UNIVERSITI PUTRA MALAYSIA

ISOLATION AND STRUCTURAL CHARACTERIZATION OF MANNANOLIGOSACCHARIDES FROM AQUEOUS PALM KERNEL CAKE EXTRACT

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By

NAVEENA REDDY KALIDAS

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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DEDICATION

This thesis is dedicated to my family

for their love, endless support, and encouragement

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the Degree of Master of Science

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June 2016

Chairman : Professor Khozirah Shaari, PhD
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Palm kernel cake (PKC) is the most valuable by-product obtained from the palm kernel oil extraction process. It has been used widely as an animal feed owing to its high protein content. PKC also contains about 81% of non-starch polysaccharides, mainly in the form of mannan-based polymers. Hydrolysis of mannan into low molecular weight mannanoligosaccharides (MOS) has been claimed to have prebiotic properties. The biological mechanism and activities of the MOS are associated with its structure and molecular weight. However, very little information was available on the structural characteristics of MOS of different molecular weights from PKC. Therefore, the objectives of the present study were to isolate MOS of different degree of polymerization (DP) from the crude PKC extract and structurally characterize the MOS using chemical derivatization and spectroscopic methods. The crude PKC extract was obtained by hot water extraction method, followed by delipidation and deproteinization step. The deproteinized PKC extract containing mixtures of MOS were then separated into individual compounds based on their molecular weights using refractive index high performance liquid chromatography (RI-HPLC). The molecular weights of the MOS were determined using electrospray ionization mass spectrometry (ESI-MS/MS). The structures of the isolated MOS were investigated using a combination of chemical analyses such as sugar composition analysis and methylation analysis followed by gas chromatography mass spectrometry (GC-MS), and other spectroscopic methods such as nuclear magnetic resonance spectroscopy (NMR). The MOS mixtures were separated into four individual major compounds with different DP, designated as **MOS-III**, **MOS-IV**, **MOS-V** and **MOS-VI**. The molecular weights of the isolated MOS as determined by ESI-MS/MS were 689, 851, 1013 and 1151 Dalton (Da) corresponding to tetra-, penta-, hexa- and heptasaccharide of the **MOS-III**, **MOS-IV**, **MOS-V** and **MOS-VI**, respectively. Sugar analysis of the isolated MOS indicated the presence of mannose in each of the oligomers. Methylation and 1D/2D NMR analysis showed that the MOS have a linear structure consisting of (1→4)-*β*-D-mannopyranosyl residues with DP ranging from 4 to 7. They were identified as: a) *β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*, b) *β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-

Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*, c) *β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*, d) *β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*. In conclusion, the present study revealed a successful application of chromatography, chemical analysis, ESI-MS/MS and NMR to the isolation and characterization of MOS fraction from PKC.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENGASINGAN DAN PENCIRIAN STRUKTUR MANNANOLIGOSAKARIDA DARIPADA ISIRONG KELAPA SAWIT

Oleh

NAVEENA REDDY KALIDAS

Jun 2016

Pengerusi : Profesor Khozirah Shaari, PhD
Institut : Biosains

Isirong kelapa sawit (PKC), yang diperolehi daripada proses pengekstrakan minyak isirong kelapa sawit merupakan hasil sampingan yang paling berguna. PKC digunakan secara meluas sebagai makanan haiwan kerana mempunyai kandungan protein yang tinggi. Selain itu, PKC juga mengandungi kira-kira 81% polisakarida bukan kanji dalam bentuk polimer mannan. Hidrolisis polimer mannan kepada karbohidrat berat molekul rendah seperti mannanoligosakarida (MOS) mempunyai ciri-ciri prebiotik. Mekanisme biologi dan aktiviti MOS bergantung kepada struktur dan berat molekul. Walau bagaimanapun, maklumat tentang struktur pecahan MOS yang terdiri daripada berat molekul yang berbeza daripada PKC adalah sangat sedikit Oleh itu, objektif kajian ini adalah untuk mengasingkan MOS daripada ekstrak PKC mengikut darjah pempolimeran (DP) dan mencirikan struktur MOS dengan menggunakan kaedah kimia dan spektroskopi. Ekstrak PKC diperolehi melalui pengekstrakan air panas, dan kemudiannya diasingkan daripada lipid dan protein. Ekstrak PKC yang mengandungi campuran MOS kemudian dipisahkan kepada pecahan-pecahan MOS berdasarkan kepada berat molekul dengan menggunakan kromatografi cecair berprestasi tinggi (HPLC). Berat molekul MOS ditentukan dengan menggunakan teknik pengionan elektrosemburan spektroskopi jisim (ESI-MS/MS). Struktur MOS dikaji melalui gabungan analisis kimia seperti analisis komposisi gula dan analisis metilasi diikuti dengan kromatografi gas spektroskopi jisim (GC-MS), dan kaedah spektroskopi lain seperti spektroskopi resonans magnetik nuklear (NMR). Campuran MOS telah diasingkan kepada empat pecahan utama yang mempunyai DP berbeza, dinamakan sebagai **MOS-III, MOS-IV, MOS-V** dan **MOS-VI**. Berat molekul **MOS-III, MOS-IV, MOS-V** dan **MOS-VI** seperti yang ditentukan oleh ESI-MS/MS adalah 689, 851, 1013 dan 1151 Dalton (Da) yang dikenal pasti sebagai tetra-, penta-, hexa- dan heptasakarida, masing-masing. Analisis gula daripada MOS menunjukkan kehadiran mannosa dalam setiap satu oligomer. Metilasi dan analisis 1D/2D NMR menunjukkan bahawa MOS terdiri daripada satu struktur linear yang terdiri daripada (1→4)-β-D-mannopiranosil yang mempunyai DP 4–7. Struktur MOS telah dikenal pasti sebagai: a) β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Man*p*, b) β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Man*p*, c) β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Man*p*-

(1→4)-*β*-D-Man*p*, d) *β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*-(1→4)-*β*-D-Man*p*. Kesimpulannya, hasil kajian mendapati bahawa kromatografi, analisis kimia, ESI-MS/MS dan NMR berjaya mengasingkan dan mencirikan campuran MOS daripada PKC.

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I certify that a Thesis Examination Committee has met on 16 June 2016 to conduct the final examination of Naveena Reddy a/p Kalidas on her thesis entitled "Isolation and Structural Characterization of Mannanoligosaccharides from Aqueous Palm Kernel Cake Extract" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

°C	- Degree Celcius
^{13}C	- Carbon-13
^{1}H	- Proton
ACN	- Acetonitrile
API	- Atmospheric pressure ionization
C_5D_5N	- Deuterated Pyridine
$CDCl_3$	- Deuterated Chloroform
CI	- Chemical Ionization
D_2O	- Deuterium Oxide
DMSO	- Dimethylsulfoxide
DP	- Degree of Polymerization
DSS	- 3-(trimethylsilyl) propane-1-sulfonic acid
EI	- Electron Impact Ionization
ESIMS	- Electrsospray Ionization Mass Spectroscopy
eV	- Electron Volt
FAB	- Fast Atom Bombardment
FTIR	- Fourier Transform Infra-Red
GC-MS	- Gas Chromatography Mass Spectroscopy
HCl	- Acid Hydrocholoric
HMBC	- Heteronuclear Multiple Bond Correlation
HPAEC	- High Performance Anion Exchange Chromatography
HPLC	- High Performance Liquid Chromatography
HSQC	- Heteronuclear Single Quantum Coherence
Hz	- Hertz
IgA	- Immunoglobulin A
IgG	- Immunoglobulin G
m/z	- Mass per charge
MALDI	- Matrix-Assisted Laser Desorption Ionization
MHz	- Megahertz
MOS	- Mannanoligosaccharides
MW	- Molecular Weight
$NaBH_4$	- Sodium borohydride
NaOH	- Sodium hydroxide
NCP	- Non Cellulose Polysaccharide
NMR	- Nuclear Magnetic Resonance Spectroscopy
NSP	- Non Starch Polysaccharides
PAD	- Pulse Amperometric Detector
PKC	- Palm Kernel Cake
PMAA	- Partially Methylated Alditol Acetates

TFA	- Trifluoroacetic acid
TMS	- Tetramethylsilane
TOCSY	- Total Correlation Spectroscopy
TSP	- Trisodium Phosphate

CHAPTER 1

INTRODUCTION

Lignocellulosic biomass is the most abundant and renewable biopolymer in nature (Zhou *et al*., 2011). It is composed predominantly of polysaccharides such as cellulose, hemicellulose, and lignin. It has been estimated that 10–50 × 10^9 tons of lignocellulosic biomass are produced annually from agro-based industries (i.e., forestry, agricultural activities, timber industries, and pulp and paper industries) (De Vries and Visser, 2001). Much of these lignocellulosic wastes are usually disposed through burning activity, which imposes serious environmental pollution issues (Levine, 1996). However, the vast amount of plant biomass considered as waste could potentially be converted into various value added bio-products including animal feed, organic chemicals, biofuel, energy sources for fermentation and human nutrients (Howard *et al*., 2003).

Malaysia, being the second largest palm oil producer produces huge quantities of oil palm by-products. Among the many secondary products from oil palm, palm kernel cake (PKC) has been regarded as the most promising. It has been reported that PKC contains about 81% of non-starch polysaccharides (NSPs), mainly in the form of mannan-based polymers (Knudsen, 1997). Mannans are presumably characterised as anti-nutritional polysaccharides, which are highly crystalline, extremely hard and insoluble in water (Sundu and Dingle, 2002). Moreover, other anti-nutritional substances such as galactomannan, xylan, and arabinoxylan are also most likely present in PKC. Several studies have reported that guar and wheat also contain these anti-nutritional compounds which could increase the viscosity of the animal feeds due to its high water absorbing tendency; thus causing poor nutrient absorption in the gastrointestinal tract (Dingle, 1995; Kumar *et al*., 1997). Owing to its high fibre content, coupled with the presence of anti-nutritional compounds, application of PKC as feed supplements in non-ruminant diets is limited. However, recent studies have found that hydrolysis of mannan polymers from PKC into lower level carbohydrates such as oligosaccharides have been proven to improve the nutritive value of PKC.

Oligosaccharides are sugar polymers consisting of 3 to 10 monosaccharide residues linked by O- or N-linked glycosidic bonds (Tomomatsu, 1994). However, the degree of polymerization (DP) can go up to 60 for some oligosaccharides, for example chicory inulin, or down to 2, for example lactulose (Crittenden and Plain, 1996). Most oligosaccharides are low molecular weight carbohydrates. Oligosaccharides could be attached to amino acids or lipids, forming glycoprotein and glycolipids, respectively. Oligosaccharides have greater structural diversity due to the number of monomers, differences in the anomeric configuration, glycosidic linkages, ring size, substitution points, and branching points. As a result of the structural diversity, the flexibility, compactness, physical, biological and biochemical properties among the oligosaccharides are varied.

Oligosaccharides have been reported to exhibit significant biological properties such as immunostimulatory, antiviral, and antitumor activities (Kamasuka *et al*., 1968; Tizard *et al*., 1989; Ezekowitz *et al*., 1989). However, the biological activities and mechanisms of action of oligosaccharides are not fully known but believed to be very closely related to its structure. For example, mannan from *Saccharomyces cerevisiae*, which is composed of high molecular weight partially phosphorylated mannose residues linked via α-(1→6), α-(1→3), and α-(1→2) linkages was not active in stimulating the interferon release. Whereas, mannan from *Candida albicans*, which is composed of low molecular weight and highly branched molecule with short chains of α-(1→2), linked mannose monomers, joined by α-(1→6) linkages and attached to peptides, are very active in the interferon-inducing assay (DeClercq *et al*., 1970). Suzuki *et al*. (1968) postulated that the differences in the interferon activity could be due to small structural or size variations among the polymers.

In recent years, there has been a growing interest in the use of oligosaccharides as prebiotics. Oligosaccharides are classified as prebiotics because it can withstand the digestion enzymes in the stomach and pass into the large bowel where it is selectively metabolized by beneficial bacteria. Prebiotics are defined as “selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health” (Roberfroid, 2007). Fermentation of oligosaccharides by the microbiota are strongly influenced by the chemical structures and the identity of the monomeric sugar units, DP, type of glycosidic linkages between the monomers, complexity of the fraction (branched or linear) and possible linkage to non-carbohydrates. Several types of oligosaccharide substances, which have been reported to possess prebiotic effects, such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), glucooligosaccharides, mannanoligosaccharides (MOS), isomaltooligosaccharides (ISO), xylooligosaccharides (XOS), gentioligosaccharides (GTO) and inulin (Crittenden and Playne, 1996; Rycroft *et al*., 2001; Desai *et al*., 2004; Pennacchia *et al*., 2006). Recently, some studies have shown that enzyme-treated PKC releases mannose and mannose-based oligosaccharides, which could be a potential source of prebiotic (Saenphoom *et al*., 2011; 2013). Chen *et al*. (2015) demonstrated that all three *Lactobacillus* strains showed better growth rate in the enzyme-treated crude PKC, owing to low DP oligosaccharides produced during the hydrolysis of NSPs. However, the nature and the exact type of oligosaccharides which are responsible for the growth of *Lactobacillus* could not be confirmed. Although, there have been some structural studies conducted on the mannans of PKC, the available information is only on the overall carbohydrate polymer with little information on the structural characteristics of MOS fractions of different molecular weights. Therefore, this study was conducted to provide this lack of structural information. To achieve this, the study was initiated with the following objectives:

1. To isolate and purify the oligosaccharides from the aqueous PKC extract.
2. To characterize the oligosaccharides using chemical and spectroscopic methods.

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LIST OF PUBLICATIONS

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Proceedings of Paper Presented in Conferences, Symposia, Congresses and Seminars

Naveena Reddy Kalidas, Khozirah Shaari, Intan Safinar Ismail, and Faridah Abas. (2015). Isolation, Fractionation, and characterization of oligosaccharides extracted from Palm Kernel Cake (PKC) using Infrared Spectroscopy (IR). International National Conference of Natural Products, pp 54.

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