UNIVERSITI PUTRA MALAYSIA

NUTRITIONAL COMPOSITION AND ANGIOTENSIN-CONVERTING ENZYME INHIBITORY ACTIVITY OF BLUE LUPIN (Lupinus angustifolius L.)

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By

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Angiotensin-converting enzyme (ACE) plays a dominant role in blood pressure regulating system. Synthetic ACE inhibitor is designed as an antihypertensive drug to restrict ACE activity. However, the usage of synthetic drug may cause several adverse effects. Hence, a natural food protein with ACE inhibitory activity may be promising as a safer alternative to the synthetic drug. Blue lupin (*Lupinus angustifolius*) is one of the legumes that rich in protein. It has the potential to be a good source of ACE inhibitory peptides. However, blue lupin is usually served as an animal feed and consumption by human is rare. Therefore, this study was carried out to investigate the nutritional, protein and amino acid composition of blue lupin flour, and to evaluate the ACE inhibitory activity of its protein hydrolysates. The results revealed that the lupin flour was abundant in protein (43.3 g/100 g) and dietary fibre (33.5 g/100 g). According to the Osborne classification, plant storage protein was characterised into four categories based on their solubility in different solvents. Results from sequential Osborne extraction procedure showed that lupin protein comprised of 46% salt-soluble globulin, 27% water-soluble albumin, 18% alkaline-soluble glutelin and 7% alcohol-soluble prolamin fractions. Furthermore, lupin protein was rich in lysine but limiting in methionine. In this study, Alcalase and Flavourzyme were used to hydrolyse the lupin protein isolate for different hydrolysis times (4, 10 and 16 h). Gel electrophoresis analysis demonstrated that protein hydrolysis catalysed by Alcalase was more effective compared with Flavourzyme as evidenced by the lower molecular weight peptides presented in the hydrolysates prepared using Alcalase. The ACE inhibitory activity of hydrolysates was determined using an *in vitro* method. Hydrolysates prepared using Alcalase exhibited higher ACE inhibitory activities compared with those prepared using Flavourzyme, showing IC_{50} values ranging from 0.10 to 0.21 mg/mL. However, there was no significant difference in IC_{50} values between the hydrolysates prepared using Alcalase for 4, 10 and 16 h of hydrolysis times. The results suggested that globulin was the major contributing protein fraction on ACE inhibitory effect in lupin protein. In addition, the information obtained in this study is important to reveal the nutritional values and health benefits of blue lupin protein and to recognise the lupin

protein hydrolysates as nutraceuticals or functional foods. The lupin protein hydrolysates with potent ACE inhibitory activities can be incorporated into the daily diet to prevent hypertension. They can also be consumed by hypertensive patients to reduce the dosage of antihypertensive drugs needed, thereby lowering the risk of side effects.

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

KOMPOSISI NUTRIEN DAN AKTIVITI PERENCATAN ENZIM PENUKAR ANGIOTENSIN DALAM LUPIN BIRU (*Lupinus angustifolius* L.)

Oleh

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September 2017

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Enzim penukar angiotensin (ACE) memainkan peranan penting dalam sistem pengawalan tekanan darah. *Synthetic ACE inhibitor* direka sebagai ubat anti-hipertensif untuk merencat aktiviti ACE. Akan tetapi, penggunaan ubat sintetik berkemungkinan menyebabkan beberapa kesan sampingan. Oleh itu, protein makanan semulajadi yang mempunyai aktiviti perencatan ACE, berkemungkinan menjadi alternatif yang lebih selamat berbanding dengan ubat sintetik. Lupin biru (*Lupinus angustifolius*) adalah kekacang yang kaya dengan protein. Ia mempunyai potensi sebagai sumber peptida dalam perencatan ACE. Walaubagaimanapun, lupin biru biasanya digunakan sebagai makanan haiwan dan jarang sebagai makanan manusia. Oleh itu, kajian ini dijalankan untuk mengkaji komposisi nutrien, protein dan asid amino dalam lupin biru dan menilai aktiviti perencatan ACE protein hidrolisatnya. Hasil kajian menunjukkan bahawa tepung lupin menpunyai kandungan protein (43.3 g/100 g) dan serat (33.5 g/100 g) yang tinggi. Menurut klasifikasi Osborne, protein penyimpanan tumbuhan dicirikan dalam empat kategori berdasarkan kelarutannya dalam pelarut yang berbeza. Hasil daripada prosedur pengekstrakan Osborne menunjukkan protein lupin terdiri daripada 46% globulin, 27% albumin, 18% glutelin dan 7% prolamin. Protein lupin juga kaya dalam lysine tetapi kandungan methionine adalah terhad. Dalam kajian ini, *Alcalase* dan *Flavourzyme* digunakan untuk menghasilkan hidrolisat daripada protein lupin pada masa hidrolisis yang berbeza (4, 10 dan 16 jam). Analisis gel elektroforetik menunjukkan hidrolisis protein yang menggunakan *Alcalase* telah menghasilkan peptida molekul yang lebih ringan. Ini menunjukkan *Alcalase* sebagai enzim yang lebih berkesan berbanding dengan *Flavourzyme*. Aktiviti perencatan ACE hidrolisat telah ditentu dengan menggunakan kaedah *in vitro*. Hidrolisat yang dihasilkan dengan menggunakan *Alcalase* menunjukan aktiviti perencatan ACE yang lebih tinggi berbanding *Flavourzyme* dengan nilai IC_{50} antara 0.10 hingga 0.21 mg/mL. Walau bagaimanapun, tidak ada perbezaan ketara nilai IC_{50} antara hidrolisat yang disediakan dengan masa hidrolisis *Alcalase* yang berbeza (4, 10 dan 16 jam). Hasil kajian mencadangkan bahawa globulin merupakan protein penyumbang utama ke atas kesan perencatan ACE. Maklumat yang diperolehi daripada kajian ini adalah penting untuk menunjukkan nilai pemakanan dan manfaat kesihatan protein lupin biru, dan untuk

mengenegahkan hidrolisat protein lupin sebagai sumber nutraseutikal atau makanan berfungsi. Hidrolisat protein lupin dengan aktiviti perencatan ACE yang kuat boleh dimasukkan ke dalam diet harian untuk menggelakkan tekanan darah tinggi. Ia juga boleh digunakan oleh pesakit hipertensi untuk mengurangkan dos ubat darah tinggi yang diperlukan.

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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

AABA	aminobutyric acid
ACE	angiotensin-converting enzyme
APS	ammonium persulphate
CVD	cardiovascular diseases
DASH	dietary approach to stop hypertension
DBP	diastolic blood pressure
DH	degree of hydrolysis
DW	dry weight
EAA	essential amino acid
FW	fresh weight
HA	hippuric acid
HCl	hydrochloric acid
HHL	N-hippuryl-L-histidyl-L-leucine
HPLC	high performance liquid chromatography
IDF	insoluble dietary fibre
LPI	lupin protein isolate
MW	molecular weight
NaCl	sodium chloride
NaOH	sodium hydroxide
NEAA	non-essential amino acid
OPA	ophthaldialdehyde
SBP	systolic blood pressure
SDF	soluble dietary fibre
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SHR	spontaneously hypertensive rats
TAC	total available carbohydrate
TDF	total dietary fibre
TEMED	N,N,N',N'-tetramethylethylenediamine

CHAPTER 1

INTRODUCTION

1.1 Background

Cardiovascular diseases (CVDs) are leading causes of death, which contribute around 31% of total deaths worldwide in 2012 (WHO, 2016). The risk of developing CVDs is highly associated to high blood pressure. According to World Health Organisation (WHO, 2016), high blood pressure is responsible for approximately 45% of deaths by coronary heart disease, and 51% of deaths by stroke. High blood pressure, also known as hypertension, is a condition in which the blood vessels show persistently elevated blood pressure (WHO, 2013). A person with systolic blood pressure over 140 mmHg and diastolic blood pressure higher than 90 mmHg is regarded to have hypertension. In Malaysia, the prevalence of hypertension for population aged 30 and above had increased 32% from 1996 to 2011 (Naing et al., 2016). The National Health and Morbidity Survey 2015 stated that the overall prevalence of hypertension, for both known and undiagnosed, among Malaysian aged 18 and above was 30.3% (Institute for Public Health, 2015). These findings suggest that policy makers should give additional attention to individual awareness enhancement and public health control.

Hypertension can be controlled by drug therapy. The treatment goal for hypertensive patients is to lower the risk of blood pressure-related clinical complications, such as chronic kidney diseases and retinopathy (Weber et al., 2014). More importantly, hypertension treatment can reduce the risk of cardiovascular mortality by maintaining the blood pressure at normal levels. There are several types of blood pressure lowering drugs available in the market. Angiotensin-converting enzyme (ACE) inhibitors are one of the blood pressure lowering drugs widely used by hypertensive patients (Weber et al., 2014). In the human body, ACE plays a significant role in regulating blood pressure. It converts angiotensin I to angiotensin II and deactivates bradykinin (Fallo & Ermolao, 2012). Angiotensin II is an activated peptide hormone responsible for blood vessel constriction, while bradykinin is a vasodilator that widens blood vessels and causes blood pressure reduction (Fallo & Ermolao, 2012). Inhibition of ACE can lower the blood pressures by restricting the production of angiotensin II to repress vasoconstriction and increasing the level of bradykinin to promote vasodilatation. Therefore, synthetic ACE inhibitors are developed as pharmaceutical drugs to treat hypertension. Usage of synthetic ACE inhibitors can cause several side effects, such as dry cough and angioedema (Weber et al., 2014). Hence, research and development on discovering safer and economical food-derived ACE inhibitors are necessary for both prevention and treatment of hypertension.

Bioactive peptides are specific protein fragments that positively affect body function and health condition (Kitts & Weiler, 2003). They are usually small and only consist of two to twenty amino acid residues in length. These protein fragments are usually inactive within their primary protein structure (Walther & Sieber, 2011). They can be released from their protein precursors through enzymatic hydrolysis. Numerous studies

had documented the ACE inhibitory effect of hydrolysates and bioactive peptides derived from protein-rich foods, such as milks, fishes, meats and legumes (Jang & Lee, 2005; Ottea, Shalabya, Zakoraa, Prippa, & El-Shabrawyb, 2007; Salampessy, Reddy, Kailasapathy, & Phillips, 2015). For example, soybean protein hydrolysate prepared using pepsin was found to have an ACE inhibitory activity with an IC_{50} value of 224 µg/mL (Boschin, Scigliuolo, Resta, & Arnoldi, 2014a). Barbana and Boye (2010) reported that the chickpea protein hydrolysate prepared using Alcalase and Flavourzyme exhibited an ACE inhibitory activity with an IC_{50} value of 272 µg/mL.

Legumes are the main sources of plant protein. Apart from soybean and chickpea, lupin also has the potential to provide ACE inhibitory peptides as it has higher protein content compared with many other legumes (Bähr, Fechner, Hasenkopf, Mittermaier, & Jahreis, 2014; Saastamoinen, Eurola, & Hietaniemi, 2013). Lupin is the common name of *Lupinus*, which belongs to the *Fabaceae* family. It is abundant in protein with biological value of 91% of egg proteins (Egana, Uauy, Cassorla, Barrera, & Yanez, 1992). This makes lupin a valuable source to produce bioactive peptides with ACE inhibitory property. In addition, an inverse relationship between lupin consumption in the diet and the condition of blood pressure was found by various researches (Bähr, Fechner, Krämer, Kiehntopf, & Jahreis, 2013; Pilvi et al., 2006). However, lupin is usually cultivated as animal feed and consumption by human is rare. This is probably due to the scarcity of information about nutritional value and physiological function of lupin seed. In this study, the nutritional, protein and amino acid compositions as well as ACE inhibitory activity of lupin were determined to reveal its nutritional value and biological activity for human consumption.

1.2 Problem Statements

The common synthetic ACE inhibitors used for hypertension treatment are captopril, enalapril, benazepril, lisinopril, perindopril and fosinopril. Although drug therapy is considered to be a useful approach to treat hypertension, several adverse effects have been documented with the use of synthetic ACE inhibitors. For example, the most common adverse effects are dry cough and skin rash, while angioedema, headaches and dizziness have been reported from the long term usage of synthetic ACE inhibitors (Atkinson & Robertson, 1979; Rasmussen, Mey, & Bygum, 2014; Weber et al., 2014). Angioedema is a swelling of deep layer of skin or mucosa. It has been reported in 0.2 to 2.5% of hypertensive patients who received synthetic ACE inhibitors to control their elevated blood pressures (Rasmussen et al., 2014). Besides, severe angioedema at upper airways may cause death due to lack of oxygen (Rasmussen et al., 2014; Tharayil et al., 2014). Apart from medical treatment of hypertension, diet therapy may be also considered as a safe and non-pharmacological approach to control the elevated blood pressure. Hence, a food protein with ACE inhibitory property has gained its market value to be used as a potential preventive agent for hypertension treatment.

In the past decade, the search and development for natural food-derived ACE inhibitors as safer alternatives to synthetic ACE inhibitors have sparked a great amount of interest. ACE inhibitors derived from food proteins are believed to have abilities in both preventing and controlling hypertension. Among the food originating sources, plant

proteins show a potential by having economical and agricultural advantages compared to animal protein sources. Lupin is one of the richest protein sources among legumes. Several *in vivo* studies reported a favourable impact of lupin protein in blood pressure reduction. Pilvi et al. (2006) found that the systolic blood pressure of spontaneously hypertensive rats was reduced by 18.6 mmHg after two weeks of high sodium diet containing 20% (weight/weight; w/w) of lupin protein isolate. Besides that, both systolic and diastolic blood pressures of hypercholesterolemic adults were significantly reduced after 8 weeks of supplementation of 25 g lupin protein isolate per day (Bähr et al., 2013). Lupin protein, therefore, holds a potential as a good source of ACE inhibitory peptides.

Lupin seed is nutritious for human body as it provides high protein and dietary fibre contents (Australian Government, 2013). Researchers believe it has a great potential to be developed as a nutraceutical or functional food (Arnoldi, Boschin, Zanoni, & Lammi, 2015; Kohajdová, Karovičová, & Schmidt, 2011). Lupin seed has a long history of utilisation in Mediterranean region, starting with the ancient Greeks who grew lupin as an agricultural crop (Gladstones, 1970). Nowadays, Australia is the world largest producer of lupin seed, which accounts for 80% of the global lupin production (FAOSTAT, 2015). Blue lupin (*Lupinus angustifolius*) is the major species of lupin production in Australia. However, lupin seed is mostly served as animal feed to livestock and poultry (Australian Government, 2013). Lupin, especially blue lupin, is not popular for human consumption. Australian Government (2013) reported that less than 4% of global lupin production is being consumed as human food. This is probably due to the limited information on nutritional and functional properties to justify the development of blue lupin protein as a nutraceutical or functional food. Therefore, nutritional, amino acid and protein composition as well as ACE inhibitory activity of blue lupin will be examined in this study to provide detail understanding and information about its health benefits.

1.3 Significance of the Study

Information about nutritional and amino acid compositions of blue lupin is important to reveal its nutritional value for human consumption. The findings of this study will redound to the benefit of Malaysian diet considering that blue lupin is a good source of protein and dietary fibre. From that, blue lupin can be recognised as a nutritious food or ingredient to be included in the daily diet. For example, blue lupin seed can be eaten raw or cooked. It can also be ground and applied into several food productions. Lupin flour can be used as a substitution for wheat flour to produce baked dishes or pastries with lower glycaemic index as it contains minimal starch content, making them great choices for diabetic patients. Besides that, lupin protein isolate and lupin protein hydrolysates can be utilised as plant protein sources in formulation of food products, such as dairy products and meat analogue. Both lupin protein isolate and hydrolysate can also be used as egg substitutes in food application to improve the nutritional value of food product for vegetarian (Drakos, Doxastakis, & Kiosseoglou, 2007; Papavergou, Bloukas, & Doxastakis, 1999; Pollard, Stoddard, Popineau, Wrigley, & MacRitchie, 2002).

Previous *in vivo* studies demonstrated that lupin protein consumption could contribute to blood pressure lowering effect in spontaneously hypertensive rats and hypercholesterolemic adults (Bähr et al., 2013; Pilvi et al., 2006). Hence, this study is performed to justify the antihypertensive property of blue lupin protein. Antihypertensive effect of blue lupin can be explained by studying the ACE inhibitory activity of blue lupin protein. The knowledge and information about physiological function of blue lupin protein is important for it to be recognised as a nutraceutical or functional food. Blue lupin protein with ACE inhibitory activity can be incorporated into Malaysian diet to prevent the development of hypertension, and thus reducing the prevalence of CVDs. In addition, supplementation of blue lupin protein with ACE inhibitory effect into hypertensive patient's diet could be considered a safe and non-pharmacological approach to control hypertension. This could help to reduce the dosage of antihypertensive drugs, therefore reducing the risk of adverse effects on hypertensive patients.

According to Hernández-ledesma, Contreras and Recio (2011), ACE inhibitory peptides are usually short-chain peptides with two to twelve amino acids. In this study, blue lupin protein was hydrolysed with two commercial food proteases, which are Alcalase and Flavourzyme, to generate small peptides with possible ACE inhibitory activity. Commercial food proteases are preferable to chemical processes as enzymatic hydrolysis shows environmental and technical advantages over alkaline or acid hydrolysis (Tavano, 2013). Hence, the knowledge of effective production obtained in this study will be helpful to the food industry in preparing protein hydrolysate with antihypertensive property. Furthermore, results from amino acid and protein compositions analysis can help to provide better understanding on the ACE inhibitory activity of lupin protein. The findings will also serve as a reference for researchers on further study of ACE inhibitory activity of blue lupin protein.

1.4 Objectives

1.4.1 General Objective

To investigate the nutritional and protein composition of blue lupin (*L. angustifolius*) seed flour and its hydrolysates on ACE inhibitory activity.

1.4.2 Specific Objectives

i. To determine the nutritional composition of blue lupin seed flour.
ii. To characterise the major protein fractions in blue lupin seed flour.
iii. To determine the amino acid composition of blue lupin seed flour and protein fractions isolated from blue lupin seed flour.
iv. To determine the ACE inhibitory activity of hydrolysates prepared from lupin protein isolate and major protein fractions.

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BIODATA OF STUDENT

The student, Chin Ying Yee was born on 3rd of July, 1992 in Kuala Lumpur, Malaysia. She completed her secondary school education at Kuen Cheng Girls' High School, Kuala Lumpur in 2010. She further pursued her tertiary education at Taylor's University, Subang Jaya, Malaysia, and graduated with a Bachelor of Science (Honours) (Food Science with Nutrition) in 2014. For her final year project, she studied the carotenoid profile and antioxidant capacities of six Malaysian seaweed species. She was an intern at Cerebos (Malaysia) Sdn. Bhd., working as an industrial trainee in Quality Assurance Department to assist on daily microbiology testing and hygiene inspection.

She previously worked as a research assistant at Taylor's University, managing two projects focused on "Feasibility Studies on the Development and Evaluation of Probiotics and Angiotensin I-converting Enzyme Inhibitory Peptides Incorporated Tropical Juice" and "Exploiting The Nutraceutical Potential of Bioactive Peptides Derived from Food Proteins".

A year later, she enrolled as a Master student at Faculty of Medicine and Health Sciences, Universiti Putra Malaysia under the supervision and guidance of Professor Amin Ismail and Associate Professor Azrina Azlan. During her postgraduate study, she received a scholarship, namely Graduate Research Fellowship (GRF), from University Putra Malaysia.

PUBLICATION

Journal

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