# Production Optimization and Sensory Evaluation of Egg White Protein Hydrolysate with Angiotensin I Converting Enzyme Inhibitory Activity

by

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**ABSTRACT**

Egg proteins are a well-known rich source of bioactive peptides with inhibitory activity against angiotensin I converting enzyme (ACE), a key enzyme responsible for the regulation of blood pressure. Peptides with ACE inhibitory activity could have potential application for prevention of high blood pressure, a chronic condition affecting about one third of Canadian adults. However, the lack of cost-effective methods of large-scale production and the presence of an unpleasant bitter taste limit the utilization of bioactive peptides and protein hydrolysates in functional food applications. The objectives of this study were to develop a scale-up method to prepare egg white protein hydrolysate with ACE inhibition activity and to investigate the applicability of egg white protein hydrolysate in two different food matrices. The optimal condition for preparing egg white protein hydrolysate was first determined by Taguchi's method and then applied for scale-up preparation. ACE inhibitory activity and peptide yield of the egg white protein hydrolysates prepared in laboratory scale and large scale were 30 μg hydrolysate/mL and 77.5%, and 55 μg hydrolysate/mL and 53%, respectively. Egg white protein hydrolysate was incorporated into protein bars and protein beverages at up to 20% (w/w) and 2% (w/w), respectively. Protein beverages formulated with up to 1.5% (w/w) protein hydrolysate were found to be acceptable by study participants while all protein bar prototypes were not liked in general. Further research is needed to improve the consumer acceptability of protein hydrolysate.

## PREFACE

This thesis is an original work of Qiyi Li. The consumer acceptability testing of protein bars and beverages enriched with egg white protein hydrolysate, which is a part of this thesis, received research ethics approval from University of Alberta Human Research Ethics Boards (Protocol # 00045746, approved on July 23, 2014). Written consent was granted from all participants before participation in the study. The concept of this thesis originated from my supervisor Dr. Jianping Wu, and the research was funded by Alberta Livestock and Meat Agency Ltd. (ALMA).

This thesis consists of four chapters: Chapter 1 provides a literature review on several topics related to this research work and the objectives of the thesis; Chapter 2 aims to optimize the conditions for enzymatic hydrolysis of egg white protein and to scale-up prepare the hydrolysate based on optimal conditions established; Chapter 3 evaluates consumer acceptability of egg white protein hydrolysate in solid and liquid food matrices; and Chapter 4 gives concluding remarks and discussion on future research direction.

Dr. Jianping Wu contributed to experimental design, data interpretation, thesis preparation and edits. I was responsible for the literature search, experimental design and performing the experiments, data collection and analysis, and thesis preparation. Ms. Jing Zheng performed the mass spectrometry of egg white protein hydrolysates, and Dr. Yuchen Gu helped analyze the results of LC-MS/MS in Chapter 2. Mr. Daniel Hung from the Food Processing Development Centre provided assistance in HACCP plan development in Chapter 2. Dr. Jimmy Yao from the Food Processing Development Centre provided guidance and technical assistance in scale-up production of egg white protein hydrolysate in Chapter 2. Dr. Wendy Wismer provided guidance in experimental design of consumer acceptability testing in Chapter 3.

# DEDICATION

This thesis is dedicated to my beloved parents,

Haoqiang Li and Yanyun Mei

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# CHAPTER 1 LITERATURE REVIEW

## 1.1 Introduction

### *1.1.1 Functional food overview*

Food is essential and fundamental for human survival as well as pleasure. Safety, nutritiousness, taste and variety are the basic requirements of food products perceived by most consumers. In the last three decades, diet has been consistently documented as an important contributor to human health (Jew, AbuMweis, & Jones, 2009); as consumers are becoming increasingly health conscious, food for health is a growing trend of the food industry. The concept of functional foods was first introduced in Japan in 1984, which started as a national project, and later developed into a new food category named “foods for specified health uses” (FOSHU) (Arai et al., 2001). Functional foods are the fastest growing food category in the world. As reported by Leatherhead Food Research (2014), the global market of functional foods, limited to foods and drinks with specific functional health claims, was estimated to be $43.27 billion in 2013. The United States accounted for more than 50% of global sales, the largest functional food share in the world (Basu, Thomas, Acharya, & Canada, 2007; Siró, Kápolna, Kápolna, & Lugasi, 2008); while Canada has generated a revenue of more than $6 billion in functional food sector in 2011 (Statistics Canada, 2011).

There is no universally accepted definition of functional foods. Health Canada (2013) defined functional foods as “similar in appearance to, or may be, a conventional food, is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions”. Fortification (e.g. fruit juice fortified with calcium), addition of bioactive ingredients (e.g. margarine with added phytosterols) and enhancement with bioactive components through different techniques (e.g. omega-3 eggs) were

the means recognized by Health Canada to develop functional foods (Agriculture and Agri-Food Canada, 2015). After two decades of development, the functional food market become more mature and products can be found in all food categories to meet consumers' demand such as functional dairy products, functional drinks, functional cereal and bakery products, and functional meat (Siró et al., 2008). As the definition and regulations of functional foods vary among national authorities, claims or statements that are allowed on product packages also vary. Functional food claims that are associated with cure/treatment/prevention of a disease or condition, risk reduction in development of a chronic disease or condition, affecting structure or function in humans, and maintenance of good health have been proposed by Health Canada (2013).

The research of functional foods has mainly focused on searching, purifying and characterizing bioactive ingredients from food sources, and different functional components have been identified including bioactive peptides, carotenoids, dietary fibers, fatty acids, phenolic compounds, plant sterols and more (International Food Information Council, 2011). Bioactive peptides are among the most studied functional food ingredients. They are demonstrated to exert different physiological properties such as antimicrobial, antioxidative, antihypertensive, immunomodulatory, antithrombotic and other activities after released from their parent proteins by gastrointestinal digestion, fermentation or enzymatic hydrolysis (Hernández-Ledesma, Del Mar Contreras, & Recio, 2011; Korhonen & Pihlanto, 2006).

### *1.1.2 Bioactive peptides*

*Antimicrobial peptides*

Antimicrobial peptides are a group of molecules with diverse structures, specificities and modes of action (Parisien, Allain, Zhang, Mandeville, & Lan, 2008). The majority of these peptides

possess common physical features, cationic and amphipathic, which allow them to interact with negatively charged cytoplasmic membrane and eventually exert their bactericidal activities (Epand & Vogel, 1999). Antimicrobial peptides have been identified from different food sources and bacteria with the emphasis in milk proteins and egg proteins.

Milk protein lactoferrin, a member of the transferrin family, has been well documented for its bacteriostatic and bactericidal activity (Farnaud & Evans, 2003). Lactoferricin B, a peptide isolated from pepsin-digested bovine lactoferrin, was reported to have potent antimicrobial activity against various Gram-positive and Gram-negative bacteria, and was suggested to be responsible for the bactericidal activity of its parent protein (Bellamy et al., 1992). As another well studied member of transferrin family, egg white ovotransferrin was reported to exert antimicrobial activity, which was attributed to OTAP-92, a 92-residue peptide that was generated by partial acid proteolysis (Ibrahim, Iwamori, Sugimoto, & Aoki, 1998).

*Antioxidative peptides*

Antioxidation-oxidation balance is suggested to play an important role in maintaining good health, slowing down the aging process, and prevention of age-related disease. This balance could be disturbed by excess formation of free radicals which eventually results in cell damage and increased susceptibility to diseases such as diabetes and atherosclerosis (Xiong, 2011; Power, Jakeman, & FitzGerald, 2013). Food protein derived antioxidant peptides, considered as safe alternatives to synthetic antioxidants, have been identified from various animal and plant sources such as milk proteins (Hogan, Zhang, Li, Wang, & Zhou, 2009), eggs (You, Udenigwe, Aluko, & Wu, 2010), soy protein (Park, Lee, Baek, & Lee, 2010) and barley glutelin (Xia, Bamdad, Gänzle, & Chen, 2012). The antioxidant activities of these peptides could be implemented

through ion chelating, radical scavenging and lipid peroxidation inhibition (Memarpoor-Yazdi, Asoodeh, & Chamani, 2012).

*Immunomodulatory peptides*

Peptides that have the ability to suppress or stimulate certain specific and non-specific immune responses are categorized as immunomodulatory peptides (Agyei & Danquah, 2012). The immune responses that can be modulated by these peptides include lymphocyte activation and proliferation, antibody production, cytokine expression, macrophage function, and natural killer cell function (Gauthier, Pouliot, & Saint-Sauveur, 2006). Different peptide fragments released from bovine milk whey proteins and casein have been reported to possess immunomodulating activities (Agyei & Danquah, 2012), as well as egg white proteins ovalbumin, lysozyme and ovotransferrin. Addition of pepsin-digested and chymotrypsin-digested ovalbumin peptides was found to improve phagocytic activity of macrophages (Mine & Kovacs-Nolan, 2006).

### 1.2 Hypertension and angiotensin I converting enzyme (ACE) inhibitory peptides

Hypertension, or high blood pressure, is one of the most common chronic conditions worldwide (Kaplan, Huguet, Feeny, & McFarland, 2010). Globally, there has been estimated 1.5 billion people affected by raised blood pressure in 2008 (Chockalingam, 2008), of which approximately 6 million Canadian adults were living with diagnosed hypertension (Robitaille et al., 2012). Listed as the leading cause of death worldwide by World Health Organization (Chockalingam, 2008), hypertension is known to increase the risk of stroke, coronary artery disease, heart and kidney failure and other chronic diseases (Robitaille et al., 2012). In addition to genetic factors, hypertension is closely related to various environmental factors, since unhealthy lifestyle, such as high sodium intake, high alcohol consumption, lack of physical activity and overweight, are the risk factors that contributing to the development of hypertension (Touyz et al., 2004).

The renin-angiotensin system is involved in numerous pathophysiological mechanisms including hypertension, in which it plays an important role in blood pressure regulation. Renin cleaves the N-terminal of its substrate angiotensinogen and releases decapeptide angiotensin I, which is later broken down into potent vasoconstrictor octapeptide angiotensin II and dipeptide His-Leu by angiotensin I converting enzyme (ACE) (Erdös, 1976; Haque & Chand, 2008). At the same time, ACE inactivates potent vasodilator bradykinin by removing dipeptide Phe-Arg from its C-terminal and results in an inactive heptapeptide (Erdös, 1976). Therefore, inhibiting the activity of ACE could increase the level of bradykinin and reduce the formation of angiotensin II, which result in reduction of blood pressure. Synthetic ACE inhibitors such as captopril, lisinopril and enalapril are used in pharmacological therapy to treat hypertension (de Barros Silva, 2012). However, the use of these antihypertensive agents is usually reported with adverse side effects such as cough, skin rash and oedema (Vercruysse, Van Camp, & Smagghe, 2005; Weisser, Mengden, Vetter, & Du, 1998). Therefore, the investigation of safe alternatives to synthetic ACE inhibitors such as food protein derived ACE inhibitory peptides has expanded rapidly.

ACE inhibitor was first identified from snake venom. A peptide mixture with bradykinin potentiating activity, named bradykinin-potentiating factor, was extracted from venom of *Bothrops jararaca* by Ferreira (1965). Later Ferreira and his colleagues (1970) isolated nine biologically active peptides from bradykinin-potentiating factor, and one of these was identified to possess the amino acid sequence pyrrolidonecarboxyl-Lys-Trp-Ala-Pro. Bradykinin-potentiating factor was demonstrated to possess the ability to inhibit the activity of bradykinin-inactivating enzyme, and also shown to exert *in vitro* inhibitory activity against partially purified ACE prepared from dog lung (Ferreira, Bartelt, & Greene, 1970). Similar or identical peptides with ACE inhibitory activity were isolated and identified from the same venom by Ondetti et al

(1971). To date, bioactive peptides with ACE inhibition activity have been successfully isolated from different plant (e.g. soybean protein, wheat, buckwheat and garlic) and animal (e.g. milk proteins, egg proteins, muscle proteins and marine proteins) protein sources.

*ACE inhibitory peptides from plant sources*

Soybean protein was one of the protein sources that has been extensively studied. A peptide fraction separated from pepsin-digested soybean hydrolysate was reported to exhibit blood pressure lowering activity in spontaneously hypertensive rats (SHR) at a dose of 2.0 g/kg body weight, which was further separated and five peptides with potent *in vitro* ACE inhibitory activity were identified (Chen, Okada, Muramoto, Suetsuna, & Yang, 2002). Wu and Ding (2002) isolated two ACE inhibitory peptides, Asp-Leu-Pro and Asp-Gly, from soy protein hydrolysate prepared by Alcalase 2.4 L. ACE inhibitory peptides have been also discovered in other grains. An antihypertensive tripeptide, Ile-Ala-Pro, was isolated from wheat gliadin hydrolysate after treatment with pepsin and protease M (Motoi & Kodama, 2003). Rice protein hydrolysate prepared by Alcalase was reported to exert *in vitro* ACE inhibition and *in vivo* antihypertensive activity in SHR after single oral administration, while a tetrapeptide (Thr-Gln-Val-Tyr) isolated from this hydrolysate was also shown to exhibit blood pressure lowering activity at a lower effective dose (Li, Qu, Wan, & You, 2007).

*ACE inhibitory peptides from animal sources*

Milk is an excellent source of bioactive peptides, which have been reported with multiple physiological functions including ACE inhibitory activity. Two well-known tripeptides, Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), were isolated from Calpis sour milk (Calpis Co., Ltd, Tokyo, Japan), which was fermented by *Lactobacillus helveticus* and *Saccharomyces cerevisiae*

(Nakamura et al., 1995). These two tripeptides have been shown to exhibit *in vitro* ACE inhibitory activity (Nakamura et al., 1995) and *in vivo* antihypertensive effect in SHR after oral administration (Nakamura, Yamamoto, Sakai, & Takano, 1995). Ingestion of Calpis sour milk containing VPP (1.5 mg/100 g of sour milk drink) and IPP (1.1 mg/100 g of sour milk drink) was reported to significantly decrease the systolic and diastolic blood pressure of hypertensive subjects after eight weeks of treatment, and the decrease in blood pressure remained in the subjects who ingested sour milk four weeks after treatment ended (Hata et al., 1996). Other ACE inhibitory peptides derived from milk proteins by exogenous enzymes or lactic acid bacteria were also reported. An antihypertensive heptapeptide, Lys-Val-Leu-Pro-Val-Pro-Gln, was isolated from casein hydrolysate prepared by a proteinase from *Lactobacillus helveticus* CP790, which showed to exhibit strong *in vivo* antihypertensive activity in SHR after oral administration but weak *in vitro* inhibition of ACE (Maeno, Yamamoto, & Takano, 1996). Otte et al (2007) identified two potent ACE inhibitory peptides from thermolysin digested β-casein which possessed similar amino acid sequences. They also prepared thermolysin digested α-lactalbumin hydrolysate, and identified four peptide fragments that contained same sequence at C-terminal with $IC_{50}$ values ranged from 1 μM to 5 μM. Three different peptide fragments with less potent ACE inhibition were released from α-lactalbumin hydrolysate that digested by trypsin and a combination of pepsin, trypsin and chymotrypsin, respectively (Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000).

Egg proteins have been demonstrated as suitable substrates for production of bioactive peptides with ACE inhibition and antihypertensive activity. The first ACE inhibitory peptide derived from egg protein reported was ovokinin released from pepsin-digested ovalbumin (Fujita, Usui, Kurahashi, & Yoshikawa, 1995). Later Matoba et al (1999) isolated ovokinin (2-7), which

corresponded to the fragment 2-7 of ovokinin, from chymotrypic digest of ovalbumin. Both peptides were demonstrated to possess vasorelaxing activity, and antihypertensive activity after oral administration to SHR (Fujita, Sasaki, & Yoshikawa, 1995; Matoba, Usui, Fujita, & Yoshikawa, 1999). ACE inhibitory peptides have also been successfully purified from other egg proteins. Three novel peptides, Met-Lys-Arg, Arg-Gly-Tyr and Val-Ala-Trp, isolated from egg white lysozyme after treatment with pepsin, α-chymotrypsin and trypsin were shown to exhibit potent ACE inhibitory activity (Rao et al., 2012). Majumder and Wu (2011) identified three potent ACE inhibitory tripeptides, Ile-Arg-Trp, Ile-Gln-Trp and Leu-Lys-Pro, from thermolysin-pepsin digested ovotransferrin, which later were proven their *in vivo* activity in SHR model (Majumder et al., 2013, 2015).

In addition to milk and egg proteins, ACE inhibitory peptides were also reported from muscle proteins from domestic animals such as pork and chicken, and fish proteins including sardine, salmon, tuna, bonito and Alaska pollack (Vercruysse et al., 2005).

### 1.3 Optimization and scale up production of bioactive peptides

The research on bioactive peptides has been mainly focused on purification and characterization of novel peptides with multiple biological functions from different food sources and agricultural byproducts. However, from the standpoint of commercialization, a cost-effective manufacturing process for large scale production is one of the key factors that lead new products to succeed in the market. Most of the methods developed to produce and purify bioactive peptides were efficient under laboratory scale and the process parameters could be easily controlled to obtain reproducible results (Bhaskar, Benila, Radha, & Lalitha, 2008). However, the small variations in large scale application could affect the quality of final product in different extent. Thus, it would

be essential to study the critical parameters involved in the process and optimize process conditions to achieve desirable product quality and reduce cost.

There are different parameters involved in the production of bioactive peptides, such as characteristics of starting material (e.g. proximate composition, seasonal variability) and enzyme (e.g. purity, specificity of substrate cleavage, optimal pH and temperature required for maximal enzymatic activity), and process conditions (e.g. water-to-substrate ratio, enzyme dose, pH, temperature, time) (Li-Chan, 2015). Studies have shown that these parameters together or individually could significantly affect the characteristics of resulted protein hydrolysis such as degree of hydrolysis (Bhaskar et al., 2008), bioactivity (Guo, Pan, & Tanokura, 2009), yield (Cheung & Li-Chan, 2010) and amino acid composition (Kechaou et al., 2009). Therefore, investigation of the relationship between process parameters and desirable product attributes would help manufactures to develop an optimal production process.

A traditional optimization study usually investigates one parameter at a time by changing one independent variable while keeping all other variables at a fixed level, which is costly and time consuming (Kunamneni & Singh, 2005). Systematic approach design of experiment (DOE) such as response surface methodology (RSM) could help to overcome this shortage by investigating multiple variables at the same time. Response surface methodology has been widely used to optimize bioactive peptide production conditions (Kong et al., 2011; Tavares et al., 2011), which require less experimental trials compared to full factorial design and allow interaction of independent variables to be studied. The target product response of tested experimental combinations could be plotted as a predictive response surface, and then the optimized conditions could be predicted by a model equation, which should be verified as well as the model (Cinq-Mars & Li-Chan, 2007). The number of experiments conducted depends on the number of

process parameters investigated; therefore, RSM is appropriate for optimization studies where researchers have prior knowledge of which process parameters were significant (Li-Chan, 2015). Taguchi's fractional factorial design is well accepted in the engineering field, while its application in bioactive peptide optimization is limited. Taguchi's method allows researchers to prescreen a large number of process parameters for their influence on the product attributes of interest with limited experimental trials required.

**1.4 Challenges of bioactive peptides and protein hydrolysates in food application**

Although numerous bioactive peptides and protein hydrolysates were reported with proven *in vitro* and *in vivo* activities, their application as functional ingredients in the food industry is still limited, hindered mainly by their undesirable sensory properties (Hernández-Ledesma et al., 2011). The generation of peptides with bitter taste during enzymatic hydrolysis of food proteins is well documented in literature. The unpleasant bitter taste is reported to be related to their structural properties, especially the hydrophobic amino acid residues (Matoba & Hata, 1972). Different techniques have been developed to remove, reduce or mask the bitter taste from bioactive peptides and protein hydrolysates, such as activated carbon absorption, chromatographic separation, selection of enzymes that produce peptides with less hydrophobic residues, addition of masking agents and encapsulation (Saha & Hayashi, 2001; Sun-Waterhouse & Wadhwa, 2013). Debittering methods that mainly achieved by removing hydrophobic residues should be avoided when dealing with ACE inhibitory peptides as most peptides with ACE inhibition activity are reported to contain hydrophobic amino acid residues and often possess bitter taste. However, there is no solid evidence that ACE inhibition activity is correlated with bitterness intensity, even though both attributes were contributed by hydrophobic amino acids. It was also reported that there is salty off-flavor built up during enzymatic hydrolysis due to the

addition of acid or alkali to maintain the optimal pH for maximum enzyme activity (Hernández-Ledesma et al., 2011). Encapsulation would be a better technique to mask all undesirable flavors while retaining bioactivity of functional ingredients. This method also possesses several advantages over other debittering methods, such as increasing the stability of active compounds during processing and ingestion, and being able to control the release of active compounds and prolong their effect *in vivo* (Hwang, Tsai, & Hsu, 2010).

### 1.5 Hypothesis and objective

We hypothesized that egg white hydrolysate with ACE inhibitory activity could be prepared at a pilot scale, and this hydrolysate could be used as a functional ingredient or a protein source as a substitute for other commercially available proteins in commercial food products. The objectives of this study were:

- To optimize the enzymatic hydrolysis conditions for production of egg white hydrolysate with high ACE inhibitory activity and peptide yield by Taguchi's fractional factorial design;
- To investigate the feasibility of use of liquid egg white, instead of egg white powder, as the starting material for production of bioactive hydrolysate;
- To assess the feasibility of the established optimal enzymatic hydrolysis conditions in large scale production;
- To evaluate consumer acceptability of protein bars and beverages enriched with egg white hydrolysate and whey protein isolate.

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# CHAPTER 2 OPTIMIZATION AND SCALE-UP PREPARATION OF EGG WHITE HYDROLYSATE WITH ANGIOTENSIN I CONVERTING ENZYME INHIBITORY ACTIVITY

## 2.1 Introduction

There were approximately 23% of Canadian adults living with diagnosed hypertension in 2007/08, and this number was predicted to increase to 26.5% by 2012/13 (Robitaille et al., 2012). Hypertension is considered as the primary risk factor for stroke and coronary heart disease, and responsible for about 13% of all deaths worldwide (World Health Statistics, 2012). Hypertension could be prevented and controlled by lifestyle modification and medication (Chockalingam, Campbell, & Fodor, 2006); however, antihypertensive medication is often reported with side effects which could be severe enough to cause discontinuation of drug treatment (Curb et al., 1985; Weisser, Mengden, Vetter, & Du, 1998). Therefore, the public and scientists are interested in searching for safe alternatives to synthetic drugs.

Food protein derived angiotensin I converting enzyme (ACE) inhibitory peptides have been extensively studied in the past two decades. Angiotensin I converting enzyme is well documented for its important role in raising blood pressure; it activates the conversion of angiotensin I to hypertensive angiotensin II and inactivates hypotensive bradykinin by degradation (Erdös, 1976). Therefore, it is believed that inhibiting ACE could result in a reduction of blood pressure. ACE inhibitory peptides have been isolated from different animal and plant protein sources including milk proteins (Otte, Shalaby, Zakora, & Nielsen, 2007; Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000), muscle (Arihara, Nakashima, Mukaai, Ishikawa, & Itoh, 2001; Jang & Lee, 2005; Terashima et al., 2010), marine products (Byun & Kim, 2001; Matsufuji et al., 1994; Tsai, Chen, & Pan, 2008), eggs (Miguel, Recio,

Goméz-Ruiz, Ramos, & López-Fandiño, 2004), soybean (Wu & Ding, 2002), canola (Wu, Aluko, & Muir, 2008), and others.

Our previous study has isolated and identified three novel peptides, Ile-Arg-Trp (IRW), Ile-Gln-Trp (IQW) and Leu-Lys-Pro (LKP), with potent ACE inhibitory activity from egg white protein ovotransferrin, and their activity has been proven by *in vitro* assay and *in vivo* spontaneously hypertensive rat (SHR) model (Majumder & Wu, 2011; Majumder et al., 2013, 2015). However, ovotransferrin only accounts for about 12% of total egg white proteins (Stevens, 1991); it is costly to extract ovotransferrin from egg white. Egg white, as the starting material for acquisition of pure ovotransferrin, also contains other proteins including ovalbumin, ovomucoid, lysozyme, ovoglobulins, ovomucin and some minor proteins (Mine & Zhang, 2013). Miguel et al (2004) identified two ACE inhibitory peptides from pepsin-treated egg white hydrolysate which were fragments of ovalbumin. Lysozyme was also found to be a potential source of ACE inhibitory peptides (Rao et al., 2012). Our preliminary study has shown that the three novel peptides could be released from egg white using the method developed by Majumder and Wu (2011).

Although there have been a lot of food protein derived ACE inhibitory peptides reported being active *in vivo*, only a few have been commercialized due to a lack of cost-effective methods of scale-up production. Many studies have shown that conditions of enzymatic hydrolysis affect the physiochemical and physiological properties of final product. In order to develop a method that is feasible in large scale, it was necessary to investigate the relationship between hydrolysis conditions and desirable final product attributes which were ACE inhibitory activity and peptide yield in this study.

The traditional experimental design to investigate "one factor at a time" is time consuming; therefore, it would be preferred to use design of experiments (DOE) in this study which would

allow multiple factors to be investigated at the same time and reduce the number of experiments to be conducted (Wu & Hamada, 2011). Taguchi's method has been widely used in the engineering field to optimize operational processes, but its application in enzymatic hydrolysis is limited. However, compared to other fractional factorial designs, Taguchi's method can be used in a design with a large number of factors to prescreen the significant ones with least experiments conducted while researchers have no prior knowledge of the influence of experimental parameters on product attributes (Li-Chan, 2015).

Therefore, the objective of this study was to optimize the conditions of enzymatic hydrolysis to produce egg white hydrolysate with high ACE inhibitory activity and peptide yield by Taguchi's method. The feasibility of liquid egg white being used as starting material for production of functional ingredients was investigated as well as the feasibility of the established optimal method being applied to large scale production.

## 2.2 Materials and Methods

### *2.2.1 Materials*

Egg whites from chicken (powder form), angiotensin converting enzyme (ACE, from rabbit lung), and N-hippuryl-L-histidyl-L-leucine hydrate (HHL) were purchased from Sigma-Aldrich (Oakville, Ontario, CA). Thermoase PC10F (from *Bacillus thermoproteolyticus* Var. Rokko, food grade alternative to thermolysin) was purchased from Amano Enzyme Inc. (Nagoya, Japan). Pepsin (from porcine stomach, 10000 units/mg) was purchased from American Laboratories Inc. (Omaha, NE, USA). Liquid egg white for lab scale experiment was purchased from local supermarket. All other analytical grade chemicals were purchased from Fisher Scientific (Ottawa, Ontario, CA) or Sigma-Aldrich (Oakville, Ontario, CA).

Pasteurized liquid egg white for scale up experiment was purchased from Egg Processing Innovations Cooperative (Lethbridge, Alberta, CA). Food Chemicals Codex (FCC) grade hydrochloric acid and sodium hydroxide were purchased from Fisher Scientific (Ottawa, Ontario, CA).

### *2.2.2 Experimental design*

Taguchi's $L_{27}$ orthogonal array was used to study the effect of hydrolysis conditions on angiotensin I converting enzyme (ACE) inhibitory activity and peptide yield. The hydrolysis conditions being investigated included substrate-to-water ratio (2.5%, 5%, 7.5%), thermoase-to-substrate ratio (0.1%, 0.5%, 1%), pH of thermoase digestion (7, 8, 8.5), temperature of thermoase digestion (60, 65, 70 $^{o}$C), thermoase digestion time (45, 90, 180 min), pepsin-to-substrate ratio (0.1%, 0.5%, 1%), pH of pepsin digestion (1.5, 2.5, 3.5), temperature of pepsin digestion (45, 55, 65 $^{o}$C), and pepsin digestion time (45, 90, 180 min). The levels of each factor were determined according to preliminary experiments (*data not shown*). There were a total of 27 experimental runs, and each run was performed in duplicate on a different day. The experimental design and resulting responses were summarized in Table 2.1.

### *2.2.3 Preparation of egg white hydrolysate*

Egg white hydrolysis was carried out according to Majumder and Wu's method (2011). Egg white powder was mixed in designated amount of deionized water, and then subjected to sonication for 4 pulses (30 s each pulse) at 70% amplitude using Sonic Dismembrator Model 500 (Fisher Scientific, Pittsburgh, PA). After heating to denature the protein, the dispersion was transferred to a jacket beaker equipped with a water bath (Lauda-Brinkmann, Lauda-Königshofenand, Germany) and Titrando (Metrohm, Herisau, Switzerland) for temperature and

pH control. Egg white solution was first digested by thermoase for selected length of time, and then the enzyme was inactivated by adjusting pH to 3.5 or below for pepsin digestion. After hydrolysis by pepsin for selected duration, the reaction was terminated by heating the solution at 95$^{o}$C for 15 min. Egg white hydrolysate was then centrifuged at 10000g for 25 min at 4$^{o}$C and the supernatant was collected and freeze-dried for further analysis.

### ***2.2.4 Measurement of ACE inhibitory activity***

ACE inhibitory activity was measured according to the method developed by Wu et al (2002) with minor modifications. All chemicals and samples were prepared in 100 mM potassium phosphate buffer containing 300 mM sodium chloride (pH 8.3). The reaction mixture including 50 μL of 5 mM HHL, 20 μL of 100 mU ACE and 10 μL of sample solution at five different concentrations was incubated at 37$^{o}$C with continuous shaking. The reaction was terminated by adding 125 μL of 1 M hydrochloric acid after 30 min incubation. The solution was filtered through 0.2 μm syringe filter prior to injection for Ultra Performance Liquid Chromatography (UPLC) analysis. 5 μL of reaction mixture was injected into Acquity UPLC BEH $C_{18}$ column (1.7μm, 2.1*50mm, Waters, Ireland), and then eluted at a flow rate of 0.245 mL/min from 95% solvent A (0.05% trifluoroacetic acid in HPLC grade water) to 60% solvent B (0.05% trifluoroacetic acid in HPLC grade acetonitrile) within 3.49 min, stayed at 60% solvent B for 0.67 min, and then to 5% solvent B in 0.84 min. The elution was monitored by Photodiode Array (PDA) eλ Detector (Acquity UPLC, Waters, USA) at 228 nm. The ACE inhibitory activity was expressed as $IC_{50}$, the concentration of egg white hydrolysate that can inhibit 50% of ACE activity.

### ***2.2.5 Determination of peptide yield***

Nitrogen content was determined using Leco TruSpec CN analyzer (Leco Corporation, St. Joseph, MI, USA), and the protein content was calculated using a protein factor of 6.25 (Duan et al., 2013). Peptide yield was calculated using the following equation.

$$\text{Peptide yield (\%)} = \frac{protein\ content\ in\ the\ hydrolysate}{amount\ of\ egg\ white\ powder\ before\ digestion} \times 100$$

***2.2.6 Validation of optimal hydrolysis conditions***

Egg white powder and liquid egg white were subjected to enzymatic hydrolysis using the established optimal conditions, and then ACE inhibitory activity and peptide yield were measured. Liquid egg whites were also subjected to enzymatic hydrolysis according to the method mentioned previously but without sonication and increasing the heating time to 10 min at 80$^{o}$C before digestion.

**Table 2.1 Summary of experimental design and resulting ACE inhibitory activity and peptide yield of each experimental run**

| Trial # | S% | T/S (%) | pH-T | Temp-T | Time-T | P/S (%) | pH-P | Temp-P | Time-P | ACE inhibitory activity ($IC_{50}$: µg hydrolysate/mL) | Peptide yield (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 2.5 | 0.1 | 7 | 60 | 45 | 0.1 | 1.5 | 45 | 45 | 126±29 | 73.4±2.2 |
| **2** | 2.5 | 0.1 | 7 | 60 | 90 | 0.5 | 2.5 | 55 | 90 | 45±10 | 78.5±0.1 |
| **3** | 2.5 | 0.1 | 7 | 60 | 180 | 1 | 3.5 | 65 | 180 | 63±13 | 67.6±8.9 |
| **4** | 2.5 | 0.5 | 8 | 65 | 45 | 0.1 | 1.5 | 55 | 90 | 72±7 | 79.4±0.7 |
| **5** | 2.5 | 0.5 | 8 | 65 | 90 | 0.5 | 2.5 | 65 | 180 | 41±6 | 79.2±1.2 |
| **6** | 2.5 | 0.5 | 8 | 65 | 180 | 1 | 3.5 | 45 | 45 | 75±2 | 39.3±4.0 |
| **7** | 2.5 | 1 | 8.5 | 70 | 45 | 0.1 | 1.5 | 65 | 180 | 126±2 | 54.1±12.4 |
| **8** | 2.5 | 1 | 8.5 | 70 | 90 | 0.5 | 2.5 | 45 | 45 | 111±17 | 76.8±2.0 |
| **9** | 2.5 | 1 | 8.5 | 70 | 180 | 1 | 3.5 | 55 | 90 | 76±9 | 26.6±1.1 |
| **10** | 5 | 0.1 | 8 | 70 | 45 | 0.5 | 3.5 | 45 | 90 | 64±3 | 16.2±4.3 |
| **11** | 5 | 0.1 | 8 | 70 | 90 | 1 | 1.5 | 55 | 180 | 28±2 | 78.9±0.4 |
| **12** | 5 | 0.1 | 8 | 70 | 180 | 0.1 | 2.5 | 65 | 45 | 124±16 | 73.8±0.7 |
| **13** | 5 | 0.5 | 8.5 | 60 | 45 | 0.5 | 3.5 | 55 | 180 | 64±0 | 37.2±12.5 |
| **14** | 5 | 0.5 | 8.5 | 60 | 90 | 1 | 1.5 | 65 | 45 | 111±8 | 55.4±19.8 |
| **15** | 5 | 0.5 | 8.5 | 60 | 180 | 0.1 | 2.5 | 45 | 90 | 124±16 | 57.0±20.3 |
| **16** | 5 | 1 | 7 | 65 | 45 | 0.5 | 3.5 | 65 | 45 | 66±3 | 55.1±24.1 |
| **17** | 5 | 1 | 7 | 65 | 90 | 1 | 1.5 | 45 | 90 | 42±5 | 79.9±0.6 |
| **18** | 5 | 1 | 7 | 65 | 180 | 0.1 | 2.5 | 55 | 180 | 61±6 | 76.9±1.1 |

| **19** | 7.5 | 0.1 | 8.5 | 65 | 45 | 1 | 2.5 | 45 | 180 | 45±3 | 77.9±0.8 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **20** | 7.5 | 0.1 | 8.5 | 65 | 90 | 0.1 | 3.5 | 55 | 45 | 101±8 | 10.9±0.4 |
| **21** | 7.5 | 0.1 | 8.5 | 65 | 180 | 0.5 | 1.5 | 65 | 90 | 100±10 | 46.3±18.7 |
| **22** | 7.5 | 0.5 | 7 | 70 | 45 | 1 | 2.5 | 55 | 45 | 53±5 | 78.0±1.1 |
| **23** | 7.5 | 0.5 | 7 | 70 | 90 | 0.1 | 3.5 | 65 | 90 | 48±6 | 25.3±1.6 |
| **24** | 7.5 | 0.5 | 7 | 70 | 180 | 0.5 | 1.5 | 45 | 180 | 49±1 | 79.1±1.3 |
| **25** | 7.5 | 1 | 8 | 60 | 45 | 1 | 2.5 | 65 | 90 | 45±0 | 76.9±2.1 |
| **26** | 7.5 | 1 | 8 | 60 | 90 | 0.1 | 3.5 | 45 | 180 | 48±2 | 18.8±0.0 |
| **27** | 7.5 | 1 | 8 | 60 | 180 | 0.5 | 1.5 | 55 | 45 | 55±0 | 78.0±0.5 |

* S%= Substrate-to-Water ratio (%), T/S (%)= Thermoase-to-Substrate ratio (%), pH-T= pH of thermoase digestion, Temp-T= Temperature of thermoase digestion (°C), Time-T= Thermoase digestion time (min), P/S (%)= Pepsin-to-Substrate ratio (%), pH-P= pH of pepsin digestion, Temp-P= Temperature of pepsin digestion (°C), Time-P= Pepsin digestion time (min).

### *2.2.7 Verification of the presence of and quantification of tripeptides (IRW, IQW and LKP) by Reverse Phase High Performance Liquid Chromatography (RP-HPLC)*

Egg white powder hydrolysates, liquid egg white hydrolysates and ovotransferrin hydrolysates prepared with and without sonication, synthesized peptides and synthesized peptides spiked samples were analyzed by Reverse Phase High Performance Liquid Chromatography (RP-HPLC). All samples were prepared with 0.1% trifluoroacetic acid in deionized water (solvent A). Synthesized tripeptide standards were prepared in concentrations that gave proper resolution; 0.05 mg/mL of IRW, 0.05 mg/mL of IQW and 0.5 mg/mL of LKP in the final sample solution. 10 μL of sample was injected into XBridge $C_{18}$ column (5μm, 3.0*250mm, Waters, Ireland) coupled with a guard column. Then the sample was eluted at a flow rate of 0.8 mL/min with a linear gradient from 95% solvent A to 30% solvent B (0.1% trifluoroacetic acid in acetonitrile) within 50 min, and then to 60% solvent B over 5 min. The elution was monitored by PDA Detector (2998, Waters, Milford, MA, USA) at 220 nm.

Standard curves of individual tripeptide standards were prepared to quantify the amount of IRW, IQW and LKP released from different hydrolysates.

### *2.2.8 Confirmation of presence of tripeptides (IRW, IQW and LKP) by LC-MS/MS*

LC-MS/MS analysis was performed according to Majumder and Wu's method (2011) with minor modifications. Samples were injected into $C_{18}$ column without trapping, and eluted by 99% solvent A (0.1% formic acid in LC/MS grade water) at a flow rate of 0.4 μL/min for 2 min, and then to 30% solvent B (0.1% formic acid in acetonitrile) over 33 min, to 60% B over 5 min and stay for another 15 min at a flow rate of 0.5 μL/min.

### *2.2.9 Scale-up production of food grade egg white hydrolysate*

Food grade egg white hydrolysate was prepared in the Food Processing Development Centre (Leduc, AB) according to the optimal digestion conditions determined in this study with minor modifications. Liquid egg white was diluted with water at a ratio of 1:1 (v/v) to obtain a solution with 5% protein solid, and then hydrolyzed by thermoase and pepsin using optimal conditions. The hydrolysate was continuous centrifuged (GEA Westfalia Separator Group, Oelde, Germany) at 8510 rpm and average flow rate of 450 L/h, and then it was condensed to obtain approximately 10% solid by wiped film evaporator (Model: 4.2-12L-7; Pfaudler Inc., Rochester, NY, USA). The hydrolysate was then spray dried at an inlet temperature of 300°C and outlet temperature of 90°C, the powder was collected and stored in food grade freezer for further experiments.

#### *2.2.10 Statistical analysis*

The data collected were analyzed by Analysis of Variance general linear model (ANOVA-GLM) using Minitab 15 to determine the factors that significantly affected ACE inhibitory activity and peptide yield at 5% significance level (Cheung & Li-Chan, 2010). The significant differences between levels of each factor were determined by Tukey's test at $p < 0.05$. The contribution of effect was also computed, as well as the predicted responses at optimal conditions.

### 2.3 Results and Discussion

Egg white hydrolysates were prepared according to the conditions summarized in Table 2.1, and each trial was performed in duplicate on a different day. The resulting ACE inhibitory activity and peptide yield of 27 experimental runs ranged from 28-126 μg hydrolysate/mL and 10.9-79.9%, respectively. The wide range of the results suggested that one or more factors had significant effect on the responses and were responsible for the observed differences. However, these factors influenced the two responses differently; egg white hydrolysate prepared by a trial

that had high peptide yield did not necessary have potent ACE inhibitory activity and vice versa, such as trial 26.

### *2.3.1 Effect of hydrolysis conditions on ACE inhibitory activity and peptide yield of egg white hydrolysate*

The data collected from Taguchi's experimental design can be analyzed by standard analysis method, which is based on the mean of responses, and signal-to-noise ratio method (S/N ratio). S/N ratio is a performance measure that compares the effect on response caused by controllable factors to that caused by uncontrollable factors in an experimental design. A higher S/N ratio, which leads to a more desirable response with less variation, is always the aim in an optimization study. Since S/N ratio provides an objective comparison on both mean and variation of the experimental data, it is preferred for an optimization study with replications (Roy, 2010).

As shown in Table 2.2, ACE inhibitory activity of egg white hydrolysates was significantly ($p<0.05$) affected by substrate-to-water ratio, pH of thermoase digestion, thermoase digestion time, pepsin-to-substrate ratio, temperature of pepsin digestion and pepsin digestion time. Among all factors, pH of thermoase digestion (28.40%), pepsin digestion time (23.19%), and pepsin-to-substrate ratio (19.83%) contributed to 71.42% of the change observed in ACE inhibitory activity of egg white hydrolysates. These three factors exhibited significant difference between levels in terms of ACE inhibitory activity (Figure 2.1A); for example, as a function of pH of thermoase digestion, egg white that digested at pH 8.5 by thermoase had significantly lower ACE inhibitory activity than those digested at lower pH values. However, peptide yield was only significantly affected by pH of pepsin digestion which contributed to 62.15% of the change observed in this response (Table 2.3).

Previous studies have shown that a thermolysin-pepsin enzyme combination along with sonication is crucial for the production of IRW, IQW and LKP from ovotransferrin with potent ACE inhibitory activity (Majumder & Wu, 2010, 2011); therefore, the digestion conditions of both enzymes were investigated in this study. Both enzymes used in this study were food grade alternatives to purified enzymes from Sigma-Aldrich. The factor pH of thermoase digestion was one of the major contributors of ACE inhibitory activity of egg white hydrolysates. The pH levels of thermoase being investigated were within a relatively narrow range, from pH 7 to pH 8.5; however, ACE inhibitory activity significantly declined when pH changed from 8 to 8.5 (Figure 2.1A). Although the hydrolysates prepared at pH 7 and pH 8 did not show significant difference in terms of ACE inhibitory activity, those digested with thermoase at pH 8 had the lowest average $IC_{50}$ value which was consistent with the manufacturer's information that thermoase reaches its maximum activity at pH close to 8.

Pepsin also played an important role in the observed response. Pepsin-to-substrate ratio and pepsin hydrolysis time within the range studied were positively correlated to the resulting ACE inhibitory activity; an increase in pepsin dosage and digestion time led to higher ACE inhibitory activity (Figure 2.1A). Increasing pepsin dosage from 0.1% to 0.5% significantly ($p<0.05$) increased ACE inhibitory activity while the responses were not significantly different between hydrolysates prepared with 0.5% and 1% pepsin. A similar trend was observed in the hydrolysates prepared with different digestion time (Figure 2.1A). Similar results were reported by other researchers on whey protein hydrolysates digested by Corolase PP (van der Ven, Gruppen, de Bont, & Voragen, 2002) and pacific hake fillet hydrolysates digested by Protamex (Cinq-Mars & Li-Chan, 2007). However, the effect of enzyme dosage and hydrolysis time in the thermoase digestion phase was not consistent with that observed in the pepsin digestion phase.

Thermoase-to-substrate ratio did not significantly affect ACE inhibitory activity of egg white hydrolysates even though a slight increase in bioactivity was shown along with increasing enzyme concentration. Thermoase digestion time was another factor that significantly affected the resulting bioactivity but with a minor contribution (8.69%). Within the range tested, increasing digestion time did not result in reduction of $IC_{50}$ values, which may be due to further degradation of ACE inhibitory peptides after generation in the hydrolysis process, which leads to production of peptides with less ACE inhibitory potency (van der Ven, Gruppen, de Bont, & Voragen, 2002; Cinq-Mars & Li-Chan, 2007).

The maximum enzyme-to-substrate ratio and digestion time being investigated in this study were 1% and 180 min for both enzymes. These two factors were not set to extended levels as they are major contributors to operational cost. The egg white hydrolysate prepared in this study was intended to be incorporated into food products ultimately; therefore operational cost should be controlled within a reasonable range while desirable responses can still be obtained.

Substrate-to-water ratio and temperature of pepsin digestion also significantly affected the resulting ACE inhibitory activity with a contribution of 8.34% and 5.51% respectively. Substrate-to-water ratio was found to significantly affect ACE inhibitory activity of rice dregs hydrolysates in a negative correlation (He, Xuan, Ruan, Chen, & Xu, 2005), while Cheung and Li-Chan (2010) reported that water-to-substrate ratio was not a significant factor contributing to the change in ACE inhibitory activity of shrimp hydrolysates prepared by different proteases. In our study, ACE inhibitory activity increased along with an increase in substrate-to-water ratio but not significant. Increasing substrate-to-water ratio would allow more cleavage sites to be available for enzymatic hydrolysis, which may generate more peptides that are bioactive.

Peptide yield of egg white hydrolysates was only significantly influenced by pH of pepsin (Table 2.3). Egg white hydrolyzed by pepsin at pH 3.5 had significantly ($p<0.05$) lower peptide yield in comparison to those digested at pH 1.5 and 2.5, while no significant difference was observed between these two levels. The major proteins in egg white include ovalbumin, ovotransferrin, ovomucoid, ovomucin and lysozyme (Mine & Zhang, 2013). Most of these proteins have acidic isoelectric points (pI) of pH 4.1 and above, except lysozyme which has alkaline pI; therefore when adjusting the pH of egg white slurry to 3.5 after thermoase digestion, some undigested egg white proteins started to coagulate which reduced the availability of substrate to be digested by pepsin.

Taguchi design is inefficient in studying interactions among individual factors unless the interactions are included and treated as factors in the orthogonal array (Eşme, 2009). It is not practical to study all possible interactions within one design, as it requires to perform a large number of experiments which is time consuming and costly. Therefore prior knowledge or prescreening testing is necessary to determine possible significant interactions. However, the presence and strength of interaction between factors can be determined by computing the angle between two lines in the interaction plot which is named interaction severity index. The interaction severity index ranges from 0% to 100%, while 0% means parallel lines and 100% means a 90° angle between lines (Roy, 2001).

Severity index (SI) of all possible interactions on ACE inhibitory activity and peptide yield was shown in Table 2.4. The interaction with largest SI on ACE inhibitory activity (94.88%) was between thermoase-to-substrate ratio and temperature of thermoase digestion which also had a high SI (78.56%) on peptide yield, although both factors had no significant impact on either response. Similarly, the interaction between temperature of thermoase digestion and temperature

of pepsin digestion had high SI on both responses, 73.54% for ACE inhibitory activity and 73.42% for peptide yield.

**Table 2.2 Summary of ANOVA results of effect of nine factors on ACE inhibitory activity using signal-to-noise (S/N) ratio analysis**

| Factor Name | Degree of Freedom | Sum of Square | Mean Square | F ratio | *p* | Percentage Contribution |
|---|---|---|---|---|---|---|
| **Substrate-to-Water ratio** | 2 | 28.56 | 14.28 | 8.02 | 0.012* | 8.34 |
| **Thermoase-to-Substrate ratio** | 2 | 1.59 | 0.79 | 0.45 | 0.655 | 0.46 |
| **pH of thermoase digestion** | 2 | 97.28 | 48.64 | 27.32 | < 0.0001* | 28.40 |
| **Temperature of thermoase digestion** | 2 | 2.85 | 1.42 | 0.80 | 0.482 | 0.83 |
| **Thermoase digestion time** | 2 | 29.76 | 14.88 | 8.36 | 0.011* | 8.69 |
| **Pepsin-to-Substrate ratio** | 2 | 67.93 | 33.96 | 19.08 | 0.001* | 19.83 |
| **pH of pepsin digestion** | 2 | 2.00 | 1.00 | 0.56 | 0.591 | 0.58 |
| **Temperature of pepsin digestion** | 2 | 18.88 | 9.44 | 5.30 | 0.034* | 5.51 |
| **Pepsin digestion time** | 2 | 79.43 | 39.71 | 22.30 | 0.001* | 23.19 |
| **All other/error** | 8 | 14.21 | 1.78 | | | 4.17 |
| **Total** | 26 | 342.52 | | | | 100 |

*means significance at α=0.05

**Table 2.3 Summary of ANOVA results of effect of nine factors on peptide yield using signal-to-noise (S/N) ratio analysis**

| Factor Name | Degree of Freedom | Sum of Square | Mean Square | F ratio | *p* | Percentage Contribution |
|---|---|---|---|---|---|---|
| Substrate-to-Water ratio | 2 | 32.92 | 16.46 | 2.19 | 0.174 | 5.09 |
| Thermoase-to-Substrate ratio | 2 | 6.62 | 3.31 | 0.44 | 0.658 | 1.02 |
| pH of thermoase digestion | 2 | 64.38 | 32.19 | 4.28 | 0.054 | 9.95 |
| Temperature of thermoase digestion | 2 | 4.32 | 2.16 | 0.29 | 0.758 | 0.67 |
| Thermoase digestion time | 2 | 17.41 | 8.71 | 1.16 | 0.361 | 2.69 |
| Pepsin-to-Substrate ratio | 2 | 39.41 | 19.70 | 2.62 | 0.133 | 6.09 |
| pH of pepsin digestion | 2 | 402.14 | 201.07 | 26.76 | < 0.0001* | 62.15 |
| Temperature of pepsin digestion | 2 | 2.82 | 1.41 | 0.19 | 0.833 | 0.44 |
| Pepsin digestion time | 2 | 16.92 | 8.46 | 1.13 | 0.371 | 2.62 |
| All other/error | 8 | 60.10 | 7.51 | | | 9.28 |
| Total | 26 | 647.04 | | | | 100 |

*means significance at $\alpha$=0.05

**Table 2.4 Estimated interaction severity index of different factors on ACE inhibitory activity and peptide yield**

| Number # | Factors[a] | SI on ACE inhibitory activity (%)[b] | Optimal Levels on ACE inhibitory activity[c] | SI on peptide yield (%) | Optimal Levels on peptide yield |
|---|---|---|---|---|---|
| **1** | S%*T/S | 76.99 | [3,3] | 62.23 | [1,1] |
| **2** | S%*pH-T | 28.13 | [3,2] | 20.37 | [1,1] |
| **3** | S%*Temp-T | 68.97 | [3,1] | 59.79 | [1,1] |
| **4** | S%*Time-T | 61.93 | [3,1] | 79.01 | [1,2] |
| **5** | S%*P/S | 33.28 | [3,3] | 68.98 | [1,2] |
| **6** | S%*pH-P | 70.26 | [3,2] | 21.90 | [1,2] |
| **7** | S%*Temp-P | 48.36 | [3,1] | 55.81 | [1,3] |
| **8** | S%*Time-P | 32.99 | [3,3] | 24.93 | [1,3] |
| **9** | T/S*pH-T | 41.15 | [3,2] | 39.14 | [1,1] |
| **10** | T/S*Temp-T | 94.88 | [3,1] | 78.56 | [1,1] |
| **11** | T/S*Time-T | 45.72 | [1,2] | 56.87 | [2,1] |
| **12** | T/S*P/S | 48.85 | [1,3] | 63.60 | [1,3] |
| **13** | T/S*pH-P | 33.45 | [2,3] | 11.51 | [3,2] |
| **14** | T/S*Temp-P | 45.27 | [1,2] | 77.96 | [2,2] |
| **15** | T/S*Time-P | 37.74 | [1,3] | 49.64 | [1,3] |
| **16** | pH-T*Temp-T | 46.02 | [2,1] | 30.39 | [1,1] |
| **17** | pH-T*Time-T | 38.60 | [2,2] | 30.94 | [1,3] |
| **18** | pH-T*P/S | 7.55 | [2,3] | 17.83 | [1,3] |
| **19** | pH-T*pH-P | 34.75 | [2,1] | 24.94 | [2,1] |
| **20** | pH-T*Temp-P | 16.97 | [2,2] | 92.29 | [2,2] |
| **21** | pH-T*Time-P | 24.45 | [2,3] | 21.48 | [1,3] |
| **22** | Temp-T*Time-T | 42.87 | [2,1] | 63.23 | [2,1] |
| **23** | Temp-T*P/S | 37.99 | [3,3] | 30.67 | [1,3] |
| **24** | Temp-T*pH-P | 67.61 | [2,2] | 13.17 | [2,2] |
| **25** | Temp-T*Temp-P | 73.54 | [3,2] | 73.42 | [1,3] |
| **26** | Temp-T*Time-P | 28.03 | [2,3] | 78.64 | [2,3] |
| **27** | Time-T*P/S | 45.48 | [1,3] | 77.41 | [2,2] |
| **28** | Time-T*pH-P | 54.64 | [1,2] | 29.32 | [2,2] |
| **29** | Time-T*Temp-P | 31.04 | [2,2] | 49.48 | [1,2] |
| **30** | Time-T*Time-P | 57.13 | [2,3] | 54.32 | [3,3] |
| **31** | P/S*pH-P | 32.58 | [3,2] | 15.88 | [2,2] |
| **32** | P/S*Temp-P | 26.21 | [3,2] | 34.78 | [3,3] |
| **33** | P/S*Time-P | 19.63 | [3,3] | 47.72 | [3,3] |
| **34** | pH-P*Temp-P | 52.59 | [1,2] | 29.24 | [1,2] |
| **35** | pH-P*Time-P | 25.66 | [2,3] | 10.24 | [2,3] |
| **36** | Temp-P*Time-P | 33.68 | [1,3] | 51.37 | [3,3] |

a. S%= Substrate-to-Water ratio, T/S= Thermoase-to-Substrate ratio, pH-T= pH of thermoase digestion, Temp-T= Temperature of thermoase digestion, Time-T= Thermoase digestion time, P/S= Pepsin-to-Substrate ratio, pH-P= pH of pepsin digestion, Temp-P= Temperature of pepsin digestion, Time-P= Pepsin digestion time.

b. Interaction severity index was calculated according to Roy's method (2001). The absolute value of the biggest angle between two lines was reported while 100% means 90° angle between lines and 0% means parallel lines.

   Example: SI of interaction A*B was calculated using the following equation:

$$SI = \frac{\left(\overline{A_2B_1} - \overline{A_1B_1}\right) - \left(\overline{A_2B_2} - \overline{A_1B_2}\right)}{2 * constant}$$

   where constant = difference between maximum and minimum combined factor average effects of A*B. SI was calculated at all 3 levels, and the largest SI was reported in this table.

c. Indicates the desirable levels of factors at optimal conditions.

### *2.3.2 Determination and validation of optimal levels*

The optimal levels of significant factors could be easily determined by selecting the level with the biggest S/N ratio. Thus, the optimal conditions for factors S%, pH-T, Time-T, P/S, pH-P, Temp-P and Time-P were 7.5%, pH 8, 90 min, 1%, pH 2.5, 55°C and 180 min, respectively. The effect of T/S on both responses was negligible; therefore the smallest thermoase dosage was selected due to cost consideration. A higher S/N ratio of Temp-T was obtained at level 2 in terms of ACE inhibitory activity and at level 1 in terms of peptide yield (Figures 2.1B and 2.2B). However, as ACE inhibitory activity is considered as a more important characteristic than peptide yield, the optimal level of Temp-T was selected as 65°C.

ACE inhibitory activity and peptide yield were predicted to be 24±2 μg/mL and 74.3±1.7% at optimal conditions. The predicted values were computed according to Roy's method (2010) and only significant factors were taken into consideration. Egg white powder was subjected to enzymatic hydrolysis using the proposed optimal conditions, and the resulting ACE inhibitory activity and peptide yield were 30±2 μg/mL and 77.5±0.3%, which were comparable to the predicted values.

### *2.3.3 Feasibility of substitute egg white powder with liquid egg white in the production of bioactive peptides*

Egg white powder has been widely used in the food industry, and it offers several advantages such as long shelf life and low transportation cost (Lechevalier, Jeantet, Arhaliass, Legrand, & Nau, 2007). However, it was also observed to encounter difficulty in being soluble in water, which would be unfavorable in scale-up production. As liquid egg white is easier to handle and ready to use, its potential to be used instead of egg white powder was investigated.

Liquid egg white was diluted with water to reach a final substrate-to-water ratio of 7.5%, and then hydrolysis was carried out using the optimal conditions with and without sonication. As shown in Table 2.5, sonication did not affect liquid egg white hydrolysate much in terms of ACE inhibitory activity and peptide yield while other conditions were held constant. However, both liquid egg white hydrolysates had lower ACE inhibitory activity than that of egg white powder hydrolysate. Egg white proteins undergo denaturation when subjected to heat treatment, although individual egg white proteins have different denaturation temperatures, heating at 80°C was high enough to cause liquid egg white to form coagulum which may result in substrate being less accessible to enzymes (Mine & Zhang, 2013). Liquid egg white hydrolyzed at a lower substrate-to-water ratio of 5% showed less coagulation after heat treatment with improved ACE inhibitory activity and peptide yield.

**Table 2.5 Liquid egg white hydrolysis conditions and resulting responses**

| Substrate-to-water ratio (%) | Sonication | ACE inhibitory activity ($IC_{50}$: µg hydrolysate/mL) | Peptide yield (%) |
|---|---|---|---|
| 5 | Yes | 50 | 83.6 |
| 5 | No | 36 | 85.9 |
| 7.5 | Yes | 61 | 81.1 |
| 7.5 | No | 63 | 79.6 |

Previous study has shown that sonication aided in disruption of disulfide bonds in ovotransferrin and eventually led to the release of the predicted peptides (Majumder & Wu, 2010). The importance of sonication on ovotranferrin hydrolysis was reinforced by this study; while all three predicted peptides were liberated from ovotransferrin hydrolysate pretreated with sonication, IRW was not found in the ovotranferrin hydrolysate without sonication pretreatment (Figure 2.3). The results of HPLC analysis demonstrated that peptide composition of all egg white hydrolysates was similar; the impact of sonication on egg white hydrolysates was less than that on ovotransferrin hydrolysates. The quantities of IRW, IQW, and LKP generated in different hydrolysates were estimated by HPLC (Table 2.6). Only IRW and IQW were found in all egg white hydrolysates, not LKP. Table 2.6 showed that sonication enhanced the release of IRW from egg white, but had no effect on liberation of IQW. The results suggested that egg white hydrolysates containing two potent ACE inhibitory peptides could be prepared without sonication pretreatment.

**Table 2.6 Quantities of IRW, IQW and LKP generated in different hydrolysates**

| Hydrolysates | IRW (μg/mg hydrolysate) | IQW (μg/mg hydrolysate) | LKP (μg/mg hydrolysate) | ACE inhibitory activity ($IC_{50}$: μg hydrolysate/mL) |
|---|---|---|---|---|
| Ovotransferrin hydrolysate prepared with sonication | 39.15±7.81 | 5.30±0.58 | 10.78±1.48 | 14±1 |
| Ovotransferrin hydrolysate prepared without sonication | NF | 1.40±0.08 | 10.76±0.69 | 49±2 |
| Egg white powder hydrolysate prepared with sonication | 0.48 | 0.54 | NF | NM |
| Egg white powder hydrolysate prepared without sonication | 0.26 | 0.60 | NF | 35 |
| Liquid egg white hydrolysate prepared with sonication | 0.46 | 0.39 | NF | 50 |
| Liquid egg white hydrolysate prepared without sonication | 0.24 | 0.39 | NF | 36 |
| Large scale egg white hydrolysate prepared without sonication | 1.13 | 0.60 | NF | 55 |

*NF means Not Found; NM means Not Measured

### *2.3.4 Feasibility of scale-up production of bioactive egg white hydrolysate*

A large quantity of food grade egg white hydrolysate was produced to assess the feasibility of its production procedure at an industrial scale. A total of 150 L of liquid egg white was digested, and the resulting hydrolysate was dried by spray drying, which is a cheaper and faster alternative to freeze drying. The peptide composition of scale-up egg white hydrolysate was similar to those prepared at lab scale (Figure 2.3), and the estimated quantity of IRW and IQW generated was 1.13 and 0.60 μg/mg hydrolysate, respectively (Table 2.6). The presence of these two peptides was also confirmed by LC-MS/MS analysis (Figure 2.4). Egg white hydrolysate prepared at large scale showed potent ACE inhibitory activity of 55 μg hydrolysate /mL, which was comparable to those prepared at lab scale. However, the peptide yield was relatively low, an estimate of 53%, which was mainly due to sample loss during transfer between instruments.

## 2.4 Conclusion

The results of our study demonstrated the potential of egg white as starting material to produce a functional ingredient with potent ACE inhibitory activity. Taguchi's design was used to study the nine factors involved in the production process of egg white hydrolysate. Six factors, S%, pH-T, Time-T, P/S, Temp-P and Time-P, had significant effect on ACE inhibitory activity while only pH-P significantly influenced peptide yield. Egg white hydrolysate prepared with the established optimal conditions showed potent ACE inhibitory activity and high peptide yield. The production procedure showed feasibility in large scale; however, improvement of in-plant process design is needed to increase peptide yield. Our research has demonstrated blood pressure lowering effect of IRW and IQW *in vivo* (Majumder et al., 2013, 2015), it is reasonable to presume that egg white hydrolysate containing IRW and IQW would also have *in vivo* activity. The hypothesis needs to be verified by animal studies. As many ACE inhibitory peptides has been reported with bitter taste (Maehashi & Huang, 2009), the format of vehicle to deliver such functional ingredients to consumers and consumer acceptance need to be investigated in order to provide more information for future product development.

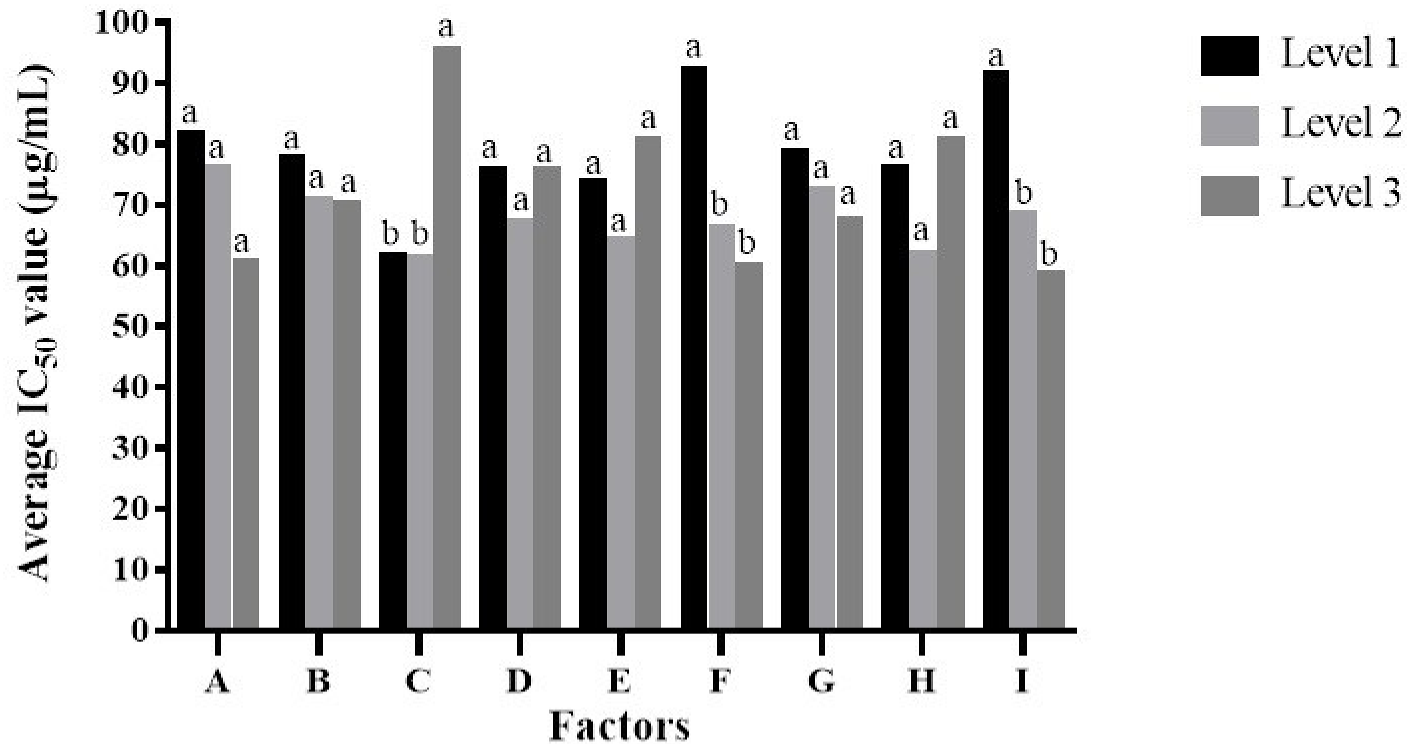



Figure 2.1A Average ACE inhibitory activity at different levels of each factor (A: substrate-to-water ratio (2.5%, 5%, 7.5%); B: thermoase-to-substrate ratio (0.1%, 0.5%, 1%); C: pH of thermoase digestion (7, 8, 8.5); D: temperature of thermoase digestion (60, 65, 70 °C); E: thermoase digestion time (45, 90, 180 min); F: pepsin-to-substrate ratio (0.1%, 0.5%, 1%); G: pH of pepsin digestion (1.5, 2.5, 3.5); H: temperature of pepsin digestion (45, 55, 65 °C); I: pepsin digestion time (45, 90, 180 min)). The bars with same letter on top within each factor were not significantly different (α=0.05).

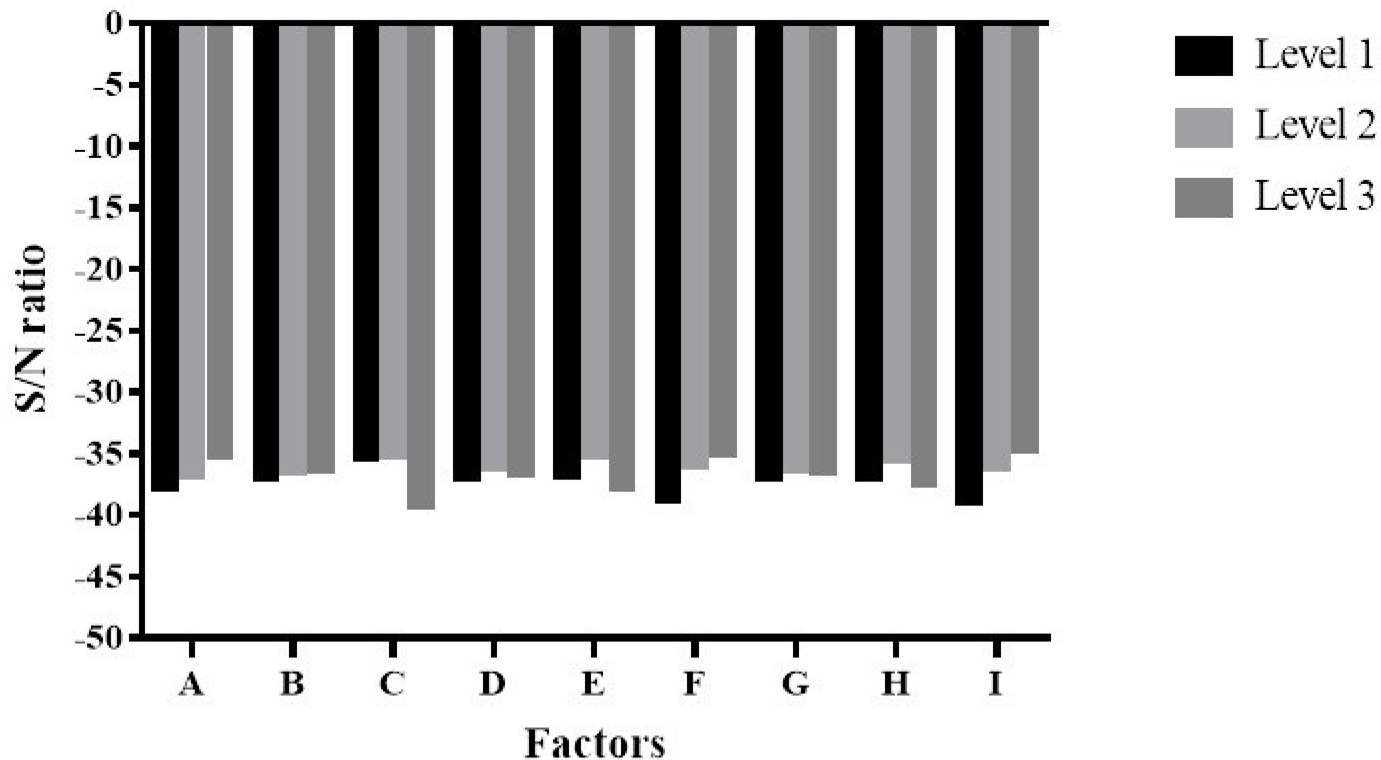



Figure 2.1B Average S/N ratio at different levels of each factor on ACE inhibitory activity, computed based on smaller is better characteristic (A: substrate-to-water ratio (2.5%, 5%, 7.5%); B: thermoase-to-substrate ratio (0.1%, 0.5%, 1%); C: pH of thermoase digestion (7, 8, 8.5); D: temperature of thermoase digestion (60, 65, 70 °C); E: thermoase digestion time (45, 90, 180 min); F: pepsin-to-substrate ratio (0.1%, 0.5%, 1%); G: pH of pepsin digestion (1.5, 2.5, 3.5) ; H: temperature of pepsin digestion (45, 55, 65 °C); I: pepsin digestion time (45, 90, 180 min)).

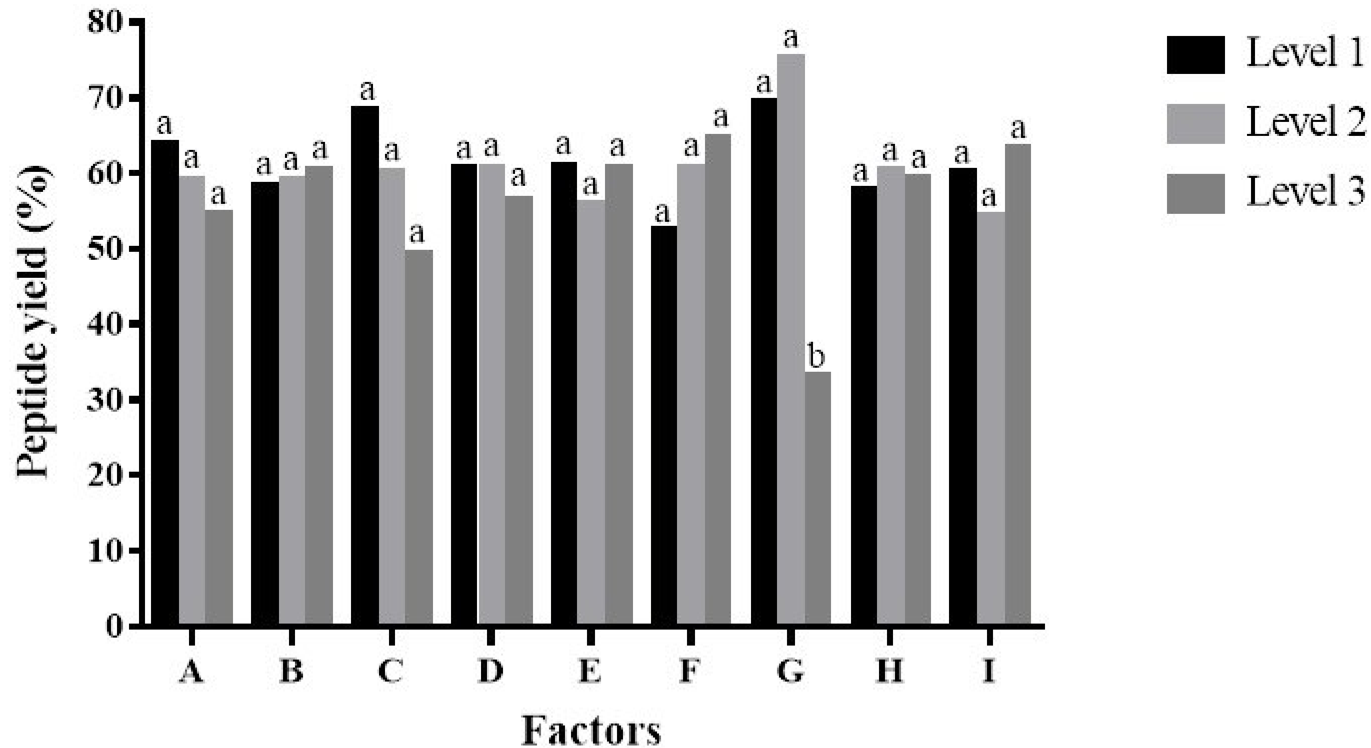



Figure 2.2A Average peptide yield at different levels of each factor (A: substrate-to-water ratio (2.5%, 5%, 7.5%); B: thermoase-to-substrate ratio (0.1%, 0.5%, 1%); C: pH of thermoase digestion (7, 8, 8.5); D: temperature of thermoase digestion (60, 65, 70 °C); E: thermoase digestion time (45, 90, 180 min); F: pepsin-to-substrate ratio (0.1%, 0.5%, 1%); G: pH of pepsin digestion (1.5, 2.5, 3.5); H: temperature of pepsin digestion (45, 55, 65 °C); I: pepsin digestion time (45, 90, 180 min)). The bars with same letter on top within each factor were not significantly different (α=0.05).

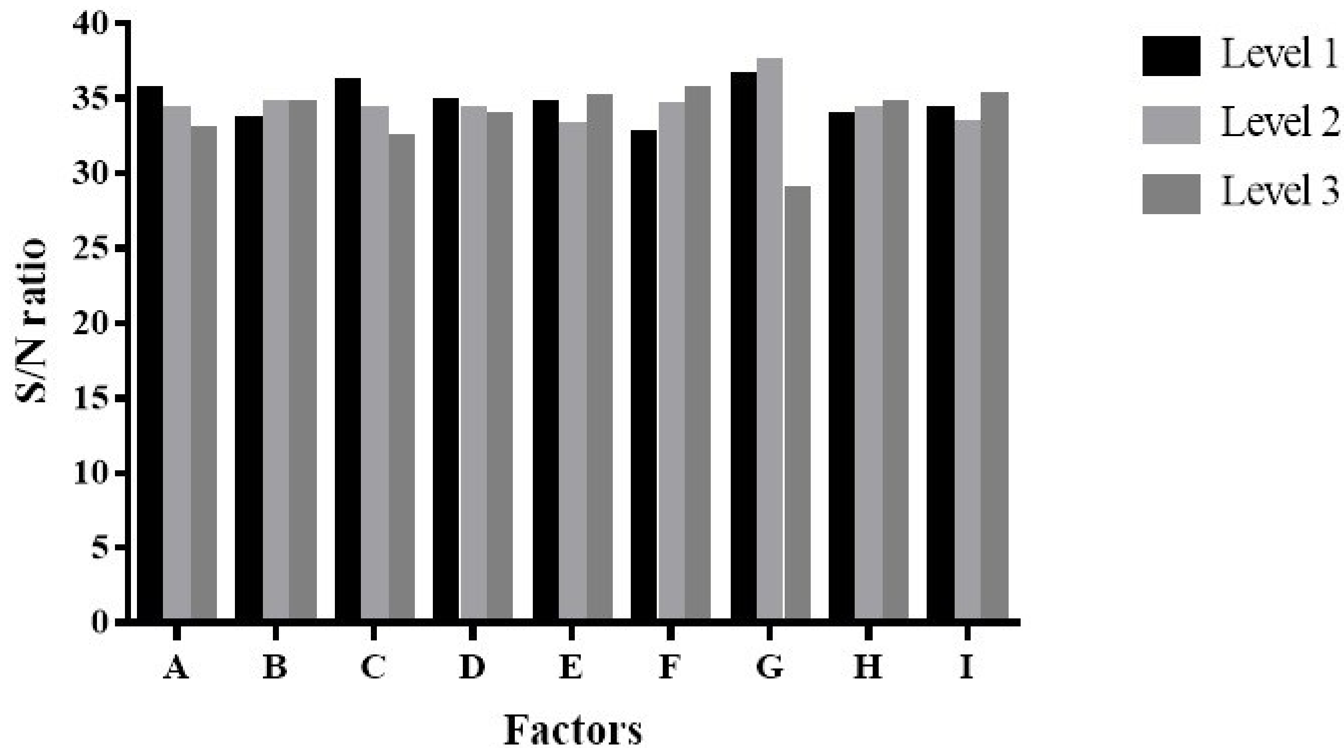



Figure 2.2B Average S/N ratio at different levels of each factor on peptide yield, computed based on larger is better characteristic (A: substrate-to-water ratio (2.5%, 5%, 7.5%); B: thermoase-to-substrate ratio (0.1%, 0.5%, 1%); C: pH of thermoase digestion (7, 8, 8.5); D: temperature of thermoase digestion (60, 65 70 °C); E: thermoase digestion time (45, 90, 180 min); F: pepsin-to-substrate ratio (0.1%, 0.5%, 1%); G: pH of pepsin digestion (1.5, 2.5, 3.5); H: temperature of pepsin digestion (45, 55, 65 °C); I: pepsin digestion time (45, 90, 180 min)).

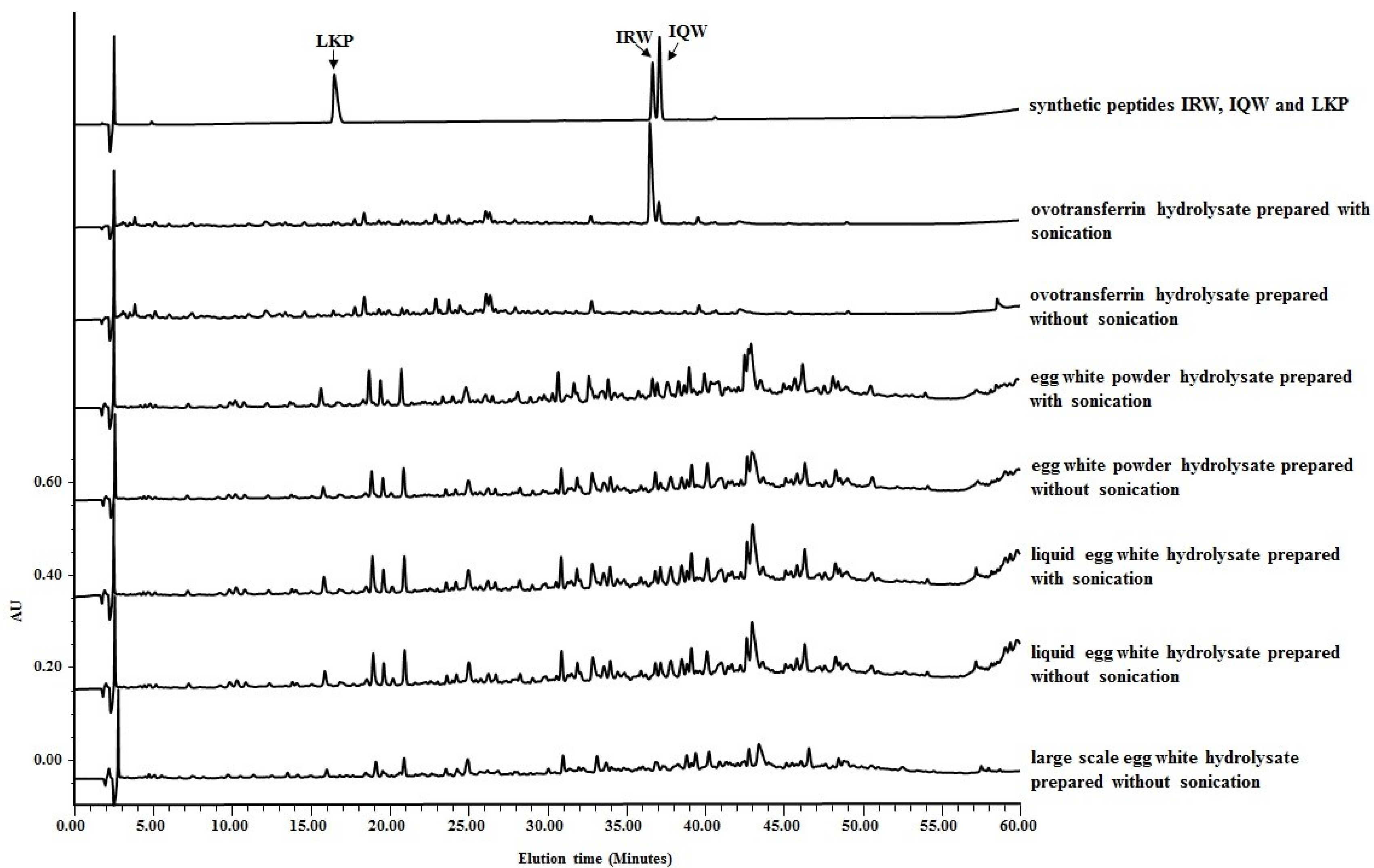
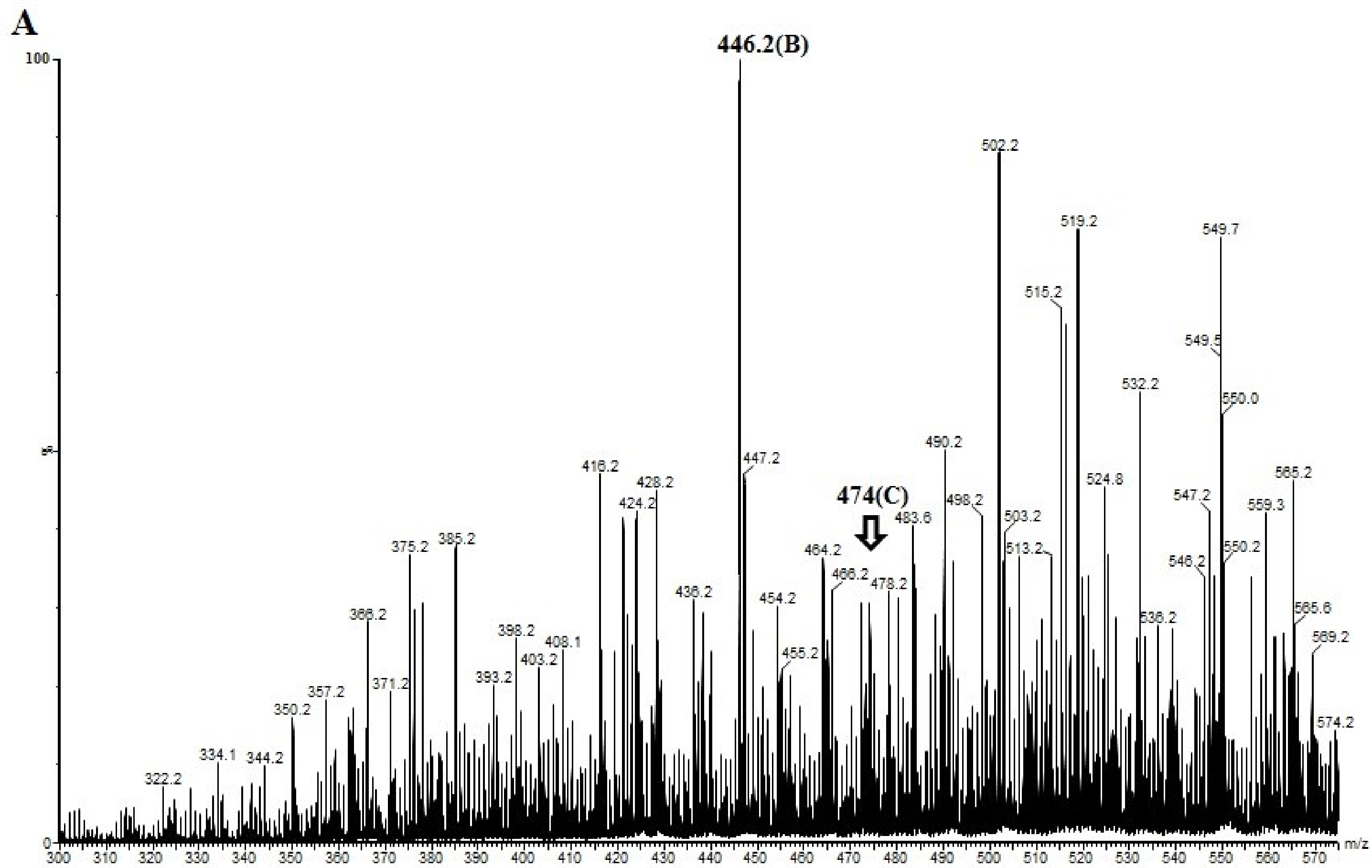
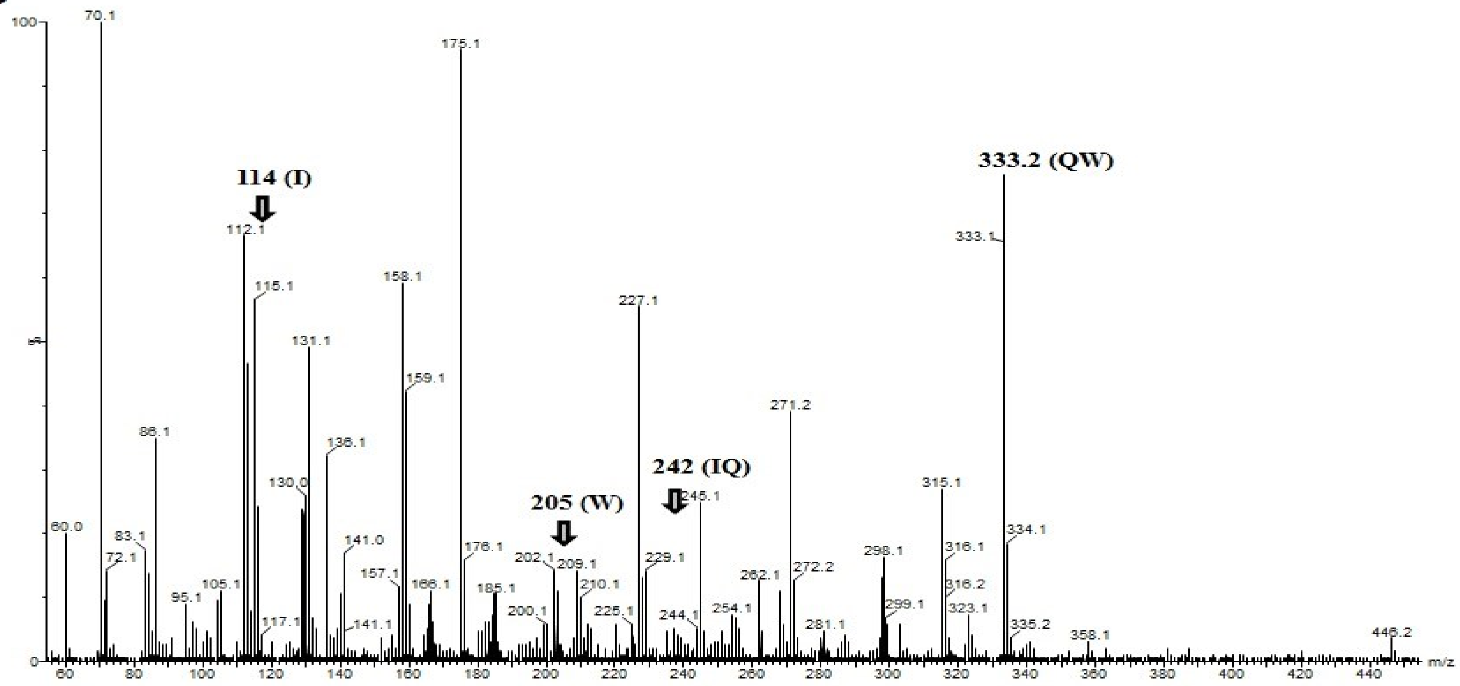



Figure 2.3 RP-HPLC chromatogram of synthetic peptide standards, ovotransferrin hydrolysates and egg white hydrolysates.


A
446.2(B)
474(C)
m/z


B
114 (I)
205 (W)
242 (IQ)
333.2 (QW)
m/z

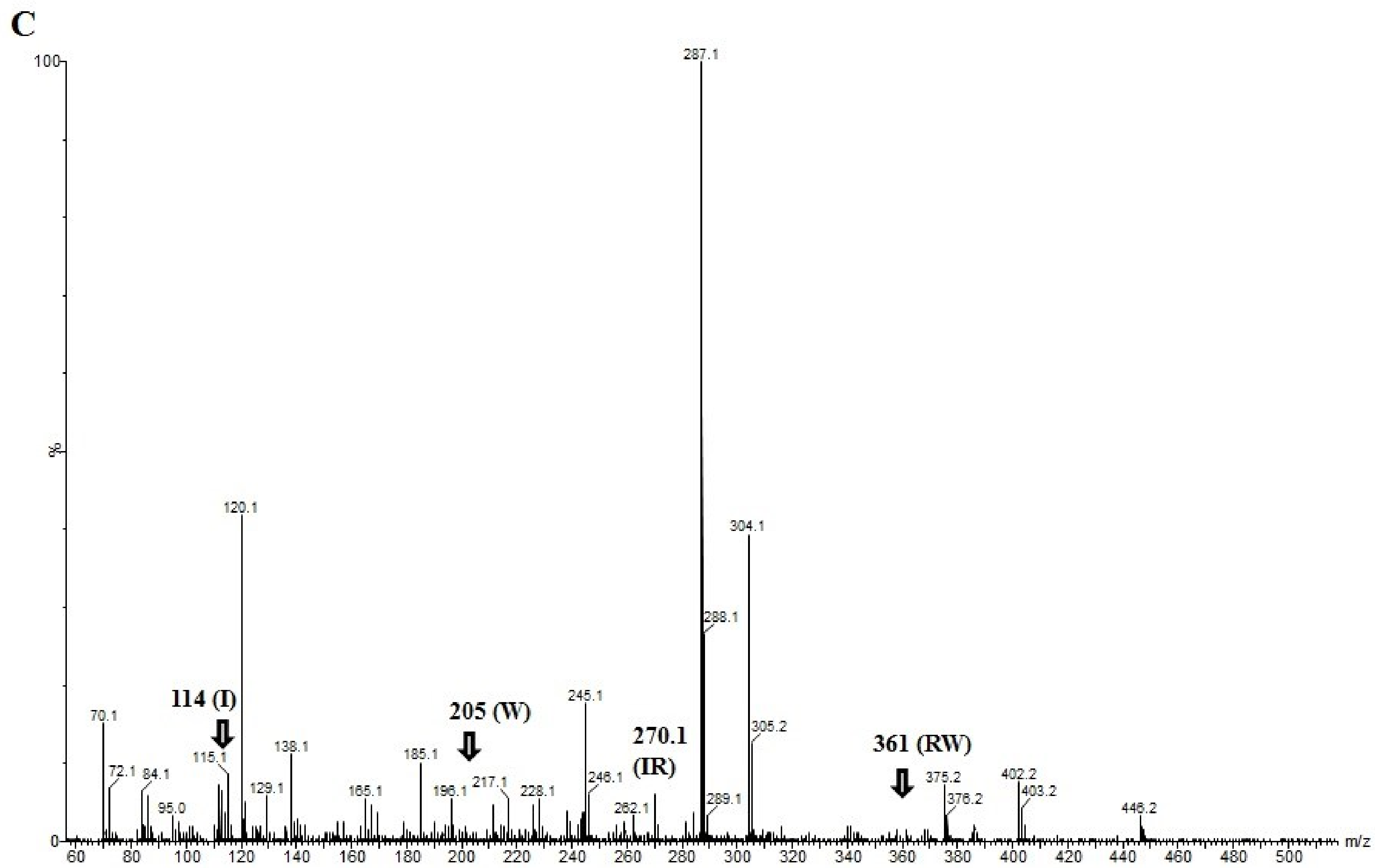



Figure 2.4 LC-MS spectra of large scale liquid egg white hydrolysate prepared without sonication; (A) LC-MS spectra of the whole hydrolysate; (B) LC-MS/MS spectra of IQW from the whole hydrolysate; (C) LC-MS/MS spectra of IRW from the whole hydrolysate.

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# CHAPTER 3 CONSUMER ACCEPTABILITY OF FOOD PRODUCTS ENRICHED WITH EGG WHITE PROTEIN HYDROLYSATE

## 3.1 Introduction

Proteins, a macronutrient of the daily diet, exhibit nutritional, physicochemical and physiological properties (Korhonen, 1998). In addition to providing essential amino acids for normal growth and maintenance, food proteins are incorporated as important food ingredients for different functionalities including solubility, water holding capacity, foaming, gelling and emulsification (Nickerson, House, & Li-Chan, 2013). Recent research has suggested various potential health benefits of food proteins beyond their nutritional values; for example soy protein has been credited for its potential to reduce the risk of coronary heart disease along with diets that are low in saturated fat and cholesterol (FDA, 2015). As consumers become more knowledgeable about the contribution of protein to overall health and wellbeing, especially in weight management and sport nutrition (Adams, 2015), protein enriched products have become a rising sector in food industry with a global sales of $16 billion in 2013 (Schmidt, 2014).

Protein ingredients have been applied in a wide range of foods such as bakery, dairy products, beverage and snacks. However, the International Food Information Council Foundation (IFIC) 2014 Food and Health Survey reported that 51% of survey participants consume protein bars as specific food products to get protein and 38% obtained protein from protein shakes or beverages.

Whey protein is one of the commonly used protein sources in protein enriched products due to its abundance, moderate processing cost and complete amino acid profile (Clemente, 2001). As another excellent animal protein source, egg white also has well-balanced amino acid profile. Our previous study has demonstrated that thermoase-pepsin digested egg white protein

hydrolysate exhibited potent *in vitro* angiotensin I converting enzyme (ACE) inhibitory activity and had potential usage in functional food and nutraceutical applications. Enzymatic hydrolysis, as a mild processing treatment, not only releases bioactive peptides from their parent proteins, it also improves digestion and absorption and reduces allergenicity (Clemente, 2001). Protein hydrolysates can be used to develop products associated with geriatric need, sport nutrition, weight management (Frokjaer, 1994), and patients with protein digestion and absorption problems, allergies and other health conditions (Clemente, 2001). However, the well documented bitter taste caused by enzymatic hydrolysis has limited the use of protein hydrolysates in food applications (Hernández-Ledesma, Del Mar Contreras, & Recio, 2011) as taste was the primary factor that influence consumers' food choice (IFIC, 2014). The objective of this study was to investigate consumer acceptance of egg white protein hydrolysate as a substitute for whey protein in protein enriched bars and beverage without compromising sensory attributes, which would allow us to compare the potential of solid and liquid food matrices as vehicles for functional ingredient delivery.

## 3.2 Materials and Methods

### *3.2.1 Materials*

Food grade egg white hydrolysate was prepared in the Food Processing Development Centre (Leduc, AB) according to the method described in section 2.2.9. Isomaltooligosaccharide (VitaFiber) was purchase from BioNeutra Global Corporation (Edmonton, AB). Whey protein isolate (whey protein isolate, soy lecithin), sugar, honey and almond granola (oat flakes, brown sugar, wheat flakes, honey, soy oil, wheat bran, almonds, sesame seeds, sunflower seeds, molasses), cocoa powder, pure vanilla extract, partly skimmed chocolate milk (2% milk fat),

vanilla-flavored frozen yogurt and instant coffee were purchased from local grocery stores (Edmonton, AB).

### *3.2.2 Samples Preparation*

Egg white hydrolysate (EWH) was used to prepare chocolate-flavored protein bars and coffee-flavored protein beverage where whey protein isolate (WPI) was replaced by 0%, 25%, 50%, 75%, and 100% for both products. The basic formulation for preparing the chocolate-flavored protein bars was as follows: 25% sugar, 0-20% WPI, 0-20% EWH, 20% isomaltooligosaccharide, 19% honey & almond granola, 10% water, 5% cocoa powder and 1% pure vanilla extract; while that of the coffee-flavored protein beverage was 62% partly skimmed chocolate milk, 32% vanilla-flavored frozen yogurt, 4% coffee, 0-2% WPI and 0-2% EWH. Both samples were prepared in the food grade laboratory located in the Agriculture/Forestry Centre at University of Alberta.

Protein bars were prepared according to the method described by Imtiaz et al (2012) with minor modifications. To prepare protein bars, whey protein isolate, various levels of egg white hydrolysate, granola, and cocoa powder were weighed and mixed in a mixing bowl (Whirlpool, USA). Isomaltooligosaccharide, sugar, water, and vanilla extract were heated in a saucepan with continuous mixing until the temperature of the mixture reached 100°C. The syrup mixture was poured into the dry mixture and then mixed by KitchenAid 4.5 quart tilt-head stand mixer (Whirlpool Corporation, USA) for 30 s at speed 1. The mixer was then stopped and the sides of mixing bowl were scraped down. The mixing action was repeated 2 more times to make a uniform mixture and then the dough was placed in a baking pan and pressed to have a flat surface. The dough was wrapped and packed in sealed plastic bags and stored in freezer for

overnight before cut into bars of approximately 20 grams. The bars were placed in individual 96.1 mL translucent plastic soufflé cups with clear plastic lids and thawed to room temperature before serving to participants. Protein bars were prepared one day before sensory testing.

The protein beverages were prepared on the day of sensory testing. Instant coffee was dissolved in hot water according to manufacturer's instruction. Then chocolate milk, vanilla-flavored frozen yogurt, instant coffee, whey protein isolate and egg white hydrolysate were weighed and combined in KitchenAid KSB560MC blender (Whirlpool Corporation, USA). The mixture was mixed for 3 times (15 s each time) until smooth, the sides of blender were scraped down between each mixing period. Protein beverage was poured into individual 96.1 mL translucent plastic soufflé cups with a serving size of 30 mL, the sample cups were covered with clear plastic lids and stored in refrigerator until present to participants.

### *3.2.3 Sensory evaluation procedure*

The consumer acceptability testing of protein bars and protein beverage was conducted at the sensory evaluation room located in the Agriculture/Forestry Centre at University of Alberta. This study was approved by a University of Alberta Human Research Ethics Board. A total of 140 participants were recruited from University of Alberta through emails and fliers; 70 subjects (32 male and 38 female) participated in the sensory evaluation of protein bars and the other 70 subjects (30 male and 40 female) participated in the sensory evaluation of the protein beverages. The participant recruitment and sensory testing of each sample was held on different days. Only potential participants who were not allergic to any ingredients in the samples and were interested in high protein food products were recruited to this study. All subjects were explained the benefits and risks of participating, and their consent were collected before sensory testing.

Participants were asked to sit in individual sensory booths with white fluorescent light, and they were presented with a tray consisting of samples with random three-digit codes, a glass of distilled water, a serviette, a spit cup (for protein beverage sensory evaluation only), a pencil and a questionnaire. Protein bars were served at room temperature while protein beverage was served at refrigerated temperature. There were five samples of each prototype product; however, only three samples were presented to participants each time to avoid organoleptic fatigue. A balanced incomplete block design with predetermined balanced and randomized presentation order was generated by XLSTAT 2015 (Addinsoft, Paris, France). Participants were asked to complete a demographic questionnaire first, which allowed us to collect general information of participants such as gender and age, as well as their purchase habits of high protein food products. Participants were asked to take a sip of water prior to and between each evaluation to avoid carryover of flavors. The sensory evaluation was comprised of affective testing and preference ranking. First participants were asked to rate their liking of each sample in terms of appearance, flavor, texture/mouthfeel, and overall opinion using 9-point hedonic scale (1= dislike extremely and 9= like extremely). Then participants were given the same set of samples with different random three-digit codes in balanced and randomized presentation order, and they were asked to rank the samples based on their preference with 1=liked most and 3=liked least.

### *3.2.4 Data analysis*

The data collected from affective testing were analyzed by Analysis of Variance (Proc GLM) using SAS 9.3 (SAS Institute Inc., Cary, NC, USA) to determine if there were significant differences between samples at $p < 0.05$. The data collected from the preference ranking test were analyzed by Durbin test using XLSTAT 2015 (Addinsoft, Paris, France) to determine whether one sample was more preferred than others.

## 3.3 Results and Discussion

### *3.3.1 Effect of egg white hydrolysate addition on consumer acceptability of protein bars*

Appearance, flavor, texture and overall opinion of protein bars were evaluated by seventy participants. According to ANOVA results, there was a significant difference among bars made with different protein blends with respect to appearance and flavor liking (Table 3.1). The average liking score of appearance of protein bars increased with increasing proportion of egg white hydrolysate addition, probably due to an improved bar shape at higher egg white hydrolysate addition. Protein bars made with 100% and 75% EWH had significantly higher rating in appearance liking than those made with 25% and 0% EWH ($p<0.05$). However, participants' perception of the flavor of bars showed the opposite trend; participants preferred protein bars with lower proportion of egg white hydrolysate. The bars made with 0% and 25% EWH had significantly higher liking scores on flavor than other samples but 50% EWH bars. Although 38% of participants commented that some formulations resulted in bars that were too soft and sticky, there was no significant difference between bars in terms of texture liking. In terms of overall opinion, there was no significant difference between bar samples ($p=0.08$). A trend could be observed that participants preferred bars with less EWH incorporated, although these samples had lower hedonic rating in appearance. The results showed that flavor was likely the dominant sensory attribute that contributed to consumer acceptance in this food system. When participants were asked to rank the bar samples without knowing their identities, bars made with 0% and 25% EWH were significantly more preferred than 50% and 100% EWH bars, while the three samples with higher level of EWH were not significantly preferred over each other (Table 3.2). The results were consistent with affective testing, with a trend that bars with less egg white hydrolysate incorporation were liked more.

Overall, average liking scores of bars in all sensory attributes evaluated were below 6 (6= like slightly) on 9-point hedonic scale except flavor liking score of bars with 0% and 25% EWH, which indicated that the prototype protein bars were not liked in general. The scores were distributed all over the 9-point hedonic scale with a few responses on the end points (1= dislike extremely and 9= like extremely) which showed that participants' perception of the prototype protein bars was very different. Individuals' expectations affected consumer acceptance of products being evaluated (Cardello, Maller, Masor, Dubose, & Edelman, 1985). The food products with sensory properties closer to consumer expectations would be liked more, and vice versa. Pliner (1982) has proposed that increasing exposure to an unfamiliar food could improve its affective response. As more than half of participants (55.7%) have never or rarely consumed (less than once a month) high protein bars, providing commercial products as a reference would help participants evaluate lab prototype samples and give researchers better direction to improve product formulation.

A participant who received bar samples with 0%, 50% and 100% EWH ranked the bars in terms of degree of firmness. The bar texture was perceived to be firmer as the proportion of EWH in the prototypes increased. The difference observed in bar texture was attributed to protein type, which was the only factor that changed in the controlled formulation. Similar results were reported by Childs et al (2007) who investigated the sensory properties of meal replacement bars made with whey protein, soy protein and a combination of both. Substitution of protein hydrolysate for conventional ingredients was shown to negatively affect sample texture. Replacement of mechanically deboned chicken meat with its protein hydrolysate in mortadella-type sausage resulted in samples with softer texture (Cavalheiro et al., 2014); however, addition or substitution of protein hydrolysates was reported to increase firmness of baked products

(Fitzgerald et al., 2014; Gani et al., 2015). Consequently, the texture change attributed to protein hydrolysates has resulted in negative responses in consumer acceptance testing.

**Table 3.1 Average liking scores of appearance, flavor, texture and overall opinion of protein bars using 9-point hedonic scale, with 1= dislike extremely, 5= neither like nor dislike, and 9= like extremely. Mean values with different letters within a row were significantly different (*p*<0.05) (n=70)**

| Sensory attributes | 100% WPI, 0% EWH | 75% WPI, 25% EWH | 50% WPI, 50% EWH | 25% WPI, 75% EWH | 0% WPI, 100% EWH |
|---|---|---|---|---|---|
| Appearance | $3.8 \pm 2.2^{c}$ | $4.6 \pm 2.1^{bc}$ | $5.0 \pm 1.6^{ab}$ | $5.5 \pm 1.5^{a}$ | $5.7 \pm 1.7^{a}$ |
| Flavor | $6.3 \pm 1.6^{a}$ | $6.3 \pm 1.9^{a}$ | $5.6 \pm 1.7^{ab}$ | $4.9 \pm 1.7^{bc}$ | $4.5 \pm 2.1^{c}$ |
| Texture | $4.9 \pm 1.9^{a}$ | $5.0 \pm 2.2^{a}$ | $4.8 \pm 1.6^{a}$ | $4.9 \pm 1.7^{a}$ | $5.3 \pm 2.0^{a}$ |
| Overall opinion | $5.5 \pm 1.7^{a}$ | $5.6 \pm 1.9^{a}$ | $5.4 \pm 1.4^{a}$ | $5.0 \pm 1.5^{a}$ | $4.7 \pm 2.0^{a}$ |

**Table 3.2 Sum and average rank of protein bars in preference ranking test, with 1= liked most and 3= liked least (n=70)**

| | 100% WPI, 0% EWH | 75% WPI, 25% EWH | 50% WPI, 50% EWH | 25% WPI, 75% EWH | 0% WPI, 100% EWH |
|---|---|---|---|---|---|
| Rank sum | 63 | 73 | 96 | 88 | 100 |
| Average rank | $1.5^{a}$ | $1.7^{ab}$ | $2.3^{c}$ | $2.1^{bc}$ | $2.4^{c}$ |

### *3.3.2 Effect of egg white hydrolysate addition on consumer acceptability of protein beverage*

A slight increase in sensory scores for all attributes was observed with incorporation of EWH up to 50% and a slight decrease beyond that point. There was significant difference in flavor liking and overall opinion between protein beverage samples according to ANOVA results (Table 3.3), while there was no significant difference among beverages with different protein blends in terms

of appearance and mouthfeel liking. The average liking scores of appearance and mouthfeel were in the range between 6= like slightly and 7= like moderately, which suggested that the appearance and mouthfeel of all beverage samples were generally accepted. The prototype protein beverage was coffee-flavored smoothie made with two main ingredients, chocolate milk and vanilla-flavored frozen yogurt. The former is widely available and popular in the market and familiar to milk drinkers. Therefore, there was no surprise that most participants accepted the appearance and mouthfeel of our protein beverage prototypes. The protein beverage made with 50% EWH had a significantly higher liking score in flavor than those made with 0% and 100% EWH. The same trend was observed in the overall opinion; the protein beverage made with 50% EWH had the highest overall liking score while the beverage made with 100% EWH had the lowest. The affective testing results were consistent with that of preference ranking; protein beverage made with 100% EWH was significantly less liked than other samples.

Overall, the average liking scores of protein beverage prototypes in all sensory attributes evaluated were above 6 (6= like slightly) on 9-point hedonic scale except flavor liking and overall opinion of beverage made with 100% EWH. Although the majority of participants accepted protein beverage prototypes with up to 3.75 g of egg white hydrolysate added in a serving size of 250 g, there were 23% participants that perceived one or more of these protein beverages as being too sweet for them. Studies have reported that different factors may affect individual's response to sweetness liking, such as age and ethnicity, as well as the food system evaluated (Kim, Prescott, & Kim, 2014). Health conscious consumers may consider protein enriched beverages as healthy drinks and expect them to have a less sweet taste as the perception of sweetness is always connected with sugar content. Epidemiological studies have suggested a link between overconsumption of simple sugar and diseases associated with metabolic syndrome

such as hypertension and diabetes (Lustig, Schmidt, & Brindis, 2012), which have driven consumers' desire for food products with reduced sugar. However, sugar was not added as an ingredient in the protein beverage formulation. The sweet taste was contributed by the sugar content in chocolate milk and vanilla-flavored frozen yogurt, which were chosen intentionally to mask the bitter taste of egg white hydrolysate.

Thirteen percent of participants perceived bitter/weird aftertaste in one or more of the protein beverage samples, which was consistent with the literature that bitter taste is generated during the process of enzymatic hydrolysis of food proteins (Adler-Nissen, 1984). Bitterness of peptides was reported to be related to their chemical structural properties such as hydrophobicity, peptide length, spatial structure, bulkiness and primary sequence (Kim & Li-Chan, 2006; Maehashi & Huang, 2009). As many ACE inhibitory peptides were reported to possess bitter taste due to their similar chemical structure requirements (Wu, Aluko, & Nakai, 2006), quantitative structure and activity relationship (QSAR) model was applied to predict a correlation, but not at a significant level, between bitterness and their ACE inhibitory activity of di- and tri-peptides (Wu & Aluko ,2007), while Pripp and Ardö (2007) found significant correlation between these two properties of dipeptides but not oligopeptides. Some effective bitter reduction methods have been proposed including controlled enzymatic hydrolysis to a certain degree, selection of suitable proteases that produce peptides with less hydrophobic amino acids and use of exopeptidases (Raksakulthai & Haard, 2003). As the use of exopeptidases would be resulted in release of hydrophobic amino acids from peptides, this method may compromise ACE inhibitory activity of protein hydrolysates. Thus, encapsulation and addition of masking agents may be better choices for bitter taste reduction of protein hydrolysates while retaining their bioactivity (Hernández-Ledesma et al., 2011).

There were 7% of participants perceived salty taste in one or more samples. Salt was built up during enzymatic hydrolysis of egg white as sodium hydroxide and hydrochloric acid were added to maintain appropriate pH (Hernández-Ledesma et al., 2011). Keast et al (2001) suggested that sodium salts could suppress bitterness and enhance sweetness in a mixture of bitter and sweet compounds; however, sodium salts suppress the bitterness of different compounds to different extents. Thus, the role of salt in the egg white hydrolysate may explain why the protein beverage with 50% EWH was liked most. The overall flavor profile of protein hydrolysates may be more complicated than individual peptides and free amino acids as there is a possibility of synergistic or antagonistic interactions among components in the mixture (Cheung & Li-Chan, 2010); therefore, product development researchers may need to understand the flavor profile of individual protein hydrolysate before product formulation design.

The prototype formulations were developed to incorporate the maximum quantity of egg white hydrolysate possible without severely compromising sensory attributes. Although protein bar and beverage prototypes had encountered different challenges in formulation, flavor is still considered as the primary factor that influences consumers' food choice in general (Verbeke, 2006). The level of EWH incorporated in the beverage prototypes was much lower than that in the bar prototypes as undesirable flavors were harder to mask in liquid foods. A similar result was reported by Childs et al (2007) who reported that flavors contributed by soy protein were carried through at a higher intensity in the beverage than bars even though the protein content in beverage was two times lower. Flavor release and perception were influenced by food matrix structure and individual eating pattern (Wilson & Brown, 1997). The study conducted by Wilson and Brown (1997) has demonstrated that an increase in firmness of food samples resulted in a decrease in maximum flavor intensity perceived and an increase in the time at which maximum

intensity of flavor perception occurred. Mastication is required to break down solid foods into smaller pieces to increase surface area in the mouth for flavor release and perception, while consumption of liquid foods did not require chewing to maximize surface area for flavor perception.

It is crucial for functional foods to retain their bioactivity after processing; however, processing such as heat treatment, extrusion and dehydration, may pose a detrimental effect on the bioactivity of peptides and protein hydrolysates (Hernández-Ledesma et al., 2011; Korhonen, 1998). Studies have shown that protein hydrolysates with ACE inhibition activity retained their bioactivity in bread (Fitzgerald et al., 2014) and pasta (Segura-Campos, García-Rodríguez, Ruiz-Ruiz, Chel-Guerrero, & Betancur-Ancona, 2014) after baking, extrusion and cooking. The preparation of EWH enriched protein bars and beverage in this study involved minimum processing steps; therefore, these samples would retain ACE inhibitory activity after processing.

**Table 3.3 Average liking scores of appearance, flavor, mouthfeel and overall opinion of protein beverage using 9-point hedonic scale, with 1= dislike extremely, 5= neither like nor dislike, and 9= like extremely. Mean values with different letters within a row were significantly different ($p$<0.05) (n=70)**

| Sensory attributes | 100% WPI, 0% EWH | 75% WPI, 25% EWH | 50% WPI, 50% EWH | 25% WPI, 75% EWH | 0% WPI, 100% EWH |
|---|---|---|---|---|---|
| **Appearance** | $6.6\pm1.3^{a}$ | $6.6\pm1.4^{a}$ | $6.7\pm1.3^{a}$ | $6.5\pm1.5^{a}$ | $6.1\pm1.6^{a}$ |
| **Flavor** | $6.1\pm2.1^{bc}$ | $6.7\pm1.4^{ab}$ | $7.0\pm1.5^{a}$ | $6.7\pm1.4^{ab}$ | $5.7\pm1.5^{c}$ |
| **Mouthfeel** | $6.2\pm1.9^{a}$ | $6.8\pm1.2^{a}$ | $6.9\pm1.6^{a}$ | $6.8\pm1.3^{a}$ | $6.2\pm1.7^{a}$ |
| **Overall opinion** | $6.1\pm2.0^{bc}$ | $6.7\pm1.2^{ab}$ | $6.9\pm1.5^{a}$ | $6.7\pm1.3^{ab}$ | $5.8\pm1.8^{c}$ |

**Table 3.4 Sum and average rank of protein beverage in preference ranking test, with 1= liked most and 3= liked least (n=70)**

| | 100% WPI, 0% EWH | 75% WPI, 25% EWH | 50% WPI, 50% EWH | 25% WPI, 75% EWH | 0% WPI, 100% EWH |
|---|---|---|---|---|---|
| Rank sum | 83 | 79 | 73 | 80 | 105 |
| Average rank | 2.0[a] | 1.9[a] | 1.7[a] | 1.9[a] | 2.5[b] |

## 3.4 Conclusion

The identification of peptides and protein hydrolysates with ACE inhibition activity from different food sources has been extensively reported, while there is limited information in regards to their application in food product formulation. Consumer acceptability of protein bars and protein beverage was investigated where egg white hydrolysate with *in vitro* ACE inhibitory activity was used to replace whey protein isolate. Overall, protein beverage with up to 1.5% EWH was accepted by participants while all protein bar samples were not liked in general. The bitter taste and salty off-flavor attributed to EWH negatively impacted participants' hedonic rating on both prototypes. Thus, it would be necessary to search for methods to efficiently mask undesirable flavors. This study has demonstrated that a solid food system can incorporate higher level of egg white hydrolysate than a liquid food system. Therefore, a solid food system such as bars would be a better vehicle to deliver high protein content, while a liquid food system like beverage is more suitable for functional food development.

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# CHAPTER 4 OVERALL CONCLUSION AND RECOMMENDATIONS

## 4.1 Key findings of the present study

Food protein derived bioactive peptides have great potential for prevention and treatment of chronic diseases. There has been extensive research on bioactive peptides; however, there are only limited bioactive peptides commercialized, probably due to the well-reported unpleasant sensory attributes of most bioactive peptides. Our previous studies have identified three novel tripeptides, IRW, IQW and LKP, from egg white protein ovotransferrin, which have shown *in vitro* ACE inhibitory activity and *in vivo* antihypertensive effect in SHR (Majumder & Wu, 2011; Majumder et al., 2013, 2015). These tripeptides have potential to be developed as functional foods for prevention and management of hypertension. The aims of this study were to develop a cost-effective method for large scale production of egg white protein hydrolysate with ACE inhibitory activity and to determine the consumer acceptance of this bioactive protein hydrolysate in solid and liquid food matrices.

Taguchi's method was applied to investigate the effect of enzymatic hydrolysis conditions on ACE inhibitory activity and peptide yield. A total of 27 experimental trials were performed, and the resulting ACE inhibitory activity and peptide yield ranged from 28-126 μg hydrolysate/mL and 10.9-79.9%, respectively. The ACE inhibitory activity of thermoase-pepsin digested egg white hydrolysate was significantly affected by six factors out of nine being studied, with pH of thermoase digestion, pepsin digestion time and pepsin-to-substrate ratio contributing the most; while peptide yield was only significantly influenced by one factor, pH of pepsin digestion. The established optimal conditions were substrate-to-water ratio 7.5%, thermoase-to-substrate ratio 0.1%, thermoase digestion pH 8, thermoase digestion temperature 65°C, thermoase digestion

time 90 min, pepsin-to-substrate ratio 1%, pepsin digestion pH 2.5, pepsin digestion temperature 55°C and pepsin digestion time 180 min. ACE inhibitory activity and peptide yield of the egg white protein hydrolysates prepared in laboratory scale and large scale were 30 μg hydrolysate/mL and 77.5%, and 55 μg hydrolysate/mL and 53%, respectively. IRW and IQW have been successfully released from egg white hydrolysate prepared with the established method but not LKP. As IRW and IQW have been shown to effectively reduce blood pressure of SHR, the egg white protein hydrolysate containing these two peptides has the potential to exert antihypertensive effect in SHR as well.

Consumer acceptance of egg white protein hydrolysate was evaluated in solid and liquid food matrices, chocolate-flavored protein bars and coffee-flavored protein beverage, respectively. Egg white protein hydrolysate was incorporated into protein bars and protein beverage up to 20% (w/w) and 2% (w/w), respectively; and substituted for whey protein isolate in both prototypes at 0%, 25%, 50%, 75%, and 100%. Participants were asked to rate their liking of each sample in terms of appearance, flavor, texture/mouthfeel, and overall opinion using the 9-point hedonic scale followed by preference ranking. The results of consumer acceptance testing have shown that bar prototypes with lower levels of egg white hydrolysate incorporated were liked more, even though all protein bar samples were not liked in general. Protein beverages formulated with up to 1.5% (w/w) protein hydrolysate were found to be acceptable by participants, and the prototype formulated with 1% (w/w) protein hydrolysate was liked most. However, the undesirable bitter taste of egg white protein hydrolysate negatively impacted the overall acceptance of both prototypes by participants. Therefore, it would be necessary to search methods to efficiently mask undesirable flavors. The results of the consumer acceptability study suggest that egg white protein hydrolysate has the potential for incorporation into functional

foods, for prevention and management of hypertension or to deliver protein to meet our daily requirement.

## 4.2 Future work recommendations

According to the results of the present study, some future work are recommended:

1. As Taguchi's method was not efficient in studying interactions among factors, fractional factorial design can be applied to further study the six significant factors including their interactions.
2. The optimization of scale-up production process of egg white protein hydrolysate to improve peptide yield is necessary as the batch prepared with the established optimal method in large scale had a relatively low peptide yield.
3. In order to improve consumer acceptance of egg white protein hydrolysate enriched food products, it is essential to develop methods to efficiently mask the bitter taste and salty-off flavors.
4. To study the effect of storage on egg white protein hydrolysate enriched food products in sensory attributes, microbial counts and stability of bioactive protein hydrolysate will provide us further information in future functional food development.
5. It is important to evaluate the *in vivo* bioactivity of egg white protein hydrolysate using animal models, and later clinical trials before new functional foods can be launched with health claims.

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