COMPARISON OF ALPHA-GLUCOSIDASE INHIBITORY ACTIVITY OF PROTEIN HYDROLYSATES OBTAINED FROM DIFFERENT TYPES OF HEN EGGS

By

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ABSTRACT

COMPARISON OF ALPHA-GLUCOSIDASE INHIBITORY ACTIVITY OF PROTEIN HYDROLYSATES OBTAINED FROM DIFFERENT TYPES OF HEN EGGS

Ku Sheau Lee

Diabetes mellitus has become a major healthcare concern not only in Malaysia but also worldwide. Despite the efficacy of the current treatments to control blood glycaemia, they could lead to adverse side effects among the patients. Therefore, focus has been given to natural products in the discovery of new source of novel bioactive peptides with alpha-glucosidase inhibitory properties such as hen eggs. Hen egg is one of the commonest dietary protein source available in the market and has been proven to exhibit various therapeutic properties. In view of this, this study was performed to compare the alpha-glucosidase inhibitory activities of three different types of eggs which were classic egg, omega 3 enriched and selenium enriched egg. The protein hydrolysates obtained from the three hen eggs were digested with pepsin and papain separately to yield bioactive peptides. The optimum pepsin hydrolysis was obtained by using 4% (w/w) of pepsin at pH 2 and at 37ºC, whereas the optimum papain hydrolysis was obtained by using 4% (w/w) of papain at pH 7 and at 37ºC. The protein concentration yielded from pepsin hydrolysis was relatively higher than papain hydrolysis for all the three types of eggs. For

pepsin digested protein hydrolysates, selenium enriched egg exhibited the most potent alpha-glucosidase inhibition (IC_{50} = 96.69 mg/mL), followed by classic egg (IC_{50} = 220.28 mg/mL) and the lowest alpha-glucosidase inhibition was achieved by omega 3 enriched egg (IC_{50} = 462.20 mg/mL). Conversely, the IC_{50} of protein hydrolysates for all the three hen egg white digested with papain were not able to be determined due to low protein concentration yielded. In conclusion, selenium enriched egg whites digested with pepsin can be a potential source of novel bioactive peptide with potent alpha-glucosidase inhibitory properties that can be used as alternative therapeutic means.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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Ku Sheau Lee

APPROVAL SHEET

The project report entitled **"COMPARISON OF ALPHA-GLUCOSIDASE INHIBITORY ACTIVITY OF PROTEIN HYDROLYSATES OBTAINED FROM DIFFERENT TYPES OF HEN EGGS"** was reported by KU SHEAU LEE and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

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(Dr. Michelle Ng Yeen Tan) Date: 25th September 2018

Supervisor

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PERMISSION SHEET

It is hereby certified that **KU SHEAU LEE** (ID No: **15ADB07590**) has completed this final year project entitled **"COMPARISON OF ALPHA-GLUCOSIDASE INHIBITORY ACTIVITY OF PROTEIN HYDROLYSATES OBTAINED FROM DIFFERENT TYPES OF HEN EGGS"** under the supervision of **DR. MICHELLE NG YEEN TAN** from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(KU SHEAU LEE)

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LIST OF ABBREVIATIONS

ACE	Angiotensin-Converting Enzyme
ADA	American Diabetes Association
BSA	Bovine Serum Albumin
DMSO	Dimethyl Sulfoxide
DPP-IV	Dipeptidyl Peptidase-4
E/S	Enzyme / Substrate
GLUT4	Glucose Transporter 4
HbA1c	Haemoglobin A1c
HCl	Hydrochloric Acid
IC_{50}	Half Maximal Inhibitory Concentration
IDF	International Diabetes Federation
IFG	Impaired Fasting Glycaemia
IgE	Immunoglobulin E
IGT	Impaired Glucose Tolerance
MS	Mass Spectrometer
Na_2CO_3	Sodium Carbonate
Na_2HPO_4	Disodium Phosphate
NaH_2PO_4	Monosodium Phosphate

NaOH	Sodium Hydroxide
NCD	Non-communicable disease
NHMS	National Health and Morbidity Survey
OAD	Oral Antidiabetic Drug
PBS	Phosphate Buffer Solution
pNPG	p-nitrophenyl-α-D-glucopyranoside
PPAR-γ	Peroxisome Proliferator Activated Receptor Gamma
RP-HPLC	Reversed Phase High Performance Liquid Chromatography
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder caused by inherited or acquired deficiency in the production and effectiveness of insulin produced by the β-cells of Islets of Langerhans in pancreas. The most common form of diabetes mellitus is type 1 diabetes mellitus (T1DM), which is insulin dependent and type 2 diabetes mellitus (T2DM) which is insulin independent (WHO, 2018a). T1DM is caused by the absolute deficiency of insulin production by the pancreas whereas T2DM is the combination of insulin resistance and insufficient insulin secretion at later stage (Clinical Practice Guidelines Development Group, 2015).

Therefore, proper managements and timely treatments for diabetes are crucial to control the blood glucose level in the body. Besides the administration of insulin, the most common clinical treatments available in Malaysia are metformin sulphonylurea, alpha-glucosidase inhibitors, glitazones, meglitinides and other oral antidiabetic drugs (OAD) (Feisul and Azmi, 2013). These treatments are effective in the control of blood glucose level, thus reduce the risk of developing diabetic associated complications. Despite that, these drugs are expensive and tend to increase the risk of side effects among the patients (Inzucchi et al., 2012; Clinical Practice Guidelines Development Group, 2015).

This prompt the need for an alternative treatment that can overcome the disadvantages but retain the ability to treat diabetes.

One of the most effective way for glycaemic control is the control of postprandial blood glucose level (Kim et al., 2007). Alpha-glucosidase is an enzyme found in the epithelial mucosa of small intestine that functions to cleave disaccharides and oligosaccharides to glucose. The inhibition of this enzyme will retard the release of glucose, thus delay the absorption of glucose in proximal small intestine (Kim et al., 2007; Clinical Practice Guidelines Development Group, 2015). Alpha-glucosidase inhibitors are said to be a potential candidate to prevent the excessive increase in blood glucose, especially after a meal by returning it to normal range (Zhang et al., 2015).

Generally, alpha-glucosidase inhibitors is an OAD used to treat T2DM by acting as a competitive inhibitor to alpha-glucosidase (Clinical Practice Guidelines Development Group, 2015; Zhang et al., 2015). The effect of alpha-glucosidase inhibitor in controlling blood glucose is modest with minimal side effects when used in combination with other OADs (Clinical Practice Guidelines Development Group, 2015). Many papers have reported that alpha-glucosidase inhibitors are potential target in the development of drug to treat diabetes. Hence, research has been focused on the discovery of potential novel alpha-glucosidase inhibitors from natural products to suppress the postprandial hyperglycaemia in diabetic patients (Kim et al., 2007; Zhang et al., 2015; Chukwujekwu et al., 2016).

Natural products provide a huge and diversified choices and precursors, which can be a potential therapeutic agent for various diseases. Dietary proteins either exert their physiological effects directly or via the biologically active peptides produced after enzymatic hydrolysis (Korhonen and Pihlanto, 2003; 2006). Bioactive peptide is defined as a specific protein sequence that has an impact on body that may have health benefits (Kitts and Weiler, 2003). The amino acid composition and sequence will determine the activity of the bioactive peptide (Sanchez and Vazquez, 2017). The potential of bioactive peptide obtained from dietary protein to promote human health has make it a popular field in scientific research to discover more natural foods with therapeutic effect (Korhonen and Pihlanto, 2003; 2006).

Hen egg is well known as a source of dietary proteins because it has a lot of bioactive peptides (Bhat et al., 2015; Liu et al., 2017). The eggs are gaining more attention because they contain numerous biological functions that demonstrated therapeutic effects apart from their basic nutritional values (Yu et al., 2011). According to a study by Kovacs-Nolan et al. (2005), hen egg comprises of three main components which are the eggshell (9–12%), egg white (60%) and egg yolk (30–33%). They also claimed that the egg white component contains a lot of bioactive peptides that has been extensively studied to display various biological activities.

There are various types of hen eggs that are available in the market. A study has compared the composition of egg white proteins found in white shell, brown shell, organic, lutein enriched, omega 3 enriched and vitamin enriched eggs (Wang et al., 2012). They found that the abundancy but not the composition of egg white proteins are significantly different among different egg varieties. However, there is no comparison made on the egg white protein on alpha-glucosidase inhibition from different types of hen eggs.

Therefore, the objectives of this study were first to determine the optimal conditions for pepsin and papain digestion on hen egg white. The enzymatic digestion of pepsin and papain on hen egg white were also compared. The main focus of this study was to compare the alpha-glucosidase inhibitory activity for classic and enriched eggs digested with pepsin and papain. The IC_{50} on alpha-glucosidase of the protein hydrolysates obtained from classic and enriched eggs were also determined.

CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes Mellitus

2.1.1 Epidemiology of Diabetes Mellitus

Diabetes mellitus is a global health issue and was rated as the 7th leading cause of global death accounting for 1.6 million of deaths in year 2016 (WHO, 2018b). Globally, an estimated 425 million adults are living with diabetes in year 2017 and it has been foreseen that the number would increase tremendously by 48% up to 625 million by the year 2045 (IDF, 2017). Although with the increasing incidence of non-communicable diseases (NCD) such as diabetes mellitus, many international health agencies have paid less attention to them (Zimmet et. al., 2016). However, with both the number of cases and prevalence of diabetes increased tremendously over the past few decades, diabetes mellitus is now recognized as an important public health problem which requires prompt actions by world leaders (WHO, 2016b).

The National Health and Morbidity Survey (NHMS) reviewed that the prevalence of diabetes mellitus in Malaysia had increased by 31.0% from 11.6% in year 2006 to 15.2% in year 2011 (Feisul and Azmi, 2013). The prevalence of diabetes mellitus has been increasing and currently at a high level of approximately 18% by year 2015. The high prevalence rate is associated with

the major risk factor namely overweight and obesity among children and adults (Tee and Yap, 2017). The number of diabetic patients in Malaysia was 3.5 million in year 2017 and it was predicted that there would be a two fold increase in the number up to 6.1 million by the year 2045 (International Diabetes Federation, 2017). Diabetes mellitus is the major NCD ranked as the 6th leading cause of death in Malaysia (Clinical Practice Guidelines Development Group, 2015; WHO, 2016a).

2.1.2 Type 1 Diabetes Mellitus (T1DM)

Type 1 diabetes mellitus (T1DM) is also known as juvenile diabetes because only children and young adults are affected. This form of diabetes only accounts for 5% from those who are diagnosed with diabetes worldwide but the incidence is increasing every year. T1DM is an autoimmune disease which is caused by the inability of the body to produce insulin due to the destruction of pancreatic islet β-cells. T1DM can be diagnosed easily because autoantibodies can be detected in most of the patient. The autoantibodies serve as a biomarker for the detection of the disease even before the onset of symptoms. Therefore, proper management and treatment can be performed before the disease progress into critical stage.

However, there is no cure for this disease and patients have to depend on lifelong exogenous insulin therapy. The frequency of insulin injection depends on the degree of pancreatic β-cell destruction which is quite variable among

individuals (ADA, 2010). It is also shown that the autoimmune destruction of pancreatic β-cell has multiple genetic predisposition. This corresponds to a study by Katsarou et al. (2017) and ADA (2018), whereby both genetic and environmental factors play an important role in the development of T1DM but the association between them has yet to be defined.

2.1.3 Type 2 Diabetes Mellitus (T2DM)

Type 2 diabetes mellitus (T2DM) is the most common form of diabetes characterized by the inability of the body to react to the insulin action caused by insulin resistance and β-cell dysfunction (Katsarou et al., 2017). T2DM is primarily caused by insulin resistance and progressed to insulin deficiency at later stage (Clinical Practice Guidelines Development Group, 2015). T2DM accounts for the majority of people with diabetes mellitus worldwide. This disease was thought to only affect adults but at present, it was found that it also affects children and adolescences (WHO, 2016b).

The risk factor for T2DM is determined by an interplay of genetic and metabolic factors. Factors that can increase the risk of the disease are ethnicity, family history and previous gestational diabetes in addition to overweight and obesity, unhealthy diet, physical inactivity and smoking (WHO, 2016b). Recently, some genes have been reported to have an association with the increased risk for T2DM. The non-genetic risk factors of T2DM for instance lifestyle and diet can help to decrease the prevalence and cases. This will reduce the mortality rate of

diabetes mellitus with improved management, prevention and treatment (Zimmet et al., 2016).

2.1.4 Complications of Diabetes Mellitus

The complications of diabetes mellitus are caused by the long term hyperglycaemic condition in the body. Long term chronic hyperglycaemia in diabetes will cause damage, dysfunction and failure in organs for instance the eyes, kidneys, heart and blood vessels (ADA, 2010). Diabetes mellitus is strongly associated with both microvascular and macrovascular complications. Examples of microvascular complications are retinopathy, neuropathy and nephropathy whereas macrovascular complications involve ischemic heart disease, cerebrovascular disease and peripheral vascular disease (Cade, 2008).

According to a study by Inzucchi et al. (2012), diabetes mellitus is an important risk factor that leads to cardiovascular diseases, loss of vision, kidney failure and many other complications. Besides these, it will increase the risk of cancer, psychiatric disorders, cognitive decline, liver disease and other disabling or deadly conditions. The disease may also progress into the severe form of diabetes for instance ketoacidosis and non-ketotic hyperosmolar hyperglycaemic state (Alberti and Zimmet, 1998).

2.2 Current Treatments for Diabetes Mellitus

World Health Organisation (2017) suggested that the effective treatment for T2DM involves the control of diet and physical activity in order to reduce the level of blood glucose. The modification of lifestyle is the mainstay treatment with the major aim to improve glycaemic control, reduce weight and minimize the risk of diabetic complications. Pharmacological therapy will only be used when the hyperglycaemic condition becomes severe and requires medication to control the blood glucose (Inzucchi et.al, 2012; Kapoor and Thomas, 2017). Metformin (82.5%) is the most common oral antidiabetic drug used in Malaysia in the year 2012 to treat T2DM patients, followed by sulphonylureas (56.9%). On the other hand, the use of insulin therapy has increased from 11.7% in 2009 to 21.4% in 2012 (Feisul and Azmi, 2013).

Metformin, a biguanide, is the most studied antidiabetic drug and remains widely use as the first-line type 2 diabetes drug. Its predominant function is to lower fasting blood glucose by reducing hepatic gluconeogenesis. Generally, metformin alone does not increase the risk of hypoglycaemia because it does not stimulate insulin secretion. However, metformin could cause adverse gastrointestinal side effects like nausea, flatulence and diarrhoea. The long term complication of metformin therapy could lead to vitamin B12 deficiency. Patients with advanced renal insufficiency and alcoholism have also been advised to avoid this drug as it will predispose them to risk of lactic acidosis. Despite its side effect, this drug is still in used for diabetes treatment because of its low cost and mild effect on weight loss (Crandall et al., 2008; Inzucchi et.al,

2012; Clinical Practice Guidelines Development Group, 2015). Metformin-treated patients have also been shown to have improved insulin sensitivity and increase proinsulin secretion which will reduce the progression of diabetes (Lachin et al., 2007).

Sulfonylurea which is also known as insulin secretagogues acts as the second line of option for antidiabetic drug. Sulfonylurea stimulates insulin release by closing the potassium channels on β-cells in Islet of Langerhans (Bryan et al., 2005). Although it is effective in controlling blood glucose, this drug may cause modest weight gain and hypoglycaemia. The adverse effect can be seen more commonly in patient with renal impairment, liver cirrhosis and elderly (Inzucchi et.al, 2012; Clinical Practice Guidelines Development Group, 2015).

The shorter-acting insulin secretagogues, meglitinides is a derivative of sulfonylurea. Meglitinides stimulate insulin release through similar mechanisms but bind to a different site on the receptor. It has shorter half-life and is associated with lesser risk of hypoglycaemia and weight gain. However, this drug requires more frequent dosing and is commonly prescribed in combination with other OADs to act synergistically. This drug is primarily used to control postprandial hyperglycaemia (Gerich et al., 2005; Clinical Practice Guidelines Development Group, 2015).

Thiazolidinediones are peroxisome proliferator-activated receptor γ activators (PPAR-γ) agonists that enhance insulin sensitivity in skeletal muscle, adipose tissue and liver (Yki-Jarvinen, 2004; Clinical Practice Guidelines Development Group, 2015). This drug is used as the first line of therapy because it does not result in hypoglycaemia and has greater durability in blood glucose control compared to sulfonylureas and metformin (Inzucchi et al., 2012). The side effects of this drug include weight gain, heart failure, macular oedema, fluid retention and increased risk of bone fractures (Kahn et al., 2006; Clinical Practice Guidelines Development Group, 2015).

2.2.1 Uses of Antidiabetic Drugs

Inzucchi et al. (2012) stated that personalised treatment is the cornerstone of success because every patient has different needs, preferences and tolerances. In agreement with that, Clinical Practice Guidelines Development Group (2015) reviewed that a minimal dose of OAD should be given and at the same time emphasised on lifestyle modification. The dosage should be monitored and gradual increase in the dosage of the treatment should be made every 3 months if the glycaemic control is not achieved. Combination of other OADs is recommended only if monotherapy fails to control the blood glucose level.

According to a study by Feisul and Azmi (2013), patients on monotherapy in 2012 were lower compared to 2009. The switch from monotherapy to combination of OADs were also observed in their study. Metformin

monotherapy is often given first. If the HbA1c level does not reduced after 3 months, combination of OADs will be prescribed in addition to metformin. The choice of drug is individualised based on patient's condition with the final goal to improve glycaemic control while reducing any side effects. Insulin is the most effective agent as a third-line therapy (Inzucchi et al., 2012; Kapoor and Thomas, 2017). The number of patients who were on insulin-OAD combination treatment had increased from 2009 to 2012 which indicate that the demand for insulin therapy as a third line therapy is increasing over the years (Feisul and Azmi, 2013).

2.3 Alpha-Glucosidase Inhibitors

Alpha-glucosidase inhibitors are effective in glycaemic control by reducing postprandial glucose and HbA1c. It is less effective in reducing blood glycaemia compared to metformin or sulphonylurea because it has no direct effect on insulin secretion or sensitivity. However it displays synergistic effects and effective when it is used in combination with other OADs and insulin (Clinical Practice Guidelines Development Group, 2015). Besides the therapeutic effect, Van de Laar et al. (2006) reviewed that alpha-glucosidase inhibitors are used as a prophylactic agent in people at risk for diabetes. Impaired glucose tolerance (IGT) and impaired fasting glycaemia (IFG) are the intermediate categories between normal and diabetes categories that possess higher risk to develop T2DM (WHO, 2017). Therefore, alpha-glucosidase inhibitors not only can be used as therapeutic agent but also as a prophylactic agent. They are able to

decrease the progression of diabetes in subjects with pre-diabetic condition (Van de Laar et al., 2006; Godbout and Chiasson, 2007).

2.3.1 Current Medications and Side Effects

At present, synthetic alpha-glucosidase inhibitors, for instance acarbose, miglitol and voglibose have been used to treat T2DM (Zhang et al., 2015). Acarbose is a pseudo-tetrasaccharide of microbial origin that has a chemical structure identical to an oligosaccharide. Acarbose acts as a competitive inhibitor and the binding to alpha-glucosidase is reversible. However, acarbose has no effect on lactose's digestion and absorption. Most importantly, acarbose could not inhibit the absorption of monosaccharide such as glucose. The absorption of acarbose is relatively poor, which explains why its effect only limited to small intestine (Godbout and Chiasson, 2007).

These synthetic alpha-glucosidase inhibitors would cause adverse gastrointestinal side effects like flatulence, bloating, abdominal discomfort and diarrhoea due to incomplete carbohydrate absorption (Ghadyale, et al. 2011; Inzucchi et al., 2012; Clinical Practice Guidelines Development Group, 2015). A study by Crandall et al. (2008) reported that acarbose decreases the number of diabetes cases in individuals with IGT. However, the therapeutic effect of acarbose on the treatment of diabetes is not reliable and the adverse gastrointestinal side effects in acarbose-treated patients results in a major

drawback. Therefore, the use of acarbose to prevent diabetes is limited in clinical practice.

Moreover, intensive control of the blood glucose level and the rapid decrease in HbA1c levels caused by acarbose might increase the diabetic associated mortality. Hence, it is important to ensure that acarbose will not cause hypoglycaemia in acarbose treated patients. Contraindications of alpha-glucosidase inhibitors were observed in patients with diabetic ketoacidosis or gastrointestinal obstruction as well as those who are known to have hypersensitivity reaction to the drug. The drug is also not suitable to be used in patient that has chronic intestinal diseases with inefficient digestion or absorption (He et al., 2014).

Another synthetic alpha-glucosidase inhibitor, miglitol is also in used to treat diabetes which is similar to the chemical structure of a glucose. The absorption of this drug is similar to the normal glucose transport mechanism and then transport back to the small intestine to perform its function. After the absorption, miglitol will not be metabolised and subsequently be eliminated in the urine (Godbout and Chiasson, 2007). The use of miglitol to treat diabetes in clinical practice is less common as compared to acarbose. The side effect of this drug is similar to acarbose since they share similar mechanism of action.

2.3.2 Alternative Treatments from Natural Sources

Natural products are diverse sources of novel compounds and have become the best source for new drug discovery (Alam et al., 2016a). Findings from Carter et al. (2010) and Alam et al. (2016a) reviewed that natural products such as fruits, vegetables, spices, mushrooms and natural beverages have been associated with remarkable results in T2DM management. These natural products have various mechanisms such as the inhibition of alpha-amylase and alpha-glucosidase, increasing insulin secretion and activity, regulation of glucose transporter 4 (GLUT4), mimicking insulin action and many other mechanisms (Alam et al., 2016b).

Various kinds of foods have been investigated on their inhibitory effect against alpha-glucosidase and the results showed that some of the food components are more potent than the synthetic alpha-glucosidase inhibitor. Various fruits and vegetables such as white grape skin (Lavelli et al., 2016), apricot (Cui et al., 2015), passion fruit (Saravanan and Parimelazhagan, 2014), avocado pear (Oboh et al., 2014b), strawberries (Cheplick et al., 2010), onion (Kim et al., 2011), eggplant (Kwon et al., 2008), ginger (Rani et al., 2011), turmeric (Lekshmi et al., 2012), chilli (Cazzola et al., 2012), pepper and nutmeg (Adefegha and Oboh, 2012) displayed significant inhibition effects against alpha-glucosidase.

Besides these, natural food and beverages like cocoa bean (Oboh et al., 2014a), propolis (Zhang et al., 2015), mushroom (Su et al., 2013), black tea (Striegel et

al., 2015) and green tea (Kamiyama et al., 2010; Matsui et al., 2014) also displayed potent alpha-glucosidase inhibitory activity. Apart from plant based products, animal proteins such as alkaline protease hydrolysate from sardine muscle also showed alpha-glucosidase inhibitory activity (Matsui et al., 2014). Therefore, researchers now are particularly interested to discover more novel natural alpha-glucosidase inhibitors from natural products which can be a potential antidiabetic drug with minimal adverse side effects (Nair et al., 2013).

Although various natural products were tested to be antidiabetic, most of the studies were done in *in vitro.* Further study need be done in simulated *in vivo* system to reflect the actual potential of all these natural products in controlling the disease. Some of the natural products were found to be potent in *in vitro* system but less effective in *in vivo* (Oki et al., 1999). Moreover, some natural products are less effective in alpha-glucosidase inhibition as compared to the synthetic drugs. The functional foods need to be consumed in certain amount in order to achieve the therapeutic effect.

However, the acceptance and usage of natural products as alternative therapies are gaining popularity because the adverse effects and cost are lower as compared to the commercially available drugs. Inzucchi et al. (2012) stated the price for antidiabetic drugs are relatively high especially for the drugs that have better glucose control. Hence, the cost of antidiabetic drugs has become a major concern for patient compliance (Alam et al., 2016a). Natural products have also

shown to enhance the effect of other antidiabetic drugs in a combination therapy (Prabhakar e al., 2014).

2.4 Different Types of Protein Hydrolysates

Natural products have to undergo solvent extraction or protease digestion to generate active compounds and peptides with therapeutic abilities. Studies were performed on the investigation of secondary metabolites found in natural products for their biologically active chemical compounds (Elya et al., 2011; Nair et al., 2013; Zhang et al., 2015; Chukwujekwu et al., 2016). Besides secondary metabolites, previous study have shown that protein hydrolysates can also produce bioactive peptides. This correspond to a study by Kamiyama et al. (2010) that demonstrated that novel alpha-glucosidase inhibitors obtained from natural products can be sugars or derivatives of sugar and also non-saccharide compounds. The non-saccharide compounds have direct hydrophobic binding to the active site of the enzyme results in enzyme inhibition (Bharatham et al., 2008).

Protein hydrolysates are the digested products of proteins into smaller fragments of peptides obtained from enzyme digestion. Protein hydrolysate obtained from various natural products digested with different proteases have distinct biological activities. A study conducted by Chalamaiah et al. (2012) reviewed that different protein hydrolysates obtained from fish digested with different proteases display antioxidant, antihypertensive, immunomodulatory and

antimicrobial activities. Vercruysse et al. (2005) also compared the angiotensin-converting enzyme (ACE) inhibitory activity of protein hydrolysates obtained from muscle protein of meat, fish, and invertebrates. These peptides are not only able to display *in vitro* ACE inhibition, but also *in vivo* antihypertensive properties. Moreover, protein hydrolysates obtained from chicken skin digested with alcalase possess antioxidant activity (Onuh et al., 2014).

Apart from hen egg, egg white proteins obtained from other poultry species like quail, duck, pheasant and ostrich were analysed and compared. It was found that the major types of egg white proteins were found to be similar but varied in the abundancy among the poultry species (Miguel et al., 2005). This suggested that the eggs from different species may possess similar biological activities. It was found that protein hydrolysates obtained from duck egg white hydrolysed with papain and pepsin was found to display antioxidant properties (Chen et al., 2009; Bisswanger, 2014). On the other hand, protein hydrolysates obtained from ostrich egg white hydrolysed with α-chymotrypsin, pepsin, trypsin, and papain separately (Tanzadehpanah et al., 2012) yielded peptide with scavenging activity. Another study conducted by Tabrizi et al. (2015) used pepsin and pancreatin to digest ostrich egg white to produce bioactive peptides with antioxidant properties. A different study found out that peptides obtained from ostrich egg white digested with trypsin could control hypertension by inhibiting ACE (Tanzadehpanah et al., 2013).

Besides animal based protein, plant based protein has also been investigated for its biological activities. Arise et al. (2016) stated that protein hydrolysates obtained from watermelon seed digested with pepsin, trypsin and alcalase separately displayed various degree of antioxidant and alpha-amylase inhibitory activities. Peptides with antioxidant properties were obtained from walnut (*Juglans regia* L.) digested with neutrase, papain, bromelain, alcalase, pepsin and pancreatin separetly (Liu et al., 2016). Cumin seeds digested with protamex also produced antioxidative and alpha-amylase inhibitor peptides (Siow and Gan, 2017). A study conducted by Konrad et al. (2014) displayed that whey protein produced bioactive peptides with dipeptidyl peptidase-4 (DPP-IV), alpha-glucosidase and ACE inhibitory activities when digested with serine protease. The protein hydrolysates obtained from red alga *Palmaria palmate* was hydrolysed with the enzyme papain to generate peptide with renin inhibitory activity (Sanchez and Vazquez, 2017).

2.5 Hen Eggs

2.5.1 Composition of Hen Eggs

Hen egg consists of 58% of egg white with 10-12% of protein which consist of ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), lysozymes (3.5%) and ovomucin (3.5%). Proteins in egg white have a wide range of biological functions, which have high potential to be used in nutraceutical and pharmaceutical industries (Kovacs-Nolan et al., 2005; Miguel et al., 2005). Different protein components displayed different biological activities.

Ovalbumin which is a phosphoglycoprotein, is the most abundant egg white protein. When it is subjected to enzymatic digestion using proteases, the hydrolysate was found to display ACE-inhibitory, antihypertensive, antioxidant and antimicrobial activities (Kitts and Weiler, 2003; Kovacs-Nolan et al., 2005; Liu et al, 2017). Ovotransferrin is a monomeric glycoproteins which was reported to have iron-binding properties, antibacterial, antiviral, antioxidant, anti-inflammatory, ACE-inhibitory and antidiabetic activities (Kitts and Weiler, 2003; Giansanti et al., 2005; Kovacs-Nolan et al., 2005).

Ovomucoid is an egg white protein which is highly glycosylated and the major food allergen present in egg white. It can also act as a trypsin and serine inhibitor (Kovacs-Nolan et al., 2000; Kitts and Weiler, 2003; Kovacs-Nolan et al., 2005). Abeyrathne et al. (2013) stated that peptides derived from ovomucoid displayed anticancer, antimicrobial, immunomodulating, ACE-inhibitory and free radical scavenging activities. Therefore, it has high potentials to be used in the pharmaceutical, nutraceutical and food industries.

Ovomucin is a sulfated glycoprotein with poor solubility after isolation, hence chemical reduction or sonication is performed prior to use (Omana et al., 2010). Many studies have reported that ovomucin has antiviral, antitumor, antioxidant, antibacterial and anti-inflammatory potency (Kitts and Weiler, 2003; Liu et al, 2017; Sanchez and Vazquez, 2017). Lysozyme is another monomeric protein found in egg white. Enzymatic hydrolysis using proteases yielded peptides that displayed antimicrobial activities against both Gram-positive and Gram-

negative bacteria, as well as ACE-inhibitory, anticancer and antioxidant activities (Kovacs-Nolan et al., 2005; Liu et al., 2017).

Besides egg white, egg yolk is also found in hen egg. However, Liu et al. (2017) showed that egg white proteins are more frequently used as the target for peptide production rather than egg yolk proteins. This is because the major component in egg yolk is lipid (Abeyrathne et al., 2013). Thus, extensive research is performed to determine and characterize new biologically active peptides found in egg white. This can enhance the additional benefits of hen eggs as a routine and cheap source of bioactive components (Yu et al., 2011).

2.5.2 Omega 3 Enriched Hen Egg

The benefits of consuming omega 3 polyunsaturated fatty acids (n-3 PUFA) are well recognised. Unfortunately, the recommended daily intake of omega 3 fatty acids is rarely achieved. Hence, the enrichment of foods can increase the uptake of these active compounds. Hen eggs are often the target for this purpose as they are common in human diet (Trautwein, 2001). The hens' feed which includes flaxseed, chia seed, fish oil and marine algae which are rich in α-linolenic acid as well as long chain fatty acids for instance docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Ayerza and Coates, 2000). The α-linolenic acid is 30 times higher and the DHA is four times higher in the eggs when the hens are fed with flaxseed (Lewis et al., 2000).

Dietary intake of omega 3 fatty acids will reduce the risk of heart disease (Trautwein, 2001; Yashodhara et al., 2009), decrease the incidence of prostate and breast cancer, delay the loss of immunological functions and essential for normal foetal visual and brain development (Jordan, 2010). Moreover, omega 3 fatty acids helps to prevent and treat many chronic diseases such as diabetes mellitus (Trautwein, 2001; Yashodhara et al., 2009). The ability of omega 3 enriched egg to control diabetes mellitus by inhibiting alpha-glucosidase will be an additional benefit for consuming omega 3 enriched egg.

2.5.3 Selenium Enriched Hen Egg

Selenium is an essential trace element required by human for normal metabolism. The intake of selenium supplement was found to be able to protect the individual against oxidative stress and various cancers (WebMD, 2016). Fisinin et al. (2009) stated that intake of selenium results in various health benefits. Studies showed that insufficient selenium intake will result in poor health, decreased fertility and immunity against viral and bacterial infections. Selenium deficiency in the individual will result in immunocompromised and higher risk of acquiring various diseases such as cancer, cardiovascular disease, diabetes and others (Surai, 2006). Clinical studies have also shown that the sufficient intake of organic selenium will reduce the risk of cancer mortality (Fisinin et al., 2008).

Unfortunately, common foods contain low amount of selenium and selenium deficiency is a global health issue (Fisinin et al., 2009). Hence, there are several potential options to improve human selenium intake including the production of selenium enriched eggs (Surai, 2006). Generally, selenium enriched egg is considered as a good source of selenium in human diet (Fisinin et al., 2009). Therefore, the ability of selenium enriched egg to control diabetes mellitus by inhibiting alpha-glucosidase will improve the benefits of consuming selenium enriched egg.

2.5.4 Protein Hydrolysates from Hen Egg White Protein

Abeyrathne et al. (2013) have shown that not only egg white proteins but also the hydrolysed products have various biological properties. Over the years, numerous functional peptides have been obtained from egg white protein which showed remarkable health benefits. *In vitro* enzymatic hydrolysis using purified proteases has been commonly used to produce bioactive peptides from egg proteins. Protease are enzymes, which hydrolyse the egg white proteins to release the bioactive peptides (Liu, et al., 2017).

Purified proteases can be categorized into animal gastrointestinal proteases (e.g. pepsin, trypsin and chymotrypsin) and non-gastrointestinal proteases (e.g. papain, alcalase and thermolysin) from plant and microbial sources (Korhonen and Pihlanto, 2003; Liu et al., 2017). Different proteases yielded different active peptides since they cleave at different sites. These proteases differ not only in

their specificities but also the optimal conditions required for maximum enzymatic hydrolysis such as pH and temperature (Abeyrathne et al., 2013). Various studies were performed using different proteases to generate various active peptides with different biological activities.

Studies have shown that proteolysis occurs mostly during the gastric phase in gastrointestinal digestion (Nyemb et al., 2014). Therefore, researches have greater interest in the peptide production in *in vitro* gastrointestinal digestion using gastrointestinal proteases as compared to the non-gastrointestinal proteases. This is because the potency and bioavailability of bioactive peptides obtained from egg white protein tested *in vitro* may represent the actual physiological benefits in *in vivo* gastrointestinal digestion (Liu et al., 2017).

Pepsin is the most common protease used to hydrolyse egg white protein components and shown to produce peptides that display various biological functions. Kovacs-Nolan et al. (2000) showed that egg white protein hydrolysates showed Immunoglobulin (Ig) E binding activity and trypsin inhibitory activities. Protein hydrolysate obtained from egg white was also found to be able to control hypertension (Miguel and Aleixandre, 2006). This correspond to a study by Liu et al. (2017) showed that pepsin digested protein hydrolysates from egg white has the highest ACE-inhibitory activity as compared to those hydrolysed by chymotrypsin and trypsin. Besides these, pepsinolysis of egg white protein showed scavenging activities, inhibition to lipid peroxidation (Xu et al., 2007; Shen et al., 2010) and anti-inflammatory

(Majumder et al., 2015). Ibrahim et al. (2005) showed that pepsin hydrolysed egg white protein are potent bactericidal agent against several strains of bacteria.

There are also studies that argued that enzymatic hydrolysis using non-gastrointestinal proteases could generate more potent bioactive peptides (Liu et al., 2017). They claimed that non-gastrointestinal proteases have different hydrolytic characteristics, thus resulting in different peptide sequences with different bioactivities (Liu et al., 2017). Among the non-gastrointestinal proteases, papain, alcalase and thermolysin are commonly used for the digestion of egg white proteins (Memarpoor-Yazdi et al., 2012). Papain digested egg white protein produced bioactive peptides that function as a metal chelating agent, antimicrobial agent (Memarpoor-Yazdi et al., 2012), ACE inhibitor (Chen and Chi, 2011), antioxidant and lipid peroxidation inhibitor (Chen et al., 2012; Memarpoor-Yazdi et al., 2012). Besides papain, protein hydrolysate obtained from egg white digested with alcalase also has alpha-glucosidase and ACE inhibitory properties (Yu et al., 2011; 2012).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Reagents

The list of chemicals and reagents used throughout this study with their respective manufactures is attached in Appendix A.

3.2 Equipment and Laboratory Wares

The list of equipment and laboratory wares used throughout this study with their respective brands and manufactures is attached in Appendix B.

3.3 Preparation of Hen Egg White Protein Slurry

Egg white was separated from egg yolk and homogenised mechanically. In a 50 mL centrifuge tube, 1 g of egg white was dispersed in 20 mL of distilled water to obtain 5% (w/v) protein slurry. The centrifuge tube was incubated at 90ºC in a water bath for 10 min. The protein slurry was later cooled down to room temperature.

3.4 Optimisation of Hen Egg White Protein Hydrolysis

3.4.1 Preparation of 1 M Hydrochloric Acid (HCl)

In a 100 mL media bottle, 8.3 mL of 12 M HCl was added to 91.7 mL of distilled water. The solution was stored at room temperature until use.

3.4.2 Preparation of 1 M Sodium Hydroxide (NaOH)

In a 100 mL media bottle, 4 g of NaOH was dissolved in 100 mL of distilled water. The solution was stored at room temperature until use.

3.4.3 Optimisation of Pepsin Concentration

The egg white protein solution prepared as described in Section 3.3 was adjusted to pH 2. Five mL of egg white protein solution was aliquoted into four different tubes. Various concentrations of pepsin was added to the solution separately as shown in Table 3.1. The concentration of pepsin was calculated using the following formula:

$$\text{Concentration of pepsin (\%) (w/w)} = \frac{\text{weight of pepsin}}{\text{weight of egg white}} \times 100$$

Table 3.1: Preparation of different pepsin concentrations.

Tube	Concentration of pepsin (%) (w/w)	Amount of pepsin added (mg)
1	1	0.25
2	2	0.50
3	3	0.75
4	4	1.00

The solution was incubated at 37ºC in a water bath for 60 min to carry out enzymatic hydrolysis. After that, the hydrolysate obtained was incubated for 10 min at 90ºC in a water bath to terminate the enzymatic digestion. The mixture was then centrifuged at 12000 rpm for 10 min at 4ºC. The supernatant obtained was used to perform the Bradford assay for protein quantification. The optimisation was carried out in triplicates.

3.4.4 Optimisation of pH on Pepsin Digestion

Twenty mL of egg white protein solution prepared as described in Section 3.3 was aliquoted into four different tubes and the pH was adjusted to pH 1, pH 2, pH 7 and pH 8 separately. Then, 0.01 g of pepsin (4%) (w/w) was added to the egg white mixture. The mixture was incubated at 37ºC in a water bath for 60 min for enzymatic hydrolysis to take place. After that, the hydrolysate obtained were incubated for 10 min at 90ºC in a water bath to terminate the enzymatic

digestion. The mixture was then centrifuged at 12000 rpm for 10 min at 4ºC. The supernatant obtained was used to perform the Bradford assay for protein quantification. The optimisation was carried out in triplicates.

3.4.5 Optimisation of Temperature on Pepsin Digestion

The egg white protein solution prepared as described in Section 3.3 was adjusted to pH 2 prior to addition of 0.04 g of pepsin (4%) (w/w) to initiate enzymatic hydrolysis. Five mL of egg white protein solution was aliquoted into three different tubes incubated at 30ºC, 37ºC and 45ºC separately in a water bath for 60 min to carry out enzymatic hydrolysis. After that, the hydrolysate obtained were incubated for 10 min at 90ºC in a water bath to terminate the enzymatic digestion. The mixture was then centrifuged at 12000 rpm for 10 min at 4ºC. The supernatant obtained was used to perform the Bradford assay for protein quantification. The optimisation was carried out in triplicates.

3.4.6 Optimisation of Papain Concentration

The egg white protein solution prepared as described in Section 3.3 was adjusted to pH 6. Five mL of egg white protein solution was aliquoted into four different tubes. Various concentrations of papain was added to the solution separately as shown in Table 3.2. The concentration of papain was calculated using the following formula:

$$\text{Concentration of papain (\%)} = \frac{\text{weight of papain}}{\text{weight of egg white}} \times 100$$

Table 3.2: Preparation of different papain concentration.

Tube	Concentration of papain (%) (w/w)	Amount of papain added (mg)
1	1	0.25
2	2	0.50
3	3	0.75
4	4	1.00

The solution was incubated at 50ºC in a water bath for 60 min to carry out enzymatic hydrolysis. After that, the hydrolysate obtained was incubated for 10 min at 90ºC in a water bath to terminate the enzymatic digestion. The mixture was then centrifuged at 12000 rpm for 10 min at 4ºC. The supernatant obtained was used to perform the Bradford assay for protein quantification. The optimisation was carried out in triplicates.

3.4.7 Optimisation of pH on Papain Digestion

Twenty mL of egg white protein solution prepared as described in Section 3.3 was aliquoted into four different tubes and the pH was adjusted to pH 5, pH 6, pH 7 and pH 8 separately. Then, 0.01 g of papain (4%) (w/w) was added to the

egg white mixture. The mixture was incubated at 50ºC in a water bath for 60 min for enzymatic hydrolysis to take place. After that, the hydrolysate obtained were incubated for 10 min at 90ºC in a water bath to terminate the enzymatic digestion. The mixture was then centrifuged at 12000 rpm for 10 min at 4ºC. The supernatant obtained was used to perform the Bradford assay for protein quantification. The optimisation was carried out in triplicates.

3.4.8 Optimisation of Temperature on Papain Digestion

The egg white protein solution prepared as described in Section 3.3 was adjusted to pH 6 prior to addition of 0.04 g of papain (4% w/w based on the weight of egg white) to initiate enzymatic hydrolysis. Five mL of egg white protein solution was aliquoted into three different tubes incubated at 37ºC, 50ºC and 65ºC separately in a water bath for 60 min to carry out enzymatic hydrolysis. After that, the hydrolysate obtained were incubated for 10 min at 90ºC in a water bath to terminate the enzymatic digestion. The mixture was then centrifuged at 12000 rpm, 10 min and 4ºC. The supernatant obtained was used to perform the Bradford assay for protein quantification. The optimisation was carried out in triplicates.

3.5 Quantification of Protein Concentration

3.5.1 Preparation of 1 mg/mL Bovine Serum Albumin (BSA) solution

In a 0.2 mL microcentrifuge tube, 15 µL of 10 mg/mL BSA was added to 135 µL of distilled water prior to use.

3.5.2 Preparation of 5X Bradford Solution

In a 500 mL media bottle, 100 mg of Coomassie Brilliant Blue G-250 was added into 47 mL of methanol and 100 µL of 85% phosphoric acid. The solution was then topped up to 200 mL with distilled water and stored at 4ºC until use.

3.5.3 Preparation of 1X Bradford Solution

In a 50 mL centrifuge tube, 1 mL 5X Bradford solution was added to 4 mL distilled water prior to use.

3.5.4 Preparation of 0.1 M Sodium Phosphate Buffer (PBS) with pH 6.8

In a 1 L beaker, 1.312 g of NaH_2PO_4 was dissolved in 1 L of distilled water. Another 1 L beaker was used to dissolve 0.704 g of Na_2HPO_4.in 1 L of distilled water. In a 1 L media bottle, 503 mL NaH_2PO_4 solution was mixed with 497 mL

of Na_2HPO_4 to achieve pH 6.8. The solution was autoclaved and stored at room temperature until use.

3.5.5 Bradford Assay

In a 96-well flat bottom plate, BSA standard curve was obtained using 1 mg/mL BSA solution. The BSA concentration used for the standard curve ranged from 100 mg/mL to 500 mg/mL were prepared as shown in Table 3.3.

Table 3.3: Preparation of BSA standard for Bradford Assay.

BSA concentration (mg/mL)	Volume of 1 mg/mL BSA (µL)	Volume of PBS (µL)
0	0	20
100	2	18
200	4	16
300	6	14
400	8	12
500	10	10

Five µL of sample was added into each well containing 15 µL of PBS. Duplicates of standard range and samples were used. Two hundred microliters of 1X Bradford reagent was then added into each well and the plate was read at $OD_{595\ nm}$ using a microplate reader.

3.6 Enzymatic Hydrolysis on Hen Egg White

Egg white was separated from egg yolk and homogenised mechanically. In a 500 mL beaker, 15 g of egg white was dispersed in 300 mL distilled water to obtain 5% (w/v) protein slurry. The beaker was incubated at 90ºC in a water bath for 10 min. The protein slurry was later cooled down to room temperature. The solution was adjusted to pH 2 for pepsin and pH 7 for papain digestion, respectively. Then, 0.6 g of pepsin and papain (4%) (w/w) was added separately to carry out enzymatic hydrolysis. The mixture was then incubated at 37ºC in a water bath for 180 min for enzymatic hydrolysis to take place. After that, the hydrolysate obtained was incubated for 10 min at 90ºC in a water bath to inactivate the enzyme. The mixture was then centrifuged at 12000 rpm, 10 min and 4ºC. The supernatant obtained was kept -20ºC and lyophilised. The lyophilised powder was stored at -20ºC until use. The procedure were carried out on classic, omega 3 enriched and selenium enriched eggs.

3.7 Alpha-Glucosidase Inhibitory Assay

3.7.1 Preparation of 1 M Sodium Carbonate (Na_2CO_3)

In a 250 mL media bottle, 13.25 g of Na_2CO_3 was dissolved in 125 mL of distilled water. The solution was autoclaved and stored at room temperature until use.

3.7.2 Preparation of Sample Solution

In a 1.5 mL microcentrifuge tube, 0.1 g of lyophilised egg white protein hydrolysates powder was dissolved in 1 mL of 10% dimethyl sulfoxide (DMSO) to yield protein hydrolysate stock solution with a concentration of 100 mg/mL. The protein hydrolysate obtained was used to perform the Bradford assay for protein quantification. The experiment was carried out in triplicates.

3.7.3 Preparation of 1 U/mL Alpha-Glucosidase Solution

In a 1.5 mL microcentrifuge tube, 3.4 µL of 290 U/mL alpha-glucosidase stock solution was added to 996.6 µL of PBS. The alpha-glucosidase solution was stored at -20ºC until use.

3.7.4 Preparation of 10 mM p-nitrophenyl-α-D-glucopyranoside (pNPG) Stock Solution

In a 15 mL centrifuge tube, 0.032 g of pNPG was dissolved in 10 mL of PBS. The pNPG stock solution was stored at 4ºC until use.

3.7.5 Preparation of 2 mM p-nitrophenyl-α-D-glucopyranoside (pNPG)

In a 1.5 mL microcentrifuge tube, 200 µL of 10 mM pNPG stock solution was added to 800 µL of PBS. The pNPG working solution was stored at 4ºC until use.

3.7.6 Alpha-Glucosidase Inhibitory Assay

In a flat bottomed 96-well plate, 40 µL of protein hydrolysate was added and mixed with 20 µL of alpha-glucosidase (1 U/mL) and 100 µL of 0.1 mM PBS. The final concentrations of protein hydrolysate used were 100, 200, 300, 400 and 500 mg/mL for pepsin digested egg white protein. For papain digested egg white protein, the final concentration used were 2, 4, 6, 8 and 10 mg/mL. The mixture was incubated at 37ºC for 15 min. After the incubation, 40 µL of pNPG (2 mM) was added into the mixture and further incubated at 37ºC for 20 min. Then, 100 µL of 1 M Na_2CO_3 solution was added to terminate the reaction. The amount of p-nitro phenol released was measured at $OD_{410\ nm}$ using a microplate reader to measure the enzymatic activity. The IC_{50} value was defined as the half maximal inhibitory concentration of an inhibitor required to inhibit 50% of the alpha-glucosidase activity. The negative control used in this assay was prepared by replacing the protein hydrolysate with 10% of DMSO. Whereas, the blank used in this assay was 10% of DMSO. The assay was carried out in triplicates.

3.7.7 Percentage of Inhibition of Alpha-Glucosidase Activity

The percentage of inhibition of alpha-glucosidase activity was calculated using the following formula:

$$\text{inhibitory activity (\%)} = \frac{(OD_{\text{negative control}}\text{-}OD_{\text{blank}})\text{-}(OD_{\text{sample}}\text{-}OD_{\text{blank}})}{(OD_{\text{negative control}}\text{-}OD_{\text{blank}})} \times 100$$

The half maximal inhibitory concentration (IC_{50}) was determined from the graph of percentage of alpha-glucosidase inhibition against concentration of protein hydrolysates.

CHAPTER 4

RESULTS

4.1 Optimisation of Hen Egg White Protein Hydrolysis

4.1.1 Optimisation of Pepsin Concentration

Hen egg white was digested using different pepsin concentrations ranging from 1 to 4% (w/w). The concentration of protein obtained after the digestion was quantified using the Bradford assay. Based on Table 4.1, the concentration of protein increased with increasing pepsin concentration.

Table 4.1: The concentration of protein yield using various concentrations of pepsin for enzymatic digestion.

Concentration of pepsin (%) (w/w)	Concentration of protein (mg/mL)
1	81.83 ± 5.39
2	92.42 ± 2.31
3	95.78 ± 2.79
4	99.02 ± 0.12

The values are presented in mean ± standard deviation (n=3).

However, the increase in protein concentration was relatively low and constant from 2 to 4% (w/w). Based on Table 4.1, a constant increment of protein concentration of about 3.36 mg/mL was obtained when the pepsin concentration was increased from 2 to 3% (w/w). Whereas, an increment of protein concentration of about 3.24 mg/mL was observed when pepsin concentration was increased from 3 to 4% (w/w). The concentration of protein yielded at 4% (w/w) of pepsin increased by 21%, 7% and 3.3% compared to that obtained at 1, 2 and 3% (w/w) of pepsin, respectively. As 4% of pepsin concentration yielded the highest concentration of protein, thus it was chosen to be used for subsequent optimisation.

4.1.2 Optimisation of pH on Pepsin Digestion

Hen egg white was digested using different pH which were pH 1, 2, 7 and 8. The concentration of protein obtained after the digestion was quantified using the Bradford assay. The four pHs used in this study represented different conditions. For instance, pH 1 and 2 represented acidic condition, pH 7 represented a neutral condition and pH 8 represented an alkaline condition. Based on Table 4.2, the concentration of protein obtained increased from pH 1 to 2.

Table 4.2: The concentration of protein yield using various pH for pepsin digestion.

pH	Concentration of protein (mg/mL)
1	200.39 ± 29.26
2	212.28 ± 32.82
7	181.67 ± 24.69
8	160.41 ± 14.77

The values are presented in mean ± standard deviation (n=3).

The concentration of protein yielded at pH 2 was 11.89 mg/mL higher in comparison to protein concentration obtained at pH 1. However, the protein concentration obtained dropped significantly when the pH was increased to pH 7 and 8. The protein concentration yielded at pH 7 and 8 has decreased by 14.4% and 24.4%, respectively compared to protein concentration obtained at pH 2. Since the results showed that pH 2 produced the highest protein concentration yielded, thus it was chosen as the optimum pH for pepsin digestion for subsequent optimisation steps.

4.1.3 Optimisation of Temperature on Pepsin Digestion

Hen egg white was digested using different temperatures which were at 30ºC, 37ºC and 45ºC. Based on Figure 4.1, the highest concentration of protein yielded (221.83 ± 5.40 mg/mL) was at 37ºC. The protein concentration

increased drastically from 30ºC to 37ºC and dropped when the temperature was further increased to 45ºC.

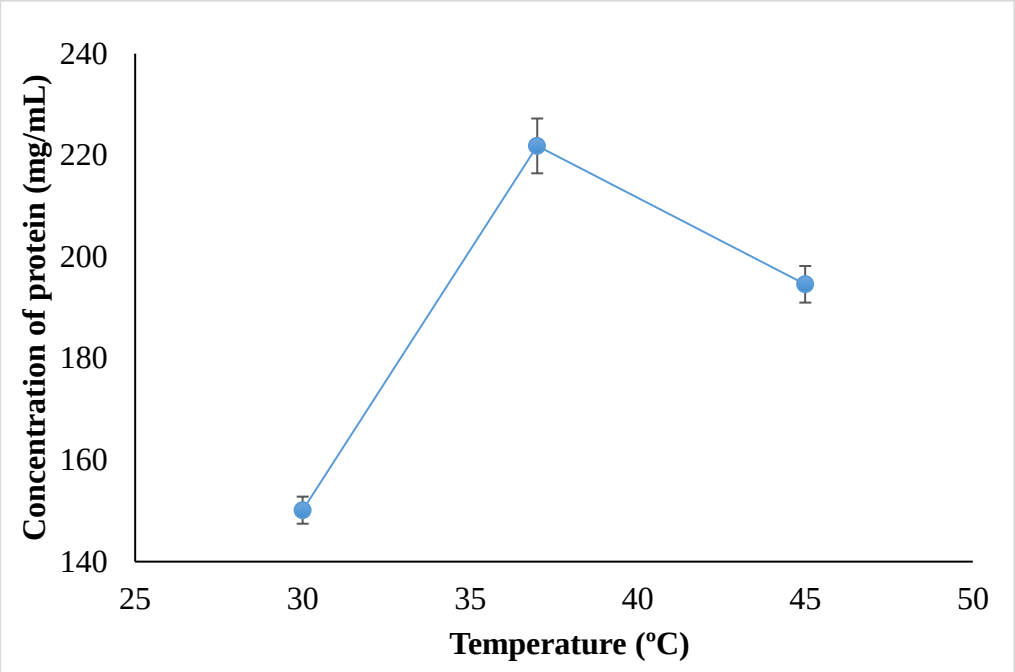

Figure 4.1: The concentration of protein yielded using different temperatures for pepsin digestion. The values are presented in mean ± standard deviation (n=3).

Based on Table 4.3, the concentration of protein obtained increased by 47.8% when the temperature was increased from 30ºC to 37ºC. However, the protein concentration yielded dropped by 12.3% when the temperature was increased to 45ºC. Therefore, this study showed 37ºC was the optimum temperature to use for pepsin digestion, thus it was chosen to be used in this study.

Table 4.3: The concentration of protein yield using various temperatures for pepsin digestion.

Temperature (ºC)	Concentration of protein (mg/mL)
30	150.13 ± 2.67
37	221.83 ± 5.40
45	194.59 ± 3.60

The values are presented in mean ± standard deviation (n=3).

4.1.4 Optimisation of Papain Concentration

Hen egg white was digested using different papain concentrations ranging from 1 to 4% (w/w). Based on Figure 4.2, the concentration of protein increased linearly from 1 to 4% (w/w) with increasing papain concentration. However, a plateau stage for their enzymatic reaction was not achieved.

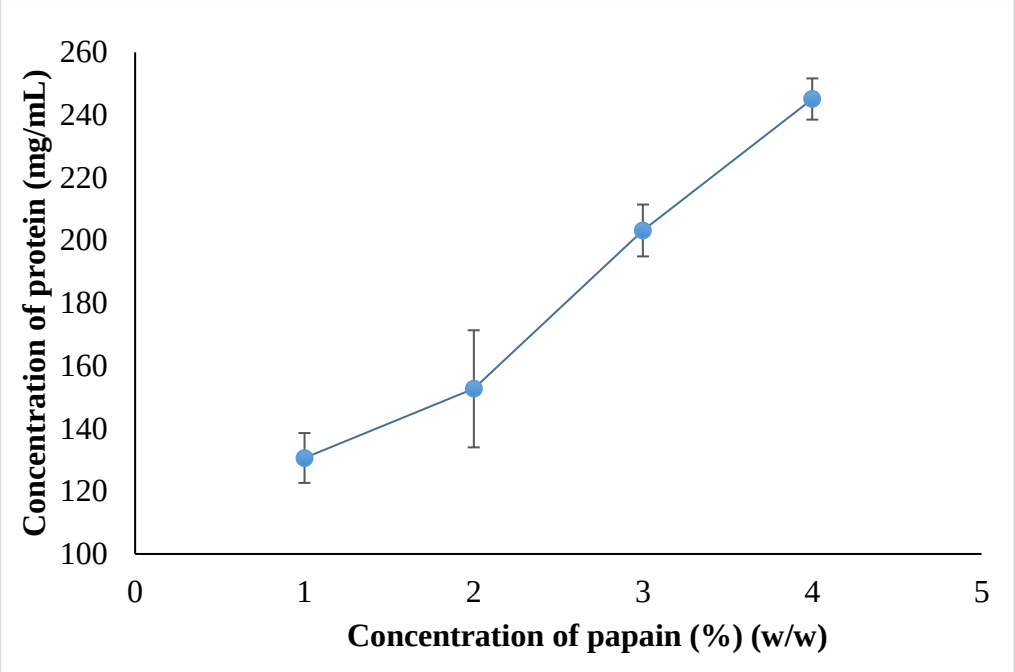

Figure 4.2: The concentration of protein yielded using various concentrations of papain for enzymatic digestion. The values are presented in mean ± standard deviation (n=3).

According to Table 4.4, the concentration of protein yielded increased by 16.9% when the concentration of papain was increased from 1 to 2% (w/w), followed by an increment of 33.1% from 2% to 3% (w/w) and lastly the protein concentration increased by 17.11% from 3 to 4% (w/w). The concentration of protein produced at 4% (w/w) of papain was the highest (245.09 ± 6.57 mg/mL), which was approximately two fold higher than the one yielded at 1% (w/w). Since 4% (w/w) of papain concentration yielded the highest concentration of protein, it was chosen to be used in the subsequent optimisation.

Table 4.4: The concentration of protein yield using various concentrations of papain.

Concentration of papain (%) (w/w)	Concentration of protein (mg/mL)
1	130.61 ± 7.96
2	152.67 ± 18.67
3	203.17 ± 8.22
4	245.09 ± 6.57

The values are presented in mean ± standard deviation (n=3).

4.1.5 Optimisation of pH on Papain Digestion

Hen egg white was digested using different pH ranging from pH 5 to 8. Based on Figure 4.3, the highest protein concentration was achieved at pH 7 (122.13 ± 6.23 mg/mL).

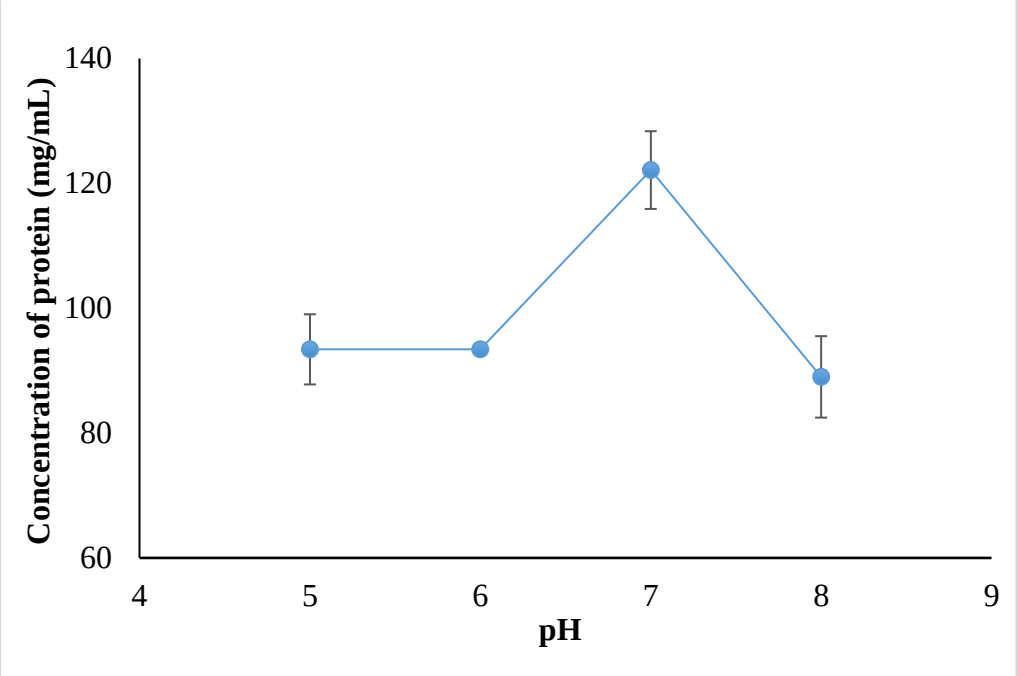

Figure 4.3: The concentration of protein yielded using various pH for papain digestion. The values are presented in mean ± standard deviation (n=3).

Based on Table 4.5, the concentration of protein produced was relatively constant between pH 5 (93.41 ± 5.62 mg/mL) and 6 (93.43 ± 0.85 mg/mL). The difference in protein concentration obtained at the two points was only 0.02 mg/mL. The concentration of protein yielded at pH 7 was 30.7% higher than the protein concentration yielded at pH 5 and 6. When the pH was increased to pH 8, the protein concentration decreased by 37% compared to that obtained at pH 8. The results showed that pH 7 was the optimum pH for papain digestion, thus it was chosen to be used for the next step of optimisation.

Table 4.5: The concentration of protein yield using various pH for papain digestion.

pH	Concentration of protein (mg/mL)
5	93.41 ± 5.62
6	93.43 ± 0.85
7	122.13 ± 6.23
8	89.00 ± 6.52

The values are presented in mean ± standard deviation (n=3).

4.1.6 Optimisation of Temperature on Papain Digestion

Hen egg white was digested using different temperatures which were at 37ºC, 50ºC and 60ºC. Based on Figure 4.4, the concentration of protein was the highest at 37ºC (95.74 ± 3.04 mg/mL). The protein concentration decreased when the temperature was increased from 37ºC to 50 ºC and then to 60 ºC.

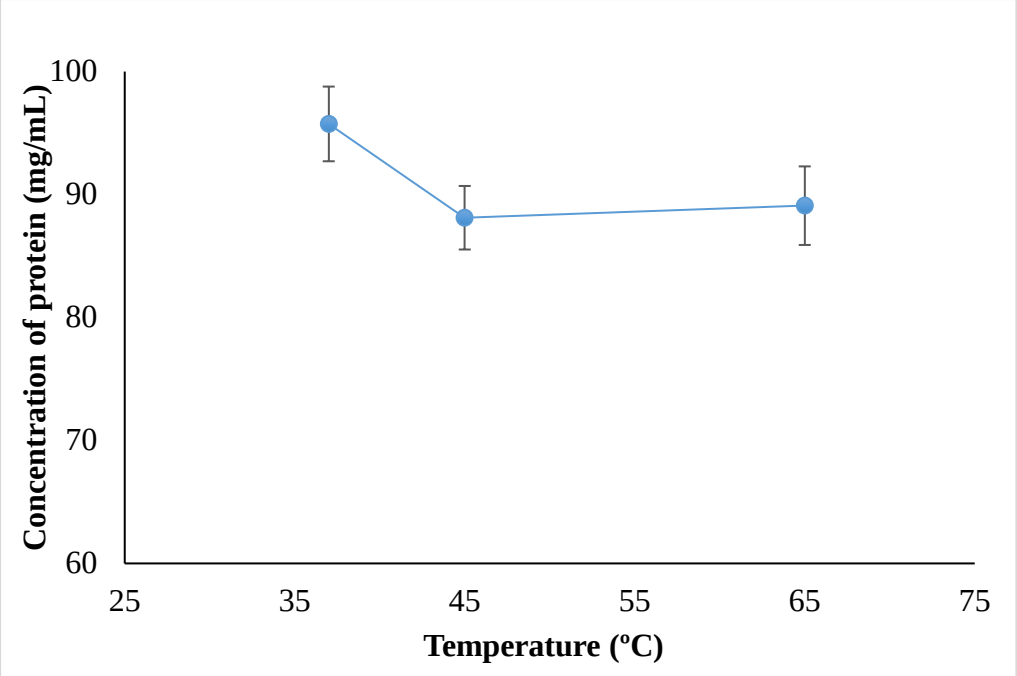

Figure 4.4: The concentration of protein yielded using various temperatures for papain digestion. The values are presented in mean ± standard deviation (n=3).

The protein concentration yielded at the tested temperatures did not vary significantly. The highest protein concentration was obtained at 37ºC (95.74 ± 3.04 mg/mL), followed by 65ºC (89.09 ± 3.19 mg/mL) and the lowest concentration achieved at 50ºC (88.11 ± 2.58 mg/mL). Since the highest protein concentration was achieved at 37ºC, therefore it was selected as the optimum temperature for papain digestion in this study.

4.2 Enzymatic Hydrolysis on Hen Egg White Protein

Hen eggs were digested by pepsin and papain separately by using the optimized conditions as described in Section 4.1. Based on Figure 4.5, the concentration of protein yielded from pepsin digestion was significantly higher than papain digestion for all the three egg variants.

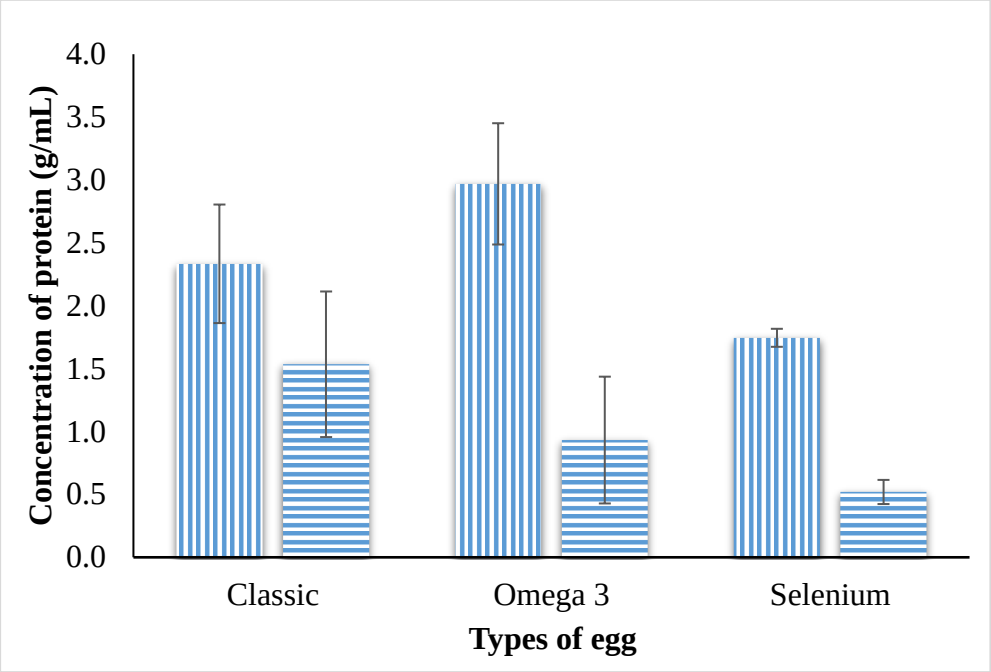

Figure 4.5: The concentration of protein yielded using pepsin and papain for hen egg white protein hydrolysis quantified using the Bradford assay. The values are presented in mean ± standard deviation (n=3).

Based on Table 4.6, the pepsin digested omega 3 enriched egg white generated the highest concentration of protein (2.97 ± 0.48 g/mL), followed by classic egg (2.33 ± 0.47 g/mL) and lastly the selenium enriched egg (1.75 ± 0.07 g/mL). The concentration of protein produced from pepsin digested omega 3 enriched

egg was 27.5% higher than the classic egg and 69.7% higher than selenium enriched egg. However, papain digestion produced a slightly different result from pepsin digestion. For papain digestion, classic egg produced the highest concentration of protein (1.53 ± 0.28 g/mL), followed by omega 3 enriched egg (0.93 ± 0.12 g/mL) and lastly the selenium enriched egg (0.52 ± 0.11 g/mL). The concentration of protein yielded from papain digested classic egg was 64.5% higher than omega 3 enriched egg and approximately three fold higher than selenium enriched egg. Overall, the selenium enriched egg yielded the lowest concentration of protein for both pepsin (1.75 ± 0.07 g/mL) and papain (0.52 ± 0.11 g/mL) digestion.

Table 4.6: The concentration of protein yield using different enzymes for hen egg white protein hydrolysis.

Types of egg	**Concentration of protein (g/mL)**	
	Pepsin	**Papain**
Classic	2.33 ± 0.47	1.53 ± 0.28
Omega 3 enriched	2.97 ± 0.48	0.93 ± 0.12
Selenium enriched	1.75 ± 0.07	0.52 ± 0.11

The values are presented in mean ± standard deviation (n=3).

4.3 Alpha-Glucosidase Inhibitory Assay

The lyophilised hen egg white protein was used to test on the alpha-glucosidase inhibitory activity. The percentage of inhibition was calculated using the formula stated in Section 3.7.6. The negative control used in this study was conducted in the absence of protein hydrolysates, thus would not show any inhibitory activity against alpha-glucosidase.

4.3.1 Pepsin Digested Egg White Protein Hydrolysates

The concentration of protein hydrolysates ranging from 100 to 500 mg/mL for pepsin digestion was tested. Based on Figure 4.6, the results showed that the inhibitory activity increased with increased in protein hydrolysates concentration. Pepsin digested selenium enriched egg showed the highest percentage of inhibition (97.17 ± 2.85%) compared to classic egg and omega 3 enriched egg.

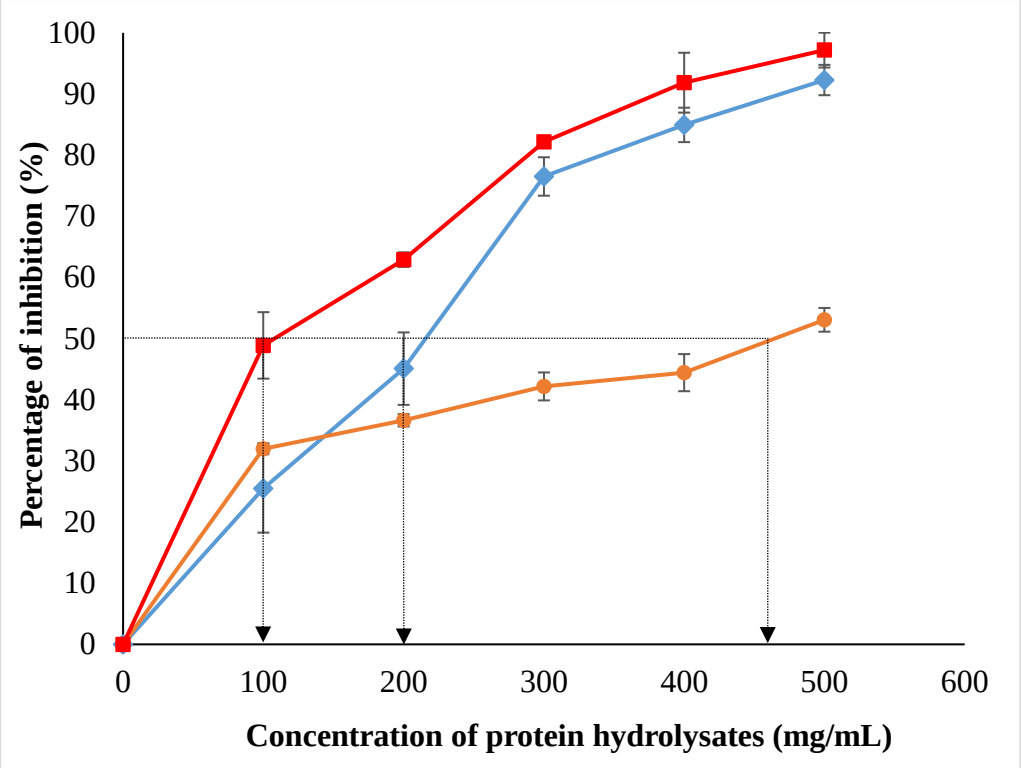

Figure 4.6: Alpha-glucosidase inhibitory activity by using different concentrations of pepsin digested protein hydrolysates from classic ◆, omega 3 enriched ● and selenium enriched ■ egg. The results were expressed as mean ± standard deviation (n=3).

Footnote: The indicated arrow represent the half maximal inhibitory concentration (IC_{50}) for different egg varieties.

As observed in Table 4.7, the highest percentage of inhibition achieved in this study using 500 mg/mL of protein hydrolysates for selenium enriched egg was 97.17%, followed by classic egg (92.28%) and the lowest inhibitory activity was obtained from omega 3 enriched egg (53.07%). The inhibitory effect exhibited by both classic and selenium enriched eggs were approximately two fold higher than the inhibitory effect of omega 3 enriched egg at 500 mg/mL of

protein hydrolysates. However, the selenium enriched egg showed 4.9% higher inhibitory effect compared to classic egg at 500 mg/mL of protein hydrolysates.

Table 4.7: The percentage of alpha-glucosidase inhibitory activity of different egg variants by using different concentrations of pepsin digested protein hydrolysates.

Concentration of protein hydrolysates (mg/mL)	Percentage of inhibition (%)		
	Classic egg	Omega 3 enriched egg	Selenium enriched egg
100	25.47 ± 7.20	31.99 ± 0.90	48.89 ± 5.44
200	45.09 ± 5.93	36.64 ± 1.01	62.92 ± 1.19
300	76.51 ± 3.14	42.18 ± 2.29	82.18 ± 0.60
400	84.94 ± 2.82	44.43 ± 3.04	91.85 ± 4.89
500	92.28 ± 2.48	53.07 ± 1.9	97.17 ± 2.85

The values are presented in mean ± standard deviation (n=3).

The IC_{50} for the three egg variants based on Figure 4.6 and the values are tabulated in Table 4.8. Selenium enriched egg displayed the highest alpha-glucosidase inhibitory activity with IC_{50} at 96.69 mg/mL, followed by classic egg with IC_{50} at 220.28 mg/mL and lastly the omega 3 enriched egg with IC_{50} at 462.20 mg/mL.

Table 4.8: The half maximal inhibitory concentration (IC_{50}) of various egg variants in alpha-glucosidase inhibitory assay.

Variant of eggs	IC_{50} of alpha-glucosidase inhibitory assay (mg/mL)
Classic	220.28
Omega 3 enriched	462.20
Selenium enriched	96.69

4.3.2 Papain Digested Egg White Protein Hydrolysates

The concentration of protein hydrolysates ranging from 2 to 10 mg/mL for papain digestion was tested. Based on Figure 4.7, the results showed that the inhibitory activity increased with increased in protein hydrolysates concentration. The increase in protein hydrolysate concentration has resulted in a linear increase in percentage of inhibition. Papain digested selenium enriched egg showed the highest percentage of inhibition (28.06 ± 1.42%) compared to classic egg and omega 3 enriched egg.

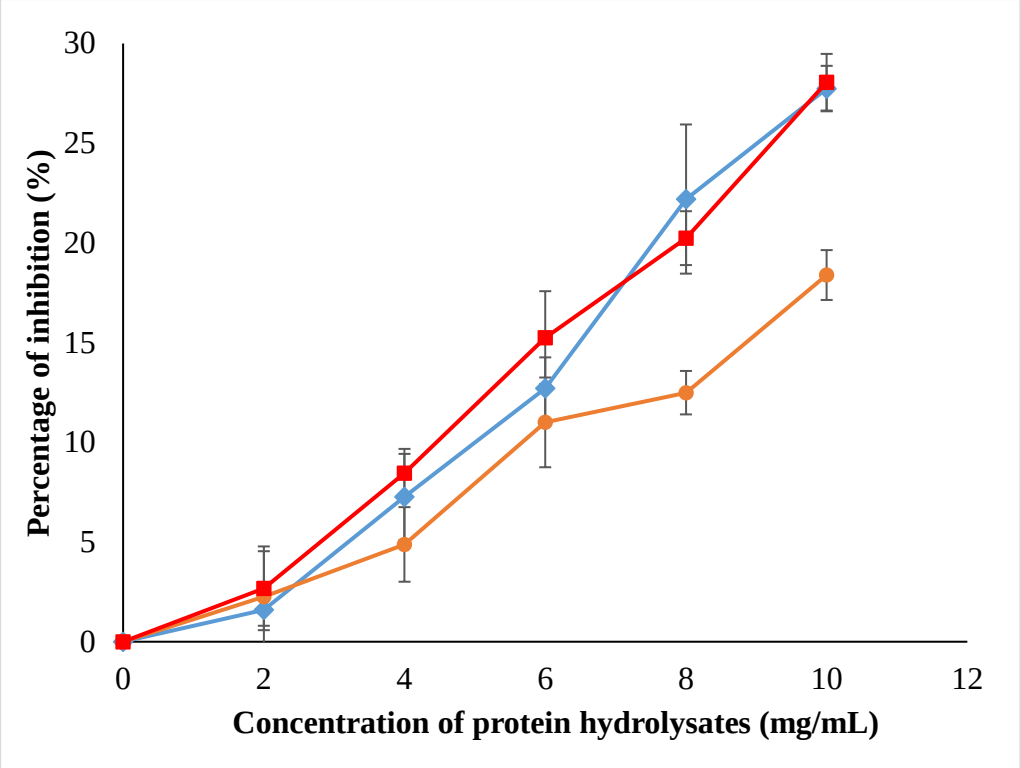

Figure 4.7: Alpha-glucosidase inhibitory activity by using different concentrations of papain digested protein hydrolysates from classic , omega 3 enriched and selenium enriched eggs. The results were expressed as mean ± standard deviation (n=3).

Based on Table 4.9, at 10 mg/mL of protein hydrolysates, selenium enriched egg showed the highest percentage of inhibition at 28.06 ± 1.42%. This was then followed by classis egg with a percentage of inhibition at 26.74 ± 1.14%. Lastly, omega 3 enriched egg achieved the lowest percentage on inhibition in this study which was at 18.39 ± 1.25%.

Table 4.9: The percentage of alpha-glucosidase inhibitory activity of different egg variants by using different concentrations of papain digested protein hydrolysates.

Concentration of egg white protein (mg/mL)	Percentage of inhibition (%)		
	Classic egg	Omega 3 enriched egg	Selenium enriched egg
2	1.59 ± 1.01	2.26 ± 2.52	2.67 ± 1.87
4	7.26 ± 2.16	4.88 ± 1.87	8.46 ± 1.21
6	12.70 ± 1.56	11.00 ± 2.25	15.25 ± 2.33
8	22.20 ± 3.74	12.49 ± 1.09	20.24 ± 1.35
10	26.74 ± 1.14	18.39 ± 1.25	28.06 ± 1.42

The values are presented in mean ± standard deviation (n=3).

However, the IC_{50} value for the three egg variants were not able to be determined in this study because the concentration of protein hydrolysates digested with papain obtained after freeze drying was too low. High concentration of protein hydrolysate could not be prepared from the low protein concentration yielded from papain digestion.

CHAPTER 5

DISCUSSION

5.1 Optimisation of Hen Egg White Protein Hydrolysis

5.1.1 Optimisation of Pepsin Concentration

The higher the enzyme concentration, the faster the rate of substrate hydrolysis. However, this only applies when a sufficient concentration of the substrate is available (Ruan et al., 2010) as observed in this study. When the enzyme concentration was increased from 1 to 4% (w/w), the protein concentration yielded has also increased as observed in Table 4.1.

Based on Table 4.1, the optimum concentration pepsin obtained in this study was 4% (w/w) because the highest concentration of protein (99.02 ± 0.12 mg/mL) was produced. The use of 4% (w/w) of pepsin to digest egg white protein in this study successfully generated bioactive peptides with alpha-glucosidase inhibitor properties. This is similar to that reported by Lacroix and Li-Chan (2013) which demonstrated that 4% (w/w) of pepsin was the optimum concentration used to digest whey protein to generate bioactive peptides with alpha-glucosidase and dipeptidyl peptidase (DPP-IV) inhibition.

Higher pepsin concentration above 4% (w/w) was not tested in this study because the hydrolysis as shown in Table 4.1 produced a relatively constant protein concentration. The protein concentration yielded at 2% (w/w) and 3% (w/w) of pepsin concentration were only 6.7% and 3.3%, respectively and lower compared to that obtained at 4% (w/w). High concentration of enzyme is not essential to produce high concentration of protein. This could be due to substrate limitation and cleavage specificity when high concentration of enzyme is used (Ruan et al., 2010).

An optimum enzyme concentration is crucial to yield high degree of hydrolysis to produce high concentration of protein (Chen et al., 2009). The increase in enzyme concentration that exceeds certain range may result in decrease in the degree of hydrolysis. This is because high concentration of enzyme could result in excess amount of enzyme binding to low amount of available substrate, which makes the substrate concentration a limiting factor for the enzymatic reaction (Shu et al., 2016). In a nutshell, when the substrate concentration is low, increase in enzyme concentration will not increase the rate of hydrolysis resulting in higher protein concentration.

5.1.2 Optimisation of pH on Pepsin Digestion

Pepsin digestion on hen egg white protein produced higher concentration of protein at acidic pH (pH 1 and 2) as compared to neutral (pH 7) and alkaline (pH 8) pH. Based on Table 4.2, the highest protein concentration was obtained

at pH 2 (212.28 ± 32.82 mg/mL). The result obtained was similar to that reported by Ruan et al. (2010) and Lacroix and Li Chan (2013), whereby the highest degree of pepsin hydrolysis was achieved at pH 2. In accordance to that, it was demonstrated that the lower the pH, the faster the rate of reaction with the highest rate obtained between pH 1 to 3 (Liu et al., 2017). This may be explained as pepsin is an acidic protease that functions the best at acidic condition. Pepsin is also a gastrointestinal protease present in stomach with the normal acidity of gastric juice at around pH 2 (Liu, 2010).

Based on Table 4.2, increased in pH has resulted in the decreased in concentration of protein yielded in this study. This is similar to that reported by Bisswanger (2014), whereby the enzymatic activity of pepsin decreased when the pH reached an alkaline state due to denaturation and inactivation of the enzyme. Furthermore, pH could affect the conformation of an enzyme and the substrate molecular dissociation (Shu et al., 2016). When the pH used for enzymatic digestion is out of the optimal pH range, the enzyme tend to function less effectively. Similar to this study, pepsin may undergo irreversible inactivation due to protein unfolding and loss of active sites when the pH tested exceeded pH 7.

5.1.3 Optimisation of Temperature on Pepsin Digestion

The optimum temperature for pepsin digestion obtained in this study was 37ºC that yielded the highest concentration of protein (221.83 ± 5.40 mg/mL) (Table

4.3). On the contrary, several studies demonstrated that the highest degree of hydrolysis by pepsin was obtained at 45ºC (Liu, 2010; Ruan et al., 2010). However, the concentration of protein yielded at 45ºC in this study was 194.59 ± 3.6 mg/mL which was only 27.24 mg/mL lower than that obtained at 37ºC. The use of human body temperature to study the pepsin digestion on egg white could relate better to the actual mechanism in gastrointestinal tract (Bisswanger, 2014). Therefore, various studies have performed pepsin digestion at 37ºC to generate bioactive peptides (Lacroix and Li Chan, 2013; Abeyrathne et al., 2014).

Based on Figure 4.1, the lowest protein concentration (150.13 ± 2.67 mg/mL) was achieved at 30ºC. The result is similar to that reported by) and Ruan et al. (2010). Enzymatic hydrolysis at low temperature would not be able to completely activate the enzyme to perform at its maximum activity (Liu, 2010). Thus, increase in temperature could provide sufficient energy for the substrate to be converted to product in the presence of enzyme. Higher temperature but still within the optimal temperature range will aid in substrate protein unfolding, increase in enzymatic activity and lower the activation energy required in substrate to product conversion stage (Whitaker, 2000).

5.1.4 Optimisation of Papain Concentration

Based on Figure 4.2, the concentration of protein yielded increased linearly with the increased in enzyme concentration from 1 to 4% (w/w). The optimum papain

concentration obtained in this study was 4% because the protein concentration yielded was the highest (245.09 ± 6.57 mg/mL) (Table 4.4). The optimum papain concentration and enzyme to substrate (E/S) ratio for hen egg white digestion has yet to be published. There is no similar papain concentration reported in hen egg white digestion so far.

Different papain concentration was used to digest hen egg white in previous studies. The result obtained in this study contradicted to the papain concentration used which was 1% (w/w) (Abeyrathne et al., 2014), 2% (w/w) (Panyanuan et al., 2014) and 3% (w/w) (Chen and Chi, 2011) to digest hen egg white. This could be explained as previous studies did not perform the optimisation on enzyme concentration. The enzymatic hydrolysis carried out in their study was modified from other studies which also used papain for digestion. In fact, the optimisation of E/S ratio is the most crucial parameter to obtain high degree of hydrolysis (Chen et al., 2009).

However, it was reported that residual insoluble proteins were produced using 1% (w/w) of papain after heat inactivation. Thus, this suggested that this concentration is unable to hydrolyse ovalbumin found in hen egg white completely (Abeyrathne et al., 2014). Panyanuan et al. (2014) also reported that 2% (w/w) of papain generated low protein yield and nitrogen recovery from hen egg white. In this study, 4% (w/w) of papain was also unable to generate bioactive peptides with potent alpha-glucosidase inhibitor effect. The most plausible explanation could be due to the requirement of higher papain

concentration above 4% (w/w) for the enzymatic digestion to yield bioactive peptides.

5.1.5 Optimisation of pH on Papain Digestion

Based on Figure 4.3, the optimum pH for papain digestion obtained in this study was pH 7 with the highest protein concentration produced at 122.13 ± 6.23 mg/mL. The result is similar to previous studies whereby the optimum pH for papain digestion was also reported at pH 7 on hen egg white (Panyanuan et al., 2014) and duck egg white (Chen et al., 2009). Many studies have shown that papain functions optimally at pH close to neutral such as pH 6 and pH 6.5 (Chen et al., 2012; Abeyrathne et al., 2014). This can be explained as different types of protease will have different optimal pH range for proteolytic activity (Panyanuan et al., 2014). Since different proteases have different active site and cleavage specificities, they will have their specific optimum pH range to ensure the stability of the enzyme.

The concentration of protein yielded at pH 5, 6 and 8 were not significantly different from pH 7 (Figure 4.3). The protein concentrations obtained at these pH were approximately 70% of that achieved at pH 7. This corresponds to a study conducted by Butterfield and Lee (1994), which reported that the active site of papain has a more exposed binding structure at intermediate pH (pH 4.2 to 8) than at higher (> pH 8) or lower (< pH 4.2) pH. Furthermore, it was suggested that the optimum pH range for papain digestion is between pH 6 to 8

(Gul et al., 2006). Kusumadjaja and Dewi (2005) demonstrated that the papain enzymatic activity was very low at acidic (pH 4) and alkaline (pH 9) conditions. A drastic shift from the optimum pH will alter the active site of enzyme, thereby causing inactivation of the enzyme, as observed in this study.

5.1.3 Optimisation of Temperature on Papain Digestion

Papain digestion produced the highest protein concentration (95.74 ± 3.04 mg/mL) at 37ºC (Figure 4.4). The result was similar to that reported by Abeyrathne et al. (2014), whereby papain digestion on hen egg white was carried out at 37ºC to evaluate the functional properties of protein hydrolysates from egg white. However, this is in contrast to the previous studies which claimed that papain functioned the best at 40ºC to digest duck egg white (Chen et al., 2009) and hen egg white (Panyanuan et al., 2014). It was also reported that the temperature used to digest goat milk casein using papain peaked at 55ºC when tested in the range of 30ºC to 65ºC (Shu et al., 2016). These studies showed that the optimum temperature for papain varies with different substrate used (Ghosh, 2005). This can be explained as different substrate has different amino acids content, hence possess different cleavage sites and peptide bond strength.

The concentration of protein decreased insignificantly when the temperature increased from 37ºC to 50ºC (88.11 ± 2.59 mg/ mL) and 65ºC (89.09 ± 3.2 mg/mL) (Figure 4.4). The result obtained in this study contradicts with the

optimum temperature of papain digestion reported by Kilara and Shahani (1977), which was at 65ºC. Papain is a thermal stable enzyme up to 70ºC (Pinto et al., 2011). The ability of papain to resist heat to function does not mean that it would not undergo certain degree of inactivation at high temperature. Generally, extreme high temperature will result in inactivation of the enzyme, which then reduce the speed of enzymatic activity (Shu et al., 2016). Therefore, the temperature used for enzymatic hydrolysis must be within the enzyme stability range even if the enzyme does not display maximum activity (Bisswanger, 2014). For instance, Gul et al. (2006) showed that 30ºC was used for papain digestion and it was able to generate functional peptides.

5.2 Enzymatic Digestion on Hen Egg White Protein

Pepsin relatively showed higher enzymatic hydrolysis as compared to papain digestion (Figure 4.5) based on the high concentration of protein produced as described in Section 3.4. Pepsin is responsible in cleaving peptide bonds between hydrophobic and aromatic amino acid such phenylalanine, tryptophan and tyrosine (Budryn et al., 2012). Since hen egg white is rich in protein consisting of both hydrophobic and hydrophilic amino acids, pepsin will be able to cleave the peptide bonds to generate more free peptides.

This in agreement with a study by Abeyrathne et al. (2014), which claimed that pepsin digestion on hen egg white produced a total of 183 peptides. They also showed that pepsin digested egg white produced peptides with the highest

angiotensin converting-enzyme (ACE) inhibitory activity compared to other proteases. Moreover, protein hydrolysates obtained from hen egg white digested with pepsin generated bioactive peptides with potent antioxidant activity (Chen et al., 2012).

This study also showed that papain digestion was less efficient than pepsin digestion similar to that reported by Panyanuan et al. (2014). This is because proteins tend to aggregate and precipitate at neutral pH due to increase in surface hydrophobicity at neutral pH (pH 7) (Panyanuan et al., 2014). Abeyrathne et al. (2014) also demonstrated that insufficient papain and alcalase concentration will result in the formation of residual insoluble protein after heat inactivation of enzyme. In the present study, the supernatant containing only the soluble proteins was used to measure the protein concentration yielded from the enzymatic digestion without considering the insoluble protein. Therefore, this explains why low protein concentration was generated using papain digestion in this study.

However, the results obtained in this study contradicted to a study which claimed that non-gastrointestinal proteases from plant or microbial source could generate more bioactive peptides from egg white protein due to its different and broader cleavage specificity (Liu et al., 2017). A study by Chen et al. (2009) has shown that papain digested duck egg white protein hydrolysate produced higher protein concentration because cysteine protease is more efficient in hydrolysing duck egg white.

This study has shown that papain digestion was less effective on hen egg white. This is because cysteine protease inhibitor obtained from hen egg white has shown to inhibit ficin and papain (Fossum and Whitakker, 1968; Sen and Whitakker, 1973). They believed that the presence of these inhibitors would protect the hen egg against the invasion of enzyme from plant or microbial source. However, the actual mechanism of the inhibition and the functional peptide has yet to be discovered. The inhibition of the papain activity could reduce the cleavage of peptide bond, thus low protein concentration yielded as observed in this study.

For pepsin hydrolysis, the highest protein concentration was yielded from protein hydrolysates of omega 3 enriched egg (2.97 ± 0.48 g/L), followed by classic egg (2.33 ± 0.47 g/L) and lastly the selenium enriched egg (1.75 ± 0.07 g/L). Whereas for papain hydrolysis, classic egg yielded the highest concentration of protein (1.53 ± 0.28 g/L), followed by omega 3 enriched egg (0.93 ± 0.12 g/L) and lastly the selenium enriched egg (0.52 ± 0.11 g/L). However, it was demonstrated that high concentration of protein did not exhibit high alpha-glucosidase activity in the current study.

The protein hydrolysates obtained from selenium enriched egg digested with both pepsin and papain yielded the lowest protein concentration but displayed the highest alpha-glucosidase inhibitory activity among the three tested types of hen egg. This phenomenon can be explained as the digested protein may have been degraded into inactive peptide fragments or free amino acids, which lead

to reduced biological activity of the egg white protein (Shu et al., 2016). Abeyrathne et al. (2014) also suggested that the study on ACE inhibitory, iron chelating and antioxidant activity of hen egg white protein depends on the composition of bioactive peptides generated rather than the number of the peptides produced.

5.3 Alpha-Glucosidase Inhibitory Assay

5.3.1 Pepsin Digested Egg White Protein Hydrolysates

This study demonstrated that increased in pepsin digested egg white protein hydrolysates resulted in increased inhibitory activity against alpha-glucosidase regardless of the types of eggs. Different types of eggs have been shown to display different degree of inhibition against alpha-glucosidase in the current study. As observed in Table 4.7, the concentration of protein hydrolysates at 500 mg/mL of selenium enriched egg achieved the highest inhibition (97.17 ± 2.85%), followed by classic egg (92.28 ± 2.48%) and the lowest inhibitory activity was obtained from omega 3 enriched egg (53.07 ± 1.94%).

The results are in accordance to a study by Wang et al. (2012), which reported that different types of hen egg white have similar protein types but different in the abundancy among the six tested egg varieties in their study which also include classic egg and omega 3 enriched egg. Among the 23 total proteins, 19 proteins were identified to be significantly different in different egg varieties.

For instance, ovotransferrin, which is one of the most abundant protein types found in egg white is one of the 19 proteins that is significantly different among different types of egg. It was reviewed that ovotransferrin present in higher amount in classic egg as compared to omega 3 enriched egg. Also ovotransferrin was found out to be existed in three different types namely ovotransferrin BB type, ovotransferrin CC type and ovotransferrin chain A (Wang et al., 2012).

However, so far there is no study that compares the protein content in selenium egg and classic egg. Based on the results obtained from this study, it was shown that the alpha-glucosidase inhibitory activity was higher in selenium egg compared to the other two eggs, which could be possibly due to the presence of more bioactive peptides in the pepsin digested egg white.

IC_{50} is the half maximal inhibitory concentration of an inhibitor needed to inhibit 50% of the activity of alpha-glucosidase. The lower the IC_{50} value, the more potent the agent is in alpha-glucosidase inhibition. Selenium enriched egg was found to be the most potent alpha-glucosidase inhibitor among the three types of egg in this study. The IC_{50} value of selenium enriched egg was 96.69 mg/mL (Table 4.8), which is relatively greater than the IC_{50} of acarbose (38.25 mg/mL) (Qaisar et al., 2014).

This is in contrast to a study conducted by Yu et al. (2011), which demonstrated that the peptide sequence RVPSLM generated from ovotransferrin of egg white protein achieved an IC_{50} of 0.016 mg/mL, which was lower than the of IC_{50}

acarbose, thus more effective in alpha-glucosidase inhibition. This can be explained as the egg white protein hydrolysates used in this study was a crude mixture of proteins whereas the previous study involved purified bioactive peptides. Therefore, protein purification is important to study the biological function of only one bioactive peptide at one time (Berg et al., 2002).

In the present study, all the three types of eggs tested in this study showed higher IC_{50} value as compared to the synthetic alpha-glucosidase inhibitor, acarbose (Table 4.8). This means that the bioactive peptides obtained from pepsin digestion on hen egg white are less effective in alpha-glucosidase inhibition. In a study conducted by Matsui et al. (2014), the alpha-glucosidase inhibitory activity of the protein hydrolysate obtained from alkaline protease digested sardine muscle (IC_{50} = 48.7 mg/mL) was also weaker than the synthetic inhibitor, voglibose (IC_{50} = 2.6 mM). However, they suggested that the sardine muscle can be taken as a functional food and the intake of 48.7 mg/mL may be acceptable for its prophylactic agent.

Based on the results obtained in this study, hen eggs can also be taken as a functional food and the intake of 96.69 to 462.2 mg/mL of egg white can be achieved by the consumption of one hen egg (Abeyrathne et al., 2013; Iqbal et al., 2014). Despite of its high cholesterol and fat content in egg, the nutritional benefits of eggs are well recognized (Abeyrathne et al., 2013). Thus, the consumption of one egg per day to prevent the onset of diabetes is advisable for

normal individual. The consumption of natural food for prevention would lead to less desirable side effects as compared to synthetic drug.

5.3.2 Papain Digested Egg White Protein Hydrolysates

The concentration of protein yielded from papain hydrolysis was significant lower than the protein concentration produced by pepsin hydrolysis (Figure 4.5). The low protein concentration produced from the reaction of enzymatic hydrolysis limited the concentration of protein hydrolysates that can be used to perform alpha-glucosidase inhibitory assay. The concentrations of papain digested egg white protein hydrolysate used in this study ranged from 2 to 10 mg/mL (Figure 4.7).

This is in accordance to a study by Memarpoor-Yazdi et al. (2012) that reported the use of low concentrations of papain digested egg white protein hydrolysates (0.187 to 2 mg/mL) for antioxidant study. Similarly, low concentration of papain digested egg white protein hydrolysate ranging from 0.8 to 12 mg/mL were used for the lipid peroxidation assay (Chen and Chi, 2011). However, previous studies used purified peptides for the assays which is in contrast with the current study. Since a crude protein mixture was used in this study, thus higher protein hydrolysates concentration has to be used.

Despite that, higher concentration of protein hydrolysates could not be prepared in this study. This is because low amount of protein concentration was produced by papain hydrolysis. It was shown in this study that high concentration of hen egg white protein hydrolysate turned the protein samples to cloudy state which resulted in false positive result. Furthermore, papain digested egg white protein has shown to produce yellow powder after freeze drying in this study. Similar observations was reported by Lee and Chen (2002). This could be due to the reaction between protein and glucose through Maillard reaction, which enhanced the yellow-brown colour formation during freeze drying.

The IC_{50} value of all the three types of egg digested with papain were not able to be achieved in this study. Based on Table 4.9, the alpha-glucosidase inhibition achieved at 10 mg/mL of protein hydrolysate was the highest for selenium enriched egg (28.06 ± 1.42%), followed by classic egg (26.74 ± 1.14%) and the lowest alpha-glucosidase inhibition was achieved by omega 3 enriched egg (18.38 ± 1.25%). The procedure for papain hydrolysis may need to be improved in order to achieve sufficient protein concentration in order to determine the IC_{50} value on alpha-glucosidase.

5.4 Limitations

The limitation in this study is the inability to compare the competency of egg white protein against alpha-glucosidase inhibition to acarbose, a synthetic alpha-glucosidase inhibitor. By comparing with acarbose, the inhibitory effect of the three eggs against alpha-glucosidase can be evaluated simultaneously. Crude protein hydrolysates containing a mixture of peptides may act synergistically or antagonistically to each other, hence could not reflect the actual potential of the peptide to inhibit alpha-glucosidase. Moreover, the choice of protease used in this study was only limited to pepsin and papain. There are other gastrointestinal and non-gastrointestinal protease that can be used to produce bioactive peptides with alpha-glucosidase inhibitory activity.

5.5 Future Recommendations

This study can be improved by using a combination of protease such as pepsin followed by papain for enzymatic hydrolysis rather than only one single protease to yield higher concentration of bioactive peptides. This is because each protease has its own specific cleavage site that will produce certain unique peptide sequence. A combination of proteases will definitely generate higher concentration of peptides as compared to a single protease hydrolysis.

Furthermore, protein hydrolysates obtained from selenium enriched egg can be purified using reversed phase high performance liquid chromatography (RP-HPLC) to determine the specific peptide that is responsible for the alpha-

glucosidase inhibition. The bioactive peptide can be characterized using mass spectrometer (MS) to identify the peptide sequence, which can be used in further studies. The current study only utilized alpha-glucosidase obtained from yeast to investigate the effect of different alpha-glucosidase inhibitors. In the future studies, the purified and identified bioactive peptide obtained from protein hydrolysate of selenium egg can be tested for its alpha-glucosidase inhibition against mammalian alpha-glucosidase to demonstrate the actual inhibitory effect.

CHAPTER 6

CONCLUSION

The optimum condition for enzymatic digestion is a crucial factor to ensure maximum protein hydrolysis. Different substrate may require different conditions for digestion, thus optimisations are needed to be carried out for each parameter involved. The common optimisation parameters are enzyme concentration, pH and temperature. The optimum pH and temperature for human protease pepsin obtained in this study were at pH 2 and 37ºC, respectively. On the other hand, the optimum pH and temperature for plant protease papain were obtained at pH 7 and 37ºC, respectively. The optimum enzyme concentration obtained in this was 4% (w/w) for both pepsin and papain in the enzymatic digestion.

This study also showed that the alpha-glucosidase inhibitory effect of protein hydrolysates of different egg variants varied accordingly. Among the tested protease, pepsin digested egg white protein hydrolysates displayed a more potent inhibitory activity than papain digested egg white protein hydrolysates. Moreover, it was found that the classic and enriched eggs possessed different protein contents as they displayed different degree of alpha-glucosidase inhibitory activity in the current study. The selenium enriched egg protein hydrolysate obtained from pepsin digestion showed the highest alpha-glucosidase inhibition effect with an IC_{50} value at 96.69 mg/mL as compared to

the other two types of eggs. Hence, protein hydrolysates obtained from selenium enriched egg may possess bioactive peptides that can be a potential potent alpha-glucosidase inhibitory agent as well as a potential prophylactic and treatment agent for diabetes mellitus.

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Appendices

Appendix A

List of Chemicals and Reagents

Chemicals/ Reagents	Manufacturers/ Company
Alpha-glucosidase from *Saccharomyces cerevisiae*	Sigma-Aldrich, USA
Classic egg	Lay Hong, Malaysia
Coomassie blue G-250	Bio Basic Canada Inc, Canada
Dimethyl sulfoxide	Merck, USA
Disodium phosphate	Merck, Germany
Hydrochloric acid 37%	AnalR NORMAPUR, France
Monosodium phosphate	Merck, Germany
Omega 3 enriched egg	Lay Hong, Malaysia
Papain	Merck, USA
Pepsin	Bio Basic, Canada
p-nitrophenyl-α-D-glucopyranoside	Calbiochem, USA
Selenium enriched egg	Lay Hong, Malaysia
Sodium carbonate	Classic Chemicals, Malaysia
Sodium hydroxide	Merck, Germany

Appendix B

List of Equipment and Laboratory Wares

Chemicals/ Reagents	Manufacturers/ Company
Aluminium foil	Diamond, Malaysia
Benchtop centrifuge machine	Sigma-Aldrich, USA
Centrifuge tube	Greiner Bio-One, UK
Collection flask	PLT Scientific, Malaysia
Deionised water system DV25	ELGA, UK
Flat bottom 96-well plates	Greiner, Germany
Freeze-drying machine	Martin Christ, Germany
Freezer (-20ºC)	Acson, Malaysia
High speed centrifuge	Beckman Coulter, USA
Incubator (37º C)	Memmert, Germany
Media bottle	SCHOTT DURAN®, Germany
Microcentrifuge tubes	Copens Scientific, Malaysia
Micropipette (10 µL)	Gilson, Canada
Micropipette (200 µL)	Labmate, India
Micropipette (1000 µL)	Proline® Plus, Germany
Microplate reader	BMG Labtech, Germany
Parafilm	SAMEMAX, Malaysia

PCR tubes	Axygen, USA
pH meter	Sartorius, Germany
Refrigerator (4ºC)	SAMEMAX, Malaysia
Vortex mixture	Gemmy Industrial Corp, Taiwan
Water bath	Memmert, Germany
Weighing balance	BMG Labtech, Australia