida, penampung

kapasiti minyak dan aktiviti antioksida berbanding pengekstrakan menggunakan selulase. Oleh itu, dicadangkan bahawa kaedah pengekstrakan bantuan enzim adalah bersesuaian, mesra alam dan kos efektif untuk menghasilkan polisakarida bersifat pektin yang berfungsi dengan sifat antioksidan tambahan yang boleh digunakan dalam sistem makanan.

ENZYME-ASSISTED EXTRACTION AND PHYSICO-CHEMICAL CHARACTERIZATION OF PECTIN-LIKE POLYSACCHARIDES FROM *THEOBROMA* COCOA POD HUSKS

ABSTRACT

Pectin-like polysaccharides were extracted from cocoa pod husk. The aim of this study was to develop a sustainable enzyme-assisted extraction approach in order to improve the extraction yield. Several extraction conditions (i.e. type of enzyme, concentration of enzyme, pH and temperature) were investigated followed by characterization of the extracted pectin-like polysaccharide based on physico-chemical and antioxidant properties. The results showed that high extraction yield of up to 41.5% (w/w) with the content of galacturonic acid ranged from 5.14 to 20.91% (w/w) and polysaccharide contents ranged from 6.12 to 61.18% were obtained. Different degrees of esterification (35 to 58%) were observed in these pectin-like polysaccharides. Other than that, the extracted pectin-like polysaccharide was found to possess high oil holding capacity ranging from 6.26 to 29.57 g oil/g extract. These extracts were found to have an ability to form gels and exhibited non-Newtonian shear-thinning behavior. Also, it was found that the extracts contain phenolic component with content ranging of 7.90 to 47.29 µg GAE/mg, percentages of 1,1-diphenyl-2-picryhydrazyl (DPPH) from 0.95 to 51.93% and FRAP values ranged from 0.43 to 3.07 µmol/mg extract. FTIR results showed that the extracted pectin-like polysaccharide were different to the commercial citrus pectin standard. In general, extraction using hemicellulase with concentration of 0.1% (w/v) at 50 °C and pH 4.5 gave better properties to the pectin-like polysaccharide in terms of extraction yield, total polysaccharide content, oil holding capacity and antioxidant

activity compared to extraction using cellulase. Thus, it was suggested that enzyme-assisted extraction method was reliable, environmental friendly and cost effective method to produce functional pectin-like polysaccharides with additional antioxidant properties that could be applied in food system.

CHAPTER 1

INTRODUCTION

1.1 Research background

Theoroma cocoa L. (Sterculiaceae) is a domestic crop typically grown in the humid tropical countries for chocolate production. At present, Malaysia is the fifth largest cocoa processor in the world with an estimated increase area of cultivation by 2,000 hectares annually, growing up to 40,000 hectares almost doubled compared to 20,543 hectares in 2011 (Jackson, 2012). *Theoroma cocoa* L. consists of cocoa beans which constitute about 10% (w/w) of the total weight, is separated from the pod and processed into cocoa products (ICCO, 2011). After this separation process, the cocoa pod husks (CPH) which constitute between 52-76% (w/w) of the whole matured cocoa fruit is treated as waste (Donkoh et al., 1991).

3.53 million tons of CPH were discarded as wastes annually (World Cocoa Foundation, 2010). The waste produced pose an environmental problem due to the foul odors emitted during decomposition and also a probability source of plant disease inoculants (Figueira et al., 1993; Donkoh et al., 1991). Many attempts have been done to transform these agro-industrial by-products into useful products such as pectin (Masmoudi et al., 2008; Biliaderis et al., 2007; Wu et al., 2007). The alternative usages as feed for livestock and fertilizer is limited due to high theobromine content which is an anti-nutritional factor (Aregheore, 2002) and also a humus forming base factor (Prabhakaran, 2010).

To overcome this serious waste management of the CPH, it has been widely suggested that studies should look into the potential of processing the waste into potential marketable bio-products. Studies showed that CPH could be a source of pectin (Vriesmann et al., 2012; Ramli & Asmawati, 2011; Mollea et al., 2007) which has extensive applications in food industry as thickener, stabilizer, texturizer and emulsifier. Pectin is also extensively use in pharmaceutical and biomedical industries. The demand of pectin consumption continued to increase and now has exceeded more than 20,000 tons/year (Ptitchkina et al., 2008). Commercially available pectin is mainly manufactured from apple pomace and citrus peel, although many investigations have been conducted to extract pectin from other possible sources from agricultural wastes (Gan & Latiff, 2011; Liu et al., 2006). However, the low yield and low quality of pectin extracted have not fulfilled the industrial requirements. These attributes were due to the extraction methods and isolation conditions which are some of the major challenges in the procurement process from sources with low pectin content.

Generally, there are three ways to extract pectin: mechanical treatment, chemical, and/or enzymatic methods (Panouille et al., 2006) and by combinations of these methods. The common extraction methods to isolate pectin polysaccahrides includes conventional acid extractions (Yapo, 2009), Soxhlet method (Liu et al., 2006), ultrasound-assisted (Bagherian et al., 2011) and microwave extraction (Yeoh et al., 2008), autoclave method (Voragen et al., 2007) and extrusion assisted (Fishman et al., 2004). However, these extraction methods have few drawbacks which include giving low yield, require longer processing time and high energy consumption hence these methods were considered inefficient in producing pectin on

industrial scale. Furthermore, the use of acid will produce corrosive effluent that required further treatment which is hazardous to environment.

Instead of using conventional hot-diluted mineral acid extraction, the method used in this study is environmental friendly, extraction with combined water based or warm-organic acid (citric acid) with enzymatic treatments was used to produce safe pectin products. The enzymatic mediated method provides high yield, low cost and energy consumption (Li et al., 2006) and was reported to be more efficient, easy to perform and preserves the physicochemical characteristics of pectin without damaging its structures (Zu et al., 2009). This environmental friendly method also fulfil the consumers' demand for "green" products known as "green labelled" pectin (Panouille et al., 2006). Moreover the citric acid used in the study provides an economically and environmentally safe alternative (Canteri-Schemin et al., 2005). In addition, pectin with various abilities to gel or stabilize is in high demand, especially those types or derivatives with tailored properties in fruits and dairy products (Rosenbohm et al., 2003).

To date, there is no trial to extract pectin from CPH by combined chemical-enzymatic or water based-enzymatic extraction methods. The approach consists of using citric acid hydrolyze pod husk for liquefaction and releasing carbohydrates prior to enzymatic treatment by cellulase or hemicellulase. In addition, revelation by Mollea (2007) on the pectin obtained from whole and minced Ghana and Venezuela origin CPD by hot hydrochloric acid extraction showed the highest yield (9.0%) was obtained at pH 2.5 with one hour extraction incubation. This study recommended more researches should be performed on investigation of extraction variables and analysis of pectin to look into undesirable properties and to improve the quality through chemical alteration.

In this study, pectin-like polysaccharides were extracted from CPH with cellulase and hemicellulase enzymes, varying amount of enzymes and temperatures in the water and acid with varying pH were employed. The yield of pectin-like polysaccharides from crude CPH were assessed for its efficiency and the effect of various conditions on physico-chemical, functional and antioxidant properties of CPH pectin-like polysaccharides were discussed. Compared to other methods, combination of enzyme-mediated method in citrate acid and water based is anticipated to be an efficient and reliable technique for extraction of safe pectin-like polysaccharide products in future.

1.1. Objectives

The main objective of this study was to extract pectin from CPH which is considered as waste to value added product using enzyme-mediated approach. The specific objectives of this study were to:

1) Develop a method by using enzyme-mediated to extract pectin-like polysaccharides from CPH.
2) Evaluate the effects of extraction methods (enzymatic-acid and enzymatic-water) and various conditions (types of enzymes, concentration of enzymes, temperature and pH) on the yield of pectin-like polysaccharides from CPH.
3) Identify, characterize and compare the physico-chemical, functional and anti-oxidant properties of pectin-like polysaccharides from CPH with commercial citrus pectin.

CHAPTER 2

LITERATURE REVIEW

2.1. *Theobroma Cacao* L.

Theobroma cacao L. is a domestical crop under genus of flowering plants in the mallow family, belonging to the family of Malvaceae which originated from South America. *Theobroma* has been divided into twenty-two species in which *Theobroma cacao* L is the only species of commercial value. *Theobroma cacao* L. is from the family Sterculiaceae and is divided into three major distinct varieties which are Criollo, Forastero and their hybrid, Trinitario (Nagai et al., 2009).

The cocoa was discovered by the Mayas and Aztecs in South America who found the existence of cocoa believed to be *Theobroma cacao.* The fruit was considered as of divine origin and was presented during royal and religious events (Engler & Engler, 2006). The plantation of cocoa tree soon began to spread and was introduced to Indonesia and Sabah in the early 18^{th} century. The history of cocoa in Malaysia was first discovered with the finding of cultivated cocoa tree in Melaka, 1778 (Malaysian Cocoa Board, 2004). In 1853 to 1959, the most commonly cocoa, Amelonado type was first commercialized and cultivated at Jerangau, Terengganu involving a vast cultivation area of 403 hectares (Malaysian Cocoa Board, 2004).

Now, Africa is the world's largest producer of cocoa, producing 70% cocoa of total production, with Ivory Coast is the highest producer (40 % of Africa's total) followed by Ghana, Nigeria and Cameroon. While, Asia and Oceania collectively supply one fifths (19%) of the world cocoa and the rest of the world cocoa comes

from the America. The major producer in Asia and Oceania is Indonesia, followed by Malaysia and Papua New Guinea (Cocoa Market Update, 2010).

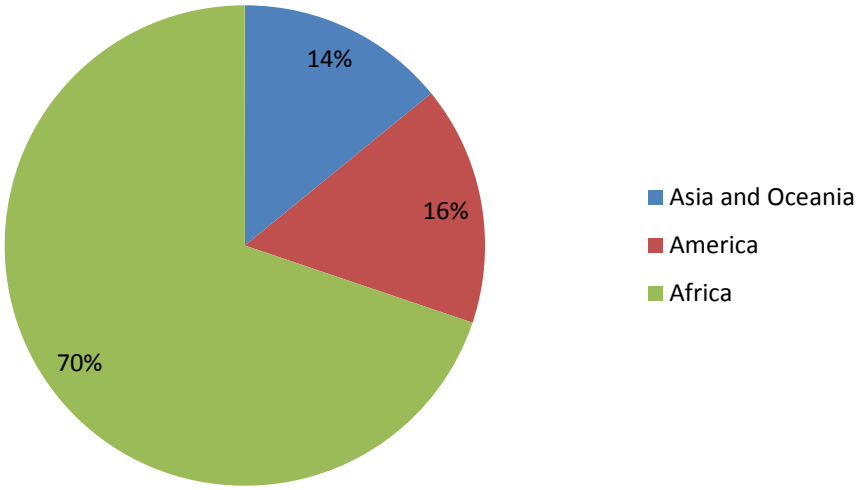

Figure 2.1 World production of cocoa. Africa: Ivory Coast (40% globally), Ghana, Nigeria, Cameroon, Asia and Oceania: Indonesia, Malaysia, Papua New Guinea, Americas: Brazil, Ecuador, Colombia (Cocoa Market Update, 2010).

Theobroma cacao L. is a long-lived tree with the height between 5 to 8 m tall. Normally, cocoa trees were grown under shady trees to resemble their natural habitat in the rainforest. The trees will start to bear fruits after five years, the fruits are 15 to 25 cm long, ovum-like shape and enclosed by red to brown pod with lumpy surfaces. The pod commonly consists of 30 to 40 seeds (or cocoa beans) which are surrounded by a mucilaginous pulp. The beans then will be removed, dried and fermented under direct sunlight. The cocoa beans will turn to brownish in colour and subsequently to be used in chocolate production (Young, 2007) or cocoa powder, as an important ingredient in baked goods (Lecumberri et al., 2007).

2.1.1 Cocoa pod husk as wastes and its chemical composition

With the beans removed, the cocoa pod which constitutes 52 to 76% of the cocoa total weight is regarded as the main by-product in industry and considered as waste. The large amount of waste is a threat to the environment and will increase the cost of waste disposal (Vriesmann et al., 2011a). According to Figueira et al., (1993), for each ton of dried cocoa beans produced, the cocoa industry will creates around ten tons of cocoa pod husks (CPH) and commonly discarded.

Cocoa fruit biomass can be categorized as two main components, the flesh and pod. As shown in Figure 2.2, the flesh and pod constitutes approximately 10 % and 52-76 % of the total weight, respectively.

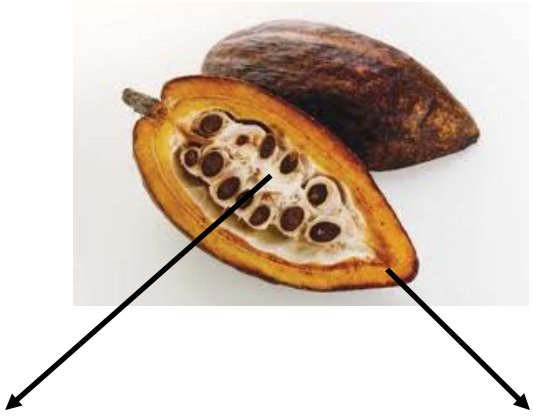

Figure 2.2 Breakdown of cocoa fruit biomass

It has been reported that the composition of CPH can be a potential source of dietary fibre, pectin and phenolic compounds (Vriesmann et al., 2011a). The content of polysaccharides types in CPH were determined as ~ 45% pectin polysaccharides, ~20% hemicelluloses and ~35% cellulose (Redgwella et al., 2003). CPH was found (gkg^{-1} dry matter) to contain 76.6 gkg^{-1} crude protein with major minerals such as calcium, potassium and sodium, 43.7 gkg^{-1} ether extract, 325.4 gkg^{-1} crude fibre, 101 gkg^{-1} ash, 414 gkg^{-1}acid detergent fibre, 522 gkg^{-1} neutral detergent fibre (Donkoh et

al., 1991), less soluble sugar content (lower than 0.5% dry matter basis) and 18.5% (dry matter basis) of fat content (Martin-Cabregas et al., 1994). Also, Aregheore (2002) and Fao (2002) have listed the composition of cocoa pod husks in their studies and the details of the chemical composition are shown in Table 2.1.

Table 2.1 Chemical compositions of cocoa pod husks

Component	**g/kg Dry Matter**
Dry matter	890
Crude protein	91.4
Organic matter	909
Crude fibre	337.4
Nitrogen-free extract	93.6
Ether extract	99.6
Neutral Detergent Fibre	598
Acidic Detergent Fibre	470
Total ash	91
Total sugar	33
Lignin	212
Hemicelluloses	128
Cellulose	262
Tannin	11.88
Nutrient content	**Kcal/kg**
Gross energy	4497
Amino-acids content	**% PROTEIN**
Lysine	5.1
Methionine	1.3
Cysteine	1.3
Minerals content	**% Dry Matter**
Calcium	0.37±0.07
Phosphorus	0.44±0.06
Potassium	2.68±0.19
Sodium	0.02±0.01
Magnesium	0.43±0.10
Copper	39
Chlorides (expressed in NaCl)	0.06±0.03

Source: Aregheore (2002) and Fao (2002)

The data from previous researches showed cocoa husk as a good source of dietary fibre with high dietary fibre such as non-starch polysaccharides (43.8 g/100 g), Klason lignin (13.7 g/ 100 g), mineral elements (10.7 g/100 g), residual level cocoa butter (43 g/100 g) and proteins (Serra Bonvehi & Escola Jorda, 1998). While, the non-starch polysaccharides consisted of cellulose (350g/kg), hemicelluloses (110g /kg) and pectin (60 g/kg) (Agyente-Badu & Oddoye, 2005).

According to Bravo (1998), polyphenolic compounds are generally embedded in the outer portions of plants including the shells and skins. Lecumberri et al. (2007) reported a total polyphenolic compounds in cocoa husks are approximately 6% of dry matter in which 1.32% are soluble polyphenols and 4.46% are found to be condensed tannins.

2.2 Plant cell walls

According to Darvill et al. (1980), plant cell wall is surrounded by hydrated wall consisted of complex of carbohydrates, glycoproteins and phenolics. The main consistuents are lignin, cellulose, hemicelluloses and pectins (Figure 2.3). While the complex polysaccharides consist of cellulose fibres with the attachment of xyloglucan (hemicellulose). The fibres in turn are surrounded by matrices of pectin, polygalacturonic acid and rhamnogalacturonan. The proximate composition of cell wall polysaccharides is varied depends on the crop or fruit sources as shown in Table 2.2.

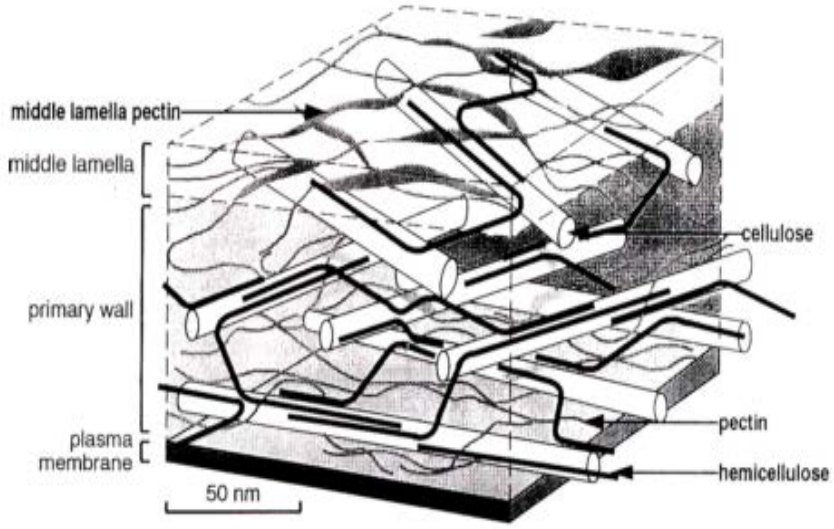

Figure 2.3 General model of primary plant cell wall (McCann & Roberts 1991)

Table 2.2 Proximate composition of plant cell wall polysaccharides of some plant material (% by weight of cell wall dry matter)

Plant materials	Pectin	Hemicellulosic			α-cellulose
		Xyloglucan	Arabinoxylan	Mannan	
Apples	31	-	20	-	40
Tomatoes	55	-	15	-	30
Carrot	37	16	11	16	21
Banana	20	-	40	-	30
Coconut kernel	6-8	-	-	61	-
Rape seed	39	29	-	-	22
Corn gem	<1	-	40	-	39
Sunflower	24	4.5	-	5	42
Palm kernel meal	-	3	3	78	12

Source: Olsen (1995)

2.2.1 Cellulose

Cellulose is the basic structure of the plant cell wall. It is also the major constituents of polysaccharides around 90% (Greil, 2001). The structure of cellulose is composed of repeating β-D-glucopyranosyl residues linked (1→4) together by hydrogen bonds to build microfibrils. However, (1,4)-β-glucans have a distinct physical organization in fibrillar structures depends on the sources (BeMiller, 2008). Figure 2.4 shows the basic structure of cellulose which is known to have great strength, fibrous, insolubility and inertness characteristics.

The presence of three free hydroxyl groups on each monomeric residue contributed to high tensile strength of inter (2 per glucopyranosyl) and intramolecular (2~3 per glucopyranosyl) hydrogen bonds among the units creates stable supramolecular fibres (Gardner & Blackwell, 1974). The combinations of hydrogen bonds and van der Waal's attractions between adjoining cellulose molecules creates a parallel arrangement and a crystalline unit which is resistant to hydrolysis.

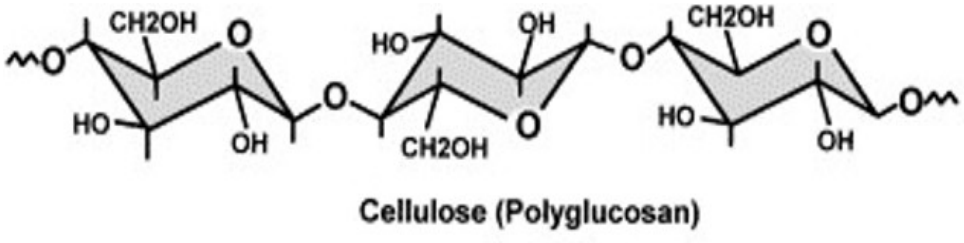

Figure 2.4 Fundamental structure of cellulose. Adapted from Greil (2001)

2.2.2 Hemicelluloses

Other than cellulose and lignin, there are hemicelluloses that are also known as araban. These galacturonate (GalA) are free group of polysaccharides that constitutes 20 to 30% dry weight in softwood (Singh & Gross, 2001). Hemicellulose consists of branched chain compounds with major units of α-1,5-linked L-arabinose and the side chain by α-1,3-linked L-arabinose (Vogel, 1991) with D-xylose, galactose and glucose as the major sugar constituents (Kobayashi et al., 1993).

This neutral or slightly acidic polysaccharide can be extracted from the plant cell wall with strong alkaline such as NaOH (Selvendran & O'neill, 1985). Hemicellulose cross linked with lignin to strongly attach to microfibrils in plant cell walls. Hemicellulose is less resistant to enzyme action compared to cellulose due to the crystalline structure of the latter (Eveleigh et al., 2009). Both play the major role as structural polysaccharides and/or energy reservoir. The fundamental structure of hemicelluloses is shown in Figure 2.5.

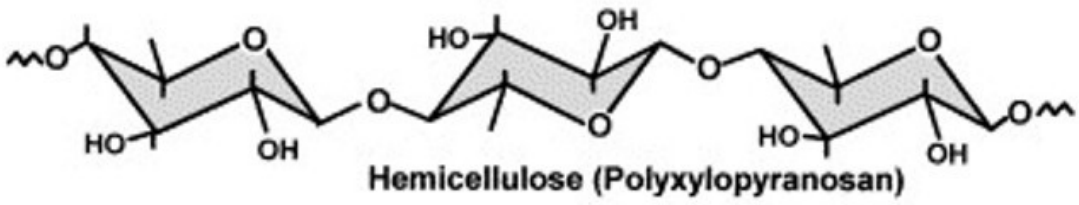

Figure 2.5 Fundamental structure of hemicellulose (Greil, 2001)

2.2.3 Pectin

2.2.3 (a) Structure and chemical composition

Pectin is a galacturonate (GalA)-rich, highly complex structural group of heteropolysaccharides. Pectin act as cementing material in the middle lamellae and thickening in the primary cell walls between cells of primary and secondary walls as well as xylem and fibre cells in woody tissue (BeMiller, 2008). It consists of 'smooth' region of a linear polygalacturonic portion and a branched region which is linked to hemicelluloses matrix. Pectin can be solubilized from plant cell walls by aqueous buffers, dilute mineral acids and calcium chelators (Yapo, 2011).

According to Mohnen (2008), pectin-like polysaccharides are a group of three polysaccharide: homogalacturonan (HG), type I Rhamnogalacturonan (RG I) and type II Rhamnogalacturonan (RG II). HG or smooth region (Figure 2.6) is the major structural domain of pectin-polysaccharide composed of a linear homopolymer chain composed of 1,4-linked α-D-galactopyranosyluronic acid units (α-$_D$-GalA) and represent around 65% of pectin (Ridley et al., 2001). Ralet et al. (2003) mentioned that the α-$_D$-GalA units with some of carboxyl groups (–COOH) are often methyl-esterified at O-6 and sometimes acetyl-esterified at C-2 and/ or C-3 in some plants. RG I that constitutes around 20 to 35% of pectin polysaccharides consists of a backbone of the repetitive disaccharide unit: (1→4)-α-D-GalA-(1→2)-α-L-Rha-, predominantly substituted at O-4 of Rha residues by neutral sugar side chain such as arabinan and galactan (Willats et al., 2006). Meanwhile, type II rhamnogalacturonan (RG II), the complex polysaccharide structure in pectin constitutes only minor of pectin (around 10%).

Pectins are classified based on their degree of esterification (DE). DE affects functional properties of pectin such as gelling mechanism. According to Willats et al. (2006), GalA in HG have different methyl-esterified and acetylated unit until to a certain degree, depends on sources. Based on the degree esterification and acetylation value, carboxyl groups esterified in pectin more than 50% are categorized as high methoxyl pectins (HMP), whereas pectin with carboxyl groups esterified less than 50% are categorized as low methoxyl pectin (LMP).

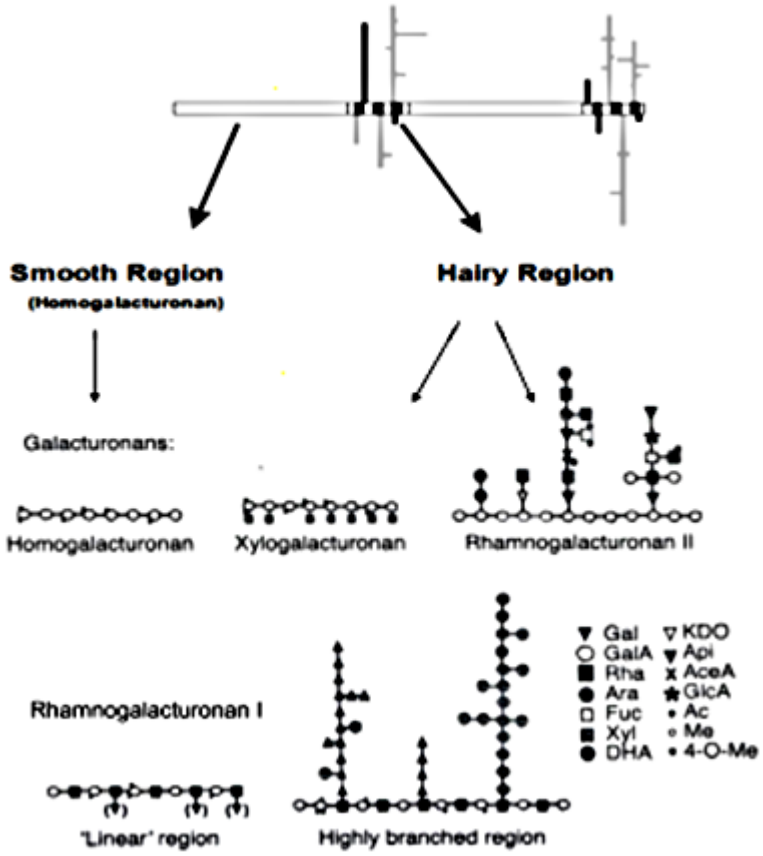

Figure 2.6 Structural elements of pectin (Voragen et al., 2001)

HMP requires co-solute such as sugar at concentration ≥ 55% and acidic condition (below ~ 3.5) for the formation of gel (Oakenfull & Scott, 1984). On the contrary, LMP needs divalent ion such as calcium (Iglesias & Lozano, 2004) within a range of pH 2.0-7.0 whether in presence or absence of sugar to form gel. Pectin solubilized by treatments with aqueous buffers and dilute acidic solutions or in the presence of calcium chelators.

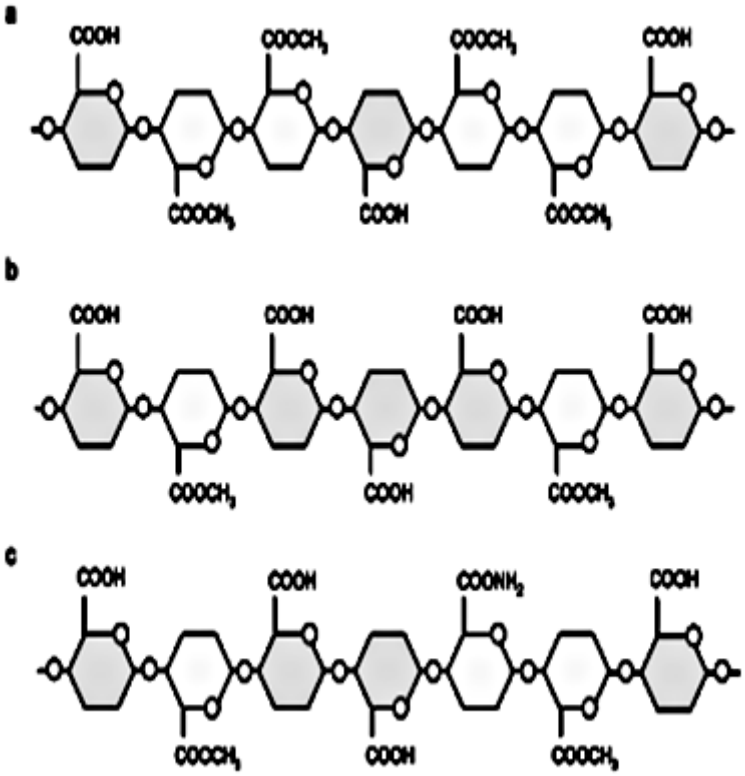

Figure 2.7 **Types of pectin**: (a) HMP (DE 70%), (b) LMP (DE 40%), (c) LM amidated pectin (DE 40%). Adapted from Yuliarti (2011)

2.2.3 (b) Pectin classes

In general, pectin is classified into three classes based on its extraction procedures from the plant cell wall. Extraction is either with mild, pH neutral extractor like water or with cyclohexane diamino tetraacetic acid (CDTA), sodium dodecyl sulphate (SDS) or with alkaline extractor like Na_2CO_3 and KOH. The classes of plant are water-soluble pectin, chelator-soluble pectin and protopectin (Van Buren, 1991). Water-soluble pectin is termed as pectin that is extractable with water or dilute salt solutions. Most water-soluble pectin is located freely in the middle lamella of the cell wall with a gradual decrease of its concentration through the primary cell wall towards the plasma membrane (Wang et al., 2002).

Contrast to water soluble pectins which is independently unattached in plant cell wall, chelator-extracts pectin are embedded in the middle lamella of the cell wall by Ca^{2+} ions. Therefore, chelator-soluble pectin can be extracted with calcium-chelating-agent such as ethylene diamino tetraacetic acid (EDTA), CDTA or hexametaphosphate. Chelator-extracts pectin are less favourable due to the residual amount of the chelating agents in the final pectin sample, thus will affect its functionality (Garna et al., 2007). These pectins contain predominantly GalA residues, with mixtures of rhamnose and neutral sugar contents (~2% and 10−20% respectively).

According to Van Buren (1991), protopectin or insoluble pectin is extractable with alkali or hot dilute acid solutions. The term protopectin are often described as the native pectin fraction in the cell wall that cannot be purified by nondegradative methods (Wang et al., 2002). Protopectin is difficult to extract because it is strongly bound in the cell wall of parenchymatous tissues (Renard et al., 1990) and has strong linkages with other polysaccharides. In industrial scale, pectin is most favorable

extracted using hot dilute acid compared to using alkali because alkali can deteriorate the degree of esterification (DE) and can diminish the length of the pectin chains by β- elimination (May, 1990).

Compared to industrial application, water-soluble and chelator-soluble pectins contents are relatively similar to commercial pectin except the low amounts of neutral sugars. According to Van Buren (1991), there is a possibility that the commercial pectins are protopectins because of their neutral sugars could be removed by hydrolysis during extraction. Different parts of plant tissues have different amount and types of pectin (Van Buren, 1991). The study showed apple tissue contains mostly protopectin (O'Beirne et al., 1982), while ripe freestone peach tissue predominantly consists of water-soluble pectin (Postlmayr et al., 1956).

2.2.3 (c) Sources of pectin

Pectin is intensively and commercially extracted from apple pomace and citrus peels (May, 1990). Pectin can also be isolated from fruits and vegetables such as apples (Loyola et al., 2011), sugar beet (Levigne et al., 2002), red beet (Fissore et al., 2012), olive fruit (Vierhuis et al., 2003) and sunflower (Iglesias & Lozano, 2004).

Agricultural wastes have also been investigated as alternative sources for pectin extraction such as from the mangosteen rinds (Gan & Latiff, 2011), chicory roots (Panouille et al., 2006), orange peels (Liu et al., 2006), dragon fruit peels (Mohd Ismail et al., 2012), passion fruit peels (Kliemann et al., 2009), mango peels (Sudhakar & Maini, 2000) as well as cocoa pod husks (Mollea et al., 2007). The composition of pectin varies depend on the plant sources. Fruits and vegetables are also known to contain high pectin according to their biomass consumed, which is

approximately 30% of the total biomass compared to 1% in higher plants and in lignified tissues (Hoondal et al., 2002). Factors such as origin, growth and maturity stages of sources that affect the degree of esterification (Herron et al., 2000) of pectin are shown in Table 2.3.

Table 2.3 Percentage and degree of esterification of pectin in various sources

Sources	Tissue conditions	Pectin (%)	DE (%)
Golden apple	Dry	16 - 22	50-58
Banana	Fresh	0.7 - 1.2	-
Apricot	Fresh	0.1 - 0.9	-
Strawberry	Fresh	0.6 - 0.7	-
Cherry	Fresh	0.2 - 0.5	-
Pea	Fresh	0.9 - 1.4	-
Carrot	Dry	6.9 - 18.6	-
Orange pulp	Dry	12 - 28	-
Potato	Dry	1.8 - 3.3	-
Tomato	Dry	2.4 - 4.6	-
Sugar beet pulp	Dry	4.1 – 16	2 - 4.2
Maracuya skin	Dry	18.5	60
Soybean husk	Dry	7 - 16	-
Sweet lemon	Dry	9 - 30	58 - 82
Peach pulp	Fresh	12	82
Peach pulp	Dry	18	38
Cocoa husk	Dry	2.6-4.7	37.94- 52.5

Source: Lara- Marquez et al., (2011)

2.2.3 (d) Pectin application

Pectin is a natural food additives and widely used as a functional food: gelling agents for production of jams, jellies and confectionery products; stabilizing agents for ice cream, fruit juice and thickening agents for sauces and ketchups in industrial food (Madhav & Pushpalatha, 2002; May, 1990). It is also used in low calories food preparations and extensively used as fat or sugar replacer. The world market demand for pectin has reached 35,000 tons per annum (Daniells, 2008) with annual production growing by about 4–5%.

HMP is employed as gelling agents in sweetener products such as jellies and marmalades. In contrast, LMP can be employed in low-sugar products to produce diet products. Many studies have been conducted on the application of pectin in the food industry as an emulsifier (Siew & Williams, 2008) as well as a stabilizer in acidified protein drinks like yogurts (Willats et al., 2006). In chemical industry, pectin is utilized as edible films, paper substitute, foams and plasticizers (Singh & Gross, 2001). Nowadays, pectins are also utilized for various pharmaceutical (Srivastava & Malviya, 2011) and biomedical purposes (Munarin et al., 2012). Pectin has been found to be non-toxic, biocompatible, biodegradable and water soluble for such use.

2.3 Extraction methods for pectin

The production of pectin generally comprises of three stages: extraction, purification and drying. Extraction is the most important stage for extracting pectin from plants, fruits, vegetables and agricultural wastes. One of the most common methods used is by thermal treatment using Soxhlet method (Liu et al., 2006), other commonly used methods include ultrasound-assisted (Bagherian et al., 2011) and

microwave extraction (Yeoh et al., 2008), autoclave method (Voragen et al., 2007) and extrusion assisted (Fishman et al., 2004). Meanwhile, chemical acid extraction has been used extensively in industrial scale (Yapo, 2009). Most methods were developed with the aim of getting the highest possible yield and producing the best quality of pectin.

2.3.1 Thermal and mechanical extraction

2.3.1 (a) Soxhlet method

Soxhlet method is performed by adding samples into the glass thimble as shown in Figure 2.8. The flask is filled with solvents and heated to boiling point. Repeating percolation towards sample is allowed with steams condensation until complete extraction is achieved (Yeoh et al., 2008). Advantages of the Soxhlet method are low cost minus the filtration steps. However, the limitations of Soxhlet methods are time consuming, require high volume of solvent and involve clean-up steps (Eskilsson & Björklund, 2000).

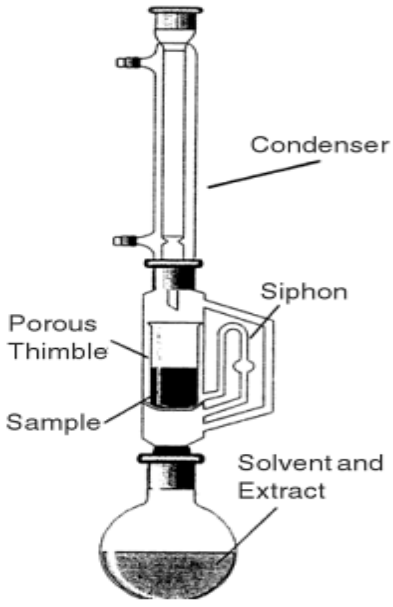

Figure 2.8 Soxhlet extraction apparatus. Adapted from Mitra (2003)

2.3.1 (b) Ultrasound-assisted extraction (UAE)

This method has the potential to be an alternative for the time consuming and low effective of the conventional acid extraction. UAE method is performed with the sample placed in a beaker on the ultrasonic clearer. The solvent is added in the presence of ultrasonic pressure waves of more than 16 kHz (Soria & Villamiel, 2010). The steps involved the selection of suitable ultrasonic power and the duration of extraction varies depending on plant sources to be extracted (Tian et al., 2012). The UAE method is rapid, conserves the structural and molecular characteristics and does not requires high temperature (Vilkhu et al., 2008). However, the downsides of UAE involved pre-treatment of samples, unable to reuse the solvents contribute to costly treatment of hazardous effluents and inconsistency in product qualities (Puri et al., 2012).

2.3.1 (c) Microwave-assisted extraction (MAE)

MAE method involves the application of microwave energy heat irradiates from between 300 to 300,000MHz to the mixture of raw materials and solvents in order to separate the target compounds from various matrices (Eskilsson & Björklund, 2000). The method also involves heating condition (80 °C) of up to 30 min (Guo et al., 2012). The solvents must have the ability to highly absorb microwave energy such as aqueous HCl.

The advantage of MAE is in its ability to produce higher yield in a shorter duration when compared to the conventional Soxhlet method (Liu et al., 2006). The downsides of this method lies in the selection of appropriate solvents having the ability to absorb microwaves heat, the clean-up process throughout the experiments

and the long duration needed for the vessel to cool down. Therefore, this method is considered to be not effective for large scale manufacturing of pectin.

2.3.2 Chemical extraction

The chemical extraction technique is extensively exploited in industrial scale pectin production. The fundamental of extraction involves treating the samples with hot dilute mineral acids with pH between 1 to 3. The temperature used is between 50 to 100°C for duration of 0.5 to 12 h (Panouillé et al., 2006; Rolin, 2002). The selection of suitable conditions is critical for this method. The process can contribute to the depolymerization and deesterification of pectin, if the condition is too mild. This acid extraction method is preferable because it gives a good yield of pectin and the ability of the acid to hydrolyze the methyl ester. Thus, DE can be tailored to the desired functionality for various kinds of food applications.

2.3.2 (a) Conventional hot dilute mineral acid method

Extraction in industrial production of pectin is commonly performed with mineral acids such as nitric acid (Aravantinos-Zafris & Oreopoulou, 1992), oxalic acid (Lim et al., 2012), hydrochloric acid (Ramli et al., 2013) and sulphuric acid (Seggiani et al., 2009). The mineral acids methods are considered as effective due to its ability to extract higher yield of pectin (Koubala et al., 2008).

Nonetheless, the strong mineral acids used have some limitations due to the high production of effluent in the filtrate that requires additional treatment in the disposition of acidic effluent (Lim et al., 2012; Min et al., 2011). Moreover, the conventional hot dilute mineral acid extraction procedures of pectin are considered time-consuming with high energy consumption (using high temperature) and thus

exposed to thermal degradation. This extreme conditions contributes to undesired alteration on physico-chemical and functional characteristics of extracts (Koubala et al., 2008).

2.3.2 (b) Eco-friendly organic acid

Due to the hazardous impacts to environment and the alteration of the final products of pectin by strong mineral acid, therefore many researches have switch to organic acid for pectin extraction. The uses of phosphoric, malic, tartaric and citric acids to replace strong mineral acids are common. The organic acids are considered as natural and safe food additive and fulfill the demand for 'green' and safe pectin products (Yapo, 2009). In addition, the use of organic acids is considered both environmental friendly and economical (Klieman et al., 2009; Pinheiro et al., 2008; Canteri-Schemin et al., 2005). Recent studies have shown that citric acid is effective for the extraction of pectin in terms of yield and the retention of physico-chemical properties (Klieman et al., 2009; Yapo, 2009; Pinheiro et al., 2008; Canteri-Schemin et al., 2005; Virk & Sogi, 2004). Canteri-Schemin et al. (2005) showed that citric acid gives highest yields among the organic acids tested (13.75 g/ 100 g yield).

2.3.3 Problems with conventional and other extraction methods

As mentioned in section 2.3.2, conventional extraction methods have many disadvantages. On industrial scale, the use of inorganic acid which is corrosive could cause adverse waste effluent that is detrimental to the environment. To overcome this waste problem, more money needs to be spent for treating the waste effluent. The huge amount of money spent is not commercially viable by the industries concerned. Moreover, during the extraction process, strong acid tends to randomly cleave the

glycosidic bonds in pectin. Thus, resulting in undesired degradation and reducing the molecular size of pectin (i.e. lower quality). In addition, the remnants are deemed as not suitable for human consumption (Ralet & Thibault, 1994).

Other method such as the extrusion extraction method involves the use of high pressure, shear and temperature that requires high energy consumption and undesirable chemical reaction that can impair the functional properties of extracted pectin (Kokini, 1993; Harper, 1981).

The uses of MAE, UAE or autoclave extraction method for the recovery of natural products have also shown many drawbacks. In these processes, the raw material is required to undergo additional pre-treatment whereas the methods are nonspecific and have batch-to-batch variation (Puri et al., 2012). The chemical and solvent use are usually not reusable, thus increase the production and waste management costs (Puri et al., 2012). In terms of product's quality, these methods of extraction showed variation in taste, frequently a 'bitter' taste can be caused by the solvent residues. Low extraction efficiency was also observed in these methods due to low accessibility of the extracting solvent into the sample (Puri et al., 2012).

Economically, most conventional methods for the extraction of usable compounds from plants related waste materials are not employed by industries due to the high cost involved and the environmental problems arising from the effluent. This has led to the increase of researches in this field over the years. However, from the literature search, few reports were found to be applicable for industrial use, more so on cocoa pod husks that are of abundance in cocoa producing countries such as Malaysia.