BIOACTIVITY STUDY OF PROTEASE DIGESTED PEPTIDES FROM *Moringa oleifera* SEEDS

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DEDICATION

This dissertation is dedicated to my father, who taught me that the best kind of knowledge to have is that which is learned for its own sake; to my mother, who taught me that even the largest task could be accomplished if it is done one step at a time. It is also dedicated to my wife, who has cooperated with me to achieve this goal, and to my brothers and sister, who always supported me.

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ABSTRACT

Moringa oleifera is an important plant for centuries due to its nutritional properties, medicinal importance, and lately, wastewater treatment. Almost all parts of this plant contribute to its nutritional properties. Many studies have reported the importance of the bioactivity of the proteins from *M. oleifera* seed like antioxidant, antidiabetic, antihypertensive and wastewater treatment, but there is no investigation on the bioactivity of digested products of *M. oleifera* fresh seed proteins that could be used for therapeutic activity. The objectives of this research were to determine the antioxidant and α-amylase inhibition activities of *Moringa oleifera* fresh seed proteins and their digested products. The seed protein was extracted using Tris-HCl buffer followed by quantification by Bradford Assay and quality checked by Sodium Dodecyl-Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were digested using proteolytic enzymes (pepsin, trypsin, and chymotrypsin). Next, the antioxidant activity was determined by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Ferric reducing antioxidant power (FRAP) assay. After that, the alpha-amylase inhibition was determined by α-amylase inhibition assay. All digested products showed higher antioxidant activity than undigested protein. Pepsin digestion products showed the highest Radical scavenging activity about 30.86±2.86 %RSA than other digestion products. But in terms of FRAP assay, trypsin digestion products showed better ability about 5.51±0.009 Fe_2^+/g than pepsin and chymotrypsin digestion. Based on the α-amylase inhibition assay, all digested products showed good inhibitory activity, but, interestingly, the undigested protein had higher inhibition activity than digestion products. In conclusion, this study showed that digestion with proteases has improved the antioxidant activity of *M. oleifera* fresh seed proteins. In addition, the digestion products have inhibition of α-amylase activity but digestion did not increase the inhibitory activity. The results provide information on antioxidant and enzyme inhibitory as well as comparison of *M. oleifera* seed protein and the effect of protein digested by different proteases that could contribute to pharmaceutical and functional food.

ABSTRAK

Moringa oleifera adalah tumbuhan yang penting sejak berabad lagi disebabkan kandungan nutrisi, kepentingan perubatan, dan akhir-akhir ini, rawatan air sisa. Hampir kesemua bahagian tumbuhan ini menyumbang kepada khasiatnya. Banyak kajian telah melaporkan kepentingan bioaktiviti protein dari biji *M. oleifera* ini seperti antioksidan, antidiabetik, antihipertensi, dan rawatan air sisa, tetapi tiada penyelidikan mengenai bioaktiviti produk pencernaan protein dalam benih segar *M. oleifera* yang berkemungkinan boleh digunakan untuk aktiviti terapi. Objektif penyelidikan ini adalah untuk menentukan aktiviti perencatan antioksidan dan α-*amylase* bagi protein benih segar *Moringa oleifera* dan yang dicerna. Protein benih segar diekstrak menggunakan penimbal *Tris-HCl* diikuti dengan kuantifikasi menggunakan *Bradford Assay* dan kualiti diperiksa menggunakan gel elektroforasis *Sodium Dodecyl-Sulfate Polyacrylamide (SDS-PAGE)*. Kemudian, protein dicerna menggunakan enzim proteolitik (*pepsin, trypsin, dan chymotrypsin*). Seterusnya, aktiviti antioksidan dianalisis menggunakan kaedah *2,2-Diphenyl-1-picrylhydrazyl (DPPH)* dan *Ferric Reducing Antioxidant Power (FRAP)*. Seterusnya, perencatan *alpha-amylase* ditentukan dengan ujian perencatan α-*amylase*. Semua produk yang dicerna menunjukkan aktiviti antioksidan yang lebih tinggi berbanding dengan protein yang tidak dicerna. Produk pencernaan *pepsin* menunjukkan aktiviti pengkautan radikal tertinggi sekitar 30,86±2,86 % *RSA* berbanding produk pencernaan yang lain. Tetapi dari segi kaedah *FRAP*, produk pencernaan *trypsin* menunjukkan kemampuan yang lebih baik sekitar 5,51±0,009 Fe_{2}^{+}/g daripada pencernaan *pepsin* dan *chymotrypsin*. Berdasarkan ujian perencatan α-*amylase*, kesemua produk yang dicerna menunjukkan aktiviti perencatan yang baik, tetapi yang menariknya, protein yang tidak dicerna mempunyai aktiviti perencatan yang lebih tinggi berbanding produk yand dicerna. Kesimpulannya, kajian ini menunjukkan bahawa pencernaan dengan *protease* telah meningkatkan aktiviti antioksidan protein benih segar *M. oleifera*. Disamping itu, produk pencernaan mempunyai perencatan aktiviti α-*amylase* tetapi pencernaan tidak meningkatkan aktiviti perencatan. Hasil kajian ini memberi maklumat antioksida dan perencatan enzim, juga perbandingan protein benih segar *M. oleifera* dan kesan protein yang dicerna dengan *protease* berbeza, yang menyumbang kepada farmaseutikal dan makanan fungsian.

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LIST OF ABBREVIATIONS

SDS	-	Sodium Dodecyl Sulphate
PAGE	-	Poly Acrylamide Gel Electrophoresis
1D	-	1-Dimension
APS	-	Ammonium persulfate
BSA	-	Bovine serum albumin
ACN	-	Acetonitrile
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetraacetic acid
HCl	-	Hydrochloric acid
IAA	-	Iodoacetamide
KCl	-	Potassium chloride
MS	-	Mass spectrometry
LC-MS/MS	-	Liquid chromatography-tandem mass spectrometry
NCBI	-	National centre for biotechnology information
Uniprot	-	Universal protein resources
NH_4HCO_3	-	Ammonium bicarbonate
PMSF	-	Phenylmethylsulphoneyl fluoride
TCA	-	Tricarboxylic acid
TEMED	-	Tetramethylethylenediamine

LIST OF SYMBOLS

℃	-	Degree Celsius
β	-	Beta
%	-	Percent
g	-	Gram
Da	-	Dalton
kDa	-	Kilodalton
kg	-	Kilogram
L	-	Litre
M	-	Molar
µL	-	Microliter
µg	-	Microgram
µM	-	Micromolar
mg	-	Milligram
mL	-	Millilitre
m/z	-	Mass per charge
rpm	-	Rotation per minute
V	-	Volt
v/v	-	Volume per volume
w/v	-	Weight per volume
α	-	Alpha

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CHAPTER 1

INTRODUCTION

1.1 Background

Moringa oleifera is a small to medium-sized tree that originates from western and sub-Himalayan tracts of India and is distributed in Pakistan, Asia, and Africa (Gopalakrishnan et al., 2016; Leone et al., 2016; Ramachandran et al., 1980). The high medicinal and nutritional values of *Moringa oleifera* makes it a multipurpose plant (Fahey, 2005). All parts of the *Moringa* tree (leaves, seeds, roots, and flowers) are usable for human and animal consumption. Leaves, seeds, roots, bark, flowers, and sap of *Moringa oleifera* are traditionally used as medicines, whereas leaves and seedpods are used as food for human nutrition.

Many reports show the medicinal uses of this plant. Aqueous and alcohol extracts of *M. oleifera* leave have many biological activities like antioxidant, anti-hyperglycemic, anti-dyslipidemic, antiulcer, antihypertensive tissue-protective activities (Stohs & Hartman, 2015), and all parts of Moringa have an antimicrobial property (Wang et al., 2016). Moringa is a rich plant source for vitamins A, B complex, vitamin C, D, E, and K. It has many important minerals, including Calcium, Magnesium, Potassium, Copper, Iron, Zinc and Iron (Mahmood et al., 2010). It could prevent malnutrition, weakness and beneficial for lactating mothers, depression, and osteoporosis (Mahmood et al., 2010). Besides nutritional value and different metabolites in Moringa, the proteins of Moringa are another significant molecule and have important biological activities. Some reports have represented that the proteins of *Moringa* have flocculation activities, and the proteins from the seed of Moringa could be used in wastewater treatment (Villaseñor-Basulto et al., 2018). Moringa has a considerable amount of protein, especially in seeds (Anwar & Rashid, 2007) but based on a report, the proteins of leaves are mostly insoluble and have low in vitro digestibility (Maria et al., 2014).

M. oleifera has significant medicinal properties. Many studies reported the antioxidant activity of different parts of this plant. Studies show that crude extract and proteins extracts of *M. oleifera* have antioxidant properties; even leaves proteins of this plant have shown better antioxidant activity than crude extract (Zulkifli & Rahmat, 2020). In addition to that, the proteins of *M. oleifera* seed have been reported to possess antidiabetic activity. It was said that insulin-like protein was recognized from seed coat of *M. oleifera*; furthermore, *M. oleifera* protein has potential for lowering blood glucose (Paula et al., 2017). Despite some study on antioxidant and antidiabetics activity of *M. oleifera* protein, no investigation has been done on antioxidant and antidiabetic activity of digested proteins. From a pharmaceutical point of view, bioactive peptides are better than traditional and chemical medicines. Bioactive peptides are very specific, non-toxicity, do not accumulate in the body, and are degradable (Castro & Sato, 2015). *M. oleifera* proteins shows better biofunctional activities when they digested with proteolytic enzymes; previous studies reported that peptides produced by digestion showed higher bioactivities like antihypertensive and antioxidants (Garza et al., 2017). Proteolytic enzymes produce most of the bioactive peptides; some of these peptides are endogenous peptides that have many important activities like regulators, hormones and many other biological functions (Castro & Sato, 2015).

Many investigations on secondary metabolites of *M. oleifera* have been done, but there is less information on proteins of *Moringa oleifera* and their digested products that have greater biological activity related to antioxidant, antimicrobial, and many other medicinal activities. However, there is limited information on antioxidant and α-amylase inhibition activities of the *M. oleifera* fresh seed proteins, and no studies have been done on their digested products until now. This study aims to determine antioxidant and α-amylase inhibition activities of *M. oleifera* seed proteins and their digested products.

1.2 Problem statement

Medicinal plants are used in traditional medicine for the treatment of different diseases. One of them is *Moringa oleifera,* which has been used as a medicinal plant for a long time. Different metabolites have been extracted from all parts of this plant (leaves extracts, bark extracts, stem extracts, root extracts, and seed extracts). Many investigations show that these metabolites can be used as bioactive components for the treatment of different diseases. Despite many investigations about these extracts and their activities, there is less information on proteins of *Moringa oleifera*, which plays a bigger role in biological processes. Protein and peptide provide binding receptor(s) for substrate or other protein in biological networks. This could provide a better solution in the medical field. On the other hand, research on protein and peptide is still behind. Although, previous studies reported significant antioxidant and α-amylase inhibition activities of protein from *M. oleifera,* not much information could be found on antioxidant and α-amylase activities of *M. oleifera* fresh seed protein and their digested peptides. This study aims to know more on determination of antioxidant and α-amylase inhibition activity of *M. oleifera* fresh seed proteins and their digested products.

1.3 Objectives

The objectives of the research are:

(a) To determine the antioxidant activity of different digested products of *Moringa oleifera* fresh seed proteins.

(b) To determine the α-amylase inhibition activity of different digested products of *M. oleifera* fresh seed proteins.

1.4 Significance of the study

Moringa oleifera seeds have a significant amount of proteins (31.65%) (Anwar & Rashid, 2007) with important medicinal activities; such as antimicrobial properties (Wang et al., 2016), antioxidative damage (Liang et al., 2020), antioxidant activity (Aderinola et al., 2018), antidiabetic activity (Vargas-Sánchez et al., 2019), and also insecticidal activity (Patrícia et al., 2016) and many others. To date, less investigation has been done on antioxidant and α-amylase activities of digested peptides of *M. oleifera* fresh seed proteins. Antioxidants are compounds that prevent from oxidative damage in cells, and inhibition of α-amylase contributes to lowering blood sugar, especially in diabetics. This project focuses on determining the antioxidant and α-amylase activities of *M. oleifera* fresh seed protein and their digested peptides. This is important because it provides us information on the importance and contribution of pharmaceutical and functional food uses.

1.5 Scope of the study

In this study, *Moringa oleifera* seeds were collected from Ulu Tiram, Johor Bahru, Johor, Malaysia. Proteins were extracted from *Moringa oleifera* seeds using the Tris-HCl buffer method (Sheoran et al., 2009). The extracted proteins were quantified and qualified by Bradford assay (Lowry et al., 1951) and the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (LaemmLi, 1970) methods, respectively. After that, extracted proteins were digested using proteases. In this study, three different kinds of proteases were used: trypsin, pepsin, and chymotrypsin, to digest the proteins and produce peptides. To evaluate the antioxidant activity of fresh seed proteins and their digested products, the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and ferric reducing antioxidant power (FRAP) assay were done. Finally, the α-amylase inhibition assay was done to determine the α-amylase inhibition activity of the proteins and their digested products.

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