PRODUCTION, CHARACTERIZATION AND IDENTIFICATION OF BIOACTIVE PEPTIDES FROM *ANNONA SQUAMOSA* L. SEEDS

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2017

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by

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Thesis submitted in fulfillment of the requirements for the degree of Master of Science

December 2017

ACKNOWLEDGEMENT

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيم

All praise and thanks to the Almighty Lord, ALLAH SUBHANANH WA TAALA for giving me health, strength, knowledge and patience to complete this work. My profound thanks to my supervisor, Assoc. Prof. Dr. Gan Chee Yuen for continuously providing me guidance, encouragement and motivation throughout my research project. With his patience and willingness to share his knowledge with me, had helped me to overcome barriers in accomplishing of this task. Apart from that, his enthusiasm in conducting research has motivated me to be determined and energetic in undergoing the research. I would never have been able to finish my dissertation without the guidance from him.

My heartfelt thanks to all the staffs of the Analytical Biochemistry Research Centre (ABrC), USM in providing an excellent atmosphere, including assistance, materials and facilities for me to undergo research. In addition, I would like to acknowledge with appreciation to RUI Grant (1001/CAATS/814257) in financially supporting my project. I am also extending my deepest appreciation to my seniors, especially Siow Hwee Leng and Ngoh Ying Yuan, who had encouraged me and given me valuable advice during the times when I encountered problems throughout the research period.

Last but not least, my warmest gratitude goes to my beloved mother and siblings for their never-ending support. Their moral and financial supports are crucial for me to pass through all difficult stages in the completion of my study.

Nurul Hidayatul Afifah Binti Saufi Sofian

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LIST OF SYMBOLS

%	Percentage
&	And
<	Smaller than
>	Greater than
±	Plus minus
®	Registered trademark
°C	Degree Celcius
μ	Viscosity
=	Equal
™	Trademark
A	Alpha
B	Beta
Δ	Delta
/	Tick
~	Approximately

LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
CAN	Acetonitrile
ANOVA	Analysis of Variance
Arg	Arginine
Asp	Aspartic acid
ASPI	Annona seed protein isolate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
C18	Octadecyl carbon chain
DASPI	Defatted Annona seed protein isolate
DNS	3,5-Dinitrosalicylic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DPPHsc	DPPH scavenging activity
e.g.	For example
eqn.	Equation
EA	Emulsifying ability
ES	Emulsifying stability
FC	Foaming capacity
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
FL	Fiber layer
FRAP	Ferric reducing antioxidant power
FS	Foaming stability
FTIR	Fourier transform infra-red
Gly	Glycine
HHLeu	Hippuryl-histidyl-leucine
His	Histidine
HLeu	Histidyl leucine
HPLC	High performance liquid chromatography

IC50	Half maximal inhibitory concentration
Ile	Isoleucine
KKS	Kinin-kallikrein system
Leu	Leucine
LVR	Linear viscoelastic region
MS	Mass spectrometry
Mw	Weight average-molecular weight
MWCO	Molecular weight cut off
ND	Not detected
O/W	Oil in water
OHC	Oil holding capacity
OP	Outer parenchyma
Phe	Phenylalanine
Pro	Proline
PTFE	Polytetrafluoroethylene
RA	*Radix asragali*
RAS	Renin-angiotensin system
RG	Rhamnogalacturonan
Rha	Rhamnose
RI	Refractive index
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
Ser	Serine
SPSS	Statistical Package for the Social Science
TAE	Tris-acetate-EDTA
TFA	Trifluoroacetic acid
TPC	Total phenolic content
TPTZ	Tripyridyltriazine
Tyr	Tyrosine
U	Enzyme unit
UV-Vis	Ultraviolet-visible
Val	Valine
W/O	Water in oil

WHC	Water holding capacity
WHO	World Health Organization
3D	Three-dimensional

PENGHASILAN, PENCIRIAN DAN PENGENALPASTIAN BIOAKTIF PEPTIDA DARIPADA BIJI *ANNONA SQUAMOSA L.*

ABSTRAK

Protein isolat biji *Annona squamosa* L. (ASPI) dan protein isolat yang dinyahlemak (DASPI) telah berjaya diekstrak dengan menggunakan penampan fosfat pada pH 8 diikuti oleh pemendakan asid pada pH 4.5. Protein utama yang terdapat dalam biji merupakan β-conglycinin dan glycinin. Profil asid amino menunjukkan protein isolat tersebut mengandungi asid glutamat, glisina, asid aspartik, dan leusina dalam jumlah yang tinggi dengan nisbah amino asid yang diperlukan kepada jumlah amino asid sebanyak 0.4. Analisis Fourier tranfrom inframerah (FTIR) mengambarkan bahawa protein isolat mempunyai kumpulan berfungsi yang sama dengan protein yang diekstrak daripada kekacang, dan titik isoelektrik (pI) terdapat pada pH 3-4. Sifat-sifat berfungsi bagi kedua-dua protein juga disiasat. Bioaktiviti dinilai berdasarkan aktiviti perangkapan radikal (DPPH), kuasa pengurangan ferik (FRAP), perencatan aktiviti α-amilase, ACE, dan lipase. kedua-dua ASPI dan DASPI mempamerkan perencatan aktiviti ACE (~87%) dan lipase (~78%) yang sama. Selain itu, ASPI memaparkan antioksidan yang lebih tinggi secara signifikan (p<0.001) (52% DPPH dan 1.71 mM $FeSO_4$ FRAP), sedangkan DASPI memaparkan prerencatan aktiviti α-amilase (73%) yang lebih tinggi secara signifikan (p<0.001). DASPI telah dipilih untuk pencirian bioaktif peptida antioksidan, antidiabetik, antihipertensi dan antiobesiti dengan menggunakan hidrolisis berenzim. Kesan masa inkubasi (30 - 300 min) dan jenis enzim (papain, neutrase, bromelain, protease kulat, and pepsin) dinilai mengikut analisis bioaktiviti. Bioaktif peptida yang diekstrak pada masa inkubasi = 60 min dengan menggunakan neutrase adalah yang tertinggi secara signifikan (p<0.05) dalam memaparkan perangkapan radikal DPPH (31.51%),

FRAP (1.10 mM $FeSO_4$), prerencatan aktiviti lipase (91.23%), and aktiviti ACE (89.44%). Oleh itu, sampel tersebut telah dipisah menggunakan membran ultraturasan dengan potongan berat molekul sebanyak 3, 10, 30, 50, dan 100 kDa. Dua pecahan berat molecular terendah memaparkan aktiviti tertinggi secara signifikan (p>0.05), seterusnya telah dipilih untuk analisis penjujukan peptida. Enam peptida telah dikenalpasti dan mekanisma aktiviti telah diulas dengan menggunakan PEPSITE2. Analisis IC_{50} untuk antioksidan, antidiabetik, antihipertensi and antiobesiti juga disiasat dengan menggunakan peptida sintetik. Kesimpulannya, protein isolat dan peptida daripada biji *A. squamosa* L. menunjukan potensi antioksidan, antidiabetik, antihipertensi, and antiobesiti yang mana boleh digabungkan dalam sistem makanan berfungsi.

PRODUCTION, CHARACTERIZATION AND IDENTIFICATION OF BIOACTIVE PEPTIDES FROM *ANNONA SQUAMOSA L.* SEEDS

ABSTRACT

Annona squamosa L. seed protein isolate (ASPI) and defatted protein isolate (DASPI) were successfully isolated using phosphate buffer at pH 8 followed by acidic precipitation at pH 4.5. The predominant proteins found in the seeds were β-conglycinin and glycinin. The amino acid profiles showed that the protein isolates contained a high amount of glutamic acid, glycine, aspartic acid, and leucine with essential to total amino acid ratio of 0.4. The Fourier transform infrared (FTIR) spectra showed that the isolated proteins exhibited a similar functional group of protein, which was extracted from legumes, and the isoelectric point (pI) was found at pH 3-4. The functionalities of both protein isolates were also investigated. The bioactivities were evaluated based on 2,2-diphenyl-1-picrylhydrazyl scavenging activity (DPPH), ferric reducing antioxidant power (FRAP), α-amylase, ACE inhibitory and lipase inhibitory activity assays. Both ASPI and DASPI exhibited a similar ACE (~87%) and lipase inhibitory activity (~78%). On the other hand, ASPI exhibited a significant (p<0.001) higher antioxidant (52% DPPH and 1.71 mM $FeSO_4$ FRAP), whereas, DASPI exhibited a significant higher α-amylase inhibition activity (73%). DASPI was selected for characterization of antioxidative, antidiabetic, antihypertensive, and antiobesity bioactive peptide using enzymatic hydrolysis. The effect of incubation time (30 - 300 min) and type of enzyme (papain, neutrase, bromalein, fungal protease, and pepsin) were evaluated based on the bioactivity assay. Bioactive peptides extracted at hydrolysis condition of incubation time = 60 min using neutrase enzyme exhibited the significant (p<0.05) highest DPPH scavenging activity (31.51%), FRAP power (1.10 mM $FeSO_4$), lipase

inhibitory activity (91.23%) and ACE inhibitory activity (89.44%). The sample was then fractionated using membrane ultrafiltration with molecular weight cutoff of 3, 10, 30, 50, and 100 kDa. A significant (p<0.05) higher activity was observed by two low molecular weight fractions, thus were subjected to peptide sequencing analysis. Six potent peptides were identified, and the mechanisms of the activities were elaborated using PEPSITE2. The IC_{50} analysis for antioxidant, antidiabetic, antihypertensive and antiobesity were also investigated by using synthesis peptides. In conclusion, the isolated protein from *A. squamosa* seeds showed antioxidant, antidiabetic, antihypertensive, and antiobesity potential, which could be incorporated in the functional food system.

CHAPTER ONE: INTRODUCTION

1.1 Research background

Annona squamosa L., which is also known as custard apple, belongs to the family of Annonaceae. The fruits are usually large pulpy fruits with green or purplish-red in color with sweet taste and scent. The pulp is milky-white in color, which is soft and edible; whereas, the seeds are brownish-black in color. The pulp, which has minty or custard flavor, is commonly used to prepare as a drink or dessert. Annona plant is one of the listed Ayurvedic medicinal plants which have been practiced in India (Neethu Simon et al. 2016). Other than India, it is also found in Malaysia, Indonesia, Thailand and Brazil. *A. squamosa* L. usually gives fruits during September to March and it may also fruit twice a year (Intaranongpai et al. 2006). There are numerous researches in evaluating the pharmacological value of *A. squamosa* L. from the seeds, fruits, bark and leaves. It is traditionally used as a medicinal plant to cure of diseases, such as diabetes, dysentery, malignant tumors, cardiac problems, worm infections, hemorrhage, dysuria, fever and constipation ulcers (Yoganarasimhan 2000; Nadkarni 1994; Andrade et al. 2001). It is interesting to note that the fruit contains a lot of seeds, however, they are usually treated as a waste after the pulp is isolated.

Peptides encoded in the parental protein are usually inert and they can be released to exert various biological activities (Korhonen and Pihlanto 2006). Extensive studies have been conducted to investigate the production, characterization and *in-vitro* or *in-vivo* evaluation of these peptides in food systems in recent years. Among the hydrolysis techniques, enzymatic hydrolysis was found to be the most popular approach to produce bioactive peptides using proteolytic enzymes (Korhonen and Pihlanto 2006). Enrichment and fractionation of bioactive peptides

are then applied to characterize and to identify the specific amino acid sequence of potential bioactive peptides. Various *in-vitro* measurements have been developed to screen and evaluate the peptides with potential bioactivities that could promote different health effects. To our knowledge, there was no negligible study to elucidate the potential bioactive peptide of the isolated protein from the *A. squamosa* L. seeds.

1.2 Problem statement

Chronic diseases such as diabetes, cardiovascular diseases, obesity, respiratory diseases, and cancers, were reported to cause 59% of the 56.5 million deaths annually and 45.9% of the global burden of disease. These numbers are expected to increase up to 25% in near future (WHO 2016a). Type 2 diabetes is a common chronic disease with an increase in blood glucose levels, disturbance in carbohydrate, lipid and protein metabolism in the body that has been acknowledged as major health issue worldwide (Goldenberg and Zubin 2013). The number of diabetes patients was reported to be 382 million in the year 2013 and expected to increase almost two times from current cases with 592 million by the year 2035 (Guariguata et al. 2014). The World Health Organization (WHO) has also estimated that diabetes as the 7^{th} leading cause of death in the year of 2030 (CDC DIVISION 2010). Drugs invented for Type 2 diabetes patient, such as acarbose, miglitol, and metformin which however, have shown undesired side effects including hypoglycemia, liver damage and diarrhea (Brunetti and Kalabalik 2012). Hence, it is crucial to develop a natural alternative medication with minimal side effects for diabetic patients. Apart from that, diabetic problem is associated with hypertension which has a direct relationship to the increasing of cardiovascular and renal diseases (Grover and Yadav 2004). It was estimated that 7.5 million deaths were due to hypertension worldwide (WHO 2013). Therefore, the useful therapeutic approach for

the treatment of hypertension is discovering potential natural antihypertensive candidates by blocking the activity of angiotensin converting enzyme (ACE). The synthetic drugs for ACE inhibitors such as enalapril, captopril and lisinopril may give some side effects, including coughing and allergies (Kim et al. 2004). These situations have triggered the progress of searching for ACE inhibitors from natural sources that contain a relatively low toxicity. There is also a well-established relationship between obesity with diabetes and hypertension. Obesity, as defined by the World Health Organization, is an enormous fat accumulation that contributes to health problem. In 2014, over 600 million people were reported as obese with overall about 13% of the world's adult (WHO 2016b). Drugs such as metformin, rimonabant, orlistat and sibutramine are currently used in treating obesity, however, these medication would also have some drawback effects, including headache, nausea and vomiting, constipation, dry mouth, and dizziness (Yanovski and Yanovski 2014b). Due to the disadvantages of these drugs, a search for a new reliable and safer medication are increasing tremendously. Natural product with effective free radicals scavenging activity provides an antioxidant ability, which could help in the prevention of these diseases (Nabavi et al. 2009). By combining these health issues, it can be clearly believed that there is a rising demand for natural and safer antioxidant, antidiabetic, antihypertensive and antiobesity agents, preferably an active component that possesses all biological activities mentioned. With the aim of solving above global health concerns, bioactive peptides, which have been widely used as functional food ingredients are drawing a great deal of attention among researchers due to its promising pharmacological and nutraceutical value. Therefore, peptides from *A. squamosa* seeds can be explored as a suitable candidate as functional protein.

1.3 Research scope and objective

The main objective of this study was to elucidate the potential of *A. squamosa* L. seeds for the production of functional and bioactive protein and peptides. The specific objectives were:

1. To extract the protein and study the physicochemical and functional properties of the defatted and non-defatted protein isolates.
2. To study and characterize the bioactivities of the extracted bioactive peptides from different types of enzymes and hydrolysis time.
3. To identify the peptides that exhibit the bioactive properties and to study their structure-activity relationships.

CHAPTER TWO: LITERATURE REVIEW

2.1 *Annona Squamosa* Linn

2.1.1 Botanical description

Annona squamosa Linn is a small, well-branched shrub that belong to family *Annonaceae*. Table 2.1 shows the native name for *A. squamosa* L. fruits based on countries. It is originated from West Indies and South America (Neethu Simon et al. 2016). However, Pinto et al. (2005) states that it is originated in lowland Central America where it is indigenous, and from there it was distributed to Mexico and throughout tropical America. It is a common species in the West Indies and has been present since at least 1689 (UK Natural History Museum 2014) It is cultivated in areas with an alternation of wet and dry seasons, such as Indonesia, Thailand, and Taiwan (Intaranongpai et al. 2006). The bark is dark brown, thin, fissured and fibrous. The shapes of the leaves are simple, alternate, ovate or oblong-lanceolate to oblong-elliptic and it has about 2.5 - 3 cm long bisexual flowers (CABI 2015).

Table 2.1: Native names of *Annona squamosa* L. from different places (CABI 2015).

Places	**Names**
The Bahamas	Sugar apple
Brazil	*fruta-do-conde*,*fruta-de-conde*, *condessa*, *fruta-pinha*, *pinha*, *ata* or *anona*.
Burmese	*Aajaa thee*
Cambodia	*Plae teib*
Ethiopia	Gishta
Germany	*Zimtapfel*
Ghana	Sweet Apple
Greece	*γλυκόμηλο* (sweet apple)
Haiti	*Kachiman*
Hong Kong	*Foreign lychee*
Iceland	*Hvaðerþetta*
India:	
Bengali	*Ata*
Gujarati	*Sitaphal*
Hindi	*Sharifa* or *sitaphal*
Kannada	*Sitaphala*
Marathi	*Sitaphal*

Table 2.1: Native names of *Annona squamosa* L. from different places. (continue…)

Places	Names
Punjabi	*Sharifa*
Tamil	Sitappalam
Telugu	sita phalamu
Indonesia	*Srimatikiya* or *srikaya*
Kenya	*Matomoko*
Madagascar	*Conicony*
Malaysia	*Buah nona*
Mali, Africa	*Hairico*
Nepal	Saripha
Nicaragua	Annona Guatemala
Northern Nigeria	*Fasadabur*
Pakistan	*Sharifa*
Philippines	*Atis*
Sri Lanka	Anoda
Taiwan	*Sakya*
Tanzania	*Matopetope*
Thailand	*noi-na*
Vietnam	*Mãng cầu ta* or *na*
Yemen	*Khirmish*

The fruits are usually in green or purplish red in color with a sweet taste and scent (Plate 2.1). It also contains a lot of seeds inside the pulp. Plate 2.2 shows the pulp is in milky white color which is soft and edible while the seeds are shiny brownish-black color (Plate 2.3). The pulp, which often has a minty or custard flavor, is considered to be rich in vitamin C and it is commonly used in preparing cool drinks, flavoring ice puddings and fermented liquor (Saha 2011). It was reported that in 100 g of its fruit pulp contains 70.8 g moisture, 20.41 g carbohydrate, 0.57 g fat, 2.78 g dietary fiber and 5.44 g protein (Bhardwaj et al. 2014). However, the seeds are treated as waste. Therefore, it would be interesting to explore the potential of the seeds.

Plate 2.1 Outer appearance of *A. squamosa* L. fruits (source from Maniac 2015)

Plate 2.2 The pulp of *A. squamosa* L. fruits (source from Bhagat 2017)

Plate 2.3 *A. squamosa* L. seeds

2.1.2 Traditional use

Plants are important sources of medicine since the beginning of human civilization. Ayurvedic medicine or Ayurveda is one of the world's oldest healing treatments that was developed in India. *A.* s*quamosa* L. is one of the Ayurvedic herbs used traditionally for health care needs. The leaves paste was used to treat ulcer, wounds and dysentery. It was also used to induce suppuration of tumors (Khare 2007). In the natives believe, the leaves were efficacious in treating hysteria and fainting spells (Pandey and Barve 2011). Other than that, the leaves of *A. squamosa* L. were made as cough remedy syrup in Brazil (Duke and Vásquez 1994). The roots were traditionally used in treating cough in India (Khare 2007). The bark of this plant was used to halt diarrhea (Khare 2007). The unripe pulp possesses a cooling effect and was used in treating burn sensation. Other than that, it was also reported to be given for treating diarrhea, dysentery, and atonic dyspepsia (Nandhakumar and Indumathi 2013).

2.1.3 Pharmacological investigation on *A. squamosa* L.

There was an interest in the scientific knowledge of *A. squamosa* L. due to its traditional use in society and also the claims for its pharmacological value. However, most of the researches conducted were related to carbohydrates, alkaloids, tannins, fixed oils and phenolic compound from its roots, bark, leaves and fruits (Yadav et al. 2010). Antidiabetic, antioxidant, antivirus, anticancer, antimicrobial, and others pharmacological value of the plant were evaluated as follows:

2.1.3 (a) Antidiabetic

Increasing research in antidiabetic activity of natural products has led to several studies of these activities from *A. squamosa* L. plant. There was an extensive use of *A. squamosa* L. young leaves by tribal men in India in treating diabetes (Sushilkumar et al. 2016). Shirwaikar et al. (2004) had proven the antidiabetic claims by inducing the aqueous leaf extract in Type 2 diabetic rats. *A. squamosa* L. aqueous leaf extract exhibited a high amount of mucilage that was documented as an agent to reduce the elevated blood glucose levels (Ajabnoor 1990). Panda and Kar (2007) reported the ability of *A. squamosa* L. aqueous leaf extract in amelioration of hyperthyroidism which are often promote to diabetes mellitus. Other than that, the root extract was also claimed to possess antidiabetic properties through the study conducted by Kaleem et al. (2006). Therefore, the existence of antidiabetic property has place an interest in evaluation of antidiabetic bioactive peptides from *A. squamosa* L. seeds.

2.1.3 (b) Antioxidant

Oxidative stress has been shown to promote others acute diseases such as diabetes, hypertension, cancer and brain damage (Pham-Huy et al. 2008). Natural antioxidant plays an important role in radical scavenging thus reducing the oxidative stress. A high antioxidant activity from *A. squamosa* L. pulp extract was reported by Nandhakumar and Indumathi (2013). The presence of flavonoids in *A. squamosa* L. leaves as a secondary metabolites was reported as a natural source of antioxidants (Kalidindi et al. 2015). The leaves of *A. squamosa* L. was also reported to demonstrate antioxidant activity. Based on a study conducted by Kaleem et al. (2006), other than lowering the blood glucose level, the leaf extract was also found to

reduce the lipid peroxidation and increase the activity of plasma insulin in experimental diabetic rats.

2.1.3 (c) Anticancer

Cancer is a common cause of death. Chemotherapy, surgery, and radiotherapy are still remained as the cornerstone of treatment. On account of their minor harmful side effects on non-targeted human cells and biological environment, herbal medicines are gaining popularity. There were a lot of evidences in the astonishing accomplishment of herbal medicine for cancer treatment. Tribe in south China used the seeds of *A. squamosa* L. as a remedy to treat "malignant sores" (cancer) (Chen et al. 2012). A study conducted by Chen et al. (2012) proves the claim of anticancer properties in the seed extract which is due to the presence of annonaceous acetogenin compounds. Wang and coworkers (2014) also reported a significant anticancer activity on human epidermoid carcinoma cell line KB-3-1 and colon cancer cell line HCT-116 from aqueous leaf extracts of *A. squamosa* L. Besides seeds and leaf, fruit pericarp of *A. squamosa* L. was also reported to have anticancer properties (Joy and Remani 2008).

2.1.3 (d) Antimicrobials

The traditional claim that concoctions of *A. squamosa* L. can be used in the treatment of bacterial diseases (Yusha et al. 2011). Substantiated scientific evidence conducted by Yusha et al. (2011) supports the claim of antibacterial properties. An active antibacterial activity was observed for the leaf extract of *A. squamosa* L. against *Staphylococcus aureus* and *Streptococcus pneumonia* due to the presence of alkaloids, flavonoids, glycosides, reducing sugars, steroids and tannins (Yusha et al. 2011). The validation in antimicrobial existence in *A. squamosa* L. leaf extract

conducted by Kalidindi et al. (2015) explained the mechanism of phytochemical compounds responsible in killing bacteria by directly attacking and damaging their cell membrane. Stem bark of these plants was also reported to carry antimicrobial properties (Kachhawa et al. 2012).

2.1.3 (e) Other medicinal properties

In regards of the traditional remedy and a strong believe in tribes of its medicinal value have encouraged researchers in exploring for other therapeutic ability from *A. squamosa* L. plant. Johns et al. (2011) have conducted an *in vitro* study of antimalarial activity from *A. squamosa* L. leaf extract to inhibit the growth of the chloroquine-sensitive strain 3D7 and chloroquine-resistant strain Dd2 of malignant Plasmodium. A promising gastro-protective effect was also revealed in the study conducted by Yadav et al. (2011) with comparison of isocorydine and N-methylcorydaline compound extracted from the bark of *A. squamosa* L. with standard gastritis drugs. *A. squamosa* L. also shows an effective potential against sexually transmitted diseases such as Neisseria gonorrhoeae (Johns et al. 2011). Nevertheless, the understanding of the therapeutic ability of *A. squamosa* L. plant is still limited. The demonstrated studies conducted were all based on the phytochemical compound existed in the plant. However, protein and peptide study have yet to be explored and the existence of pharmacological value coupled with traditional believed among folks have open an interest in this research.

2.2 Protein

Plants are major protein sources for human beings and animals with about 65% of proteins consumed by human beings are vegetal products, such as seeds and tubers. Despite of the varieties of plant protein exist, about 40% of proteins consumed in the world are cereal seed proteins (Kawakatsu and Takaiwa 2017). Many food proteins isolated from plant such as cowpea, pigeon pea, mung bean, Chia seed, and chickpea were found to have biological activities beyond basic nutritional values and have great potential as therapeutic (Butt and Batool 2010; Timilsena et al. 2016). The bioactivity of protein has mainly been focusing on immunological proteins (immunosuppressing or immunomodulatory), antimicrobial, vitamin- and mineral-binding proteins, enzymes, hormones as well as growth factor inhibitory protein from different food sources (Walther and Sieber 2011). Therefore, exploring the potential of proteins from *A. squamosa* L. seeds could be beneficial to food and nutraceutical industries.

In addition, biologically active proteins can demonstrate a physiological activity in the gastrointestinal tract, including the enhancement of nutrient absorption, inhibiting enzymes, and modulating the immune system (Jandhyala et al. 2015). The validity of these bioactive proteins such as bioactive whey protein as an effective nutritional supplement by the medical community, which can enhance both healthy and deficient immune systems as well as it is safe in overactive immune conditions, have open the door of interest to the world in exploring more bioactive protein from food sources (Walther and Sieber 2011). Other than that, lysozyme, lactoferrin A, haptocorrin, secretory immunoglobulin lactoperoxidase, bile salt stimulated lipase, α-lactalbumin, β- and κ-casein, and tumor growth factor β are also the example of commercially available bioactive protein in the pharmacological industry (Lönnerdal

2013). The significance of protein in biotechnology is not only limited to the pharmaceutical industry, but also promote a greater impact in cosmetic, food and beverage industry (Noble and Agrawal 2005). Protein and peptide derived from plant and animal have been approved by FDA as a supplementary dietary product with annual sale close to USD 20 billion in 2012 (Mezo 2013). Due to the increase demand in a safer product, the development of protein engineering in food and pharmaceutical products have therefore increased tremendously.

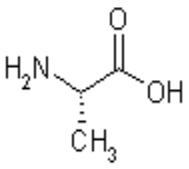
2.2.1 Physicochemical properties

Physicochemical of a protein is defined as the interrelation of physical and chemical characteristics of the protein, including structure (i.e. primary, secondary, tertiary and quaternary structure), amino acid composition and sequences, type of bonding (i.e. covalent bonding, Van der Waals, hydrophobic interaction, disulfide bridge and electrostatic interaction) and molecular weight. These characteristics will influence the solubility, stability and structural organization of the protein (Campbell and Farrell 2008). In general, the amino acid sequences from the primary structure dictate the higher level of protein structure and define their biological properties (Berg et al. 2002).

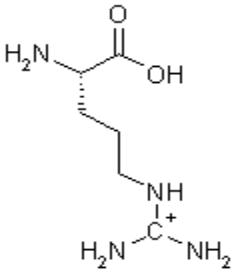
Amino acids are responsible in protein construction which contain both an acidic carboxyl group and a basic amino group (Lodish et al. 2000). This dual functional group allows the single amino acids to merge with one another through peptide bonds that consist of amide bonds between the $-NH_2$ of one amino acid and the -COOH of another (Schultz 2013). The side chain of amino acids confers different chemical, physical and structural properties foster in the differences of its

structure (Schultz 2013) and they classified the nature of amino acids as acidic, basic or neutral protein (Schultz 2013). Their functions towards the health of humans depending on two influential factors, amino acid composition and sequences (Korhonen and Pihlanto 2006). The radical scavenging and metal chelating activities were influenced by high contents of hydrophobic and aromatic amino acids (Pownall et al. 2010). The interaction between hydrophobic amino acids with hydrophobic targets, such as the cell membrane are responsible in enhancement the bioavailability such as antihypertensive, antidiabetic and immunological properties (Himaya et al. 2012). Despite these 20 standard amino acids required for synthesis of various proteins as shown in Figure 2.1, only 10 are synthesized in the human body while the remaining are called essential amino acids. Essential amino acids must be acquired in the daily diet in maintaining body homeostasis. Therefore, information on the essential amino acid composition existed in a food product is important.

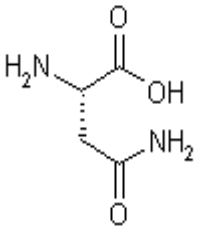
Essential amino acid



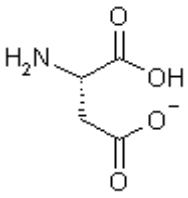
L-Alanine
Ala



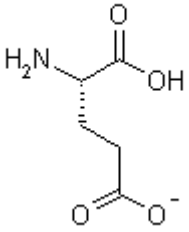
L-Arginine
Arg



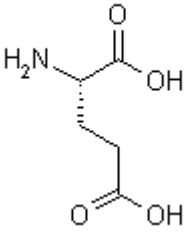
L-Aspargine
Asn



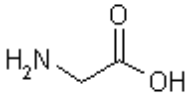
L-Aspartic acic
Asp



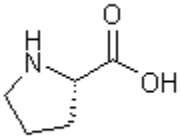
L-Glutamic acid
Glu



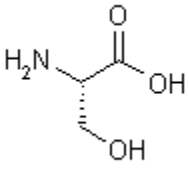
L-Glutamine
Gln



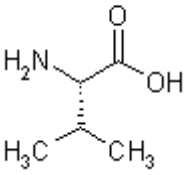
Glycine
Gly



L-Proline
Pro

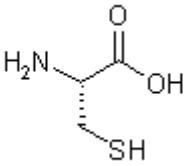


L-Serine
Ser

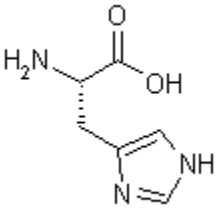


L-Valine
Val

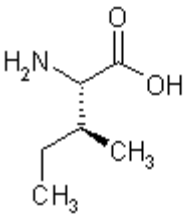
Nonessential amino acid



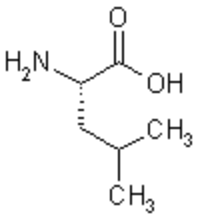
L-Cysteine
Cys



L-Histidine
His

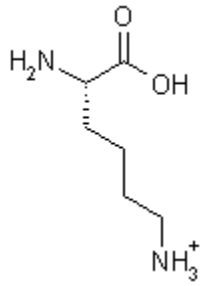


L-Isoleucine
Ile

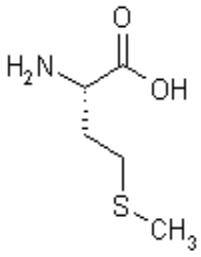


L-Leucine
Leu

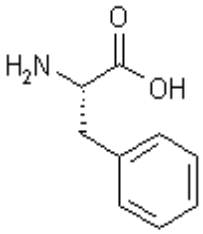
Figure 2.1: α–amino acids (Adapted from Lodish et al. 2000)



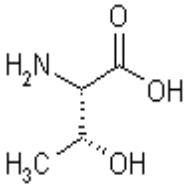
L-Lysine
Lys



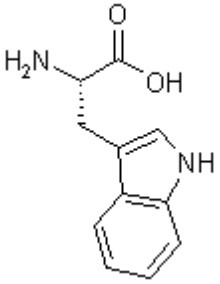
L-Methionine
Met



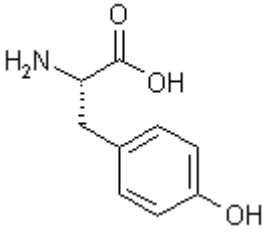
L-Phenylalanine
Phe



L-Threonine
Thr



L-Tryptophan
Trp



L-Tyrosine
Tyr

Figure 2.1: α–amino acids (continue..)

Secondary structure is defined as stretches or strands of proteins or peptides that own a specific characteristic in structural conformations. This hydrogen bond dependent structure consists of two types of conformation, α-helix and β-sheet. The α-helix structure is a right-handed coiled strand with side-chain substituents of the amino acid group extend to outside (Campbell and Farrell 2008). The stability of this structure depends on hydrogen bonding between C=O in the strand and N-H group. The carbonyl oxygens of the peptide bond point upwards toward the downward-facing amide protons as the helix turns with the N-H group point outwards from the helix (Andersen 2001). The different alignment in β-sheet is due to the hydrogen bond that is between strands (inter-strand) rather than within strands (intra-strand) (Campbell and Farrell 2008). A pair of strand lying side-by-side forms a sheet

conformation. The β-sheet can be either parallel or anti-parallel depending on whether the strand directions represented in N-terminus to C-terminus is the same or opposite direction. However, more well-aligned hydrogen bonds have made the β-sheet with anti-parallel conformation becoming more stable (Andersen 2001). Fibrous and globular proteins are defined by their secondary structure. Fibrous proteins are usually significant in forming biological structures such as collagen, fibrin and keratin (Campbell and Farrell 2008). Unlike fibrous proteins, globular proteins have a globe-like structure such as most enzymes and usually consist of a combination of the two secondary structures (Campbell and Farrell 2008). The backbone folds back on itself to produce the spherical which promote to compact tertiary and quaternary structure. Globular protein is water soluble compared to fibrous protein. Due to this, globular protein can exist as individual molecules or in small clusters in fluid and achieved a broad range of important biological functions. For example, it can act as transport vehicles for other molecules in circulating blood (Adams et al. 2004). It can also act as antibodies in the body's immune system, involved in the replication and repair of DNA. Seed protein commonly consists of globular proteins known as globulin or a storage protein (Adams et al. 2004). Therefore, *A. squamosa* L. seed protein could have the potential in contributing biological activities.

The tertiary structure of protein is the 3-D arrangement of an entire protein molecule. A maximum stability through bending and twisting of the protein molecule which is fashioned by many stabilizing forces due to bonding interactions between the side-chain groups of the amino acids, including ionic, hydrogen, hydrophobic, and disulphide bonds (Campbell and Farrell 2008). The proximity in the tertiary structure of the native protein is dependent on the folding pattern which escort the

separated residues in the amino acid sequence (Andersen 2001). The interchain associated that is made up of more than one polypeptide define the quaternary structure of protein with every single polypeptide chain having its own distinct N- and C-terminus (Thyparambil et al. 2015). Both of the tertiary and quaternary structure of a native protein create the geometry and reactive functional groups that merge together, forming the bioactive sites of the protein (Thyparambil et al. 2015).

Unfortunately, bioactivity assays are insufficient to support this claim. To address this situation, experimental approach is required to characterize the physicochemical properties of protein through the identification of amino acid composition, protein component and type of protein extracted. The purity of protein can be analysed by using High Performance Liquid Chromatography (HPLC). Other than that, analytical methods such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) could be used to determine identity of protein based on its molecular size (Suhail et al. 2011). Meanwhile, Fourier Transform Infrared Spectroscopy (FTIR) offers a qualitative analysis, which identifies functional groups in a molecule by producing an infrared absorption spectrum (Barth 2007). These physicochemical properties shown are related to various functional properties in which it provides information in the interaction of protein for the purpose of controlling functionality (Barać et al. 2011) and therefore these analyses have to be performed.

In general, the interaction of proteins with other ingredient is influenced by its structural-functional relationship (Joshi et al. 2012). Bioactive properties of a food protein are also linked by its physicochemical properties such as amino acid composition (Timilsena et al. 2016). To our knowledge, there is no relevant study conducted in characterizing Annona seed protein in terms of physicochemical and

bioactive properties, thus lead to the novelty of this research in searching for a functional food ingredient with nutraceutical properties as claimed by the Indian tribe. However, it was known that the comprehensive potential of bioactive properties of protein was encrypted in their parental structure. Digestion of these parental proteins may lead to release of peptides with high bioactive properties. Therefore, the study of bioactive peptides from *A. squamosa* L. seed protein isolate would bring to more discovery potential bioactive peptides.

2.2.2 Other properties of protein

Other than pharmacological properties, the interactions such as hydrophobic, steric and electrical influence the physicochemical properties along with its structure and functionalities and thus, varies the solubility, foaming properties, emulsifying properties, rheological properties, oil and water holding capacity of proteins. A dynamic functional food ingredient is supposed to show versatility and also provide essential amino acids, fulfilling the nutritional requirement (Muro Urista et al. 2011). In general, functional properties describe as the physical and the chemical characteristics of a specific protein that impact its behavior in the food system and drug formulation during processing, storage, cooking and consumption. The functional properties of protein involved the hydration properties (i.e. protein solubility, water and oil holding capacity), structural and textural properties (i.e. gelling and rheological properties), and surface properties (i.e. emulsifying and foaming properties). The global functional food ingredient market has estimated to be worth USD 2.5 billion by the year 2020. In coordination with the increasing demands of functional food in the global market, researchers have been urged to put more effort in exploring new raw materials as promising sources of functional food.

Protein derived from plant sources have drawn more interest among academia due to their natural sources are easier to be accepted by consumers (Timilsena et al. 2016). In this regard, protein from *A. squamosa* L. seed can be explored as a potential source of pharmaceutical and nutraceutical products. The properties examined in this study include protein solubility, water holding capacity (WHC), oil holding capacity (OHC), emulsification, foaming capacity (FC), foam stability (FS), gelling properties, and rheological properties.

2.2.2 (a) Protein solubility

Protein solubility is defined as the amount of protein in the sample that dissolves into solution (Kramer et al. 2012). It is the main characteristic of protein use in liquid foods and beverages industry (Kramer et al. 2012) because it relates to the potential utilization of functional properties, including foams, emulsions and gels. Amino acid composition, sequences, molecular weight, polar/nonpolar group content, and hydrophobicity/hydrophilicity influence the solubility of protein (Pace et al. 2004). Ionic strength, pH, temperature, type of solvent and processing condition are the example of environmental factors that could affect the solubility of proteins (Pace et al. 2004). In general, the interaction of electrostatic and hydrophobic between the protein molecules has influenced the degree of solubility in aqueous medium. Solubility of protein can be achieved when the electrostatic repulsion between the molecule is higher than hydrophobic interaction (Pace et al. 2004). The increase the solubility is based on the sensitive balance between repulsive and attractive intramolecular forces.

2.2.2 (b) Water holding capacity (WHC) and oil holding capacity (OHC)

WHC is defined as the ability of the moist material to retain the water by physically trapping the water through hydrogen bonding when subjected to centrifugal force or stress (Alfredo et al. 2009). This hydration property is vital in the perspective of the food industry, which can increase bulk volume of the food, avoid food synaeresis, as well as modify the viscosity and the texture of food product to give a desirable characteristic to the food (Rodriguez *et al.*, 2006). From the nutraceutical aspect, high WHC is able to increase faecal volume, trigger frequent peristaltic movement which results in reducing the transit time of faeces. As a result, it can relieve constipation and intestinal tract disorder (Alfredo et al. 2009). OHC is defined as the amount of oil retained by the material after mixing and centrifugation (Inman et al. 2012). OHC is another crucial functional property in formulating food products, particularly in high-fat products to confer the food with mouth feel and greasy sensation. Protein with high OHC stabilizes high fat-food products and emulsion. For example, in meat processing, water and oil binding capacities are crucial in determining the quality of meat and other meat products, especially sausages and canned ham by rendering meat tenderness and gravy. Both properties are dependent with the physicochemical characteristic of the protein (i.e. amino acid composition, number of exposed polar groups, surface hydrophobicity) and external factor (i.e. pH, temperature and protein concentration) (Chel-Guerrero et al. 2002). Guo (2013) reported that the WHC of protein is based on the protein-water interaction through hydrogen bonding allow variations of water-binding site in a protein molecule, meanwhile the OHC is due to the hydrophobic sites and surface porosity of protein. However, external factors aforementioned could also influence

the changes in WHC and OHC during protein recovery and/or measurement (Chel-Guerrero et al. 2002).

2.2.2 (c) Emulsifying ability (EA) and emulsion stability (ES)

Emulsification is the most important procedure in many formulated cosmetic and pharmaceutical industry. An emulsion can be defined as one immiscible liquid in another in the form of discrete droplets. It represents the heterogeneous mixture of fat globules. Basically, there are two types of food emulsion which are oil-in water (O/W) and water in oil (W/O). O/W emulsion describes dispersion of oil as droplets in the continuous phase in food product such as milk, cream, mayonnaise and salad dressing. While W/O emulsion refers to oils constructs a continuous phase, whereas water is the dispersed phase such as butter and margarine (Rybak 2013). The O/W emulsion commontly disclose a creamy texture while W/O system has greasy texture properties. Emulsification is a critical parameter that affects the selection of a protein in industrial process. The tension at the water-oil interface could be reduced by protein, meanwhile the stabilizing effect of protein comes from the membrane matrix that surrounds the oil droplets to prevent its coalescence (Rybak 2013). This property is essentially required in the food industry to keep the internal phase uniformly distributed between two immiscible phases during long storage and consumption. Methods of creating emulsion are generally via high shear equipment such as homogenizer in which it can emulsify oil and aqueous phase together in the presence of surface active agent. For instance, the input of mechanical energy for the formation of O/W emulsion is used to break up large oil droplets into smaller and disperse these droplets in an aqueous continuous phase for a given volume. Emulsifier, which is structurally amphiphilic, its hydrophobic groups adsorb onto oil

surface and hydrophilic groups spread out in aqueous continuous phase and therefore lower interfacial tension and result in a stabilizing interface layer. The capability of emulsion to resist any changes under extreme condition such as high temperature over time can determine the emulsion stability of emulsifier (Rybak 2013) and the condition for emulsification varies based on the properties of protein (i.e. ionic strength, viscosity, surface hydrophobicity and molecular flexibility), protein concentration and pH (Nakai 2004).

2.2.2 (d) Foaming capacity (FC) and foam stability (FS)

Foam is a colloid system with a gaseous phase stabilized in a continuous matrix to provide an aerated structure to a product, generally in the food industry and in foam drug delivery in dermatology. This foaming capacity has been employed in manufacturing beer, breads, cakes, ice cream, topping and whipped creams. Mechanical mixing of gas and liquid phase is one of the technique to create foams through stirring or whipping (Rybak 2013). Physicochemical relationship, such as protein-protein interaction, including electrostatic, hydrogen bonds and hydrophobic interaction affected foam formation and stabilization.

The ability of protein to generate foam is also an important application in industrial production. Foaming capacity has a similar principle as emulsifying capacity, which entails surface activity to allow foaming to occur by involving hydrophobic sites to adsorb onto air bubbles and hydrophilic sites orientate to aqueous continuous phase. In foam formation of protein, solubilized protein first diffused in the water/air interface, concentrate and reduce the surface tension. The unfolding at the interface was then formed an orientation of polar moieties toward water with hydrophobic and hydrophilic oriented at aqueous and non-aqueous state.

The film was formed as polypeptides interact with partially denatured protein. The cohesive film form at the water-air interface should possess sufficient viscosity to maintain stability and prevent rupture and subsequent coalescence to ensure good foam formation of protein (Kilara and Panyam 2003).

2.2.2 (e) Gelling properties

Gel is described as ‘a form of matter intermediate between a solid and a liquid’. The property is the result of the complex interaction between solvent and the molecules to form a molecular network (Gan et al. 2009). The gel formation of protein involves a development of uncoiled polypeptide segment that interact at specific points to form three-dimensional (3D) cross-linked network (Sinz 2006). The protein-protein and protein-solvent interaction develop high structural rigidity which affects the formation of the 3D protein network. The liquid in gel prevents these 3D matrices from collapse into a compact mass and the matrix prevents the liquid from flowing away. A range of physicochemical variables such as ionic strength, pH, protein concentration, and fat content have been shown to affect the progress of gelation and the final gel properties of various whey protein products (Sun and Holley 2011).

Gelation of food protein is an important function assign for various food manufacturing such as milk gels, fruit jellies, bread dough, comminuted meat and fish product. Gelation principle is commonly used to evaluate food protein such as textural properties and juiciness. Different protein is used in the food industry to produce gel or gel-containing product that shows various rheological properties, appearance and gel point. In pharmaceutical approach, gel formulation have been used as oral administration of drugs with suitable rheological characteristics for ease