IDENTIFICATION OF BIOACTIVE PEPTIDE FROM CHICKEN OVALBUMIN USING AN INTEGRATED BIOINFORMATICS-ASSISTED APPROACH AND DETERMINING THEIR FUNCTIONAL SIGNIFICANCE

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

TABLE OF FIGURES

Page

LIST OF SYMBOLS

β	Beta
Da	Dalton
h	Hour
IC_{50}	Half maximal inhibitory concentration
M	Molarity
m	Meter
mbar	Millibar
min	Minute
m/z	Mass to charge ratio
pI	Isoelectric point
p-value	Significant value
V	Voltage
^{o}C	Degree Celsius
π	Pi electron

LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ANOVA	One-way analysis of variance
BAP	Bioactive peptide
BHT	Butylated hydroxytoluene
DM	Diabetes mellitus
FM	Full match
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide
HCl	Hydrochloric acid
LC/MS	Liquid chromatography mass spectrometry
MS/MS	Tandem mass spectrometry
NaOH	Sodium hydroxide
OVA	Ovalbumin
PM	Partial match
sACE	Somatic angiotensin converting enzyme
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
tACE	Testis angiotensin converting enzyme
TBHQ	Tert-butyl hydroquinone
USDA	U.S. Department of Agriculture

LIST OF APPENDICES

IDENTIFIKASI PEPTIDA BIOAKTIF DARIPADA OVALBUMIN AYAM DENGAN MENGGUNAKAN PENDEKATAN BANTUAN BIOINFORMATIK BERSEPADU DAN MENENTUKAN KEPENTINGAN FUNGSINYA

ABSTRAK

Sebuah pendekatan bioinformatik secara bersepadu telah dikembangkan untuk mengenalpasti peptida perencat kepada enzim pengubah angiotensin (ACE) dan dipeptidil peptidase-4 (DPP-4) dari ovalbumin ayam OVA). Pendekatan ini melibatkan PeptideCutter, Peptide Ranker dan Pepsite2 untuk menghidrolisis urutan protein OVA menjadi peptida yang lebih kecil, bagi mengenalpasti kebarangkalian peptida menjadi bioaktif dan bagi menyiasat interaksi di antara peptida dan enzim sasaran masing-masing (iaitu ACE dan DPP-4) . OVA sequence was initially hydrolysed using PeptideCutter. Pepsin (P), Chymotrypsin (C) and Trypsin (T) were used in 7 different OVA (O) hydrolysis combination (OP, OC, OT, OCT, OPC, OPT and OPCT), thus, producing 71 peptides Awalnya, urutan OVA dileraikan menggunakan PeptideCutter. Pepsin (P), Chymotrypsin (C) dan Trypsin (T) telah digunakan dalam 7 kombinasi kondisi peleraian OVA (O) iaitu OP, OC, OT, OCT, OPC, OPT dan OPCT, lalu menghasilkan 71 peptida. Sepuluh peptida bioaktif baru (iaitu CF, KM, ELPF, AM, ADHPH, LPR, PR, FR, PRM dan GR) kemudiannya telah berjaya dikenalpasti dan dipilih berdasarkan urutan asid amino serta interaksi antara peptida dengan ACE dan DPP-4. Ketika menghadapi ACE, nilai IC_{50} CF, KM, `ELPF, AM, ADHPH, LPR, PR, FR, PRM dan GR secara masing-masing adalah 1.82, 1.89, 4.24, 3.07, 3.54, 1.30, 5.47, 4.35, 5.22 dan 3.11 mM. Hasil ini setanding dengan inhibitor komersial untuk ACE, captopril (IC_{50} = 3,98 mM). Namun, apabila menghadapi DPP-4, aktiviti perencatan

hanya setanding dengan peptida perencat DPP-4 lain yang telah dilaporkan seperti EK (IC_{50} = 3.22 mM) dan GL (IC_{50} = 2.62 mM). Hal ini kerana peptida tersebut secara masing-masing mendapat nilai IC_{50} 2.99 , 2.22 , 9.92 , 2.79 , 1.66 , 1.43 , 4.11 , 2.47 , 2.50 dan 2.83 mM. Keseluruhannya, kesemua peptida yang terpilih dapat merencatkan aktiviti ACE dan DPP-4 dengan jayanya. Perbandingan hidrolisis secara *in vitro* dengan kajian secara *in silico* menunjukkan bahawa semua hidrolisat OVA tidak dapat menghasilkan peptida yang sama. Walaupun, dapat menghasilkan bilangan peptide yang besar, hanya sebahagian peptida yang terhasil daripada *in vitro* sahaja yang dapat dijumpai di dalam peptida *in silico* (yakni di dalam OCT (80%) diikuti oleh OPCT (78%), OC (61%), OPC (57%, OPT (55%), OP (52%) dan OT (47%). Di antara sepuluh peptida novel yang disebut, CF dan AM dijumpai di semua hidrolisat OVA, PRM dijumpai di OP dan OPC, PR dijumpai di OP dan OPCT, dan GR dijumpai di OC, OPC, OPT dan OPCT.Peptida yang dihasilkan juga menunjukkan interaksi yang signifikan dengan ACE dan DPP-4 kerana mempunyai ciri-ciri tertentu seperti yang telah dihuraikan (contohnya, dipeptida perencat DPP-4, tryptophan, threonine atau methionine didapati berada di terminal-N dan peptida perencat ACE pula, proline dijumpai pada kedudukan terakhir, kedua terakhir atau ketiga terakhir terminal-C peptida). Kesimpulannya, kajian ini menunjukkan bahawa OVA berpotensi menghasilkan peptida perencat ACE dan DPP-4 yang dapat digunakan untuk rawatan hipertensi dan diabetes, sambil mengetengahkan manfaat besar pengurangan waktu dan kos penyelidikan dengan menggunakan pendekatan *in silico*.

IDENTIFICATION OF BIOACTIVE PEPTIDE FROM CHICKEN OVALBUMIN USING AN INTEGRATED BIOINFORMATICS-ASSISTED APPROACH AND DETERMINING THEIR FUNCTIONAL SIGNIFICANCE

ABSTRACT

A complete integrated bioinformatics approach was developed to identify angiotensin-converting enzyme (ACE) and dipeptidyl peptidase-4 (DPP-4) inhibitory peptides from chicken ovalbumin (OVA). This approach involved PeptideCutter, Peptide Ranker and Pepsite2 in order to hydrolyse OVA protein sequence into smaller peptides, to identify the probability of the peptides being bioactive and to investigate the interaction between the peptides and target enzymes (i.e. ACE and DPP-4), respectively. OVA sequence was initially hydrolysed using PeptideCutter. Pepsin (P), Chymotrypsin (C) and Trypsin (T) were used in 7 different OVA (O) hydrolysis combination (OP, OC, OT, OCT, OPC, OPT and OPCT), thus, producing 71 peptides. Top ten novel bioactive peptides (i.e. CF, KM, ELPF, AM, ADHPH, LPR, PR, FR, PRM and GR) were then successfully identified and selected based on the amino acid sequences as well as the peptide interactions with ACE and DPP-4. Against ACE, IC_{50} of CF, KM, ELPF, AM, ADHPH, LPR, PR, FR, PRM and GR were 1.82, 1.89, 4.24, 3.07, 3.54, 1.30, 5.47, 4.35, 5.22 and 3.11 mM, respectively. These results were comparable to commercial inhibitor for ACE, captopril (IC_{50} = 3.98 mM). While against DPP-4, however, inhibitory activities were only comparable to other reported DPP-4 inhibitory peptides such as EK (IC_{50} = 3.22 mM) and GL (IC_{50} = 2.62 mM), as the peptides were able to achieve 2.99, 2.22, 9.92, 2.79, 1.66, 1.43, 4.11, 2.47, 2.50 and 2.83 mM, respectively. Nevertheless, all of the selected peptides were able to

effectively inhibit the activity if ACE and DPP-4. The comparison of *in vitro* hydrolysis to the *in silico* study showed that all of the OVA hydrolysates were unable to completely produce the same peptides. Despite producing a large number of peptides, only some of the *in vitro* generated hydrolysis peptides matched with the predicted peptides from *in silico* peptides (i.e. in OCT (80%) followed by OPCT (78%), OC (61%), OPC (57%), OPT (55%), OP (52%) and OT (47%). Among the ten novel peptides, CF and AM were found in all of the OVA hydrolysates, PRM was in OP and OPC, PR was found in OP and OPCT, and GR was found in OC, OPC, OPT and OPCT. The peptides produced also showed significant interaction with ACE and DPP-4 due to the elaborated features (e.g. for DPP-4 inhibitory dipeptide, tryptophan, threonine or methionine was found to be at N-terminal and for ACE inhibitory peptides, proline was found at the ultimate, penultimate or antepenultimate position of C-terminal of the peptides). Conclusively, this study showed that OVA has the potential in producing ACE and DPP-4 inhibitory peptides that could be used for hypertension and diabetes treatments, while underlining the tremendous benefit in time and cost reduction of the research by using *in silico* approach.

CHAPTER 1

INTRODUCTION

1.1 Background

Bioactive peptides are specific fractions of protein that serve as a beneficial functional compound to living organisms especially in aiding the progression of their organ system. Depending on their sequence, they may exert different properties which lastly provide various functions such antidiabetic, antihypertensive, antioxidant, antithrombotic and immunomodulating and opioid (Toldrá, Reig, Aristoy, & Mora, 2018). Before they can function, the protein that carries these bioactive peptide sequences have to be broken down and released to our body (Rémond, Savary-Auzeloux, & Boutrou, 2016). Food proteins either from plant or animal proteins can release bioactive peptides through several means. Naturally, proteins that are consumed through eating, are digested by digestive enzymes (gastrointestinal digestion). Bioactive peptides can also be produced by enzymatic hydrolysis (microbial or plant proteolytic enzymes) or by microbial fermentation. The most progressive method to produce a bioactive peptide is to chemically synthesize it thus producing a synthetic bioactive peptide (Chakrabarti, Guha, & Majumder, 2018). Researchers have diverted some of their attention from only studying protein to discovering the essence of the protein itself (peptides). Therefore, the focus of this study was to explore the discovery of potential antihypertensive and antidiabetic bioactive peptides from a protein food source.

Hypertension or also known as high blood pressure is a condition when there is an increase in resistance in the arteries caused by the reduction of lumen diameter. This condition has particularly caused many health problems mainly cardiovascular disease.

More than 40% of the population in Malaysia in 2011 was discovered having hypertension and it is increasing in trend (Naing et al., 2016). Though there are medications that can decrease hypertension in patients, the drugs have unwanted side effects such as causing nausea, fatigue, and dizziness (Hartmann & Meisel, 2007). High blood pressure may have been regulated by different ions, receptors or enzyme proteins such as an angiotensin-II receptor, calcium channel protein, and angiotensin-converting enzyme, but this research only concentrated on the effect of peptides from ovalbumin on angiotensin-1 converting enzyme (ACE). ACE is a zinc metallopeptidase that cleaves C-terminal dipeptide from angiotensin-1 to form angiotensin-II which acts as a potent vasopressor that increases blood pressure. Many studies had been done using peptides from various source of proteins to find out their effect on ACE. This includes samples from fish, milk, cheese, casein and seaweed (Nongonierma & FitzGerald, 2017). From these studies, several peptide sequences have been determined to have a significant inhibitory effect on ACE and eventually act as an antihypertensive agent. In this study, antihypertensive peptide was one of the objectives as the study ground still has a lot to offer especially with the advancement of bioinformatics.

Aside from that, this paper will also be looking at the effect of ovalbumin bioactive peptides on a factor that contributes to diabetes mellitus (DM). Based on WHO statistics, there were 8.5% of the global population prevalence of DM in 2014 from only 4.7% in 1980 (Costanian, Bennett, Hwalla, Assaad, & Sibai, 2014). Malaysia also does not get away with this problem - 6.9% in 1996 and has risen to 17.5% in 2015 (Tee & Yap, 2017). The blood sugar level is controlled by insulin which functions mainly in regulating the absorption of glucose from the blood into body cells. Meanwhile, insulin is also being regulated by dipeptidyl peptidase-4 (DPP-4) that is responsible for deactivating incretins such as gastric inhibitory polypeptide (GIP) and

glucagon-like peptide (GLP-1) by cleaving them. Incretins are also known as insulin secretion promoters (Wang et al., 2018). Therefore, if DPP-4 is inhibited, incretins can activate insulin which will start to signal cells to take up glucose from the blood, therefore, reducing blood sugar level. As highlighted by several past studies, DPP-4 is highly regarded as a potential subject, thus, the focusing subject in this study apart from ACE. Researchers are searching for a better replacement of DPP-4 inhibitor drugs which currently producing unwanted side effects to patients. DPP-4 inhibitors have been observed to cause headache, infections in the upper respiratory tract and mostly concerned, heart failure (Imam, 2015; Packer, 2018). To date, various DPP-4 inhibitory peptides have been identified from various sources such as dairy (Lacroix & Li-Chan, 2012; Nongonierma, Paolella, Mudgil, Maqsood, & FitzGerald, 2018), oilseeds (Han, Maycock, Murray, & Boesch, 2019), rice (Hatanaka et al., 2012), tuna (Huang, Jao, Ho, & Hsu, 2012), salmon (Li-Chan, Hunag, Jao, Ho, & Hsu, 2012), quinoa (Nongonierma, Le Maux, Dubrulle, Barre, & FitzGerald, 2015), oat and barley (Wang, Yu, Zhang, Zhang, & Fan, 2015). The interaction of these peptides with the catalytic site of the DPP-4 may block off the substrate sites, thus, inhibiting the activity of DPP-4. There is a wide range of protein sources that are yet to be studied for producing DPP-4 inhibitory peptides including egg white. Moreover, a DPP-4 inhibitory peptide that can rival the synthetic DPP-4 drug has yet to be found.

Chicken egg white provides 11% of protein, 1% lipid, and 78% of water. Proteins in egg white albumen include ovalbumin, ovotransferrin, ovomucoid, lysozyme, avidin, and many more. Aside from being a protein supply for muscle building, each of these proteins has certain functional properties that contribute to various benefits to the human body. In the last 20 years, after many types of protein have been differentiated in egg white, many studies had been done towards ovalbumin

- characterisation and bioactivity. This is especially important in food processing, food biotechnology and pharmaceutical. Slowly, the benefit of ovalbumin is highlighted as having an important role in the regulation of the internal environment of the human body. Apart from being an effective protein source for nitrogen retention, once broken down, ovalbumin peptides take part in the body as antioxidant agent, antihypertensive promoter, and regulator of an enzyme in coagulation system and cell proliferation (Cuber et al., 1990; Jahandideh, Chakrabarti, Davidge, & Wu, 2016; Kaiserman, Whisstock, & Bird, 2006; Manso et al., 2008; Miguel, Recio, Gómez-Ruiz, Ramos, & López-Fandiño, 2004; Sun, Jin, Li, Yin, & Lin, 2017). However, most of the studies were using conventional methods to obtain the bioactive peptides. Therefore, this study focused on obtaining potential bioactive peptides from avian ovalbumin via *in silico* hydrolysis and making a comparison with *in vitro* hydrolysis generated peptides. Moreover, due to high potential of avian ovalbumin to producing bioactive peptides especially for inhibiting DPP-4 and ACE, therefore, prompted this study.

Recent years have been a big productive step for bioinformatics. In fact, it is a platform that connects every researcher in the world. Before the emergence of bioinformatics, the conventional approach that was used to discover bioactive peptides is very time consuming with limited references. The conventional method of discovering bioactive is mostly purely experimental which includes random digestion of a protein source and numerous sets of activity testing (Nongonierma & FitzGerald, 2017). Therefore, this approach can be considered as time and cost-inefficient. This writing proposes the usage of bioinformatics to be aligned with the bioactive peptide searching. The bioinformatics approach allows the results of other researches to be combined and presents them in a systematic and better user interface. The potential of bioinformatics is especially high when negative results of other researches can be

integrated which then can propel the results further by eliminating possible unsuitable bioactive peptide sequences for a specific protein (Giacometti & Buretić-Tomljanović, 2017). When the experimental results, together with computational biology is integrated in the bioinformatics way, not only the industry or researchers can have a concrete database but also reach a consensus method in studying the bioactive peptides.

Without a doubt, hybridising *in vitro* approach with *in silico* approach will enable researchers to simulate a condition whereby bioactive peptides and their enzymatic release can be predicted, subsequently, reducing experimental error and laboratory tests (Panchaud, Affolter, & Kussmann, 2012). Of course, to understand and to determine the biological activity of the peptides better, several factors should be considered, inter alia, digestive agents used for hydrolysis, food processing condition and the size of the resulting peptides. The discovery of new bioactive peptides from egg whites may establish not only the emergence of new functional food products but also a new paradigm in other industries such as the drug and therapeutic industries. A newly found bioactive peptide sequences might be revealed as sequences that very important in the drug delivery system as it can be adopted by a drug to dock onto a defined molecule (Khaldi, 2012). Effectively, this will help to improve human health and alleviate many medical conditions, if not understand them better.

1.2 Problem statements

The main focuses of this study were developed due to the following problem statements: -

1. In 2015, nearly two-thirds of adults were having elevated blood pressure and half of them were categorised as prehypertension symptoms (Naidu et al., 2019). While the

global hypertension rate among adults is going 29% by 2025, the rates in Malaysia had been maintained at more than 30% in recent years (Mohammed, Hassan, Suhaimi, & Ali, 2019).

2. Meanwhile, globally, diabetes had caused more than 1.6 million deaths in 2016 and diabetes prevalence is expected to increase at a rapid rate with the estimation of achieving 10% of the global population. In Malaysia, diabetes is one of the major causes of cardiovascular diseases that had caused 35% of Malaysian mortality (Rahim, Abdulrahman, Kader Maideen, & Rashid, 2020). Moreover, over the last half decade, diabetes rates had increased from 15.2% to 17.5% and this trend is alarming (Hizlinda Tohid et al., 2019).

3. Synthetic drugs that have been introduced in clinics and hospitals are not entirely safe as they pose risk of another diseases to patients. Synthetic antidiabetic drugs such as saxagliptin and alogliptin contain risks to induce cardiovascular diseases (Packer, 2018) while synthetic antihypertensive drugs such as captopril and lisinopril may induce angioedema and hyperkalaemia (Korzeniowska et al., 2017; Rosano et al., 2018)

4. The disadvantage of conventional approach is having limited sample scope and steps that are time consuming while producing low yield of peptides. Moreover, the likelihood of obtaining potent bioactive peptides remain elusive even after extensive processing. Therefore, an intervention to this approach may be beneficial (Udenigwe, 2014).

1.3 Research objectives

The main objective that is sought in this study is to find identify the bioactive peptides from avian ovalbumin using bioinformatics and *in vitro* analyses. Specific objectives for this study are as follows: -

1. To identify antihypertensive and antidiabetic bioactive peptides from avian ovalbumin using *in silico* hydrolysis and bioinformatics analyses.
2. To investigate the replication of ovalbumin bioactive peptides using *in vitro* hydrolysis.
3. To identify and compare potential bioactive peptides using bioinformatics analyses and inhibitory peptide features.

1.4 Significance of the study

It was suggested from this study that egg white ovalbumin does not only play essential role in providing protein source but also important for balancing health structure when taken into consideration in diet. This study shows potential angiotensin-converting enzyme (ACE) and dipeptidyl peptidase-4 (DPP-4) enzymes inhibitory peptides which may be beneficial in the treatment of hypertension and diabetes. This study also highlighted the basis of how the potential potent peptides were chose using bioinformatics tools and recreate the same simulation in *in vitro* analyses. The integration of *in silico* and *in vitro* approaches enabled the selection of potential bioactive peptides to be made based on the potential binding between the peptides and the proteins. Furthermore, multi-functional may be achieved by using this knowledge and can further be used to research on its application biologically.

CHAPTER 2

LITERATURE REVIEW

2.1 Chicken egg background

For humans, poultry is one of the main diets. Poultry can be defined as domesticated fowl that are farmed for their eggs, feathers and meat. The typical classes of birds in poultry are Galliformes and Anseriformes. Galliformes such as chickens and turkeys are mainly ground-feeding as they search foods on the ground while Anseriformes such as goose and ducks are mainly water-feeding birds as they are more aquatic in nature. The most domesticated species is *Gallus gallus* also known as red jungle fowl (chicken) and this species originated from the South East Asia (e.g. Burma, Malaysia and Thailand) (Coles 2009). Chickens are also regarded as the earliest domesticated poultry species. Therefore, eggs that are consumed by humans are usually farmed from this type of fowl.

2.1.1 Egg: It's importance and Malaysia

Eggs are the second highest consumption of agricultural commodities in 2017 (22kg/year) behind poultry meat in Malaysia. In fact, Malaysia's self-sufficiency ratio of chicken/duck egg in 2017 is 113.7% (Department of Statistics Malaysia, 2018). Only poultry egg and meat were able to meet the local demand and achieved more than 100% self-sufficiency level, while beef and milk followed behind at 22% and 55%, respectively. This shows that, eggs are one of important necessity in Malaysia. Furthermore, based on Egg Industry Review 2015, published by the International Egg Commission (IEC), Malaysia is considered as Newly Industrialised Country that ranked in the top 10 countries with the highest egg consumption per capita (International Egg

Commission, 2015) due to affordability of egg, easy access to production line and cultural acceptance in this country (Food and Agriculture Organization of the United Nations (FAO), 2019).

As part of daily diet, people of all ages can benefit from egg especially young children and pregnant/lactating women due to its cost-effective nutritional balance. Furthermore, the taste of cooked eggs, is easily accepted by all while cooking preparation is as simple as boiling the eggs without having to add any taste enhancer because of its unique taste. Although the prices of eggs in many countries like China, Malaysia, Turkey and India and it is as low as RM 0.27 per egg in Malaysia (International Egg Commission, 2019), the consumption of eggs is relatively small in developing country when compared to developed country. India, an example of developing country, only consumed 66 eggs per person in a year, while the consumption of egg in China per person could achieve 300 eggs per person. Several reasons had caused this especially the affordability of egg, awareness of its nutritional value and knowledge in poultry farming. These reasons are majorly applied to the rural community which has low support and access in terms of knowledge nor capital. Also, one of the contributor of the forum stated that in other parts of the world, eggs are considered as an expensive food that they were even used as currency (Food and Agriculture Organization of the United Nations (FAO), 2019).

Eggs play a major role in maintaining the food security and nutrition. In a global forum on food security and nutrition, organised by Food and Agricultural Organisation of the United Nations, it is viewed as one of the cost-effective ways to curb malnutrition and boost general health. In fact, it is one in hundreds of foods that are considered as balanced and all-around. However, there are also misconceptions about eggs such as egg could cause harm to young children and pregnant as well as increase in

cardiovascular disease when they are consumed. These misconceptions have affected the consumption of egg greatly. Also, it is about public awareness and education to the society about the nutritional value of eggs to the utilization of lost-cost production system and acceptance of eggs as food. To harmonize and achieve good execution of awareness, a good and thorough understanding regarding eggs is needed in order to spread the information clearly and convincingly. Although Malaysia is a country that regards egg as a staple food, majority of the people are not aware of the true benefits of egg nutritional value to the human's health. Research on how eggs can impact the overall health therefore has to be conducted to open up the mind of the societies.

2.1.2 Egg Features

At one time, jungle fowl can lay around 5 to 10 eggs and the eggs have to be incubated for a period of 20-21 days before it can hatch (Food and Agriculture Organization of the United Nations (FAO), 2019). According to Department of Veterinary Services, Malaysia, there are currently 6 grades of eggs ranging from AA grade to E grade (Department of Statistics Malaysia, 2018). The grades symbolise difference in size, mass and quality of the eggs subsequently affect the pricing as well. However, only grade A to C are commonly available in the normal market. The gradings are based on their mass which are 72g or more for Grade AA, 60g-71g for Grade A, 31g-59g for Grade B and 30g or less for Grade C (Bondoc et al., 2021). Regardless of the grades, ovalbumin remains the same in the protein sequence. Moreover, the grading of eggs also depends on the quality of the outer layer as well as the inner components. Inspection on the egg shell factors in the texture, shape, colour, cleanliness and the soundness of the egg shell as outlined by USDA standards. For

internal quality checks, several factors must be determined such as yolk shape, yolk strength, egg white viscosity and cleanliness and air cell size.

Eggs are considered highly nutritious food that provide balanced and versatile nutrients ranging from proteins, fats, minerals, a wide range of vitamins as well as bioactive compounds that impact the human health (Kuang, Yang, Zhang, Wang, & Chen, 2018) . The composition of egg across all avian species are about the same. The main components of an egg are yolk, albumen, shell membrane and shell. Albumen comprised of relatively 57-65% of chicken egg while egg yolk is 25-33% and the remaining percentage are the shell and shell membrane. Nutritionally, an egg consists of relatively 74% of water, 12% of protein and 11% of lipid. For chicken egg, the shells are usually white-yellow to brown in colour. The appearance of egg white (albumen) is a liquid with pseudoplastic and gel-like structure with a faint of straw-tint colour. In fact, there are small parts in the egg white that looks condensed and twisted-looking. They are chalazae, firmer layers of albumen that serves as anchors to maintain the position of yolk in the centre. The majority of protein in egg comes from the egg white (albumen) while, the proteins in egg yolk are mostly associated with lipids to form lipoproteins (Nys & Guyot, 2011).

Proteins in egg are one of the best sources for human consumption because of high amount of lysine and sulphur amino acid (Nys & Guyot, 2011). The amount of protein in egg white is 10.6% of whole composition in egg while protein in egg yolk is 16.6%. Although the amount of protein in egg yolk is higher than that in egg white, proteins in egg yolk are mostly lipid-linked proteins. There are more than 50 proteins in egg white with ovalbumin as the predominant protein constituting 54% proteins of egg white followed by other major proteins in egg white such as ovotransferrin (12%), ovomucoid (11%), ovomucin (3.5%), lysozyme (3.4%), ovoglobulin G2 and G3

(4.0%), flavoprotein (0.8%), ovoinhibitor (1.5%), ovoglycoprotein (1.0%) and cystatin (0.05%) while others are mostly considered to be in trace amount (Liu, Oey, Bremer, Carne, & Silcock, 2018; Stevens, 1991)

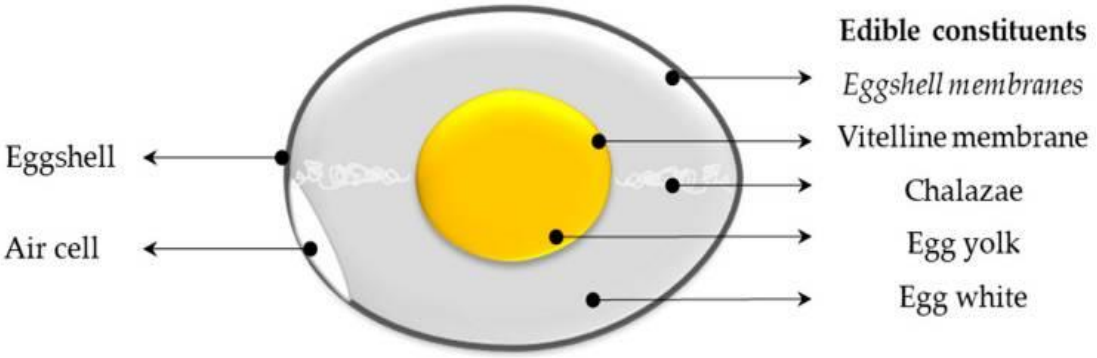

Figure 2.1 Structure of an egg. Adapted from "The golden egg: Nutritional value, bioactivities, and emerging benefits for human health," by Réhault-Godbert, S., Guyot, N., & Nys, Y., 2019, *Nutrients*, *11*(3), page 2.Copyright 2019 by the Authors.

2.1.3 Ovalbumin

Ovalbumin, the primary protein in egg white, comprises of 386 amino acid residues with relative mass of 45kDa. It has three subunits with different phosphate groups while a carbohydrate group is attached to its N-terminal, making ovalbumin a phosphoglycoprotein. Despite not being able to produce a serpin-like activity, ovalbumin comes from the serpin family (Guha, Majumder, & Mine, 2019). Ovalbumin also contains six cysteines of which there is a disulphide bond between Cys74 and Cys121 which is consider unique among egg white proteins. The isoelectric point (pI) of ovalbumin is 4.5 which is contributed by charged and hydrophobic amino acids that amount to 30% and 50% of ovalbumin, respectively (Chay Pak Ting, Pouliot, Gauthier, & Mine, 2013). Distribution of amino acids in ovalbumin can also be considered as unique compared to other glycoprotein as it does not contain classical N-terminal ladder sequence that is usually found in secretory protein. As an alternative, it contains a

hydrophobic region that may act as signalling mechanism for transmembrane translocation.

The native form of ovalbumin is trypsin-resistant. However, once it has denatured due to changes in heat or pH, it can no longer resist trypsin digestion (Guha et al., 2019). When ovalbumin is denatured due to pH or temperature change, the structure of ovalbumin changes to a more heat-stable form called S-ovalbumin. Although pH and temperature affect the conversion of ovalbumin to S-ovalbumin directly, over the time, ovalbumin would eventually convert to its stable form even when temperature was fixed at 25^{o}C during storage. In fact, it is also proposed in previous study to use s-ovalbumin as an indicator to estimate egg freshness (Huang et al., 2012)

2.1.4 Benefits of egg

In this modern world, the usage of eggs does not only to fulfil dietary requirements but also was diverted into other industries such as cosmetics, cell culturing, pet foods and most importantly gastronomy/food processing.

In gastronomy and food processing, eggs are especially important in making bakery products. Almost all bakery products are created using eggs as one of its base ingredients. This is because eggs have many functional properties that help in various textures and tastes in foods. Foaming is one of egg functional properties that is produced due to the decrease in surface tension of the air/water interface (Tan, Kanyarat, & Azhar, 2012). Foaming in egg white is caused by the rapid interaction between various proteins in egg white as well as undergoing conformational changes. The foaming properties in egg white are majorly contributed by globulins, ovalbumin, ovotransferrin

and lysozyme. Ovalbumin and lysozyme particularly, have the tendency to gain more flexibility under controlled heat-induced denaturation (Mine, 1995). Furthermore, egg whites can serve as polysaccharide emulsifier and is a better emulsifier than commercial products in 1995 (Mine, 1995). It was found that, in a controlled dry state, ovalbumin and lysozyme were able to produce conjugates with polysaccharide with very stable structure without having affected by acidic conditions nor heating. It was also tested that ovalbumin-galactomannan conjugate was not toxic and safe to be consumed. At the same time, egg yolk is a natural lipid emulsifier as part of egg yolk is mainly lipid itself. However, attempts to increase emulsifying ability of egg white proteins has been done to achieve pure protein emulsifier. One of the suggested way to increase the emulsifying property of egg white is to introduce lysophosphotidylcholine to ovalbumin as we it would produce complex that were heat stable and also improving oil-water surface tension (Mine, 1995). Other than that, heat-induced egg whites can also produce emulsifying properties and stability at a comparable rate to that of egg yolk. Furthermore, egg whites are also used as binding agent and coagulating agent. Proteins in egg can coagulate when they are exposed to heat or acid causing them to change structure from liquid to solid or semi solid, thus giving out the adhesive function.

Interestingly, egg does have beneficial role in the cosmetics industry, in particular for the skin. In fact, in 2018, there are several brands that have created egg-white-based product such as Oh K! Egg White Sheet Mask (Strusowski, 2020). In a study conducted by Faculty of Medicine, Melaka-Manipal Medical College, Malaysia, participants that were treated with egg white based facemask showed positive results towards skin problems such as skin oiliness and clogged pores (Wei, Zanuldin, Pitchai, Surukumaran, & Imran, 2020). Furthermore, homemade egg white facemask

outperforms the results of commercial products on skin oiliness. In another study, wrinkle was significantly reduced from the application of egg based facial cream as it possessed antioxidants properties that prevent the reactive oxygen species from forming up. At the same time, the egg based facial cream improves human dermal fibroblasts to produce more collagen and elastin (Jensen, Shah, Holtz, Patel, & Lo, 2016).

With these benefits of egg, it makes chicken egg a good protein source to produce bioactive peptides. This is especially worth extensively studying because the protein source is a cheap and is a staple food to the majority of the world. Although, large scale application for bioactive peptides has not yet being established because of the general traditional therapeutic peptides are deemed to be manufactured by recombinant and synthetic methods, there are suggestion to an efficient large scale operation with much cost-friendly process (Agyei & Danquah, 2011; Rizzello et al., 2016)

Egg white was also shown to be an excellent 3-D culture media in cell biology and since then, 3-D cell culture using egg white has been progressively chosen as a cost-effective culture system (Kaipparettu et al., 2008). In addition, due to the presence of antimicrobial properties of almost all proteins in egg white, development of embryo in egg is protected from "bad" microbes. One of the protein that highly responsible for antimicrobial properties is lysozyme which could hydrolyse gram-positive bacteria peptidoglycan layer which is the substrate for lysozyme (Lesnierowski & Kijowski, 2007). Therefore, not only egg is important nutritionally, but also highly potential in food processing and pharmaceutical industries as functional foods are highly sought for their safety for consumption and cost-cutting approach.

2.2 Bioactive peptides

2.2.1 The background

Biologically active peptides or commonly known as bioactive peptides (BAPs) are fragments of a protein sequence that can interact or cause a chain of reaction with other biological parts or receptors to produce certain physiological function. Organically, they are released by proteolytic enzymes from its precursor protein to form an active form fragment but BAPs can also be produced by using other food processes such as fermentation of sausages and cheese, wine production and meat tenderisation. Not all hydrolysed fragments are considered as a BAP because of its selective nature to what it can interact with. Generally, BAP is defined as peptide that contains 2-20 amino acids which can have various effects to the body physiological functions including cardiovascular, immune and digestive system (Sánchez & Vázquez, 2017; Tsuruki et al., 2003).

2.2.2 Obtaining BAPs via enzymatic hydrolysis

There are several types of proteolytic enzymes that are commonly used in producing BAPs such as pepsin, chymotrypsin, trypsin, alcalase, pancreatin, papain and thermolysin (Chalamaiah, Ulug, Hong, & Wu, 2019) Although producing BAPs using proteolytic enzymes might not be a cost-effective mechanism to achieve desired BAPs in an up-scale task; it is however, one of the best method to produce BAPs that is reasonably close to resemble the internal environment of most organism especially when they are consumed directly in the form of food (Chalamaiah et al., 2019; Cian, Garzón, Ancona, Guerrero, & Drago, 2015). This is because each BAP produced depends on the condition of the hydrolysis step and the type of proteolytic system (Li,

Liao, Fan, & Wu, 2018). For example, human can naturally receive BAPs from protein-containing food, such as meat, egg and milk. As food passes through the human digestive system, it will be digested by several enzymes from the mouth to the duodenum. These proteolytic enzymes in human are found in the stomach (i.e. pepsin) and in the duodenum (i.e. trypsin and chymotrypsin). Every proteolytic enzyme will produce different length of peptides and different sequences due to different cleavage sites.

2.2.2(a) Pepsin

Pepsin is the first endoproteinase that breaks down food dietary proteins when they reach the stomach. Pepsin is also an aspartic protease that needs an acidic environment to be active. Pepsin is initially secreted as a pepsinogen by chief cells in gastric gland in response to certain stimuli (Heda, Toro, & Tombazzi., 2020). In this form, pepsin is inactive. As the stimuli activates the production of pepsinogen, HCl is also produced by parietal cells. HCl will cause the environment of the stomach to be low in pH and thus, breaking down pepsinogen to its activated form, pepsin. Although pepsin is stable in acidic condition, it is in its most active form when it the pH of its environment in 1.5 to 2. However, upon reaching the duodenum, the pH increases up to more than 6, causing pepsin to be irreversibly inactivated.

Pepsin is not as specific in cleaving sites as it is with trypsin and other proteases. In general, pepsin cleaves hydrophobic and aromatic amino acids in the P1 and P1' positions. It usually cleaves after phenylalanine and leucine. Meanwhile, other amino acids require certain conditions for it to cleave the peptide. For example, pepsin only cleaves lysine and histidine unless they are accompanied with amino acids that are

preferred by pepsin (e.g. phenylalanine and leucine) (Ahn, Cao, Yu, & Engen, 2013). The probability of amino acids other than leucine and phenylalanine being cleaved by pepsin also depends on the amino acid that positioned at the P1 and P1' position.

2.2.2 (b)Chymotrypsin and trypsin

The pancreas secretes a number of proteases, such as trypsin and chymotrypsin, in their inactive forms (Feher, 2012). Both trypsin and chymotrypsin are from family of serine proteases that possess serine residue which is crucial for their catalysis. As the names suggest, trypsin and chymotrypsin are similar in many ways such as their tertiary structure and sequence. This class of enzymes highly dependent on their catalytic triad and oxyanion. They perform proteolysis by properly positioning the catalytic triad to pass proton to the substrates and forming catalytic intermediate. At the same time, the oxyanion functions to stabilize the intermediate (Ma, Tang, & Lai, 2005).

Although their catalytic mechanism is the same, specificity is different in which it is determined by their binding and acylation step. Residue 189 of trypsin and chymotrypsin is important that it distinguishes the difference between them especially for properties of binding pocket of both enzymes. In trypsin, a negatively charged aspartate is situated at residue 189, while chymotrypsin has a polar serine. In such, trypsin prefers lysine and arginine as they are basic amino acids, while chymotrypsin prefers phenylalanine, tyrosine and tryptophan as they are aromatic amino acids. As trypsin and chymotrypsin were released to small intestine, the alkaline environment provides optimum pH of 7 to 8 for the enzymes to cleave the peptides (Ma et al., 2005).

2.2.2(c) Cleaving potential

In order to become smaller peptides, proteins or bigger peptides have to be cleaved by proteases in which cleaving might not be done once as multiple of cleavage sites can appear in whole sequence of the peptides. As cleavage sites or specificity depends on the type and position of the amino acids, the environment must be suitable for the proteases as well. However, even with good environment condition, a protease is often not able to fully cut a protein according to its designated cleavage sites despite having sufficient time. This occurrence may have been caused by the steric hindrance of the secondary and tertiary structures of the protein. Furthermore, certain motifs are just not cleavable, causing mis cleavage or the reaction time is just too short even though the cleavage sites are accessible by the enzymes. Apart from that, active site of an enzyme can also be interfered by amino acids as such would significantly reduce the reaction speed of the catalysis. For example, the kinetics of trypsin is severely affected when lysine and/or arginine are present within the vicinity of the cleavage site. In addition, acidic residues such as glutamate and aspartate presence near the cleavage site will also affect the enzyme kinetics. Also, there are other factors of protein substrate that could affect the cleaving potential of proteases such as folding of polypeptides, disulphide bridges, glycosylation and solubility. Therefore, although controlling the external conditions for proteases can be achieved, structure of the target protein itself is the main factor for the mis cleavage, partial cleavage and even non cleavage (Šlechtová, Gilar, Kalíková, & Tesařová, 2000).

BAPs that are usually produced for their activities using other food processes or other enzymes could be affected by these aforementioned digestive enzymes because they could lose the peptide length and sequence if these BAPs are consumed orally (Yap & Gan, 2020). Therefore, it is strongly believed that producing pepsin, trypsin and/or chymotrypsin could minimize this issue.

2.2.3 The role of bioactive peptides

The world of medication by far still majored by the involvement of chemical substances in treating and medicating a disease. The usage of these substances, while being effective, are causing side effects as well. This can be commonly seen by the usage of drug such as antibiotics which can lower the host immune system, thus leading to fever or weaken body (Agyei & Danquah, 2011). Therefore, the searching for effective yet risk-free therapeutic strategies is now highly shifted to the discovery of bioactive peptides.

In fact, it is safe to assume that some BAPs are established as therapeutic agents for certain bio-functionalities as such mentioned in Table 2.1 (Hancock & Sahl, 2006; Huang, Chen, Chen, Hong, & Chen, 2010; Kim & Wijesekara, 2010; Morris, Beesabathuni, & Headey, 2018). Resistant-effect, one of the critical issues in the medical field, has always been a major slam to the medical community. The reason for this is because research community has to always be in fast-pace environment to find another cure to the newest of any bacteria, virus or any other illness. The emergence of suitable antimicrobial peptides, for example, that was studied by Hancock and co-researchers, contain potent and broad-spectrum molecules that are versatile in terms of its properties are the very much sought antimicrobial peptides (Hancock & Sahl, 2006). Though it has not been extensively studied, the real reason why peptides are sought for is because of its "evolution potential" as its production is connected to gene (Hancock & Sahl, 2006).

Table 2.1 Example of bioactive peptides discovered in recent years. (Data obtained from Xu, Hong, Wu, & Yan, 2019)

Bioactive peptides	Bioactivity	Origin	References
WDHHAPQLR	antioxidative	rapeseed protein	Xu et al., (2018)
YFCLT, GLLLPH	antioxidative	corn gluten	Ding, Wang, Zhang, Yu, & Liu, (2018)
RLSFNP	antihypertensive	whey protein	Guo et al., (2018)
LKP, IQW	antihypertensive	egg white ovotransferrin, chicken and bonito protein	Xu, Fan, Yu, Hong, & Wu, (2017)
LSW	antihypertensive, anti-inflammatory	soybean protein	Lin et al., (2017)
LY, TF, RALP	antihypertensive, renin dual inhibitory	rapeseed protein hydrolysates	Yang et al., (2017)
YWDHNNPQIR	antioxidative	rapeseed protein	Xu et al., (2017)
AHLL	antihypertensive	loach	Li et al., (2017)
IPP, LKP	antihypertensive	bovine milk β-casein, bonito fish muscle	Gleeson, Brayden, & Ryan, (2017, 2018)
Pro-HypCONHGlcN	skin and bone health	synthetic glycopeptide	Feng & Betti, (2017)
lunasin, RKQLQGVN	chemoprevention	soybean protein	Fernández-Tomé, Sanchón, Recio, & Hernández-Ledesma, (2018)
LKPTPEGDL, LPYPY, IPIQY, IPI, WR	DPP-IV inhibitory	bovine milk protein	Lacroix, Chen, Kitts, & Li-Chan, (2017)
VGPV, GPRGF	antihypertensive	bovine collagen	Fu et al., (2016)
VLPVPQK	antihypertensive, antioxidative	bovine milk casein hydrolysates	Vij, Reddi, Kapila, & Kapila, (2016)
β-casomorphin-5 (YPFPG)	opioid	bovine milk casein	Vij et al., (2016)

Another example is the potential of BAPs impact in the food-processing field especially in the case of food preservation. Preservation of foods can be done by using many methods from the usage of primitive methods such as chilling and freezing to the usage of high technology machines such as irradiation and high-pressure machines (Amit, Uddin, Rahman, Islam, & Khan, 2017). There are as well the usage of chemical additives or antioxidants which are extensively used like BHT and TBHQ which can

be harmful to the body in the long run (Bondi, Lauková, De Niederhausern, Messi, & Papadopoulou, 2017). Therefore, to search for more natural and preservative-foods, demand has brought the scientific community to bioactive peptides, where antifungal activities was shown in a wheat flour sample fermented with lactic acid bacteria (Coda et al., 2008; Coda, Rizzello, Pinto, & Gobbetti, 2012). The application of which antifungal activities identified is actually based from the proteolytic activity on the wheat protein that produce antifungal-peptides by the lactic acid bacteria. The peptides were identified and characterised to have broad inhibitory spectrum against many common fungus that contaminate bread (Rizzello, Cassone, Coda, & Gobbetti, 2011).

In this study, two diseases were of interest: (a) hypertension, and (b) diabetes. The following sub-sections will elaborate on how BAPs be used for treating these diseases.

2.2.3(a) Bioactive peptides for hypertension

High blood pressure or known as hypertension is one of the reasons for the top cases of cardiovascular diseases. High blood pressure is also the number one cause of death wide (He & MacGregor, 2007). Malaysia is also not excluded from this dilemma as it was discovered in 2011 that above 40% of the population in Malaysia is having hypertension, and it is a hot topic in the news (Naing et al., 2016). This condition is caused when there is a resistance from blood against the artery wall which may cause disease related to cardiovascular. There are many medications that can reduce hypertension such as beta blockers, alpha blockers, calcium channel blockers, angiotensin blockers and diuretics. Majority of these medications seem to have undesirable side effects including nausea, fatigue and dizziness (Hartmann & Meisel,

2007). Though blood pressure is usually regulated by different ions, receptors or enzyme proteins such as angiotensin-II receptor and calcium channel protein, the angiotensin-converting-enzyme (ACE) is the major key in controlling and regulating the vasoconstriction of the blood vessel. ACE, being a zinc metallopeptidase, can act as a vasopressor that increases blood pressure by cleaving the C-terminal dipeptide from Angiotensin-I to form Angiotensin-II. Many studies have been conducted using the peptides obtained from wide range of protein source to find out their effect, mainly inhibitory effect on ACE, as shown in Table 2.2. This includes samples from fish (Pampanin et al., 2012), milk (Sánchez-Rivera et al., 2014; van Platerink, Janssen, & Haverkamp, 2008), and cheese (Schlichtherle-Cerny, Affolter, & Cerny, 2003). Through the studies, many peptide sequences have been identified to have significant inhibitory activity on ACE. Many of them, however, were done by using conventional method.

Table 2.2 Examples of ACE inhibitory peptides from Biopep database.

Peptide	Source
LKL	Sardine (Ukeda et al., 1992)
VW	Sake lees (Saito, Wanezaki, Kawato, & Imayasu, 1994)
VWY	Sake lees (Saito et al., 1994)
YW	Sake lees (Saito et al., 1994)
RF	Sake lees (Saito et al., 1994)
GPL	Alaskan pollack skin (Byun & Kim, 2002)
PGL	Alaskan pollack skin (Byun & Kim, 2002)
LGP	Alaskan pollack skin (Byun & Kim, 2002)
PLG	Alaskan pollack skin (Byun & Kim, 2002)
LPG	Alaskan pollack skin (Byun & Kim, 2002)
GPL	Alaskan pollack skin (Byun & Kim, 2002)
IVY	Wheat germ hydrolysate (Ueno, Tanaka, Matsui, & Matsumoto, 2005)
LKA	Chicken muscle (Fujita, Yokoyama, & Yoshikawa, 2000)
LAP	Chicken muscle (Fujita et al., 2000)
FQKPKR	Chicken muscle (Fujita et al., 2000)
FKGRYYP	Chicken muscle (Fujita et al., 2000)
LTF	Tuna muscle (Meisel, Walsh, Murray, & FitzGerald, 2005)
IFG	Tuna muscle (Meisel et al., 2005)
LRY	Red algae (Meisel et al., 2005)
MKY	Red algae (Meisel et al., 2005)
MDFLI	Chickpea legumin (Murray & FitzGerald, 2007)
MFDL	Chickpea legumin (Murray & FitzGerald, 2007)
MDLA	Chickpea legumin (Murray & FitzGerald, 2007)
RIY	Rapeseed (Murray & FitzGerald, 2007)
LTPTSN	Olive seed (Esteve, Marina, & García, 2015)
LVVDGEGY	Olive seed (Esteve et al., 2015)
AFDAVGVK	Olive seed (Esteve et al., 2015)
VGVPGGV	Olive seed (Esteve et al., 2015)
LLPSY	Olive seed (Esteve et al., 2015)
LFSGGES	Olive seed (Esteve et al., 2015)
RASDPLLSV	Egg yolk (Eckert et al., 2014)
RNDDLNYIQ	Egg yolk (Eckert et al., 2014)
LAPSLPGKPKPD	Egg yolk (Eckert et al., 2014)
AGTTCLFTPLALPYDYSH	Egg yolk (Eckert et al., 2014)