EFFECT OF PRETREATMENT METHODS ON ANTIOXIDANT ACTIVITY OF EGG WHITE HYDROLYSATE

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science Program in Food Technology

Suranaree University of Technology

Academic Year 2020

ผลของกระบวนการเตรียมตัวอย่างก่อนการย่อยต่อกิจกรรมต้านอนุมูลอิสระของไข่ขาวไฮโดรไลเซท

นางสาวอาสทรี ซุร์ยานี ปราวูลานารี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
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EFFECT OF PRETREATMENT METHODS ON ANTIOXIDANT ACTIVITY OF EGG WHITE HYDROLYSATE

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. จิรวัฒน์ ยงสวัสดิกุล, 151 หน้า.

ไข่ขาวเป็นแหล่งสำคัญของเพปไทด์ที่มีฤทธิ์การต้านออกซิเดชันซึ่งได้จากการย่อยด้วยเอนไซม์ อย่างไรก็ตาม สารยับยั้งเอนไซม์โปรตีเอสในไข่ขาวเป็นสาเหตุสำคัญที่ทำให้ระดับของการย่อย (Degree of hydrolysis: DH) ไข่ขาวเกิดขึ้นอย่างจำกัดส่งผลต่อการเกิดเพปไทด์ที่มีฤทธิ์ต้านออกซิเดชัน กระบวนการเตรียมตัวอย่างก่อนการย่อยเป็นหนึ่งในกลยุทธ์ที่ช่วยเพิ่มการเกิดปฏิกิริยาไฮโดรไลซิส และกิจกรรมการต้านอนุมูลอิสระของไฮโดรไลเสทไข่ขาว คลื่นเสียงความถี่สูง คลื่นไมโครเวฟ และการให้ความร้อนภายใต้แรงดันไอน้ำจึงถูกนำมาใช้ในการเตรียมตัวอย่างไข่ขาวดิบและไข่ขาวสุก ก่อนที่จะนำมาย่อยด้วยเอนไซม์ การเตรียมตัวอย่างด้วยคลื่นไมโครเวฟ และการฆ่าให้ความร้อนภายใต้แรงดันไอน้ำมีผลทำให้ระดับการย่อยต่ำกว่าตัวอย่างควบคุม ไฮโดรไลเสทไข่ขาวที่ผ่านการเตรียมตัวอย่างด้วยคลื่นไมโครเวฟมีระดับการย่อย และปริมาณโปรตีนสูงกว่าเมื่อเทียบกับการใช้ความร้อนภายใต้แรงดันไอน้ำ ไฮโดรไลเสทไข่ขาวดิบที่ได้จากการเตรียมตัวอย่างโดยการให้ความร้อนภายใต้แรงดันไอน้ำเป็นเวลา 5 นาที และ 30 นาที สามารถจับอนุมูลอิสระเอบีทีเอสและแสดงสมบัติรีดิวซ์ได้สูงสุดตามลำดับ ถึงแม้ว่าจะมีระดับการย่อยต่ำ นอกจากนี้ตัวอย่างเหล่านี้ยังมีพื้นที่ใต้กราฟของเอไมด์ I (1600-1700 cm^{-1}) และเอไมด์ II (1500-1600 cm^{-1}) ที่ได้จากการตรวจวัดด้วยฟูเรียร์ทรานส์ฟอร์มอินฟราเรดสเปกโตรสโคปี (FT-IR) มากที่สุดอีกด้วย ผลการวิเคราะห์องค์ประกอบหลัก (Principle component analysis: PCA) แสดงให้เห็นว่า FT-IR สเปกตราของไฮโดรไลเสทที่ผ่านการเตรียมด้วยคลื่นไมโครเวฟ และการให้ความร้อนภายใต้แรงดันไอน้ำแยกกลุ่มกันอย่างชัดเจน โดยพบการเปลี่ยนแปลงที่ชัดเจนในช่วงเลขคลื่น 1577-1589 cm^{-1} แสดงให้เห็นการเปลี่ยนแปลงของแถบเอไมด์ II และเลขคลื่นที่ 1400 cm^{-1} ที่สอดคล้องกับการเพิ่มขึ้นของปลายสายคาร์บอกซิล (C-terminal end) นอกจากนี้ความแตกต่างกันระหว่างสเปกตราของไฮโดรไลเซทตัวอย่างควบคุมและไข่ขาวที่ผ่านการเตรียมตัวอย่างนั้น เห็นได้เลขคลื่น 982-1075 cm^{-1} ซึ่งแสดงถึงการสั่นแบบหักงอ (bending) ของพันธะ CO และ CC ตัวอย่างไฮโดรไลเสทควบคุมมีความสามารถในการปกป้องเซลล์ (cytoprotection) และต้านอนุมูลออกซิเดชันภายในเซลล์ (cellular antioxidant activity :CAA) ในเซลล์ตับ HepG2 ที่ถูกกระตุ้นด้วยไฮโดรเจนเปอร์ออกไซด์ (H_2O_2) ได้สูงสุด ซึ่งสอดคล้องกับการเพิ่มขึ้นของปลายสายคาร์บอกซิลของเพปไทด์

นอกจากนี้การเตรียมตัวอย่างโดยใช้คลื่นเสียงความถี่สูงที่ความเข้ม 41.5 W/cm^2 ในไข่ขาวสุก แล้วย่อยด้วยเอนไซม์อัลคาเลส 10% (10% Alcalase:US-B10%) พบว่า เป็นสภาวะที่มี

ประสิทธิภาพดีที่สุด โดยมีระดับการย่อยและความสามารถในการจับกับโลหะสูงสุด ไฮโดรไลเซท US-B10% มีความสามารถในการกำจัดอนุมูลอิสระออกซิเจน (Reactive oxygen species: ROS) ภายในเซลล์ตับ HepG2 ที่ถูกกระตุ้นด้วยไฮโดรเจนเปอร์ออกไซด์ได้สูงสุด จากการศึกษาด้วยเทคนิค FT-IR พบว่า การเพิ่มขึ้นของปลายสายคาร์บอกซิลที่เลขคลื่น 1400 cm^{-1} สอดคล้องไปในทิศทางเดียวกับระดับการย่อยสูงสุด โดยเพปไทด์ส่วนใหญ่มีโมเลกุลขนาดเล็ก <200 Da ซึ่งอาจเป็นสาเหตุที่ทำให้ไฮโดรไลเซท US-B10% มีความสามารถในการต้านอนุมูลอิสระได้ดีที่สุด ดังนั้น ผลการศึกษานี้ชี้ให้เห็นว่าการใช้คลื่นเสียงความถี่สูงเป็นกระบวนการเตรียมตัวอย่างที่ดี สามารถเพิ่มระดับการย่อยและความสามารถในการต้านอนุมูลออกซิเดชันของไฮโดรไลเสทไข่ขาว

สาขาวิชาเทคโนโลยีอาหาร
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ASTRI SURYANI PRAWULANARI : EFFECT OF PRETREATMENT METHODS ON ANTIOXIDANT ACTIVITY OF EGG WHITE HYDROLYSATE. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 151 PP.

EGG WHITE/ULTRASOUND/MICROWAVE/STEAM STERILIZATION/ ANTIOXIDANT ACTIVITY/FOURIER-TRANSFORM INFRARED SPECTROSCOPY

Egg white is a potential source of antioxidant peptides which would be released upon enzymatic hydrolysis. However, the presence of protease inhibitors in egg white is appeared as a prime cause of the limited degree of hydrolysis (DH) of egg white hydrolysate. This would hamper the release of antioxidant peptides. Pretreatment process is one of the strategies to enhance the hydrolytic reaction and antioxidant activity of egg white hydrolysate. Ultrasound (US), microwave (MW), and steam sterilization (ST) were applied to both raw and cooked egg whites prior to enzymatic hydrolysis. Microwave and steam sterilization pretreatments resulted in hydrolysates with lower DH than the control (CB). Hydrolysates prepared from MW pretreatment on egg whites possessed higher DH and protein recovery as compared to those of ST. Although lower DH values were clearly observed, hydrolysates prepared from 5-min (ST-R5) and 30-min (ST-R30) ST pretreatment on raw egg whites showed the highest ABTS radical scavenging activity and reducing power activity, respectively. High amide I (1600-1700 cm^{-1}) and amide II (1500-1600 cm^{-1}) integral area was also noticed on FT-IR spectra. Principle component analysis (PCA)

demonstrated the FT-IR spectra of hydrolysates prepared from MW and ST pretreatments were clearly separated. The pronounced changes were observed at a wavenumber range of 1577-1589 cm^{-1}, indicating the changes in amide II bands and at 1400 cm^{-1}, corresponding to an increase in C-terminal ends. In addition, the different spectra of hydrolysates prepared from controls and pre-treated egg whites was evidenced by the minor contribution of CO,CC-bending groups at wavenumber of 982-1075 cm^{-1}. The CB hydrolysate possessed the highest cytoprotection and cellular antioxidant activity (CAA) in H_2O_2-induced HepG2-cells, in concomitant with an increase in C-terminal ends.

In addition, ultrasound pretreatment at intensity of 41.5 W/cm^2 subjected to cooked egg whites followed by 10% Alcalase hydrolysis (US-B10%) was the most potential condition yielding hydrolysate with the highest DH and metal chelation ability. The hydrolysate US-B10% showed the most effective intracellular ROS scavenger in H_2O_2-induced oxidative stress HepG2-cells. FT-IR study revealed that the highest exposure of C-terminal ends at 1400 cm^{-1} was positively correlated with the highest DH, which contained peptides with a high proportion of small molecular weight <200 Da. It could be a possible reason of the excellent antioxidant activity of hydrolysate US-B10%. The results suggested that ultrasound was a remarkable pretreatment to enhance DH and antioxidant activity of egg white hydrolysate.

School of Food Technology Student's Signature _____

Academic Year 2020 Advisor's Signature _____

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my advisor, Assoc. Prof. Dr. Jirawat Yongsawatdigul for the invaluable opportunity being his student, also the excellent guidance, patience and encouragement throughout my study course and research. He taught me to deal with research problems and to sharpen the way of thinking based on scientific knowledge. Without his immense supervision and treasured advice, my thesis would not have achieved and completed.

I am also highly appreciated Asst. Prof. Dr. Ratchadaporn Oonsivilai, Dr. Thanawit Kulrattanarak, Assoc. Prof. Dr. Parinya Noisa, and Dr. Kanjana Thumanu for the insightful feedback, suggestions, and serving as my committees.

I would like to acknowledge the National Research Council of Thailand under the project of Food Innovation for Safety and Value Creation of Nakhonchaiburin, Suranaree University of Technology (SUT3-305-61-12-06). One research one grant (OROG) scholarship was also highly appreciated. I am also thankful for Suranaree University of Technology and Synchrotron Light Research Institute for supporting the laboratory facilities.

I am also extremely grateful for all members of "JY group", colleagues and Indonesian friends for the unforgettable experiences, friendship, supports, and cherish times spent together during my study.

Finally, I would to thank my family and my love for the moral support, wise counsel and sympathetic ear which were also really influential in the completion of my thesis.

Astri Suryani Prawulanari

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

ABTS	=	2,2’-azinobis(3-ethylbenzothiazoline-6-sulphonic acid
AOC	=	Antioxidant capacity
APS	=	Ammonium persulfate
AsA	=	Ascorbic acid
ATR	=	Attenuated total reflectance
BSE	=	backscattered electrons
CAA	=	Cellular antioxidant activity
CB	=	Control boiled egg white hydrolysate
CB1%	=	Control boiled egg white without ultrasound pretreatment hydrolyzed by 1% Alcalase
CB10%	=	Control boiled egg white without ultrasound pretreatment hydrolyzed by 10% Alcalase
CKD	=	Chronic kidney disease
COO^-	=	Carboxylic group
CPD	=	Critical point drying
CR10%	=	Control raw egg white without ultrasound pretreatment hydrolyzed by 10% Alcalase
CR	=	Control raw egg white hydrolysate
DCF	=	2’,7’-dichlorofluorescin
DCFH-DA	=	2’,7’-dichlorofluorescin diacetate
DH	=	Degree of hydrolysis

LIST OF ABBREVIATIONS (Continued)

DMEM	=	Dulbecco's modified eagle medium
DMRT	=	Duncan's multiple range test
EDTA	=	Ethylenediamine tetraacetic acid
EW	=	Egg white
FBS	=	Fetal bovine serum
FICC	=	Ferrous ion chelating capacity
FPLC	=	Fast performance liquid chromatography
FRAP	=	Ferric reducing antioxidant power
FT-IR	=	Fourier transform infrared spectroscopy
HepG2-cell lines	=	Human hepatocellular carcinoma
H_2O_2	=	Hydrogen peroxide
kDa	=	Kilodalton
LY	=	Lysozyme
mM	=	Millimolar
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
MW	=	Microwave
MW-B85	=	85˚C microwave-treated boiled egg white
MW-B95	=	95˚C microwave-treated boiled egg white
MW-B110	=	110˚C microwave-treated boiled egg white
MW-B121	=	121˚C microwave-treated boiled egg white
MW-R85	=	85˚C microwave-treated raw egg white
MW-R95	=	95˚C microwave-treated raw egg white

LIST OF ABBREVIATIONS (Continued)

MW-R110	=	110˚C microwave-treated raw egg white
MW-R121	=	121˚C microwave-treated raw egg white
μM	=	micromolar
NEAAs	=	Non-essential amino acids
NH_3^+	=	N-terminal of amino group
OPA	=	*o*-phthaldialdehyde
OT	=	Ovotransferrin
OV	=	Ovalbumin
PCA	=	Principal component analysis
PI	=	Isoelectric point
ROS	=	Reactive oxygen species
SDS-PAGE	=	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	=	Secondary electron
SEM	=	Scanning electron microscopy
SN-TCA	=	Trichloroacetic acid precipitation
ST	=	Steam sterilization
ST-B5	=	5 min-steam sterilization-treated boiled egg white
ST-B15	=	15 min-steam sterilization-treated boiled egg white
ST-B30	=	30 min-steam sterilization-treated boiled egg white
ST-R5	=	5 min-steam sterilization-treated raw egg white
ST-R15	=	15 min-steam sterilization-treated raw egg white

LIST OF ABBREVIATIONS (Continued)

ST-R30	=	30 min-steam sterilization-treated raw egg white
TEAC	=	Trolox equivalent antioxidant capacity
TEMED	=	N,N,N',N'-tetramethyl-ethylenediamine
TNBS	=	2,4,6-trinitrobenzene-1-sulfonic acid
US	=	Ultrasound
US-B1%	=	Ultrasound treated boiled egg white hydrolyzed by 1% Alcalase
US-B10%	=	Ultrasound treated boiled egg white hydrolyzed by 10% Alcalase
US-R1%	=	Ultrasound treated raw egg white hydrolyzed by 1% Alcalase
US-R10%	=	Ultrasound treated raw egg white hydrolyzed by 10% Alcalase

CHAPTER I

INTRODUCTION

1.1 Background and significance of the study

Chicken egg is well-known as a functional food which contains health promoting agents, including essential amino acids, vitamins, minerals, proportionally balanced of saturated and unsaturated fatty acids without any trans-fats (Molnar & Szollosi, 2020). The abundant amount of proteins scattered in egg white including ovalbumin, ovotransfferin, lysozyme, ovomucin, and ovomucoid offers health benefits especially for cancer or chronic kidney disease (CKD) patients. Previous publication reported that 40% chemotherapy patients had to deal with leucopenia; the condition of patients with the lack number of leukocytes. Egg white is commonly suggested for food diet in cancer patients who receive chemotherapy to help preventing leucopenia (Supraset et al, 2016). Albumin is also one the most prominent proteins in human body to maintain osmotic balance endogenously as its main function. Albumin metabolism is synthesized by the liver and only certain amount is filtered by glomerular membrane of kidney. Studies revealed that healthy people lose merely 10-20 mg/day of albumin through urinary process. The intensive losses of albumin often cause hypoalbuminemia; albumin level is lower than 3.0 g/dL. The main cause of hypoalbuminemia has been established over the years, either caused by the lack of hepatic albumin synthesis or abnormality of kidney glomerular filtration. The perpetual condition of hypoalbuminemia excessively leads to CKD and dialysis

treatment or kidney transplantation is necessary to suppress mortality rate. Therefore, exogenous albumin intake from egg white can be helpful to overcome the lack of albumin for the patients (Mazzaferro & Edwards, 2020; Nicholson et al, 2000).

Meanwhile, it has been established that egg white is also a high quality source of bioactive peptides which exhibits many bioactivities including antioxidant, antimicrobial, and antihypertensive activity. However, the peptides would remain in native protein and only be released during enzymatic hydrolysis. Enzymatic hydrolysis is a process of peptide bonds cleavage by proteases, yielding protein hydrolysate. Protein hydrolysate contains various chain lengths of peptide and amino acids. Short peptides are believed to be easily absorbed through intestinal digestion and exerted its bioactivity (Memarpoor-yazdi et al, 2012). Several proteases have been used to prepare egg white protein hydrolysate with antioxidant and antihypertensive activities, such as trypsin (Chen et al, 2012), papain (Chen & Chi, 2011), gastrointestinal enzymes used for lysozyme (Rao et al, 2012), microbial enzymes (Protamex, Flavorzyme, and Alcalase) subjected to ovomucin (Chang et al, 2013) and thermolysin, pepsin, and combination of two enzymes on ovotrasferrin (Shen et al, 2010). Mine et al (2004) also reported the use of pepsin and trypsin to hydrolyze lysozyme generating hydrolysate with antimicrobial activity.

Among the various bioactive activities, antioxidant is then claimed as an important bioactivity because it has ability to trigger other activities, such as ACE-inhibitory and antimicrobial activity (Sanchez and Vazquez, 2017; Zhou et al, 2016). Antioxidant is defined as a molecule or substance when presence in low concentration has the ability to prevent or reduce cellular damage. Human body naturally produces antioxidants as defense system against free radicals arising as a consequence of

biological and metabolism processes. The condition of insufficient antioxidant to counter the excessive amount of free radicals, usually known as oxidative stress, is responsible for various health problems including cancer, heart and chronic kidney diseases, neurological disorders, atherosclerosis and diabetes. Since free radicals are attacking on any cellular components with indiscriminate reactions, thus the substantial amount of antioxidant is necessary to build an extensive protection (Mamta et al, 2014; Young & Woodside, 2001). Alternatively, there are several synthetic antioxidants such as nitroxides, coenzyme Q analogues, Mn-porphyrin superoxide dismutase mimics, and dietary supplements containing ascorbate (vitamin C), tocopherols (vitamin E), carotenoid or polyphenols, as an exogenous intake to comply the need of antioxidant (Augustyniak et al, 2010).

However synthetic antioxidants are considered less safe than natural antioxidants produced by plant- or animal-based foods. In June 2004, the first International Congress of Antioxidant Methods regarding to the assessment of antioxidant capacity (AOC) was convened to concur the standardized methods of AOC (Prior et al, 2005). Antioxidant activity would not be represented only on one single mechanism, a number of antioxidant activity assays are subsequently demonstrated over the years. The antioxidant activities occur by hydrogen donating, electron transfer, transition-metal ion chelating, and active-oxygen quenching. Nowadays, chemical-based antioxidant assays are considered as an easy and short analysis time to evaluate antioxidant activity of products by certain mechanisms including free radicals scavengers, reducing power, and metal chelation ability. ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)) assay can be used as a routine antioxidant capacity assay. The principle of this technique is based on antioxidant

ability to neutralize ABTS radical either via transfer electron or donate hydrogen atom. $ABTS^{\cdot+}$ radical is soluble both in water and organic solvents, enabling wide range of application in hydrophilic and lipophilic samples. Ferric reducing antioxidant power (FRAP) assay was firstly developed by Benzie and Strain (1996) to determine the ability reduce ferric ion which occurs at low pH via electron transfer. The increase of color intensity will be monitored at 593 nm (Prior et al, 2005; Shahidi & Zhong, 2015). Ferrous ion chelating capacity (FICC) assay evaluates the chelating ability of antioxidant compound against ferrous ion (Fe^{2+}) which acts as an important catalyst for oxidation. Antioxidant compound with chelation ability would be able to disrupt the reaction of radicals. The metal-antioxidant complex will be quantified at 485 nm for ferrous sulfate or 562 nm for ferrozine (Sudan et al, 2014).

Owing to the attention of antioxidant-based peptides has rapidly expanded, some studies demonstrated the use of pre-hydrolysis process to accelerate the process and to enhance its bioactivity. For example high-intensity pulsed electric field applied prior to Alcalase hydrolysis of hen lysozyme resulted in hydrolysate with high reducing power ability at peptide size lower than 1 kDa (Lin et al, 2012), hydrolysate obtained from 10-min heat-induced egg white showed an increase in degree of hydrolysis (DH) after combination of trypsin and α-chymotrypsin (Plancken et al, 2003). Ultrasound pretreatment on egg white followed by several proteases hydrolysis showed the improvement on functional and antioxidant activity of the hydrolysates (Stefanovic et al., 2014). In addition, 15-min ultrasound subjected to egg white generating hydrolysate with DH of 39.01% after hydrolyzed by papain (Stefanovic et al, 2018).

Aside from pre-treatment, the selection of enzyme is a major factor affecting the performance of hydrolysis. Alcalase is endopeptidase which has ability to cleave

internal polypeptide chains of the peptide bonds with broad specificity. Alcalase is known to preferentially disrupt on terminal hydrophobic amino acids and has been reported to produce anti-oxidative peptides (Stefanovic et al., 2014). This study attempts to use several pre-treatment technologies subjected to the whole egg white as the publications concerning on this topic are found limited. Ultrasound (US), microwave irradiation (MW), and steam sterilization (ST) have attracted the attention. Similar concepts are offered by the pre-treatment methods in order to optimize enzymatic reaction during hydrolysis which refers to a shorter hydrolysis time and to enhance degree of hydrolysis through different mechanisms. US is proven to be involved in hydrogen bonds conformation and hydrophobic interactions of protein by giving actions such as pressure, shear, and cavitation (Stefanovic et al, 2018). The actions of US enable the exposure of protein active sites which facilitate enzyme to access them easily and smaller the particle sizes of protein which could result homogenization. Further, the degree of hydrolysis goes higher due to the faster hydrolysis process and the better accessibility of enzyme during hydrolysis (Mason et al, 1996). MW provides energy to be quickly absorbed by dipole compounds and cause rotational vibration within electric field of microwave. As effects, the temperature rises up and could disrupt the protein aggregation and promote hydrolysis process (Vanier, 2013). ST produces high pressure and temperature to extensively denature the protein, which could enhance enzyme accessibility (Sezdi & Yoleri, 2013).

Pre-treatment methods are hypothesized to have significant changes on the structure of both egg white and hydrolysates. Therefore, microstructure appearance of egg white after subjected to pre-treatments is observed by scanning electron microscopy (SEM) and the structural alteration of the hydrolysates is assessed by

Fourier transform infrared spectroscopy (FT-IR). SEM is working under electron emission principle and provides information of the surface area of the pre-treated egg white in a sharp and detail grey-scale images (Kannan, 2018). FT-IR provides information about folding, unfolding, and aggregation of proteins which is related to the changes of secondary structure of proteins by characterizing protein infrared absorption using the amide modes (Barth, 2007). Thus, these 2 techniques are suitable to monitor structural changes of a protein substrate and hydrolysate product.

Chemical antioxidant activity is not relevant as it does not mimic biological metabolism system. Cell culture model is closer to *in vivo* and found to be cheaper, quick enough, and provide reasonable metabolism prediction (Wolfe & Liu, 2007). Cellular antioxidant activity (CAA) is developed to elucidate antioxidant activity under biological systems by using cell culture. Human hepatocellular carcinoma (HepG2) cells are often selected to observe liver metabolism and toxicity and to conduct antioxidant activity assessment of compounds, in a reason that liver tissues are a first place which could absorb antioxidant compounds and distribute its biological effects (Goya et al, 2010). Therefore, the study of cytoprotection and intracellular reactive oxygen species (ROS) inhibition properties of the egg white hydrolysates is embarked in cultured HepG2-cells.

Cytoprotection is ability of antioxidants to protect cells in the presence of under free radical (Mehta et al, 2018). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay is the most common method to determine cell viability as reduction of MTT by viable cells metabolic system via mitochondrial or cytosolic enzymes form purple-blue formazan crystal which dissolves in dimethyl sulfoxide (DMSO) or isopropanol. This can be measured at 570 nm (Mc.Gaw et al, 2014).

DCFH-DA (2',7'-dichlorofluorescin diacetate) is a probe to estimate CAA due to the ability to penetrate membrane cells. In the presence of free radicals, DCFH-DA will be converted to DCF (2',7'-dichlorofluorescin). Once antioxidant substance is added to the system, it competes with radicals to inhibit DCF formation. Low fluorescence intensity indicates the success of antioxidant over radicals in preventing oxidation. This is monitored at the excitation wavelength of 485 nm and emission wavelength of 538 nm (McDowell et al, 2011).

Thus, the purpose of this study is to evaluate antioxidant activity from hydrolysates of egg white pre-treated by US, MW, and ST, as compared to the controls based on ABTS, FRAP and FICC assays. CAA on HepG2 cells is also carried out to emphasize the antioxidant activity. Microstructure after pre-treatment of the egg white is observed under SEM. The characterization on molecular weight distribution, SDS-PAGE pattern, and structural changes of the hydrolysates are also further investigated. Principal component analysis (PCA) is applied to obtain information of factors affecting antioxidant activity through second derivation of FTIR spectra from the hydrolysates.

1.2 Research objectives

1.2.1 To elucidate the effects of ultrasound, microwave irradiation, and steam sterilization on degree of hydrolysis and antioxidant activity both in chemical and cellular level of the egg white hydrolysate

1.2.2 To study the correlation between antioxidant activity and secondary structure alteration

1.3 Research hypotheses

Pre-treatment methods are likely to increase enzyme accessibility during hydrolysis by unfolding protein structure to be further cleaved by the enzyme. As an effect, egg white hydrolysate with high degree of hydrolysis is produced, indicating smaller peptides to exert high antioxidant activity. Moreover, secondary structure is suitable approach to be studied, since the alteration could emphasize the main factors affecting antioxidant activity.

1.4 Scope of the study

1.4.1 Pre-treatment methods were applied to both raw and boiled egg white prior to Alcalase hydrolysis and the hydrolysate was compared to the control. Degree of hydrolysis and chemical antioxidant activity of the hydrolysate were determined.

1.4.2 Ultrasound was used to expose protein active sites before enzymatic hydrolysis. Subsequently, the microstructure and structural changes, degree of hydrolysis, yield protein, molecular weight distribution, chemical and cellular antioxidant activity were investigated.

1.4.3 Microwave irradiation and steam sterilization also promoted protein unfolding and enzyme accessibility. The microstructure and structural changes, degree of hydrolysis, yield protein, chemical and cellular antioxidant activity were further analyzed.

1.5 Expected results

Several pre-treatment methods subjected to egg white prior to enzymatic hydrolysis may bring about a distinct antioxidant activity of each hydrolysate

obtained from pre-treated egg white which, if possible, is higher than control hydrolysates. In addition, structural changes of the egg white due to pre-hydrolysis treatment are expected to contribute in exerting antioxidant activity. Regarding to the antioxidant activity, the result could exhibit beneficial information on the development of peptides-derived egg white products which has impact in human health.

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CHAPTER II

LITERATURE REVIEWS

2.1 Egg White

Chicken egg, one of the most nutritious sources for the need of human protein intake, considering the protein content reaches up to 10-11%. Many countries have been found rising in chicken egg demands since they frequently processes eggs as primary dishes and delicacies snacks. The use of egg protein for additional ingredient in food products is mandatory choice as often, either to enhance texture or flavor as well. Looking back at the historical period, Roman times in nineteenth century was the first starting time of chicken egg consumption and industry development. The following years, demands and health concern begin to increase, so that scientists attempt to figure out the efficacy of chicken egg towards human health throughout years of research up to now. Studies stated that most of protein in chicken egg is scattered in the egg white around, while egg yolk comprises mostly lipid. They also revealed that egg protein becomes one of functional food which provides health promoting agents, such as bioactive peptides and amino acids (Elson & Vale, 2011; Guha et al, 2018; Zaheer, 2015).

Egg white is composed of 70% proteins, in which ovalbumin, ovotransferrin, ovomucoid, ovomucin, and lysozyme are the major proteins and avidin (0.05%), cystatin (0.05%), ovomacroglobulin (0.5%), ovoflavorprotein (0.8%), ovoglycoprotein (1.0%), and ovoinhibitor (1.5%) are the minor proteins (Kovacs-Nolan, Phillips, &

Yoshinori, 2005; Vachier, Piot, & Awade, 1995; Liao, Jahandideh, Fan, Son, & Wu, 2018).

Ovalbumin is the main component of egg white for about 54% of total egg white protein, considered as glycoprotein with the presence of carbohydrate group attached to N-terminal which plays critical role in gelation properties. Molecular weight of ovalbumin was found at 45 kDa with 386 amino acids, in which N-terminal amino acid is acetylated glycine and C-terminal is proline. Ovalbumin also consists of three components according to the availability of phosphate group, namely A1 (has 2 phosphate groups), A2 (has 1 phosphate group), and A3 (has no phosphate group) in ratio of 85:12:3, respectively (Stadelman and Cotterill, 1995).

Ovotransfferin accounts for 12% of total egg white protein is monomeric glycoprotein containing 700 amino acids, 15 disulfide bonds, isoelectric point (pI) of 6.1, and molecular weight of 80 kDa with the ability as iron transporter and iron scavenger, furthermore, it reported to have high potential as antimicrobial agent. Ovotransferrin consists of N-terminal and C-terminal domain and one transition metal atom, including Fe(III), Cu(III), and Al(III) and is found in two forms, apo-form; a metal-free ovotransferrin and holo-form; iron-bound ovotransferrin in which the holo-form can bind to two iron molecules at pH more than 7 and be released at pH less than 4.5 (Ko & Ahn, 2008; Kurokawa, Mikami, & Hirose, 1995; Ko and Ahn, 2008; Kurokawa et al, 1995; Stadelman and Cotterill, 1995).

Lysozyme is single polypeptide chain with 129 amino acids and molecular weight of 14.4 kDa and the widely distributed enzyme within egg white which amounts to approximately 3.5% of total egg white protein. Lysozyme contains lysine in N-terminal and leucine in C-terminal and demonstrated antimicrobial activity

because of its capability to hydrolyze bacterial cell walls which consist of peptidoglycan with β(1-4) linkage between N-acetylmuraminic acid and N-acetylglucosamine. In most cases, this antimicrobial activity has been found the effectiveness in gram positive bacteria, such as *Bacillus stearothermophilus*, *Clostridium tyrobutyricum*, and *Clostridium thermosaccharolyticum*. Lysozyme becomes the most soluble and thermal stable in dimer form compared to other egg white proteins due to its 4 disulfide bond linkages, also known as strong basic protein due to its pI of 10.7 and high potency to greatly bind to other negatively charged protein including ovomucin (Abeyrathne, Lee, & Ahn, 2013; Kovacs-Nolan, Phillips, & Yoshinori, 2005).

Ovomucin contributes to 3.5% of total egg white protein which maintains the viscosity of egg white and considered as sulphated glycoprotein. Regarding to solubility, ovomucin is highly insoluble in neutral pH, conversely ovomucin posses its solubility in alkaline condition and with addition of denaturing agents or reducing agents (Omana, Wang, & Wu, 2010). Ovomucin is responsible for the changes of egg white thinning during storage since it is mostly associated with the degradation of lysozyme-ovomucin complex or reduction of the disulfide bonds. Structurally, ovomucin comprises two components with carbohydrate attached, namely α-ovomucin is carbohydrate-poor component with 11-15% carbohydrate and β-ovomucin is the carbohydrate-rich with 50-57% carbohydrate with various large molecular weight. α-ovomucin consists of glutamic acid and aspartic acid and has two subunits with molecular weight of 150 kDa for α1 and 220 kDa for α2, and β-ovomucin consists of threonine and serine with molecular weight of 400 kDa (Huopalahti, Lopez-Fandino, Anton, & Schade, 2007; Omana & Wu, 2009).

Ovomucoid comprises not only 186 amino acids and 9 disulfide bonds, but also accounts for 11% of total egg white protein which was discovered as protease inhibitor and the food allergen in egg white. It is also recognized as the most glycosylated protein in egg white combining with theoretically molecular weight of 20.1 kDa, but the band visualized in SDS-PAGE showed in range of 30 to 40 kDa and isoelectric point (pI) at 4.82, respectively. Ovomucoid appears as single-headed inhibitor with only bind to one molecule of protease, later on, its proteolytic activity has been found to inhibit digestive enzymes such as trypsin or chymotrypsin, thereby ovomucoid is further well-known as trypsin inhibitor (Abeyrathne, Lee, & Ahn, 2013; Huopalahti, Lopez-Fandino, Anton, & Schade, 2007).

2.2 Enzymatic hydrolysis

Hydrolysis of protein can be conducted by chemical or enzymatic methods. Mechanism in conducting chemical hydrolysis seems to be more complicated than enzymatic does, because of its high cost for both reagents and instruments, also some reagents are presumably contained hazardous risks either for human health or environments (Li et al, 2019). Chemical hydrolysis involves alkaline or acid which is difficult to control the reaction. Acid hydrolysis condition is using 6 M HCl at 110 ˚C for 24 h to measure amino acids content while damage tryptophan. Alkaline hydrolysis could lower the content of cystine, arginine, threonin, serine, isoleucine, and lysine, while produce amino acids residues, namely lysinoalanine or lanthionine (Tavano, 2013). Therefore, enzymatic hydrolysis is currently chosen for hydrolysate production, since this process would avoid the reduction of nutritional value. In addition, advantages offered by this process included non chemical residual content,

ease control of conditions, considered as fast reaction, reproducible method, decrease size of peptides, molecular charge changes, enhance interaction of protein-protein, improve functional and nutritional properties of proteins, and result in high yield and quality of end-products (Barberis et al, 2018; Kuddus, 2019; Marciniak et al, 2018).

Enzymatic hydrolysis is defined as the process to hydrolyze peptide bonds from native protein using proteases. Determination of the number of peptide bonds cleavage during hydrolysis is further expressed as degree of hydrolysis (DH). There are several methods to calculate degree of hydrolysis (DH), such as pH-stat, osmometric, soluble nitrogen after trichloroacetic acid precipitation (SN-TCA), 2,4,6-trinitrobenzenesulfonicacid (TNBS), *o*-phthaldialdehyde (OPA), amino acid nitrogen, and formol titration methods (Rutherfurd, 2010). TNBS is firstly used method to evaluate peptide content since this method is based on the reaction between TNBS reagent and *N*-terminal amino groups at room temperature at pH 7-8, which will convert the free *N*-terminal amino acids to trinitrophenyl-amino acid derivatives, producing yellow color and monitoring its absorbance at 340 nm. Available amino groups largely depend on the presence of ε-amino group of lysine and α-amino groups of N-terminal amino acids in proteins. 2,4,6-trinitrobenzene 1-sulfonic acid (TNBS) reagent can be able to react with primary amino acid group specifically. This method is used to determine the concentration of α-amino group in hydrolyzates and lysine contents in proteins, even the presence of few lysine residues in low molecular weight proteins. Therefore, TNBS assay is used to determine degree of hydrolysis by measuring the concentration of primary amino group released during hydrolysis (Adler-Nissen, 1979).

Since enzymatic hydrolysis involves enzyme namely protease/peptidase/ peptide hydrolase as catalyst to promote the peptide bonds cleavage reaction, then the selection of appropriate enzyme has to be the main focus prior to hydrolysis. Also, main factors affecting the enzyme should be noticed in order to raise the optimum condition of hydrolysis, such as preparing exact pH, temperature, and substrate-enzyme concentration depending on the enzyme requirement (Noman, et al., 2018; Tapal & Tiku, 2019). Sources of enzyme can be obtained from plant, animal, and microorganism with different characteristics and biological activities output for each source. Animal source enzyme has broader range of specificity during cleavage the bonds than plant source, for example that papain is able to cleave only at phenylalanine or lysine bonds, but pancreatin is competent in cleaving at tryptophan, arginine, tyrosine, leucine, phenylalanine, and lysine bonds. Microbial source enzyme provide divers characteristics which are not owned by the other two sources, including various kind of enzyme activity, inexpensive cost of production, can be predicted and controlled. Microorganism proteases as well as Alcalase, Neutrase, Protamex, and Flavorzyme are currently available on market and widely used for industrial field. Even though coming from same sources, still they produces varies of action and specificity. Alcalase is alkaline protease originated from *Bacillus licheniformis* and classified as endopeptidase with broad range of peptide bonds specificity. Flavorzyme, enzyme which releases its action under neutral or acidic condition is obtained from *Aspergillus oryzae* and known as fungal protease. Neutrase is extracted from *Bacillus amyloliquefaciens* and categorized as neutral protease with broad range specificity (Chew et al, 2019; Kuddus, 2019).

The selection of proper enzyme for hydrolysis is needed since each enzyme has specific cleavage site resulting in different sequences of amino acid and leading to different peptide bioactivity (Huang et al, 2016; Ruan et al, 2013). Alcalase, trypsin, pepsin, pancreatin, and thermolysisn are common used enzymes to release antioxidant effect from hydrolysate (Liao et al, 2018). Endopeptidase enzymes cleave peptide bonds right in the middle of the chains and exopeptidase enzymes cleave amino acids at the end position of peptide chains. Alcalase is classified as endopeptidase enzyme with broad range activity to hydrolyze protein into short chain peptide and high antioxidant activity (Zhang et al, 2018). Samaranayaka & Li-Chan (2011) have reported the use of Alcalase enzyme to reveal antioxidant activity from many sources of foods-derived hydrolysate, such as lecithin-free egg yolk (Park et al, 2001), chickpea protein (Li et al, 2008), soybean hydrolysate (Zhang et al, 2018).

2.3 Ultrasound

Ultrasound (US) is acoustic waves which has frequency higher than human hearing range; about >16 kHz. According to frequency and intensity, ultrasound can be classified into low and high power, in which low power US consists of low intensity below 1 W/cm^2 with high frequencies range of 1-10 MHz, otherwise, high power US consists of high intensity up to 1000 W/cm^2 with low frequencies range of 16-100 kHz. High power US is usually used for physically, chemically, and mechanically disruption purposes including extractions, depolymerization, aggregates breakage into nanoscale, deflocculating, and colloidal formation (Koshani & Jafari, 2019). While low power US is often used for detection purposes, including sonography, storage and processing quality control of foods, and ingredients

observations of fermented-derived food (Awad, Moharram, Shaltout, Asker, & Youssef, 2012).

Figure 2.1 represents the main reaction produced by ultrasound, namely cavitation. When low frequency is applied to the sample, ultrasound will generate sound/acoustic waves, furthermore, interaction between bubbles of samples and acoustic energy occurs, resulting in an acoustic cavitation. Cavitation can be defined as phenomenon of formation, increase size, and subsequently rupture of the bubbles because of given compression and fluctuation pressure of the acoustic waves. After bubbles rupture, cavitation obtains physical and chemical effects to the samples, such as shock waves, agitation, turbulence, and microjetting (Bhangu & Ashokkumar, 2016). Frequency can be equalized to the capacity of cavitation effects on the bubbles. Once the frequency rises up, the cavitation effect will occur quickly, resulting in fast bubbles rupture. Intensity is also involved in cavitation effect, in which high intensity would produces larger and more stable bubbles (Nora & Borges, 2017).

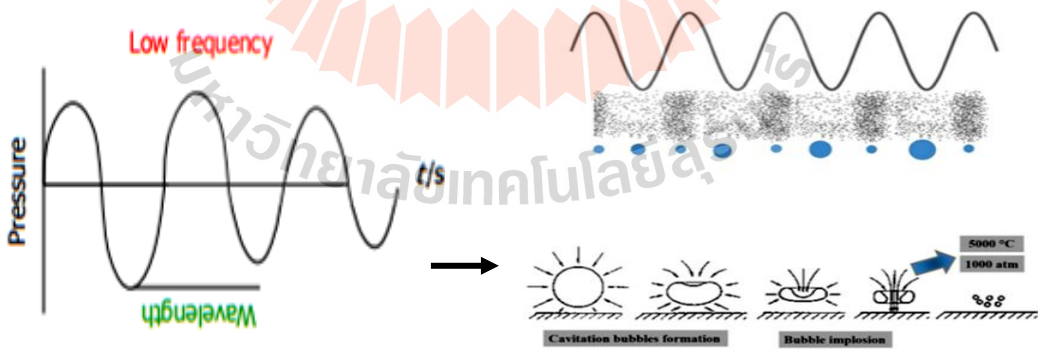


Figure 2.1 Cavitation effect generated by US into the gas bubbles.

Source: Nora & Borges (2017).

The energy distribution from ultrasound into the samples can be expressed calorimetrically as ultrasound power (W) or ultrasound intensity (W/cm^2), or acoustic energy density (W/cm^3 or W/ml). However, ultrasound power is commonly used to determine the absolute level of ultrasonic power (P) and power intensity (I) given to the samples by following the equation:

$$P = m\, C_p\left(\frac{dT}{dt}\right) \qquad \text{(i)}$$

$$I = \frac{P}{S_a} \qquad \text{(ii)}$$

whereas m is the mass of liquid sample (g), C_p is the specific heat capacity at constant pressure (3.8 J/gK^{-1}), dT/dt is the increase of temperature during sonication, and S_a is the surface are of the ultrasonic probe (1.1304 cm^2) (O'Sullivan et al 2015; Ozuna et al, 2015).

There are two type of ultrasound instruments; ultrasonic bath and probe as shown in Figure 2.2. In fact, the components of ultrasound instrument provide a clear explanation about the working principle of ultrasound. Generally, both US bath and probe comprise of three major components with different functions, which are a generator to generate electricity with power set up, a transducer to convert electric waves into acoustic waves at certain frequency through vibrational motion, and an emitter (titanium probe or bath) to transfer the acoustic waves to sonicated medium (Koshani & Jafari, 2019).

Ultrasound technology is an attractive discovery for researchers, considering its characteristics, such as environmentally friendly, not involving thermal, low time consuming, and used to optimizing quality and safety of products (O'Sullivan et al, 2015). High power ultrasound is often applied to food fields, especially for food-

derived proteins since US technology has a great advantage in accelerating physical or chemical properties of food materials by generating acoustic flow, shock waves, turbulence, pressure, and shear upon material only in few seconds or minutes. High power ultrasound promotes protein structure alterations or aggregate forming in which exposure of protein cleaving-site, hydrogen bonding, electrostatic and hydrophobic interaction may be the most likely cause of the breakage of protein structure (Alarcon-Rojo et al, 2018). High power US can be applied to US probe with frequencies range of 20-40 kHz, while high power US is only applicable for US bath at frequency around 40 kHz (Stefanovic, et al., 2018).

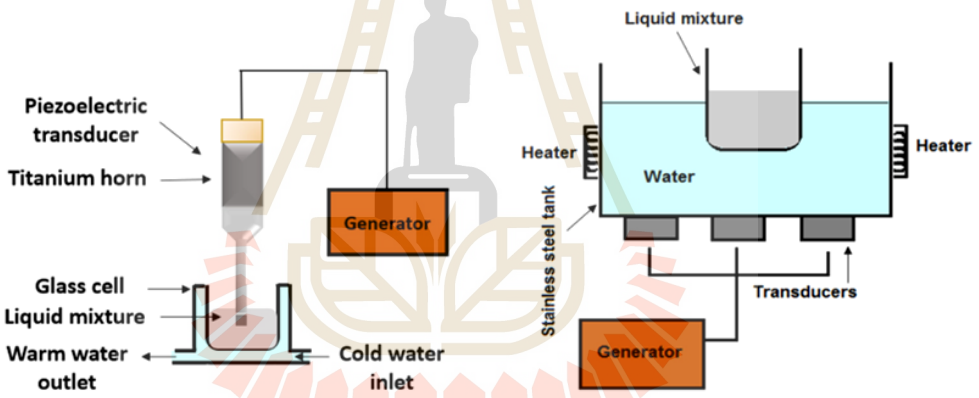


Figure 2.2 Illustration of ultrasonic probe (a) and ultrasonic bath (b).

Source: (Koshani & Jafari, 2019).

Expensive cost might belong to ultrasonic probe rather than ultrasonic bath, but the ability to set up varying parameters and advantages offered by US probe could be consideration on proper instrument selection. US probe is able to control sonication time, intensity, and frequency, also it produces satisfactory intensity in order to elevate structural conformation and shorter reaction since the probe will be directly sonicated to the samples. Besides, the possibility to lower the increased temperature

can be done by putting on cooling ice or mantle (Koshani & Jafari, 2019). Stefanovic et al (2014) reported that egg white protein hydrolyzed by Alcalase with US probe pre-treatment at 20±0.2 kHz for 10 to 15 min showed the increase of proteolytic activity and enhanced the degree of hydrolysis.

2.4 Microwave irradiation

Microwave is low energy waves which located in wavelength range of 1 mm-1 m and frequencies range of 0.3-300 GHz. Microwave electromagnetic radiation consists of electric and magnetic components to conduct energy absorption interaction. Samples must dissolve in polar solvent which consists of dipole moment, in which it would be able manage itself to spin within the rotational moves produced by the field. So that, dipole moment could absorb microwave energy and use the energy to heat right on the sample, therefore, the increased temperature will be evenly same within sample. Pressure system normally equips microwave instrument in order to generate faster temperature elevation, and resulting in only seconds or minutes heating. This phenomenon is usually called as dipolar polarization mechanism. An illustration of the effort of dipole moment to spin under rotational field is shown in Figure 2.3 Invalid source specified.

Figure 2.3 Dipolar polarization mechanism.

Source: Nora & Borges (2017).

Radiation frequency setting and viscosity of samples are directly affected energy absorption process through rotational moves of dipolar molecules. The higher frequency, the faster rotation produced by the field, therefore, the dipole moment unable to control itself to follow field rotation and temperature goes down because of the absence of energy absorption. Oppositely, at low frequency, dipole moment would be easily followed rotational moves of the field, and energy absorption occurs (Lidstrom et al, 2001).

Microwave method is widely used prior hydrolysis to overcome conventional hydrolysis method which is time consuming, since it offers high end products, easy and short time process, and maintain antioxidant activity from samples and elevate degree of hydrolysis (Huang et al, 2016). As mentioned previously, when dipole moment of sample rotates around the electric field of microwave, the temperature will be definitely increased, then the destruction of peptide bonds and exposure of protein active site could not be avoided (Vanier, 2013). Solvent selection of samples has also to be decided thoroughly to prevent the risk of toxicity and any explosions during process (Lin et al, 2005).

High power microwave radiation up to 250 W demonstrates the decrease in peptide size, which also affects the molecular weight of peptide, lower hydrolysis time, elevate the degree of hydrolysis, and produce high antioxidant activity from sea cucumber (*Acaudina molpadioides*) collagen (Jin et al, 2019; Li, Li, Lin, Yang, & Jin, 2019). The improvement of degree of hydrolysis along with stability activity of antioxidant from mackerel *Scomberomorus niphonius* protein were found after microwave digestion with power of 400 W for 5 min (Huang et al, 2016). Microwave showed its ability to increase the degree of hydrolysis and minimize allergicity of

bovine whey protein hydrolysate produced by Papain, Corolases 7089, PN-L 100, Alcalase, and Neutrase enzymes (Izquierdo et al, 2008).

2.5 Steam Sterilization

Steam sterilization or autoclave is a chamber that produces steam working by the combination reaction of temperature and pressure. The steam is generated due to the boiling process of water filled in the chamber by the sufficient pressure. Subsequently, the chamber is surrounded by steam pressure to reach the desired temperature; 121 or 135˚C. Temperature and pressure are kept stable during sterilization process (Sezdi & Yoleri, 2014).

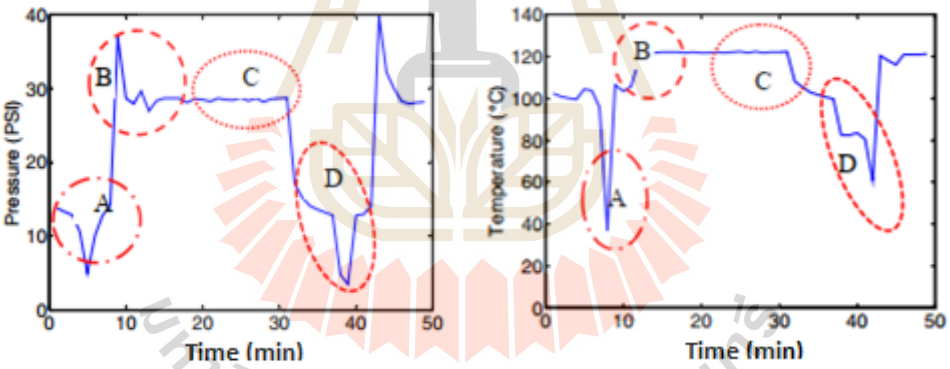


Figure 2.4 Stages of sterilization process.

Source: Angel et al. (2016).

As presented in Figure 2.4, at the beginning chamber produces uniform temperature and pressure inside. Sterilization process is started with the surged of pressure that leads to the increase of sterilization temperature. Temperature and pressure are maintained in 121˚C and 29 Psi respectively in nearly 20-30 minutes. At

the end of the process, chamber is pushing down the steam out around, resulting pressure decrease and temperature as well (Angel, Viola, Vega, & Restrepo, 2016).

Protein denaturation can be defined as structural alterations of protein from their native state, either by unfolding its secondary and tertiary structure or forming aggregation. Literatures reported that thermal processing and protein denaturation are interconnected. Moreover, the fact that steam sterilization has combination of temperature and pressure, its effect could lead to massive destruction of protein structure. Sterilization process is responsible for turning out intermolecular β-sheets into random coil in abundance, further leading to protein aggregation. In some cases, compound containing protease inhibitor may have high digestibility after sterilization as inactivation of the inhibitor simultaneously occurs during process which subsequently optimizes proteolysis using protease enzyme. However, formation of protein aggregation is sometimes willing to inhibit accessibility of enzyme as new bonds formation begins. Unfolded protein with non-covalent and/or disulfide bonds or electrostatic and hydrophobic amino acids are the examples of formation involving low protease digestibility (Salazar-Villanea, Hendriks, Bruininx, Gruppen, & van der Poel, 2016).

2.6 Fourier Transform Infrared (FT-IR) spectroscopy

Protein is divided into four structural levels, which are primary, secondary, tertiary, and quaternary (as shown in Figure 2.5). Primary structure is considered as the sequence and amount of amino acids content in protein, further, is connected by covalent bonds of peptide linkages. Secondary structure represents the configuration of amino acids and tertiary structure is defined as three dimensional structure of

protein. Secondary and tertiary structures are maintained by hydrophobic interactions, hydrogen and electrostatic bonds, and

van der Waals forces. Quaternary structure is obtained by formation of one functional protein from aggregation of polypeptides, through given forces, such as hydrogen and electrostatic bonds, hydrophobic interaction, and van der Waals. When subunits in quaternary structure are dissociated, protein is going to lose its function (Vasudevan et al, 2017).

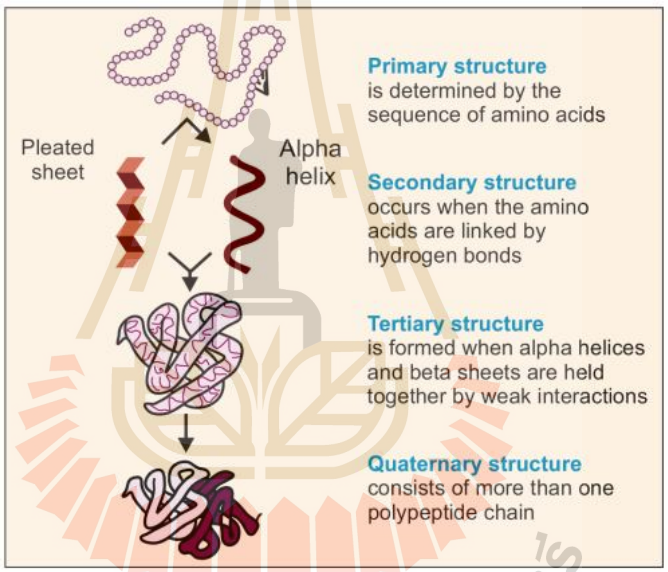


Figure 2.5 Protein Structure.

Source: Vasudevan et al. (2017).

Molecular vibration is the basic idea of infrared spectroscopy, since many bonds exhibit various vibrations, including rotting, twisting, and stretching. The energy of molecular vibration is similar to the energy of infrared region in electromagnetic spectrum (Haris & Severcan, 1999). The changes of secondary structure become crucial for overall structure of protein. Currently, one of techniques

to study protein alteration is Fourier transform infrared spectroscopy (FTIR), due to its ability in providing information about folding, unfolding, and aggregation of proteins which is related to the changes of secondary structure of proteins by characterizing protein infrared absorption using the amide modes. The sensitivity of secondary structure highly depends on amide I vibration, since most of them absorbs infrared in range of amide I region of ~1600-1690 cm^{-1}. Near wavenumber 1650 cm^{-1} with broad peak represents protein unfolding. In that, protein aggregation would be shown a peak near or below 1620 cm^{-1} which is related to intermolecular β-sheets. Wavenumber in range of 1510-1550 cm^{-1} is for amide II vibration (Barth, 2007).

Amide bonds are polypeptide chain of protein backbone which can absorb infrared radiation and excite the vibrational mode. There are two kinds of vibrational modes, which are amide I vibration resulting in stretching of carbonyl (C=O) double bonds and amide II vibration resulting in the transformation of N-H bonds. Secondary structure of protein is the major cause of the frequencies formed by amide I and amide II bands (Kumosinski & Farrell, 1993).

This technique is categorized as powerful, accurate, and precise. Many samples have varied characteristics and identities and been interpreted as infrared spectra, namely fingerprint. The benefits offered by this technique including only small amount of protein (10 μg) is needed, considered as low cost technique compared to NMR and X-Ray diffraction, and there is no fluorescence effects and light scattering (Kumosinski & Farrell, 1993; Tranter, 2017). The possibility of using FTIR to analyze samples dissolved in aqueous media needs more attention, since spectra of H_2O has strong absorption in range of amide I vibration, around 1600-1700 cm^{-1} leading to overlapping peak with amide I peak, as a consequence, the higher amount

of protein is required. Therefore, to anticipate the water interference, instrument should be purged using liquid nitrogen. Besides, water spectrum is firstly recorded and then subtracted from the spectrum of protein (Haris & Severcan, 1999).

2.7 Scanning electron microscopy (SEM)

Scanning electron microscope (SEM) is a tool, technique, and analysis working under electron emission principle and currently used to analyze materials even in atomic level. SEM has high magnifications and produces grey-scale images in sharper and more detail way than older microscope. Nowadays, SEM has been used in many fields, such as food, microbiology, and textile due to its wide range applications to display topography, morphology, composition, and crystallographic information of the specimens (Kannan, 2018; Mohammed & Abdullah, 2018).

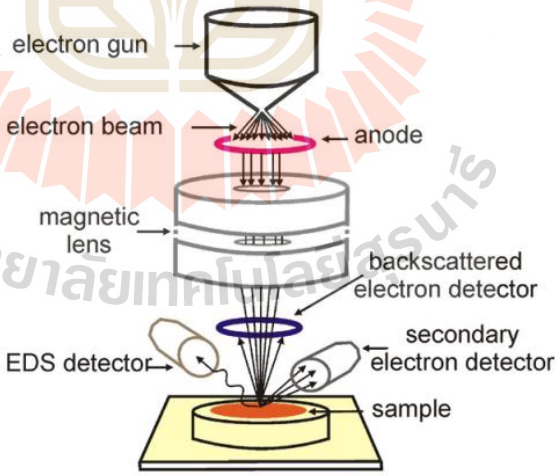


Figure 2.6 Schematic of scanning electron microscopy.

Source: Eric (2017).

The main components of scanning electron microscopy are electron column, detector, and sample board as presented in Figure 2.6. There are three major parts in electron column, including the top column, filament, and Welhnet (electron beam). Reaction of emitted electrons with non-sample particles is not allowed otherwise electron collide may occur. As prevention, electron column must be conditioned in vacuum and is focusing electron right to the sample as well. Electrons are generated by the top electron column which also consists of heating part usually made from tungsten wire. The powerful heating enables electrons released as many as possible through the column, followed by detection of electron emission by the filament. As electron emissions are uncontrollable, a Welhnet starts to manage the gun in liberating a number of electrons and direct them to anode and pass through the column. Magnetic lenses consist of condenser and objective lens which are attached to electron column. Condenser lens is used to generate sufficient electron beam scattering to sample, while objective lens evenly scans the sample surface. The detectors, secondary electron (SE) and backscattered electrons (BSE), receive signal given by scanning electron onto sample which is further transformed as image of morphology and contrasts of the sample (Eric, 2017).

2.8 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is an irreversible reaction using polyacrylamide gel which requires denaturation of protein sample, usually by heating at 90-100˚C. Acrylamide and bis-acrylamide are common chemical compounds to form polyacrylamide gel as seen in Figure 2.7.

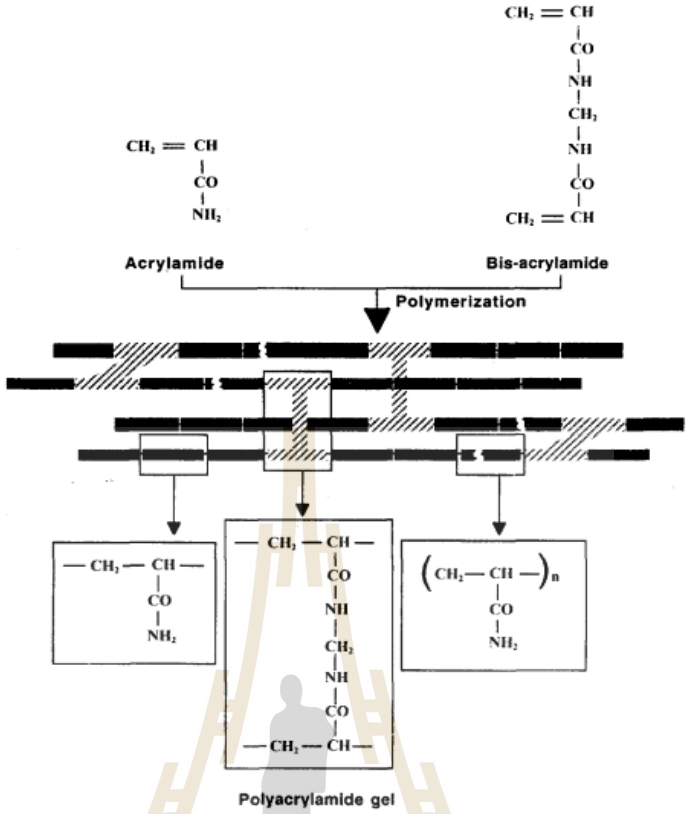


Figure 2.7 Polymerization of acrylamide and bis-acrylamide.

Source: Hames (1998).

The cross-linking formation of the compounds are initiated by ammonium persulfate (APS) and N,N,N',N'-tetramethyl-ethylenediamine (TEMED). APS and TEMED are related to each other, APS induces acrylamide activation and polymerization starts to occur by addition of TEMED as catalyst to support the reaction by bringing electron. As a result, polyacrylamide gel consisting of long polymer chain is produced and further defined as gel pores. The higher concentration of polyacrylamide gel, the smaller pore is formed due to the abundant amount of long polymer chain. Protein sample is allowed to travel along the polyacrylamide gel in an electric field with the help of buffer SDS addition by converting the net charge of

protein into negative charge. Polyacrylamide gel consists of two gels arrangement, stacking gel to handle concentrated protein sample, while separating gel permits concentrated protein molecule to migrate through the gel. It acts like a sieve which has power to permit large molecule with slow mobility moves down earlier than the small counterparts. Therefore, SDS-PAGE is employing separation only based on protein sizes (Hames, 1998).

Even though SDS-PAGE is recognized as a powerful method to estimate molecular weight of protein sample based on molecular size separation. However, applying SDS-PAGE on another approach could be possible, for example on determination of level and pattern of protein hydrolysates. Studies reported that hydrolysis performs different behavior depending on the role of enzymes used, along with that SDS-PAGE enables to quantify conformation pattern of protein under controlled condition. As reported by Alarcon et al (2001), SDS-PAGE profile showed complete and quick hydrolysis of animal protein by several digestive enzymes, presenting high coefficient protein degradation (CPD). Another evident is revealed by Zhou et al (2016), SDS pattern could distinguish inhibition activity and degree of hydrolysis of BSA hydrolyzed by trypsin in different controlled conditions.

2.9 Fast performance liquid chromatography (FPLC)

Chromatographic separation of protein dependent on the size difference is known as size exclusion or gel filtration. Chromatographic column usually contains of stationary phase which has porous beads with range of molecular weight. The column is equipped by mobile phase alongside made from buffer to undertake elution. During elution protein sample is filling the column beads with none of large molecule

entering the pores, this would be released from column afterwards by the force of mobile phase and considered as big molecular weight, while small molecule is retained in pores and eluted later (Pontis, 2017).

High-performance size exclusion system exists with intermediate pressure and is designed to determine molecular weight distribution of protein known as fast performance liquid chromatography (FPLC) (Irvine, 2009). It is important to note that molecular weight distribution could represent the effectiveness of enzymatic hydrolysis. Hydrolysis expected to produce hydrolysate with bioactivity or low undesirable components as often. Molecular weight distribution could help to interpret factors affecting the activity, as the profile is mostly correlated with the molecular size.

Yang et. al (2011) reported that soy sauce hydrolysates with varied of hydrolysis time exhibited different characteristic of molecular weight distribution profile. The main fraction was generated peptide at range of molecular weight of 3-10 kDa. Nevertheless, hydrolysis times (30 and 60 min) produced hydrolysate with high percentage of lower than 3 kDa peptide. Similar result has reported by Hong et al (2001) that 24 h hydrolysis of wheat gluten generated hydrolysate with lower number of molecular weight than initial hydrolysis time. It can be presumed by only observing molecular weight distribution profile that hydrolysate may contain different level of amino acids.

2.10 Antioxidant activity

Antioxidants are known to be beneficial to both food and human health because of their protection against free radicals, action in preventing lipid and protein oxidation, and maintaining color, aroma, taste, texture, and nutrition of food products.

The antioxidant activities occur by hydrogen donating, free radicals scavenging, transition-metal ion chelating, and active-oxygen quenching. Generally, antioxidant peptides contain from 3 to 20 amino acid residues, and their antioxidant activities are based on structural properties, amino acid composition and sequence, and hydrophobicity. These short chains of amino acids are inactive within the sequence of the parent protein, but can be released during gastrointestinal digestion, food processing, or fermentation, and also during food processing, such as cooking, fermentation, ripening.

Antioxidant activity is mainly defined as the capacity or potency of compound to protect systems from reactions that cause oxidation generated by reactive oxygen species (ROS) (Arnao, 2000), in which this activity needs to be proven by some antioxidant activity assays. Nowadays, chemically antioxidant assays are considered as an easy and short analysis time to evaluate antioxidant activity of products by certain mechanisms including free radicals scavengers, reducing power, and metal chelation ability (Shahidi & Zhong, 2015). ABTS, FRAP, and metal ion chelation are then chosen as methods to evaluate antioxidant activity because of the applicable consideration of each method to chicken egg white hydrolysate. Also, the presence of ovotransferrin within egg white which is considered to have antioxidant activity by binding or chelate ferric ion and transport to the body can be proven using FRAP or metal chelation assay (Abeyrathne et al, 2013; Chalamaiah et al, 2016).

Trolox equivalent antioxidant capacity (TEAC) is method to determine total antioxidant capacity through hydrogen donation, in which the interaction between antioxidant against $ABTS^{\cdot+}$ (2,2'-azinobis(3-ethylbenzothiazoline-6-sukphonic acid)) (Figure 2.8) (Prior, Wu, and Schaich, 2005) radical mono-cation will be monitored by reading the absorbance of the blue/green color complex at maximum wavelength of 734 nm against Trolox (water soluble vitamin E analogue) as a standard (Miller,

1996). The decrease of color intensity indicates the higher activity of antioxidant upon radicals, hence this method is familiar with the name of decolorization antioxidant assay. $ABTS^{\cdot+}$ radical is soluble both in water and organic solvents, which means it enables wide range of application in hydrophilic and lipophilic samples. The reaction between antioxidant and free radical is also considerably short and is applicable on broad range pH (Mahalgaes et al, 2008; Prior et al, 2005).

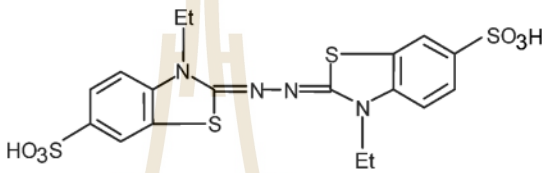


Figure 2.8 Structure of 2,2'-azinobis(3-ethylbenzothiazoline-6-sukphonic acid) ($ABTS^{\cdot+}$).

Source: Prior et al. (2005).

Ferric reducing antioxidant power (FRAP) assay was firstly developed by Benzie and Strain (1996) and currently used to determine the antioxidant ability to lower or neutralize ferric ion which occurs at low pH through electron transfer. Many reports informed that there was correlation between reducing power and antioxidant activity, thus bioactive peptides or protein hydrolysate with high antioxidant activity may have high possibility to have reducing power by donating electron (Bernardini, et al., 2011; Lassoued, et al., 2015). Basically, ferrous formation is established by the reduction of ferric ion and 2,3,5-ti-pheny-1,3,4-triaza-2-azoniacyclopenta-1,2-diene chloride (TPTZ) complex which shows color enhancement at 593 nm (Figure 2.9) (Antolovich et al, 2002). FRAP assay is considered as quick and low cost method and carried out in acidic environments, mostly in pH 3.6 to keep iron atom dissolved and enable the electron transfer. One of the limitations of this method is not able to measure the

antioxidant activity through hydrogen donation, whereas some antioxidants conduct two actions (hydrogen donation and electron transfer) in same time together (Prior et al, 2005).

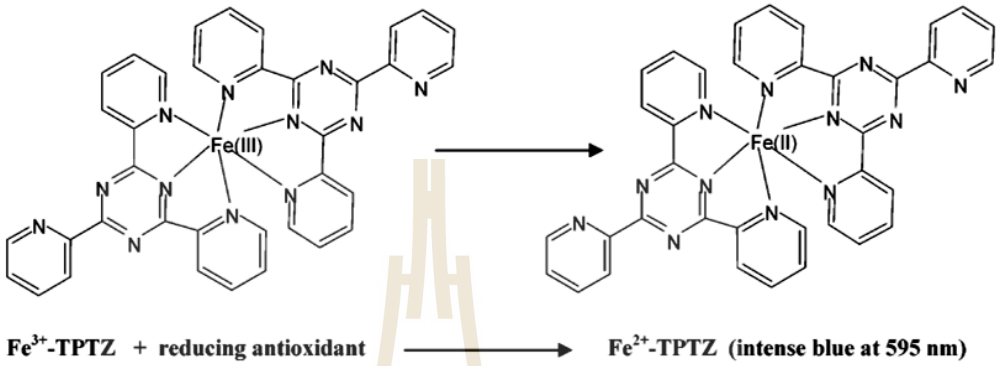


Figure 2.9 FRAP assay reaction.

Source: Prior et al. (2005).

Ion Fe^{2+} is categorized as an oxidizing agent, which would induce the formation of reactive oxygen species (ROS) and easily convert into radicals. ROS is also responsible for further reaction of radicals which can cause the damage in cell and protein membranes, and DNA mutation, leading to many diseases such as cancer, diabetes, and coronary heart (Gupta, 2015). Ferrous ion chelating capacity (FICC) assay evaluates the chelating ability of antioxidant against ferrous ion (Fe^{2+}) which acts as important catalyst for oxidation. The chelating activity of antioxidant towards free radicals is to balance the electron of radicals and metal ions chelation using chelation agents in order to disrupt the reaction of radicals (Sudan et al, 2014).

In this assay, ferrous sulfate and ferrozine are usually used as a source of ion Fe^{2+}. When antioxidant is introduced to ion Fe^{2+}, the metal-antioxidant complex is

formed. Later on, the antioxidant chelating capacity, represented in color complex formation of metal-antioxidant can be monitored spectrophotometrically by reading the absorbance at 562 nm for ferrozine and at 485 nm for ferrous sulfate against EDTA since it is standardized as a synthetic metal chelator (Shahidi & Zhong, 2015).

2.11 Cellular antioxidant activity (CAA) assay using HepG2-cells

Antioxidant activity tested by chemical assay is still irrelevant to mimic physiological condition therefore evaluation of antioxidant activity on cellular-based model should be prioritized. CAA assay was developed by Wolfe & Liu (2007) to elucidate antioxidant activity under biological systems by using cell culture derived from human hepatocellular carcinoma (HepG2) cells. Human hepatocellular carcinoma (HepG2) cells are often chosen as cell to observe liver metabolism and toxicity and to conduct antioxidant activity assessment of compounds, in a reason that liver tissues are found to be a first place which could absorb antioxidant compounds and distribute its biological effects (Goya et al, 2010).

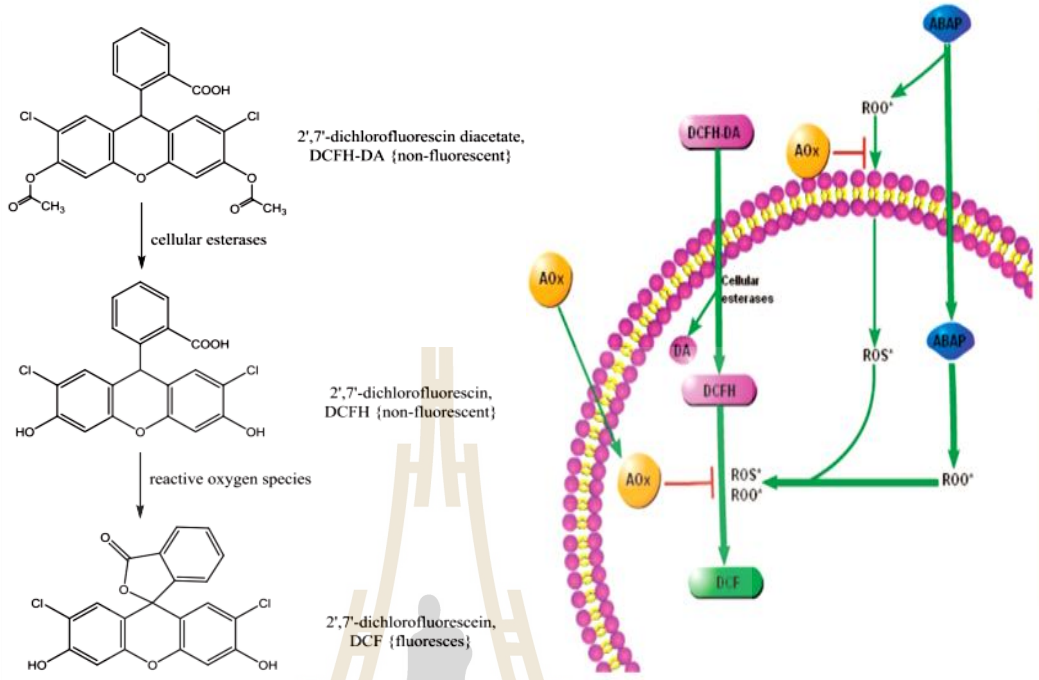
CAA assay is primarily preceded by cytotoxicity test of antioxidant compounds. As stated by Mahto et al (2010) cells may express various responses towards foreign chemical substances passing through its system, including membrane damage, genotoxicity, metabolic disruption, inhibit cell growth and proliferation reduction which all lead to cell death. Cytotoxicity is defined as toxic impact given by toxic compounds to cells. It is likely early screening to reveal effect of substances in cellular behavior and also considered as a preliminary test to design course of further research. There are several assays used to determine cytotoxicity summarized by Mc.Gaw et al (2014). The most common method namely 3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyltetrazolium or MTT assay representing cytotoxicity as percent cell viability since it has positive correlation with cell death. The principle of MTT assay is based on reduction of MTT by viable cells metabolic system via mitochondrial or cytosolic enzyme to form purple-blue formazan crystal which dissolves in dimethyl sulfoxide (DMSO) or isopropanol and is measured the absorbance at wavelength of 570 nm.

Aside from cytotoxicity, antioxidant compounds may perform efficacy to protect cells from oxidative stress in certain concentrations, namely cytoprotection effect. Mehta et al (2018) stated that antioxidant compound has two main mechanisms against radicals to avoid cell death; direct and in direct mechanisms. Direct mechanism utilizes redox reaction from antioxidant compound which sacrifices electron to stabilize radicals, while indirect mechanism involve cell system to fight against radicals through defense action triggering by the antioxidant compounds. Cytoprotection attempts to limit cell death and the survival cell against radicals is illustrated by percent cell viability using MTT assay. This is important to reveal ability of antioxidant compounds to protect cells as in some conditions they may have no sufficient barrier over excessive free radicals. Foods, drugs, and pharmaceuticals industries have succeeded to apply cytoprotection assay in their field prior to commercialize their products.

In recent years, CAA assay using HepG2-cells becomes popular because the cells have ability to detect the oxidants and to exhibit their potency which have strong antioxidant defense against radicals through inhibition (Mersh-Sunderman et al, 2004). HepG2-cells are well-differentiated transformed cell line, easy-cultured cells, easy-characterized, and suitable cell for antioxidant assessment study in which HepG2

cells responses upon radicals could be well-detected (Alia et al, 2016).



(a) (b)

Figure 2.10 Mechanism of conversion DCFH-DA to fluorescence DCF (a) (Wolfe & Liu, 2007), principle of cellular antioxidant activity (CAA) assay (b) (Kellett et al, 2018).

Literally, the principle of cellular antioxidant assay is based on the conversion of non-fluorescence DCFH-DA (2',7'-dichlorofluorescin diacetate) to fluorescence DCF (2',7'-dichlorofluorescin) because of oxidation process by free radicals. The conversion process is presented in Figure 2.10a. DCFH-DA is known as a tool to observe the mechanism of free radicals oxidation in cells. DCFH-DA shows its capability to pass through cell membrane due to its non polarity and non ionic characteristics. Once DCFH-DA succeeds to pass through cell membrane, it will be deacetylated by cellular esterase and change to non-fluorescence DCFH. In this form,

non-fluorescence DCFH is more oxidizable, thereby it would be easier to be converted to fluorescence DCF because of the presence of free radicals (Kellett, Greenspan, & Pegg, 2018).

Figure 2.10b represents the mechanism of CAA assay in which antioxidant will compete with radicals to inhibit fluorescence DCF formation. Cell itself can produce radical endogenously, but the amount seems quite small to be quantified in this assay. Therefore, free radicals should be added to system due to its competency to pass through cell membrane. When ABAP which is peroxyl radical producer has been entered the cells, it starts to form large amount of peroxyl radicals and oxidation of DCFH become fluorescence DCF is began. Once the cells are treated with antioxidant compounds and able to get in to the cells, antioxidants will inhibit and decrease the oxidation process of radicals to form fluorescence DCF in many ways. The level of oxidation can be quantified according to the level of fluorescence on excitation wavelength (Wolfe & Liu, 2007). The fluorescence intensity could be monitored at excitation wavelength of 485 nm and emission wavelength of 538 nm, in which low fluorescence intensity indicates that the antioxidant is capable to prevent oxidant of radicals (McDowell et al, 2011).

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CHAPTER III

EFFECT OF EGG WHITE PRE-TREATMENT ON ANTIOXIDANT ACTIVITY OF ITS HYDROLYSATE

3.1 Abstract

Egg white is considered as one of the best food proteins and hydrolysis can be applied to increase its nutritional value and reveal antioxidant activity. Low molecular weight of egg white hydrolysate was also beneficial for the chronic kidney disease (CKD) sufferers. However, hydrolysis of egg white protein typically results in relatively low yield. Thus, pre-treatment methods to increase yield of hydrolysate should be sought. The objective of this research was to compare the use of ultrasound, microwave, and steam sterilizer (121˚C) prior to hydrolysis of egg white by 10% (w/w protein) Alcalase on degree of hydrolysis (DH) and antioxidant activity of the hydrolysate. Ultrasound at intensity of 41.5 W/cm^2 applied to boiled egg white was found to produce hydrolysate with the highest DH of 45.4%. Antioxidant activities of egg white hydrolysate were examined by 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity, ferric reducing antioxidant power (FRAP), and ferrous ion chelating capacity (FICC) assays. Raw egg white subjected to sterilization for 5 min resulted in the hydrolysate exhibiting the strongest ABTS radical scavenging activity with approximately 64% inhibition. In addition, steam sterilization for 30 min became the most powerful technique yielding peptides with FRAP of 22 µM Trolox equivalent. Boiled egg white subjected to ultrasound at

intensity of 41.5 W/cm^2 obtained hydrolysate with FICC of 1800 µM EDTA equivalent. Since there were several measured parameters, and pre-treatment methods affected these parameters differently.

Keywords: egg white hydrolysate, ultrasound, microwave, steam sterilization, antioxidant.

3.2 Introduction

Chicken egg white is assumed as the best source of protein, because many proteins are developed within the egg white, such as ovalbumin, ovotransfferin, lyzozyme, ovomucin, and ovomucoid (Abeyrathne et al., 2018). Ovalbumin contains nearly 54% of total protein of egg white, considering as the major protein presence in the egg white. High amount of protein has been found to be beneficial as diet therapy for several diseases, including chronic kidney disease (CKD). Albumin is one the most prominent protein presence in human body to maintain osmotic balance endogenously as its main function. Albumin metabolism is synthesized by the liver and only certain amount is filtered by glomerular membrane of kidney. Studies revealed that healthy people loss merely 10-20 mg/day of albumin through urinary process. The intensive losses of albumin often cause hypoalbuminemia, bearing a meaning for a state of albumin lower than 3.0 g/dL (Mazzaferro and Edwards, 2020). The main cause of hypoalbuminemia has been established over the years, either caused by the lack of hepatic albumin synthesis or abnormality of kidney glomerular filtration. The perpetual condition of hypoalbuminemia excessively leads to CKD and dialysis treatment or kidney transplantation is necessary in the nearly future to suppress mortality rate. Therefore, exogenous albumin intake from egg white can be helpful

to overcome the lack of albumin for the sufferers (Nicholson et al., 2008).

Hydrolysis of egg white into protein hydrolysate is expected to have high nutritional value with various bioactivities, including antioxidant activity (Chen et al., 2012). Aside from that, low molecular weight of protein hydrolysate also has a vital contribution in albumin absorption due to degradation of liver capability of the sufferers to synthezise albumin (Mazzaferro and Edwards, 2020). Nevertheless, conventional enzymatic hydrolysis process sometimes comes up with relatively low yield and time consuming. Nowadays, there is a huge attraction in technology to be developed and become the most suitable approach in hydrolysate production which is often applied prior to hydrolysis, including maximizing hydrolysis process, shortening hydrolysis time, enhancing degree of hydrolysis (DH), and promoting antioxidant activity (Ketnawa and Liceaga, 2016).

The most commonly used technologies in research area are high hydrostatic pressure, microwave, and pulsed electric field (Marciniak et al., 2018). Nevertheless, the research of using technology as pre-treatment method prior to hydrolysis is still limited. Therefore, ultrasound, microwave, and steam sterilization are proposed in this research. Ultrasound is proven to generate smaller particle sizes of protein and exposure protein cleavage sites, which facilitates better enzyme accessibility during hydrolysis. This action is attributed to high shear force and cavitation generated during ultrasound (Stefanovic et al., 2014). Microwave irradiation is absorbed by dipole compounds, which cause molecular rotation within the electric field. Consequently, the temperature rises up rapidly and could disrupt protein aggregation, leading to greater enzyme accessibility (Vanier, 2013). Steam sterilizer produces high pressure and temperature to extensively denature the protein. Protein denaturation enhances

enzyme accessibility (Sezdi and Yoleri, 2013).

By improving enzymatic cleavage during hydrolysis through pretreatment technologies could increase DH that may give impact on antioxidant activity (Mason, 1996). In this study, several chemical-based antioxidant assays are evaluated, including 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay, ferric reducing antioxidant power (FRAP) assay, and ferrous ion chelating capacity (FICC) assay. Therefore, the aim of this research was to compare DH and antioxidant activity of egg white hydrolysate prepared from 3 various pre-treatments aforementioned.

3.3 Material and Methods

3.3.1 Materials

Chicken eggs were purchased from Suranaree University of Technology Farm. The egg white was separated from the yolk and used as raw egg white sample. In addition, cooked egg white sample was prepared by boiling at 95˚C for 30 min and used as a control. Raw and cooked egg white samples were added deionized water to ratio of 1:20 (w/v protein). For ultrasound pre-treatment, samples were sonicated using ultrasonic probe with a 12-mm titanium tip (Qsonica Ultrasonic Processor, LLC, Newtown, CT 06470, USA) at frequency of 20 kHz with an intensity of 4.6, 22.1, 41.5 W/cm^2 for 10 min (pulse duration of on-time 5 s and off-time 5 s). For microwave irradiation, samples were subjected to microwave digestion unit (Ethos One, Milestone Srl, Sorisole (BG), Italy) at different temperatures (121˚C for 2 min, 110˚C for 30 s, 85 and 95˚C for 1 min). Temperature was controlled manually by the microwave system. Subsequently, a raised temperature could be detected by subjecting a probe directly to the sample. Microwave quickly increased to the desired

temperature prior to significant decrease to its initial temperature. The time required to reach the desired temperature was recorded by the system, further written as aforementioned. For steam sterilization (Tomy SX-700, Tomy Digital Biology Co., Ltd., Tagara, Nerima-ku, Tokyo, Japan), samples were steamed at 121˚C for 5 15 and 30 min.

Ultrasound, microwave, and steam sterilization treated samples and controls were hydrolyzed by 10% (w/w protein) Alcalase at 60^{o}C pH 8.5-9 for 4 h. Subsequently, the reaction was inactivated at 95^{o}C for 10 min and sample was subjected to centrifugation process at 10,000 x g for 10 min at 4^{o}C. The supernatant was then adjusted to pH 7 and analyzed for the α-amino acid content using TNBS assay to determine DH according to the method of Adler-Nissen (1979).

3.3.2 Antioxidant activity assay

ABTS stock solution was made from 7.4 mM 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt mixed with 2.6 mM $K_2S_2O_8$ dissolved in 10 mM phosphate buffer pH 7.4. ABTS working solution was prepared from 1 ml ABTS stock solution and 40 ml of 10 mM phosphate buffer pH 7.4 ($A_{734\ nm}$ = 0.730±0.05). ABTS assay was carried out according to the method of Arnao (2000), in which 1.98 ml of ABTS working solution was added to 20 µl of diluted hydrolysate sample. The mixture was then kept in dark room for 5 min and the absorbance was then measured at 734 nm against blank. Trolox was used as a standard for ABTS and the result was expressed as percent scavenger activity at the same peptide concentration of 0.01 mg Leucine/ml.

FRAP assay was carried out according to the method reported by Benzie and Strain (1996). FRAP reagent was prepared from 25 ml of 300 mM acetate

buffer solution pH 3.6 ml, 2.5 ml of 10 mM TPTZ solution, and 2.5 ml of 20 mM $FeCl_{3.6}H_2O$ solution. FRAP assay was determined by adding 1 ml FRAP reagent into 100 µl of diluted sample and incubated in water bath at 37˚C for 15 min. The absorbance was read at 593 nm and result was expressed as µM Trolox equivalent at the same peptide concentration of 0.091 mg Leucine/ml.

FICC assay was performed based on method reported by Carter (1971) by adding 1400 µl of DI water and 100 µl of 2 mM $FeCl_2$ into 100 µl of diluted samples. The mixed solution was then incubated in dark room temperature for 3 min. Subsequently, 400 µl of 5 mM ferrozine was added to the mixed solution and incubated at dark room temperature for 10 min. The absorbance was read at 562 nm and results were expressed as µM EDTA equivalent at the same peptide concentration of 0.05 mg Leucine/ml.

3.3.3 Statistical analysis

All experiments were tested in triplicate and analyzed using one-way ANOVA. The difference among means was performed using Duncan's multiple range test (DMRT) at 95% confidence level using SPSS software (SPSS Inc., Version 21.0, Chicago, IL, USA).

3.4 Results and Discussions

preliminary study demonstrated that 10-min US pre Intensity was important feature involving optimum US treatment. Our -treatment on egg white showed the highest DH and antioxidant activity as compared to those of either lower or higher sonication times (data not shown). One of variable to calculate intensity was obtained from amplitude. The setting condition was programmed based on previously reported

by Stefanovic et al. (2014) modified by various range of intensities. Intensity was mostly associated with the wave energy produced by US, as intensity rose up, the cavitation occurred in more violent fashion, generating smaller size of boiled egg white. This resulted in larger surface area of boiled egg white particles, allowing greater extent of enzyme accessibility (Wang et al., 2018).

Ultrasound at intensity of 41.5 W/cm^2 on boiled egg white increased DH to approximately 45.4% ($p<0.05$; Table 1), indicating that peptide bonds were cleaved to a greater extent in ultrasound-treated sample. In contrast, control of raw egg white, raw egg white at intensity of 4.6 and 22.1 W/cm^2 turned into a gel-like material after 4 h hydrolysis. Therefore, DH of these samples could not be determined because of the lack of the supernatant. Intensity of 4.6 and 22.1 W/cm^2 generated low shear force and cavitation which might be insufficient to break particles of raw egg white and induce gel network formation.

There was a variety of DHs regarding the effect of intensity. The DH value of hydrolysate increased when boiled sample was treated with US at 4.6 W/cm^2 as compared to the control after 1 h hydrolysis. However, the increase of intensity to 22.1 W/cm^2 resulted in lower DH value ($p<0.05$, Table 1). The collapse bubble due to shear force and cavitation of US at intensity of 22.1 W/cm^2 was accumulated as larger aggregate speculating faster cross-linking reaction might occur, reducing hydrolysis rate. Ma et al. (2016) reported there was a decrease on hydrolysis process along with the increase of US intensity due to intensive destruction on pectinase structure. The subsequent increment of DH was clearly observed at maximum intensity of 45.1 W/cm^2. It indicated complete degradation of protein structure, leading to optimum hydrolytic reaction. Similar behavior was also monitored after 3 h and 4 h hydrolysis.

The results demonstrated that US at intensity of 22.1 W/cm^2 was insufficient pre-treatment as DH was found unstable. Ultrasound intensity could be one of prominent factor determining DH of hydrolysates, however, another important factor could be attributed to hydrolysis time.

The highest DH values for all hydrolysate samples were found after 4 h hydrolysis (Table 1), indicating that DH was dependent on hydrolysis time. The control sample (boiled egg white) hydrolysate showed about 38.55% DH after 4 h hydrolysis. But, hydrolysates prepared from boiled egg white subjected to US showed higher DH than that of control (p<0.05, Table 1). Longer hydrolysis time of 4 h allowed enzyme on optimum peptide bond cleavage more efficiently than those of shorter times. The result was similar to those reported for raw egg white (Stefanovic *et al.*, 2018) and poultry meal (Nchienzia *et al.*, 2010). Using similar enzyme/substrate concentration and exposure US time, the discrepancy of DH was caused by hydrolysis time. Therefore, 4 h was selected as a suitable hydrolysis time for other pre-treated egg white.

Apparently, DH values were also affected by the boiling process as all pre-treatment methods for boiled egg white hydrolysate showed higher DH than raw egg white counterparts. Heating process causes protein unfolding, leading to exposure of protein active site and thus clearly increased enzyme accessibility of boiled egg white (Stefanovic et al., 2014).

Table 1 Degree of hydrolysis of egg white hydrolysates prepared from ultrasound pre-treatment.

Hydrolysis time (h)	Hydrolysates				
	Control	**Boiled EW at 4.6 W/cm^2**	**Boiled EW at 22.1 W/cm^2**	**Boiled EW at 41.5 W/cm^2**	**Raw EW at 41.6 W/cm^2**
1	26.58± 0.62^{dD}	31.49± 0.11^{cB}	29.31± 0.07^{dC}	34.05± 0.03^{dA}	19.32± 0.02^{cE}
2	32.21± 1.09^{cC}	31.36± 0.03^{dC}	33.72± 0.04^{cB}	35.63± 0.55^{cA}	19.32± 0.02^{cD}
3	37.35± 0.01^{bB}	36.98± 0.01^{bC}	35.19± 0.01^{bD}	38.29± 0.01^{bA}	19.58± 0.16^{bE}
4	38.55± 0.01^{aC}	39.05± 0.01^{aB}	35.58± 0.07^{aD}	45.37± 0.40^{aA}	20.05± 0.01^{aE}

Different uppercase letters in each row indicate significant difference in mean±standard deviation (n=3) values of hydrolysates as an effect of various ultrasound intensities. Different lowercase letters in each column indicate significant difference in mean±standard deviation (n =3) values of hydrolysates as an effect of hydrolysis time.

Ovoglobulin G2 and G3 as protease inhibitors were reported to denature at 92.5˚C (Guha et al., 2018). Thus, microwave at several range of temperatures were assessed to study effect of the presence and inactivation of protease inhibitors (at 85˚C and 95˚C), combination effect of inactivated protease inhibitors and particle explosion (at 110˚C), and comparison temperature to that of steam sterilization (at

121˚C). Among hydrolysates of MW-treated raw egg white, 85˚C showed slightly higher DH ($p<0.05$, Figure 3.1a), indicating that the presence of protease inhibitors was likely inactivated by MW. In contrast, the combination effect aforementioned at 95˚C and 121˚C succeeded to produce boiled egg white hydrolysate with relatively high DH (Figure 3.1a).

Microwave (Figure 1b) seemed to lower DH values of both raw and boiled egg white when compared to the control ($p<0.05$). High power of microwave could possibly increase temperature within a short time, resulting in exposure of protein cleavage site. The main cause of low DH of hydrolysates prepared from MW-treated egg white was high microwave power accelerated protein aggregation, hampering enzymatic hydrolysis (Huang et al., 2017). Nevertheless raw egg subjected to MW resulted in hydrolysates with lower DH than those of boiled egg white. To our knowledge, microwave heated from within part of sample which could directly absorbed by polar compound (Ketnawa and Liceaga, 2016), generating huge explosion of protein particles. The effect of MW seemed difficult to perform on raw egg white particle due to the increase of collision between unfolded molecules after being exploded. It led to form more compact structure (Gladovic et al., 2020). Enzyme accessibility was found limited on this form, thus, low DH was a significant effect.

Steam sterilization at 121˚C for 15 min was commonly used for sterilization process. Since steam sterilization was working under combination of steam and pressure (Zhang et al., 2018), minimizing the dramatic changes of protein properties was carried out by adjusting sterilization time to 5 min. In contrast, 30-min sterilization pre-treatment was used to study the effect of excessive structural changes

at prolonging time. All of those conditions were expected to involve in antioxidant activity. Sterilization pre-treatment produced hydrolysates with much lower than that of control ($p<0.05$, Figure 1b). It was presumably due to protein denaturation which induced protein aggregation, decreasing enzyme accessibility (Sezdi and Yoleri, 2013).

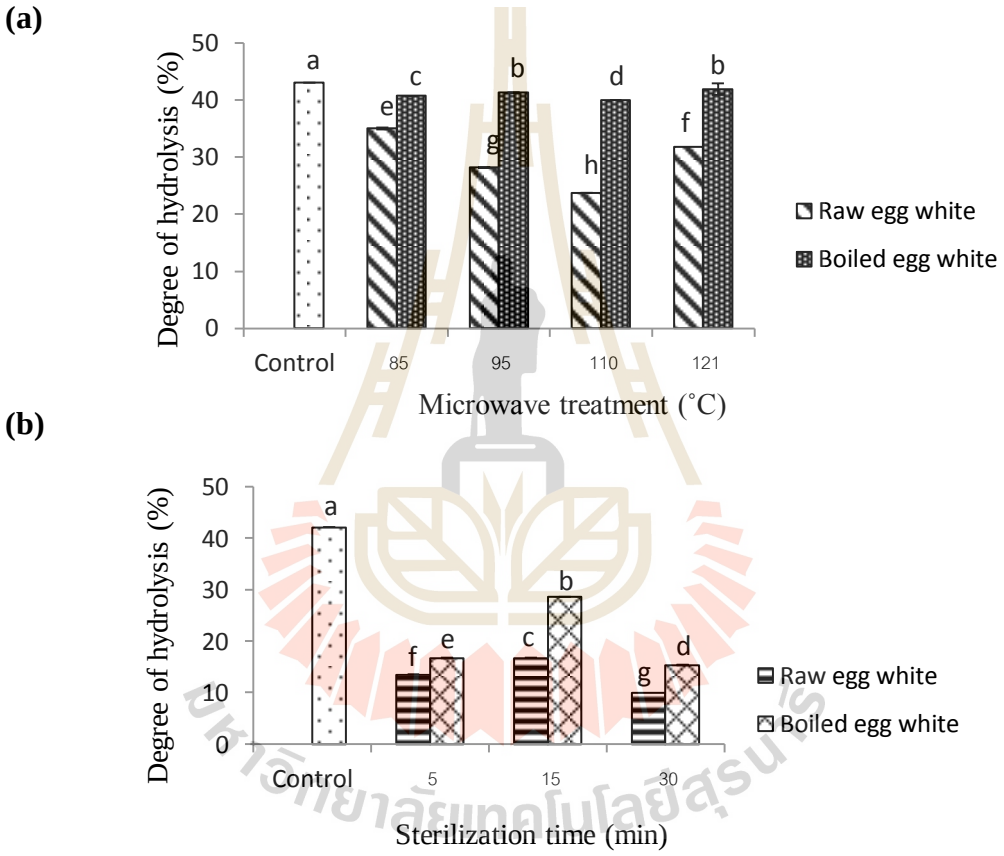

Figure 3.1 Degree of hydrolysis of egg white hydrolysates. (a) effect of microwave pre-treatment at 1400 W hydrolyzed for 4 h; (b) effect of steam sterilization pre-treatment at 121˚C hydrolyzed for 4 h. Control was boiled egg white without any pre-treatments. Mean±standard deviation (n=3) values were presented. Different letters indicate significant differences ($p<0.05$).

At the same temperature of 121˚C, DH values of ST-treated sample were lower than that of MW-treated. Note that energy of ST, which was transferred from outside into the inside part of protein (Ketnawa and Liceaga, 2016), likely promoted aggregation, further impeding enzyme mobility. As mentioned, hydrolysates of ST-treated boiled egg white showed slightly higher as compared to those of raw egg white. Steam sterilization mostly affected in protein solubility (Zhang et al., 2018). In coagulated form of boiled egg white it enabled the steam to breakdown protein aggregates into more soluble components. Meanwhile the extreme condition of sterilization likely led to insoluble aggregate formation. Therefore, our study revealed that among three pre-treatment technologies, ultrasound became the most potential treatment to be applied in boiled egg white prior to hydrolysis because it yielded hydrolysate with the highest DH value.

ABTS assay is the most common method of antioxidant activity evaluation via electron or hydrogen donation, which was applicable on wide range sample characteristics due to high solubility both in water and organic solvent (Mahalgaes et al., 2008). ABTS scavenging activity of ultrasound-treated hydrolysate was illustrated in Figure 2a, in which boiled egg white with ultrasound treatment at intensity of 41.5 W/cm^2 and hydrolyzed for 4 h was the highest with about 50% scavenging activity. As mentioned previously, intensity induced cavitation to produce small peptides upon enzymatic hydrolysis, which could contribute in ABTS scavenging activity. Therefore, the higher ultrasound intensity, the stronger inhibition of ABTS radicals would be generated. This result was in agreement with Guerra-Almonacid et al. (2019) who reported that *Erythrina edulis* hydrolysates treated by ultrasound exhibited stronger

ABTS radical scavenging activity than control. It presumably contains tyrosine at C-terminal of peptides which acts as electron donor to stabilize ABTS radical.

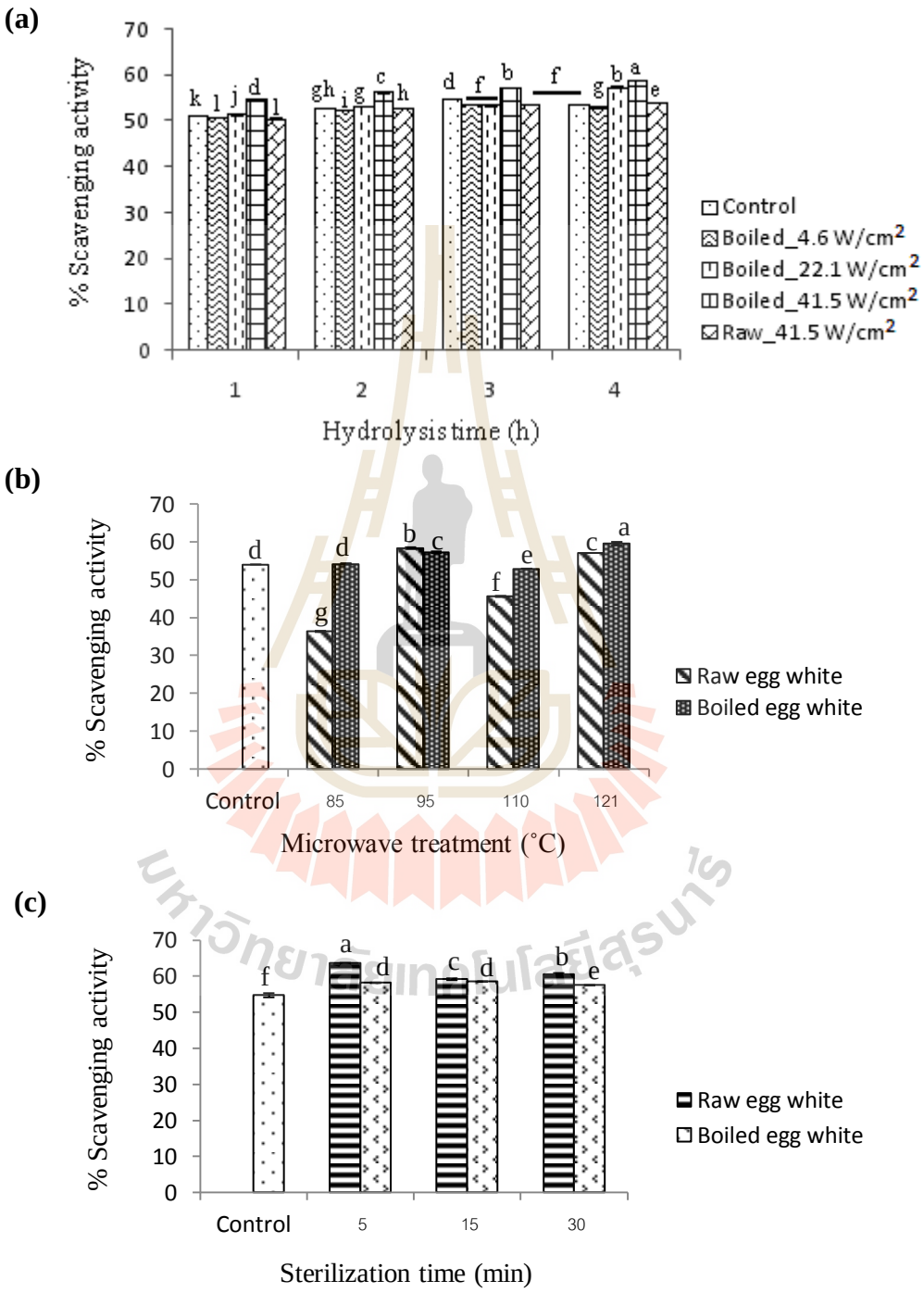

Figure 3.2 ABTS radicals scavenging activity of egg white hydrolysates. (a) effect of ultrasound pre-treatment; (b) effect of microwave pre-treatment; (c)

effect of steam sterilization pre-treatment; percent inhibition was at 10 µg Leucine/ml peptide concentration in reaction and used Trolox as a standard. Mean±standard deviation (n=3) values were presented. Different letters indicate significant differences (p<0.05).

Moreover, hydrolysate prepared from US-treated raw egg white possessed the lowest DH (Figure 3.1a), showing relatively high scavenging activity (Figure 3.2a), regardless of hydrolysis time. The result was an evident that peptide size was not a major factor affecting scavenging activity, high proportion of hydrophobic amino acids (e.g. His, Trp, Phe, Pro, Gly, Lys, Ile, and Val) might contribute to such property. Zou et al. (2016) reported that hydrophobic amino acids showed ability to form hydrophobic environment which enables peptides to convert ABTS radical into stable product.

Microwave pre-treatment on boiled egg white at 121˚C resulted in the hydrolysate exhibiting the highest ABTS radicals scavenging activity about 60% ($p<0.05$; Figure 3.22b). However, 5-min ST pre-treatment yielded raw egg white hydrolysate exhibiting ABTS radicals scavenging activity higher than those treated by microwave at the same temperature ($p<0.05$; Figure 3.2c). Similar to ABTS scavenging activity, peptide chain length was not an important indicator on reducing power ability. Dryakova et al. (2010) also reported that there was no direct correlation between DH of whey protein hydrolysates and antioxidant activity. Hydrolysates with high scavenging ability might contain abundant amount of peptides with His residues. Zou et al. (2016) reported that imidazole ring in His showed strong contribution as proton donor to scavenge radical in oxidation reaction.

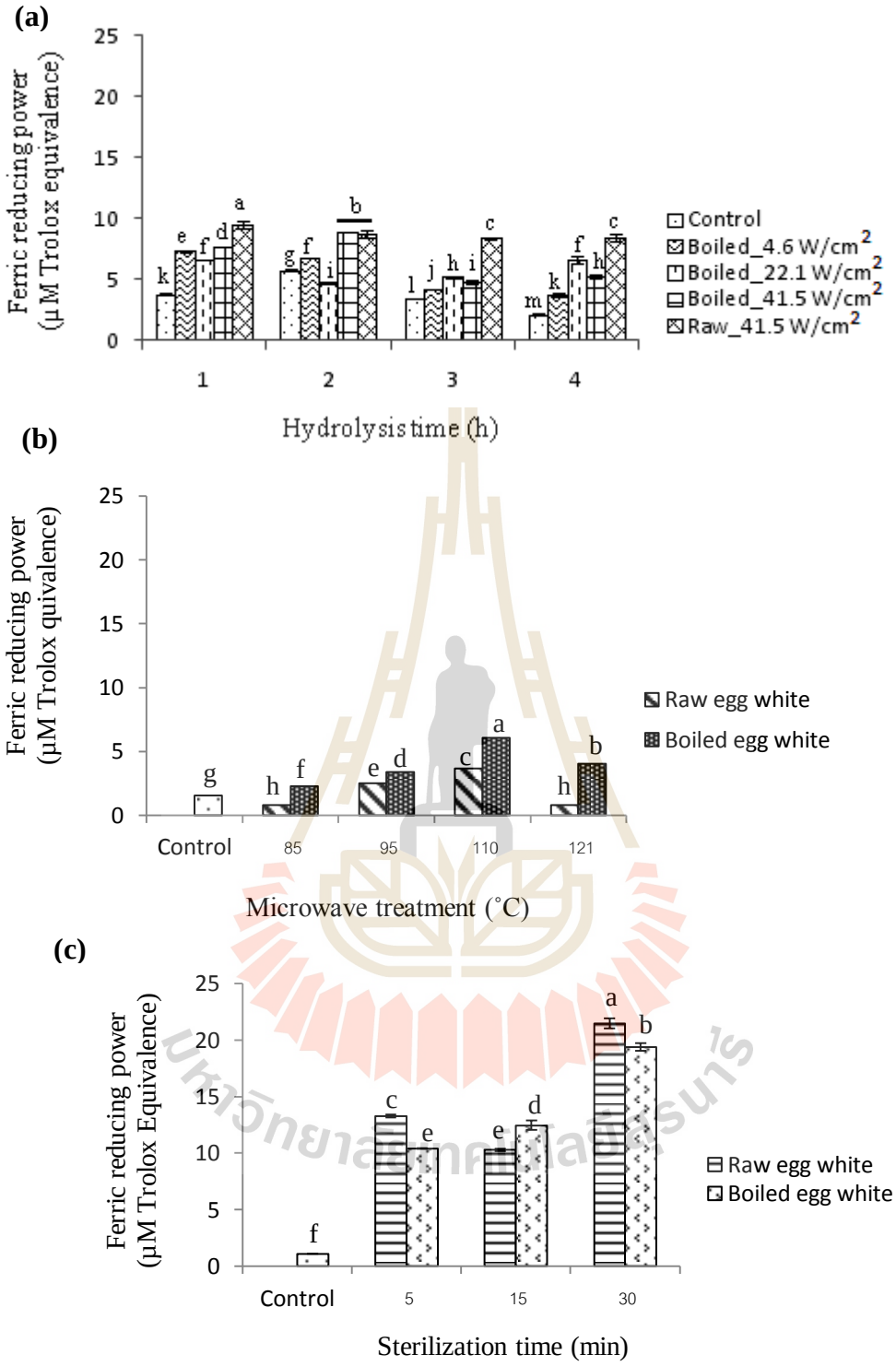

Figure 3.3 FRAP of egg white hydrolysates. (a) effect of ultrasound pre-treatment; (b) effect of microwave pre-treatment; (c) effect of steam sterilization pre-treatment. Peptide concentration was 91 µg Leucine/ml in rection.

Mean± standard deviation (n=3) values were presented. Different letters indicate significant differences (p<0.05).

This result revealed that steam sterilization could be used to improve ABTS radicals scavenging activity of egg white hydrolysate. Low molecular weight peptides and certain amino acid sequence were responsible for antioxidant activity (Lin et al., 2013; Chalamaiah et al., 2016). Therefore, the forthcoming research in either amino acid analysis or molecular weight determination of all hydrolysates was needed to explain some critical factors governing antioxidant activity of egg white hydrolysate.

FRAP assay is typically used to establish the possible correlation between reducing power and antioxidant activity of hydrolysates by donating electron (Lassoued et al., 2015). Although DH was the lowest (Table 1), US pre-treatment on raw egg white at intensity of 41.5 W/cm^2 resulted in hydrolysate with the highest reducing power ability of 9 µM Trolox equivalence after 1 h hydrolysis (Figure 3.3a). Similar to that of US pre-treatment, majority of hydrolysates prepared from MW-treated egg white with higher DH (Figure 3.1a) showed much lower reducing power ability (Figure 3.3b). Apparently, amino acid composition was the major factor involving reducing power ability. Zou et al. (2016) reported that hydrolysates with Asp and Gln residues were potential as electron donor which contributed in reducing power ability. In the case of reducing power, thus, peptide sizes could be ruled out. Finally, microwave was the least effective pre-treatment to obtain hydrolysate with reducing power ability.

In contrast, 30-min ST pre-treatment on raw egg white yielded hydrolysate with lower DH ($p<0.05$; Figure 3.1c), but the highest reducing power ability of 22

μM Trolox equivalence was obvious ($p<0.05$; Figure 3.3c). Moreover, at the same temperature of 121˚C, lower reducing power of hydrolysates prepared from MW pre-treatment was clearly observed as compared to those of ST. The result was similar to that reported by Sonklin et al. (2018) revealing that mungbean meal protein hydrolysate with the highest molecular weight of >10 kDa exerted high FRAP ability. However, Lin et al. (2013) reported that egg white protein hydrolysate prepared from Alcalase with molecular weight <1 kDa exhibited excellent FRAP ability. Therefore, peptide size was not the only factor controlling reducing power of hydrolysate. Amino acid sequence could also play a vital role in reducing power ability of the egg white protein hydrolysates. Among 3 pre-treatment technologies, steam sterilization was the most promising method, yielding hydrolysate with the highest reducing power ability.

The presence of ovotransferrin within egg white which was considered to have antioxidant activity by binding or chelate ferrous ion and transport to the body could be proven using metal chelation assay (Abeyrathne et al., 2013). Ultrasound-treated boiled egg white at intensity of 41.5 W/cm^2 showed the potent metal chelation activity after 4 h hydrolysis (Figure 4a), indicating that the highest metal chelation was accompanied by the highest DH (Table 1). Shorter peptides presumably contained concentrated carboxyl groups of acidic amino acids which was essential as metal ion chelator (Hou et al., 2019).

Hydrolysates prepared from MW pre-treatment at 121˚C, with higher DH ($p<0.05$; Figure 3.1a), also showed higher metal chelation activity as compared to those of ST ($p<0.05$; Figure 3.4b and 3.4c). It was likely a shorter peptide might contain strong metal-binding ability. A study by Korczek et al. (2019) showed that an increase in DH was correlated to metal chelating ability. In addition, lower DH of all hydrolysates prepared

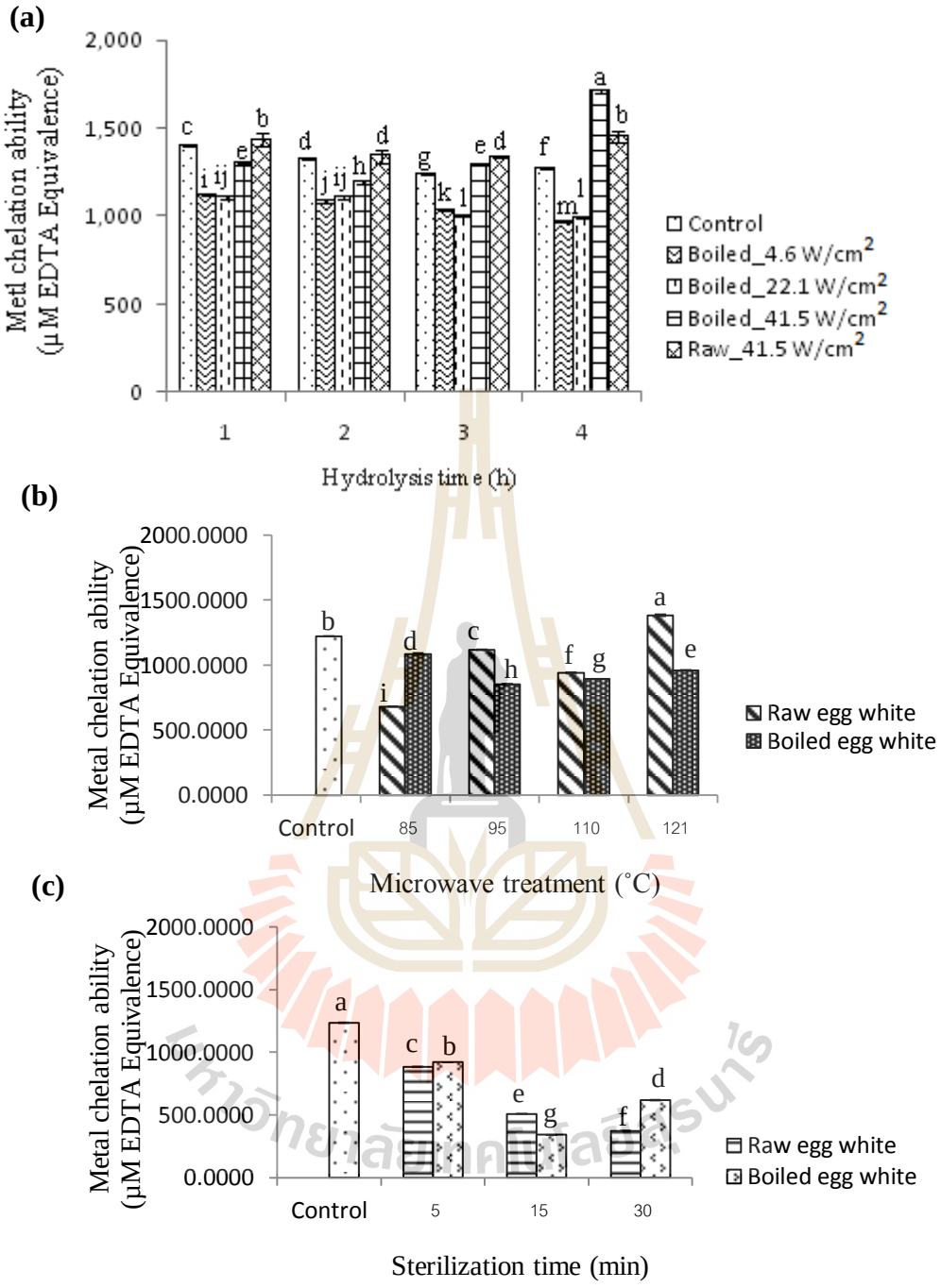

Figure 3. 4 FICC of egg white hydrolysates. (a) effect of ultrasound pre-treatment; (b) effect of microwave pre-treatment; (c) effect of steam sterilization pre-treatment at peptide concentration of 0.05 mg Leucine/ml in reaction. Mean ±standard deviation (n=3) values were presented. Different letters indicate significant differences (p<0.05).

from ST pre-treatment was concomitant with the lower metal ion chelation activity. The extreme condition of ST pre-treatment enhanced the cross-linking reaction of denatured protein being more resistant to enzyme during hydrolysis (Salazar-Villanela et al., 2016). It likely impeded the exposure of amino acids containing peptides as ion chelator.

Nevertheless, microwave and steam sterilization pre-treatments on egg white did not improve chelating activity of the hydrolysates (Figure 3.4b and 3.4c) as compared to the control without pre-treatment. The difference of chelation ability might be dependent on peptide structure and chain length (Saiga et al., 2003). This result was in same agreement with Carrasco-Castilla et al. (2012) in which purified phaseolin hydrolysate exhibited strong copper chelating ability at peptide size of <1 kDa. Short peptide of palm kernel kernel expeller glutelin-1 (PKEG-1) also showed remarkable chelating capacity due to interaction between the charged groups and metal ion (Zheng et al., 2017). Alcalase hydrolysis of Nile tilapia resulted in hydrolysate with DH of 40%, exhibiting the most potent ferrous ion chelator capable to lower metal ion oxidation (Yarnpakdee et al., 2014).

3.5 Conclusions

Ultrasound at intensity of 41.5 W/cm^2 of boiled egg generated hydrolysate with the highest DH of 45.4% and FICC about 1800 µM EDTA equivalence. In addition, steam sterilization for 5 min yielded hydrolysate with the highest ABTS radical scavenging activity of 60% inhibition and steam sterilization for 30 min yielded hydrolysate with the highest FRAP about 22 µM Trolox equivalence.

Microwave did not yield any effects on both DH and antioxidant activities. Therefore, DH and antioxidant activities of egg white hydrolysate were varied with pre-treatment method applied on it.

3.6 Acknowledgements

This work was financially supported by the Food Innovation for Safety and Value Creation of Nakhonchaiburin, Suranaree University of Technology (SUT3-305-61-12-06). One research one grant (OROG) scholarship was also highly appreciated.

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CHAPTER IV

INFLUENCE OF ULTRASOUND PRETREATMENT ON ANTIOXIDANT ACTIVITY OF EGG WHITE HYDROLYSATES

4.1 Abstract

The use of ultrasound-treated egg white prior to Alcalase hydrolysis to produce hydrolysate with antioxidant activity was investigated. Two levels of Alcalase at 1% and 10% (w/w protein) were applied. Despite different enzyme concentrations, hydrolysis and antioxidant activity of hydrolysate increased by the help of ultrasound (US) pretreatment at intensity of 41.53 W/cm^2. The hydrolysate prepared from US treatment on raw egg white hydrolyzed by 1% Alcalase (w/w protein) (US-R1%) showed the lowest DH, however, ABTS and FRAP were the highest. In contrast, the highest cytoprotective effect and intracellular ROS scavenging activity were more obvious on hydrolysate with the highest DH and metal chelation ability of hydrolysate prepared from US treatment on boiled egg white hydrolyzed by 10% Alcalase (w/w protein) (US-B10%). It was noticed that hydrolysate possessing CAA showed the highest proportion of small molecular weight peptides (<200 Da). FT-IR spectroscopy revealed an increase of N-terminal ends at amide II and C-terminal region in concomitant with a decrease of amide I. PCA illustrated the positive relationship between metal chelation, DH, C-terminal ends,

cytoprotection and CAA, while negatively correlated to ABTS and FRAP. Thus, DH and metal chelation ability of hydrolysate was predominantly correlated with cellular antioxidant activities. Our findings suggested that cooked egg white followed by ultrasound pretreatment was beneficial to produce hydrolysate potentially containing high antioxidant activity.

Keywords: egg white, ultrasound, CAA, FT-IR, PCA.

4.2 Introduction

Chicken egg white remains one of the most nutritious sources for the need of human protein intake. Egg white is composed of 10-11% proteins containing hundreds of different proteins. Ovalbumin is the most abundant protein in total of 54% protein content, followed by ovotransferrin, ovomucoid, lysozyme, and other minor proteins (Kovacs-Nolan et al., 2005). Egg white proteins are required for cancer patients who receive chemotherapy. It prevents leucopenia; a lack condition of white blood cells. Proteins in egg white are also source of energy to build strength and maintain weight due to the fast energy burning during chemotherapy (Supraset et al., 2016).

Egg white is also a potential source of bioactive peptides which would be released during enzymatic hydrolysis. It is employed to hydrolyze peptide bonds from native protein using proteases, yielding protein hydrolysate with various chain lengths of peptide and amino acids. Short peptides are believed to be easily absorbed through intestinal digestion and exerted its bioactivity (Memarpoor-yazdi et al., 2012). However, hydrolysis of egg white typically resulted in low yield. The presence of protease inhibitors in egg white is appeared as a prime cause of the limited degree of

hydrolysis in egg white. The appropriate pretreatment process is one of strategies to enhance the hydrolytic reaction. Ultrasound (US) is acoustic waves which has frequency higher than human hearing range; about >16 kHz utilizing mechanical cavitation (Jovanovic et al., 2016; Koshani & Jafari, 2019). US treatment was reported to induce protein unfolding, smaller protein molecular sizes, and exposure protein catalytic sites to enzyme. All of those actions eventually enhanced hydrolytic reaction (Arzeni et al, 2012; Wu et al, 2018; Yu et al, 2020). US pretreatment on egg white followed by several proteases hydrolysis showed the improvement on functional and antioxidant activity of the hydrolysates (Stefanovic et al., 2014), ultrasound treatment of raw egg white using 25.65 W/cm^2 intensity for 15 min generated hydrolysate with the highest DH of 39.01% after Alcalase hydrolysis (Stefanovic et al., 2018).

Cellular antioxidant activity (CAA) is more relevant to biological system. Determination of cytoprotective effect and intracellular reactive oxygen species (ROS) scavenging activity of the hydrolysates would be more reliable to illustrate peptide containing antioxidant activity. In addition, molecular weight distribution is also one of the most important factors determining antioxidant activity of the hydrolysates, due to general understanding that antioxidant activity somehow depends on the peptides sizes (Liu et al., 2018).

Another effect of US pretreatment is secondary structure alteration of the peptides, which is likely responsible for antioxidant activity. Although the application of Fourier-transform infrared (FT-IR) spectroscopy is found limited due to lack ability determining specific peptides, however, it is still a great approach to characterize antioxidant activity through different FT-IR spectra (Liu et al., 2018).

Therefore, a novelty of this study was aimed to evaluate antioxidant activity of hydrolysates prepared from US-treated egg white and its correlation with structural changes constructed by principle component analysis (PCA).

4.3 Materials and methods

4.3.1 Materials

Chicken eggs were purchased from Suranaree University of Technology Farm. Egg white was manually separated from the yolk, subsequently proposed as control raw egg white. In addition, boiling was performed by cooking raw egg white at 95˚C for 30 min, further referred to as control boiled egg white. In hydrolysis reaction, deionized water was added to either raw or cooked samples in the ratio of 1:1, yielding protein content of the mixture approximately 5% (w/v) protein.

4.3.2 Ultrasound pretreatments and enzymatic hydrolysis

Egg white mixtures were exposed to ultrasonic wave using a 12-mm diameter titanium probe equipped with 20 kHz (Qsonica Ultrasonic Processor, LLC, Newtown, CT 06470, USA) intensity of 41.53 W/cm^2 for 10 min (pulse duration of on 5 s and off 5 s). Ultrasound-treated egg white samples and controls were hydrolyzed for 4 h using 1% and 10% (w/w protein) Alcalase at 60^{o}C and pH 8.5. Enzyme inactivation was carried out by heating at 95^{o}C for 10 min. Subsequently the mixture was centrifuged at 10,000 x g for 10 min at 4^{o}C. The supernatant was adjusted to pH 7 prior to determination of degree of hydrolysis using TNBS assay (Adler-Nissen, 1979).

4.3.3 Yield protein determination

Yield protein of ultrasound-treated and control egg white hydrolysates were

determined by Kjeldahl method according to AOAC (2000). The conversion factor of 5.74 was used (Mariotti et al, 2008).

4.3.4 Scanning electron microscopy (SEM)

The morphology of ultrasound-treated egg white samples was observed using field emission scanning electron microscope (FE-SEM, Carl Zeiss, Zeiss, Germany). Initially, raw and US-treated raw egg whites were submerged in liquid nitrogen and lyophilization afterwards. While boiled and US-treated boiled egg white were treated by two steps of fixations; glutaraldehyde followed by osmium tetroxide, subsequently dehydrated by series of acetone, and critical point-dried prior to SEM analysis. All dried samples were placed in a metal stub with conductive carbon/silver tape and coated by gold-palladium using a sputter coated machine, further observed under SEM at 3.0 kV accelerating voltage.

4.3.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The pellet of hydrolysates was weighed out for 50 mg each and solubilized in 5 ml of 10% (w/v) SDS solution. Total protein was determined using Lowry method (Lowry et al, 1951). The mixture was added by the treatment buffer containing β-merchaptoethanol at the ratio of 1:1 (v/v) and heated at 90˚C for 5 min. Subsequently, the mixture of 20 μg protein each was loaded onto polyacylamide gels made from 4% stacking and 12.5% separating gels (mini-PROTEAN, Bio-Rad Laboratories Inc., Alfred Nobel Drive Hercules, California). PageRuler™ unstained protein ladder (Thermo Fisher, Life Technologies Inc., Van Alley, Carlsbad, California) was applied as a marker and protein separation was carried out at 80 V. Gels were stained using Commassie brilliant blue R-250 for 30 min and were

destained using destaining solution (25% methanol and 10% acetic acid) until the bands were clearly visible. Gels imaging was analyzed using UVITEC imaging system (Fire reader V4, Uvitec Ltd., Cambridge, UK).

4.3.6 Molecular weight distribution

Molecular weight distribution of hydrolysates was evaluated by fast performance liquid chromatography on Superdex peptide 10/300 GL (10x300 mm, GE Health care, Piscataway, NJ, USA) with UV detection at 215 and 280 nm. Mobile phase for elution was made from 30% acetonitrile containing 0.1% TFA at flow rate of 0.5 ml/min. Molecular weight standard was prepared from cytochrome C (12,000 Da), aprotinin (6,512 Da), AGNQVLNLQADLPK (1,461 Da), NTFLFFK (897 Da), Hip-His-Leu (429 Da), and tryptophan (204 Da). Molecular weight distribution was estimated from area under the peak using UNICORN version 7.1 software (GE Healthcare Bio-sciences AB, Bjorkgatan, Sweden).

4.3.7 Chemical-based antioxidant activity assays

All hydrolysate samples were measured the antioxidant activity by three different modes of actions, namely hydrogen donation using 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, electron transfer to reduce ferric ion using ferric reducing power (FRAP) assay, and ability to chelate ferrous ion using ferrous ion chelating capacity (FICC) assay. Trolox was used as a standard for ABTS and FRAP assay, while EDTA was used for FICC assay. All assays were in triplicate and statistically analyzed using one-way ANOVA. Duncan's multiple range test (DMRT) was used to evaluate the difference among means at 95% confidence level using SPSS software (SPSS Inc., Version 21.0, Chicago, IL, USA).

ABTS assay was determined according to Arnao (2000) with slight

modifications. ABTS radical was produced from the reaction between 7.4 mM 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and 2.6 mM $K_2S_2O_8$ in 10 mM phosphate buffer pH 7.4, raising absorbance of 0.730 (±0.005) at wavelength of 734 nm. The assay was carried out by the addition of 1.98 ml of ABTS solution to 20 µl of hydrolysate containing 0.01 mg Leucine/ml. The mixture was incubated for 5 min in dark prior to absorbance reading at 734 nm. Scavenging activity was calculated using the following equation.

$$\% \text{ Scavenging activity } = \frac{\text{Abs control-abs sample}}{\text{Abs control}} \text{ x } 100\%$$

Reducing ferric ion power ability of hydrolysates was measured by method of Benzie and Strain (1996) with slight modifications. FRAP reagent consisted of 25 ml of 300 mM acetate buffer solution pH 3.6 ml, 2.5 ml of 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution, and 2.5 ml of 20 mM $FeCl_3.6H_2O$ solution. The reaction between 100 µl hydrolysates containing 0.091 mg Leucine/ml peptide concentration and 1 ml FRAP reagent resulted in blue-purple color changes, which was monitored at wavelength of 593 nm. A standard of Trolox (0 to 400 µM) was used for calculation. Results were expressed in µM Trolox equivalence.

The ability of hydrolysates to chelate ferrous ion was assessed by colorimetric assay modified from Carter (1971). Initially, 100 µl hydrolysate containing 0.05 mg Leucine/ml peptide concentration was mixed with 1400 µl of DI water and 100 µl of 2 mM $FeCl_2$ and incubated in dark for 3 min at ambient temperature. Ferrozine solution in amount of 400 µl was subsequently added to the reaction and further incubated for 10 min, subsequently measured the absorbance at 562 nm. A standard curve made from EDTA at concentration of 0-900 µM was used

for calculation and the activity was reported in μM EDTA equivalence.

4.3.8 Cellular-based antioxidant activity assays

Human hepatocellular carcinoma (HepG2) cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Briefly, cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin, 1% (v/v) non-essential amino acids (NEAAs), and 1% (v/v) L-glutamine in 95% humidified incubator at 37˚C and atmosphere of 5% CO_2. Cells were maintained by replacing the culture medium in thrice a week. Sub-culture was carried out at approximately 90% confluence using 0.05% trypsin-EDTA in 1x PBS.

HepG2 cells were seeded in a clear 96-well micro-plate (Corning, Jiangsu, China) at density of $2x10^4$ cells/well and incubated for 24 h until it reached 70-80% confluence. Cytotoxicity of hydrolysates was determined. Briefly cells were exposed to various concentrations of samples (1.5625-100 mg/ml of peptides) and ascorbic acid (AsA) (0.015624-0.5 mg/ml), which were dissolved in DMEM without FBS. Cell viability on HepG2 cells was determined by MTT assay after 24 h incubation.

To study protective effect of peptides against H_2O_2, HepG2 cells were seeded following the protocol described above. Initially, cells were pretreated with hydrolysates at concentrations of 3.125-12.5 mg/ml for 24 h. Pretreated cells were rinsed thrice with 1x PBS and subsequently added 1.8 mM H_2O_2 dissolved in DMEM without FBS, and further incubated for 3 h. Viable cells were examined by MTT assay.

MTT solution in concentration of 0.5 mg/ml in 1x PBS was added to the treated cells and incubated for 4 h. Subsequently, DMSO was added to solubilize

formazan crystal. The absorbance was read at 570 nm using a SPECTROstar NANO system (BMG Labtech GmbH, Ortenberg, Germany).

Intracellular ROS was evaluated using 2',7'-dichlorofluorescein-diacetate (DCFH-DA) probe. Cells were cultured in 96-wells black plate (Corning, NY, USA) with the same protocol as described above. Culture medium was firstly discarded and washed thrice with 1x PBS prior to the addition of DMEM containing 3.125 mM H_2O_2 and incubated for 3 h. Cells was then treated with either hydrolysates or AsA dissolved in the medium without FBS at a non-toxic concentration for 3 h. Subsequently, cells were washed thrice with 1x PBS and 25 μM DCFH-DA was added then incubated for 1 h. Fluorescence intensity was detected using CLARIOstar microplate reader (BMG Labtech, GmbH, Ortenberg, Germany) at excitation and emission wavelengths of 490 and 535 nm, respectively.

4.3.9 Fourier-transform infrared (FT-IR) spectroscopy

FT-IR spectra of ultrasound-treated and control egg white hydrolysates was obtained from FT-IR spectrometer (Bruker Tensor-27, Karlsruhe, Germany) with attenuated total reflectance (ATR) diamond crystal. Lyophilized hydrolysates were scanned in wavelength range of 400-4000 cm^{-1} at resolution of 4 cm^{-1}. The spectrum of each hydrolysate was obtained from 64 scans in absorbance format and analyzed by OPUS 7.5 software (Bruker Optics GmbH, Ettlingen, Germany). Background interference was removed from each spectrum, subsequently smoothed, normalized, corrected baseline and calculated the average spectra. Principal component analysis (PCA) was determined using UnscramblerX version 10.4 software (Camo Analytics, AS) to obtain correlation between antioxidant activity and structural alteration of egg white hydrolysates. Spectra representative was examined by Sigmaplot version 13.0 software (Systat Software Inc., UK).

4.4 Results and Discussion

4.4.1 Degree of hydrolysis and yield protein

DH values of egg white hydrolysates were varied between US-treated samples and the controls. At 1% Alcalase, DH values of boiled egg white hydrolysate prepared from US treatment (US-B1%) was considerably higher than the control without US (CB1%) (p<0.05, Table 4.1). The protein recovery was also markedly increased approximately from 12% to 31% (p<0.05). A possible reason was attributed to the cavitation effect of US which turned protein into small soluble particles, providing sufficient area for protease. Literatures stated that US increased solubility and exposure of surface hydrophobicity, further enhanced substrate susceptibility to enzyme, resulting in optimum hydrolysis (Arzeni et al., 2012; Yu et al., 2020).

DH values of hydrolysates of US-treated raw egg white (US-R1%) and the control without US (CB1%) egg white were found comparable (Table 4.1). The protein recovery from CB1% was slightly higher than US-R1% (p<0.05, Table 4.1), indicating that protein remained insoluble at 1% Alcalase hydrolysis with relatively low protein recovery. The presence of protease inhibitors in raw egg white was a major factor hampering hydrolytic reaction (Julia et al., 2007). The result indicated that ultrasound pretreatment on raw egg white could be as effective as heating treatment in accelerating hydrolytic reaction. However, it should be noted that DH values of these samples were relatively low.

It was presented in Table 4.1 that the difference of DH values was conspicuous between the control without US prepared from raw (CR10%) and the boiled (CB10%) egg white. As denaturation occurred due to boiling process, proteins started to unfold, exposing cleavage sites for the enzyme. In addition, heating also

inactivated protease inhibitors. All of these changes lead to an increase in protein hydrolysis. Along with the increase of DH, protein recovery was found higher (Table 4.1). It clearly showed that boiling lowered the resistance of substrate to enzyme, allowing the production of soluble protein during hydrolysis.

Table 4.1 Degree of hydrolysis and protein recovery of egg white hydrolysates.

	Enzyme concentration	**Egg white hydrolysates**	**Degree of hydrolysis (%)**	**Protein recovery (%)**
Raw	1%	US-treated (US-R1%)	0.49±0.95 [f]	9.07±0.00 [g]
Boiled	1%	Control (CB1%)	1.21±0.54 [f]	11.70±0.00 [f]
Boiled	1%	US-treated (US-B1%)	9.26±3.37 [e]	30.69±0.41 [e]
Raw	10%	Control (CR10%)	15.28±0.36 [d]	48.67±0.00 [d]
Raw	10%	US-treated (US-R10%)	23.90±0.89 [c]	58.90±0.00 [c]
Boiled	10%	Control (CB10%)	41.58±1.77 [b]	77.45±0.70 [b]
Boiled	10%	US-treated (US-B10%)	45.58±2.05 [a]	83.74±0.00 [a]

* Egg white samples were subjected to ultrasound at intensity of 41.53 W/cm^2 and hydrolyzed by 1% or 10% Alcalase. Different superscripts in column indicated significant differences. Mean ± standard deviation values were presented (n=3)

The effect of US was evident in the raw egg white hydrolyzed by 10% Alcalase (CR10% vs. US-R10%), which the difference DH was pronounced (Table 4.1). Apparently, US pretreatment participated on protease inhibitors inactivation (Lei et al, 2011) and by the addition of high concentration Alcalase at 10% enabled more effective hydrolysis, leading to higher DH value. Similar results of higher DH was

observed in boiled egg white hydroysates of US treatment (US-B10%) than the control without US (CB10%) (p<0.05, Table 4.1). The US treatment was responsible for secondary structure of egg white protein alteration (Wu et al., 2018), which exposed active sites for enzyme hydrolysis. Therefore, US-treated raw and boiled egg white showed remarkable differences in DH values as compared to the controls. Stefanovic et al. (2014) stated that 15-min ultrasound treatment of raw egg white proteins at power of 21.3 W and 40 kHz frequency enhanced Alcalase hydrolysis with the highest DH of 31.44±0.251%. However, prolong ultrasound treatment tremendously decreased DH as aggregation of protein formed which hampered the hydrolytic reaction. Wu et al. (2018) recognized that 15-min ultrasonic treatment at frequency of 20 kHz at 300 W could help Alcalase to breakdown whey protein structure, resulting in almost 25% DH which was notably higher than the control hydrolysate without ultrasound.

The effect of boiling and US was clearly studied from the comparison of US-R10% and US-B10%, in which US-B10% showed the highest DH and protein recovery (p<0.05, Table 4.1). Boiling started to perform partial denaturation on egg white. The following US pretreatment subsequently promoted protein unfolding which further facilitated enzyme to act on it. Thus, our study suggested the combination of boiling and ultrasound treatment on egg white to increase hydrolytic reaction and contributed to high DH and protein recovery.

4.4.2 Microstructure of egg white

Different sample preparation led to various microstructure characteristics. Lyophilization frequently produced artifact due to ice crystal formation, limiting SEM observation at high magnification of >50x (Figure 4.1a & 4.1b). Unlike lyophilization, fixation and CPD seemed to minimize structural damage, enabling

SEM to experience high magnification of 5000x (Figure 4.1c & 4.1d), which provided higher image resolution.

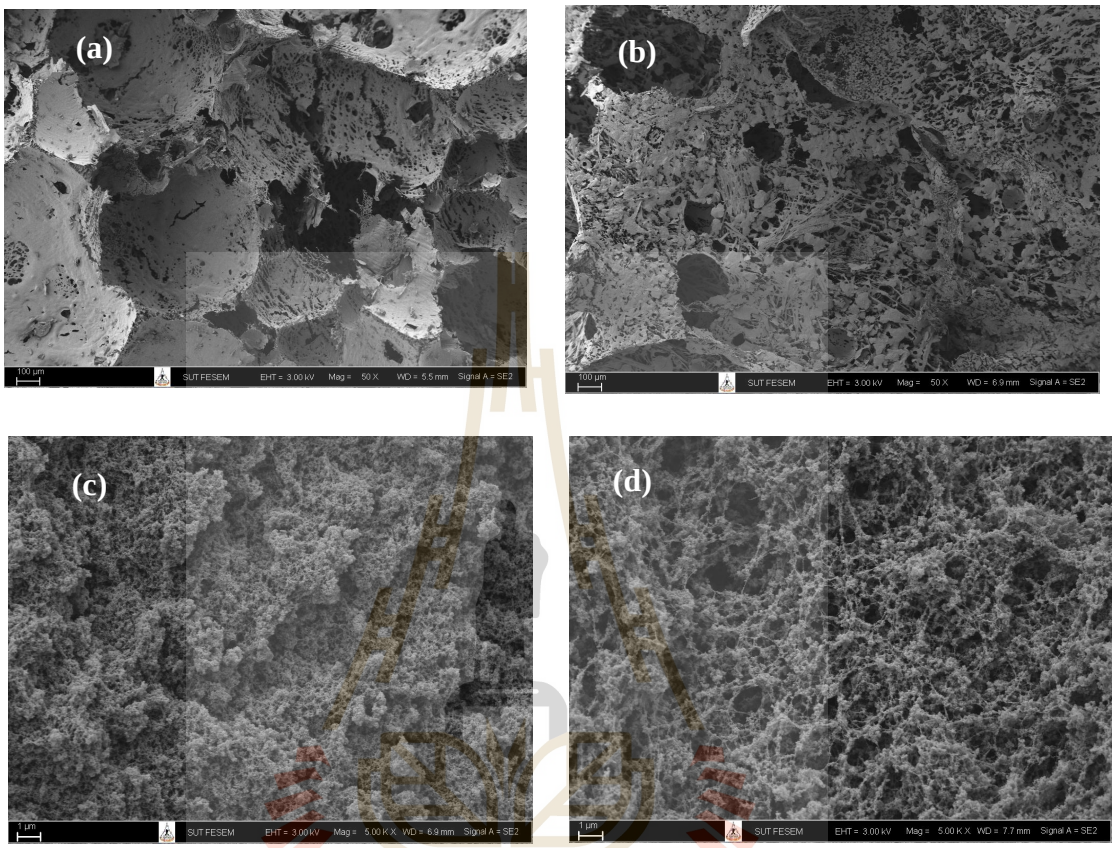

Figure 4.1 SEM images of raw egg white (a) and US-treated (b) raw sample at magnification of 50x, boiled egg white (c) and US-treated (d) boiled sample at 5000x magnification.

Ultrasound seemed to modify surface morphology of egg white. The lyophilized raw egg white showed smooth and dense surface (Figure 4.1a), while US treatment resulted in cracks on surface with more appearance of opening pores (Figure 4.1b). In contrast, boiled egg white showed aggregate appearance (Figure 4.1c), whereas after US treatment, the disintegration of aggregates was evident (Figure 4.1d). This might help increasing more enzyme accessibility.

Numerous disulfide bonds in raw egg white likely appeared as a dense structure (Figure 4.1a), which was likely interfere sonication effect. The structure of US-treated raw egg white showed more pores (Figure 4.1b), but yielded hydrolysate with low DH at 1% Alcalase (US-R1%) (Table 4.1). This was likely due to relatively low enzyme unit. Some studies reported that ultrasound treatment had no impact on enzymatic hydrolysis. Stefanovic et al. (2014) reported that 60 min exposure time of ultrasound treatment on egg white protein showed minimal effect on DH value due to the formation of protein aggregation which could impede enzyme accessibility. Moreover Lei et al. (2011) stated that there was no enhancement in hydrolysis of ovotransferrin even though sulfhydryl group was exposed due to ultrasound treatment.

In contrast, inactivation of protease inhibitor in boiled egg white seemed to increase enzyme accessibility (Figure 4.1c) and was probably beneficial to enhance hydrolysis, even at low enzyme concentration of 1%. Stefanovic et al. (2018) demonstrated that ultrasound disturbed disulfide, hydrogen, covalent and non-covalent bonds, resulting in small particles. This would in turn increasing surface area for enzyme accessibility (Figure 4.1d). This explained why US-treated boiled egg white hydrolyzed by 10% Alcalase (US-B10%) exhibited the highest DH (p<0.05, Table 4.1).

4.4.3 SDS-PAGE

SDS-PAGE patterns of egg white proteins as compared to egg white hydrolysates after hydrolysis of US-treated egg white by 1% and 10% Alcalase were presented in Figure 4.2. Three intense bands illustrated in raw egg white were ovotransferrin (OT) (85 kDa), ovalbumin (OV) (50 kDa), and lysozyme (LY) (13 kDa). Ovotransferrin disappeared in the boiled egg white, while other 2 proteins remained.

It indicated that boiling at 95˚C caused denaturation of some portions of egg white proteins resulted in the loss of OT with the contrary of OV and LY were heat-stable and not degraded.

Most residues after hydrolysis showed molecular weight lower than 10 kDa indicating shorter peptides remained after Alcalase hydrolysis. Larger proteins were observed in residues obtained after 1% Alcalase hydrolysis, particularly of hydrolysates of control (CB1%) and US-R1% (Figure 4.2a). In the case of CB1%, high molecular weight of peptides in range of 13-49 kDa was clearly noticeable (Figure 4.2a), indicating suboptimal hydrolysis rate. The effect of ultrasound was evident by US-B1% which contained smaller protein fragments without any big bands appeared than that of CB1% (Figure 4.2a). The decrease of high molecular weight peptides also arose after US pretreatment on raw egg white, yielding hydrolysate (US-R1%) residue with only a visible band at 25 kDa (Figure 4.2a). It showed that ultrasound involved in better hydrolysis process than that of without ultrasound. The SDS-PAGE pattern was likely explained higher DH and protein recovery of US-B1% as compared to those of CB1% and US-R1% (p<0.05, Table 4.1) due to a cavitation effect of US which caused the damage of protein aggregation and exposure of catalytic sites, further increasing hydrolysis rate.

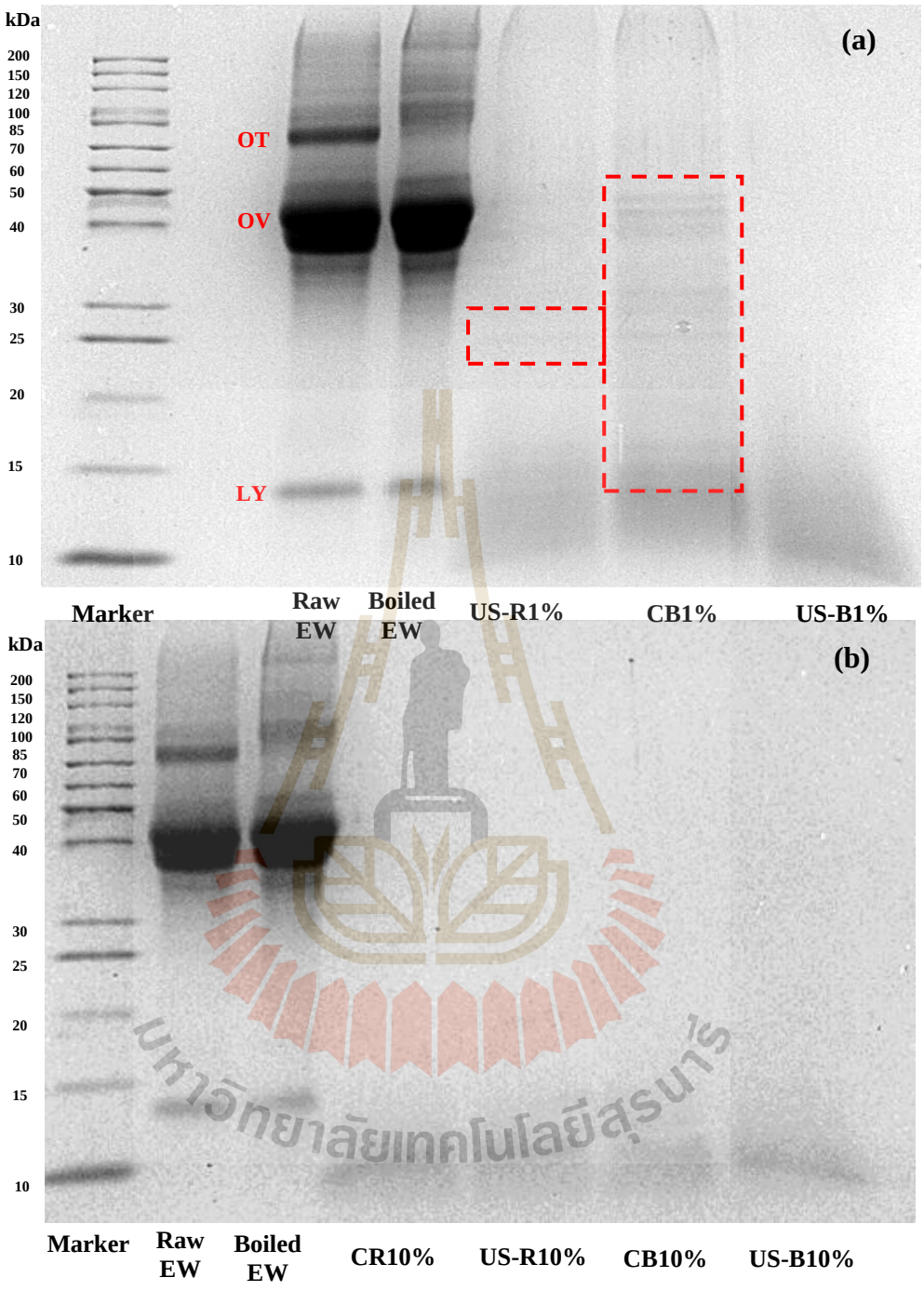

Figure 4.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of residues after hydrolysis of ultrasound-treated egg white using 1% (a) and 10% (b) Alcalase at 20 µg protein concentration.

On the contrary, only peptides with molecular weight less than 10 kDa were observed in residues of samples hydrolyzed by 10% Alcalase (Figure 4.2b). Higher DH of hydrolysates obtained from 10% Alcalase (Table 4.1) resulted in smaller fragments of proteins/peptides in the residues. Although US-treated boiled egg white generating hydrolysate (US-B10%) with the highest DH and protein recovery (p<0.05, Table 4.1), SDS-PAGE pattern of all residues were comparable. The effect of US was inconspicuous from the electrophoretic patterns, as assumed that 10% Alcalase potentially performed excellence hydrolysis. Therefore, hydrolysis using 10% Alcalase produced hydrolysates with considerably higher DH and protein recovery than those of 1% Alcalase (p<0.05, Table 4.1).

4.4.4 Molecular weight distribution

Table 4.2 Molecular weight distribution of egg white hydrolysates.

Samples	>7,000 Da	7,000-1,000 Da	1,000-200 Da	<200 Da
US-R1%	9.58±0.04 ^{c}	66.02±0.18 ^{c}	24.44±0.11 ^{d}	-
CR10%	28.83±0.81 ^{a}	52.92±0.04 ^{e}	18.25±0.09 ^{f}	-
US-R10%	11.61±0.39 ^{b}	53.51±0.54 ^{d}	30.74±0.67 ^{c}	4.55±0.10 ^{b}
CB1%	8.16±0.03 ^{d}	70.79±0.09 ^{b}	21.13±0.14 ^{e}	-
US-B1%	9.51±0.14 ^{c}	73.24±0.11 ^{a}	17.33±0.07 ^{g}	-
CB10%	1.34±0.11 ^{e}	47.65±0.14 ^{g}	51.11±0.11 ^{a}	-
US-B10%	2.93±0.06 ^{f}	49.91±0.11 ^{f}	41.54±0.09 ^{b}	5.56±0.07 ^{a}

* Different superscripts in column indicated significant difference (p<0.05). Mean± standard deviation (n=3) values were presented.

Raw egg white hydrolyzed by 10% Alcalase produced hydrolysate (CR10%) with the highest proportion of large molecular weight peptides (>7,000 Da). In contrast, control (CB10%) and US-treated boiled (US-B10%) egg white showed the least amount of large peptides (Table 4.2). The result indicated that hydrolysis of raw egg white was limited, resulting in relatively low DH (Table 4.1) and large molecular weight peptides (Table 4.2).

Pile of peptide fractions on range of 1,000-7,000 Da was found abundantly in hydrolysates prepared from control (CB1%) and US-treated boiled egg white (US-B1%) hydrolyzed by 1% Alcalase, accounting for 70.79% and 73.24%, respectively (Table 4.2). At the same fraction range, other hydrolysates were found approximately 47-65% (p<0.05). Boiling process of egg white was observed to promote hydrolysis as egg white protein was partially denatured and protease inhibitors were inactivated. Even though low enzyme concentration was utilized in this experiment, cooked egg white gained smaller molecular weight species as compared to raw egg white hydrolysates.

Hydrolysates of CB10% and US-B10% showed particularly low molecular weight peptides in the range of 200-1,000 Da; approximately 51.11% and 41.54% (Table 4.2). In contrast, other hydrolysates appeared not to contain peptides at this range, but rather peptides with molecular weight >200 Da.

As expected, boiled egg white combined with ultrasound generated a hydrolysate (US-B10%) with a complete peptide bonds cleavage, resulting 5.6% peptides with molecular weight <200 Da. In addition, hydrolysate of US-R10% also contained peptides with lower proportion of small molecular weights than that of US-B10% (p<0.05). Ultrasound pretreatment followed by hydrolysis using 10% Alcalase

showed strong tendency in producing small peptides content on the hydrolysates with a different proportion depending on the initial protein substrate (raw or boiled egg white) and it seemed conspicuous, especially on boiled egg white.

4.4.5 Chemical antioxidant activity

Table 4.3 Chemical antioxidant activity of egg white hydrolysates.

	% Scavenging activity	FRAP (μM Trolox equivalence)	FICC (μM EDTA equivalence)
US-R1%	80.77±0.44 ^{a}	50.83±2.59 ^{a}	637.10±26.80 ^{d}
CB1%	68.89±1.61 ^{b}	46.49±2.70 ^{b}	765.41±16.96 ^{e}
US-B1%	51.17±1.43 ^{de}	6.31±0.05 ^{c}	907.24±27.41 ^{de}
CR10%	53.20±0.03 ^{de}	4.21±1.18 ^{cde}	1285.10±39.24 ^{bc}
US-R10%	54.51±0.16 ^{cd}	3.33±0.12 ^{de}	1477.56±32.13 ^{b}
CB10%	53.56±3.01 ^{de}	1.56±0.12 ^{e}	1373.79±36.87 ^{c}
US-B10%	56.92±10.45 ^{c}	5.84±0.65 ^{cd}	1718.73±5.18 ^{a}

* Different superscripts in each assay indicate significant difference (p<0.05). Mean± standard deviation (n=3) values were presented. ABTS, FRAP, and FICC were executed at peptide concentration of 10, 91, 50 μg Leucine/ml respectively

DH value was conversely proportional with ABTS and FRAP assay (Table 4.3). US-R1% possessed the highest ABTS scavenging activity and reducing power in a dramatically low DH, approximately 0.49%. Dryakova et al. (2010) reported that there was no direct correlation between DH and antioxidant activity found in whey protein hydrolysates. However, egg white protein subjected to high-

intensity pulsed electric field treatment hydrolyzed by Alcalase resulted in low molecular weight peptides with high ABTS capacity by neutralizing ABTS cation (Lin et al., 2013). Our study demonstrated that US-B10% with the highest DH (Table 4.1) exhibited lower ABTS scavenging activity than those of hydrolysates possessing low DH. Zou et al. (2016) stated that hydrophobic amino acids have a significant role on scavenging free radicals. The hydrophobic interactions with membrane lipid bilayers facilitated penetration of the peptides to organ tissues. High content of hydrophobic amino acids was closely related to low solubility governing low ABTS activity. Hydrolysate of US-B10% possibly contained large amount of hydrophobic amino acids which signified its less contribution on ABTS radical scavenging activity.

Reducing power ability of egg white hydrolysate was in agreement with scavenger activity in which hydrolysate with the lowest DH (p<0.05, Table 4.1), US-R1%, possessed the highest reducing power (p<0.05, Table 4.3). However Lin et al. (2013) reported that egg white protein hydrolysate with molecular weight <1 kDa exhibited excellent FRAP ability. Sonklin et al. (2018) also reported that mungbean meal protein hydrolysate with the highest molecular weight of >10 kDa exerted high FRAP ability. Although molecular weight of peptides was an important factor in determining antioxidant activity, amino acid sequences likely also contributed to antioxidant activity significantly. Some of Alcalase hydrolysates were reported to contain aspartic acid and glutamine residues which may have significant role in electron donation and a tendency to contribute to reducing power activity (Zou et al., 2016).

Metal chelation ability of egg white hydrolysate was in a positive correlation with DH value. US-B10% experienced the highest DH value (p<0.05,

Table 4.1) with the strongest ferrous chelator (p<0.05, Table 4.3). The result was in agreement with Saiga et al. (2003) who declared that metal chelation correlated with peptides structure and molecular weight. High DH was mostly related to short peptide chain length, corresponding to strong ferrous ion chelating ability. The chelation activity was gradually decreased along with a decrease of DH. The result demonstrated that boiling egg white followed by ultrasound promoted the production of hydrolysate with presumably a high affinity binding to metal ion.

4.4.6 Cellular antioxidant activity

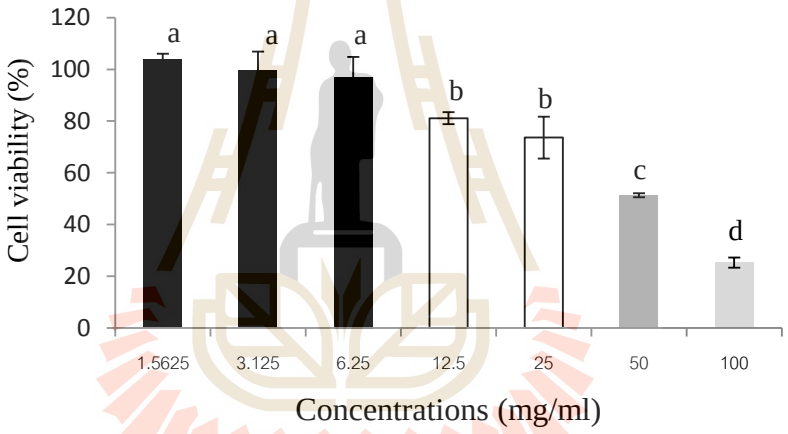

Figure 4.3 Cytotoxicity of ultrasound treated boiled egg white hydrolysates (10% w/w protein Alcalase) at various concentrations. The results corresponded to the mean±standard deviation (n=3). Different letters indicated significant differences (p<0.05)

According to Figure 4.3, boiled egg white treated by ultrasound produced hydrolysate (US-B10%) was nontoxic to HepG2 cells up to 6.25 mg/ml. High concentrations at >50 mg/ml appeared to increase toxicity, causing more than 50% cell lethality. Hydrolysate US-B10% was considered eligible for cytotoxicity

screening due to the positive correlation between DH, protein recovery, and metal chelating ability properties which was eventually an expected result on cellular level. Cytotoxicity assay was carried out for controls and all ultrasound-treated egg white hydrolysates and the results revealed similar trend for all samples. Therefore, concentrations ranging from 1.5625 to 6.25 mg/ml were selected for cytoprotective studies.

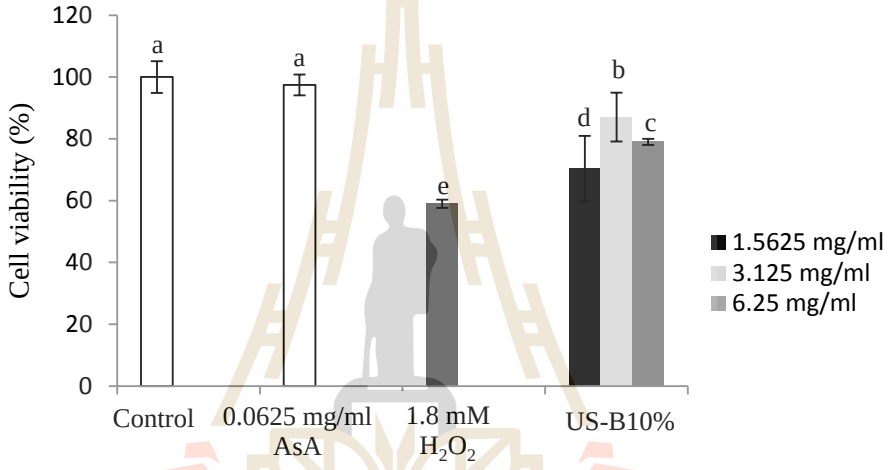

Figure 4.4 Cytoprotective effect of egg white hydrolysates against H_2O_2 at various concentrations. Mean ± standard deviation (n=3) were presented. Bars marked with different letters indicated significant differences (p<0.05).

Our preliminary study revealed that cell viability decreased to 48.025% ±4.191 after incubation of 1.8 mM H_2O_2 for 3 h. Thus, it was selected for further studies. Ascorbic acid (AsA) concentrations of 0.015624-0.0625 mg/ml were found to be nontoxic. Therefore 0.0625 mg/ml AsA was considered as an appropriate concentration for further studies.

Cytoprotective assay was carried out for all US-treated hydrolysates and cell viability at various concentrations for all hydrolysates showed similar trend.

Results of US-B10% were presented as a representative sample (Figure 4.4). Low concentration of hydrolysate showed ability to protect cells against H_2O_2 with the optimum concentration at 3.125 mg/ml. Cytoprotection effect appeared to decrease as concentration increased to 6.25 mg/ml.

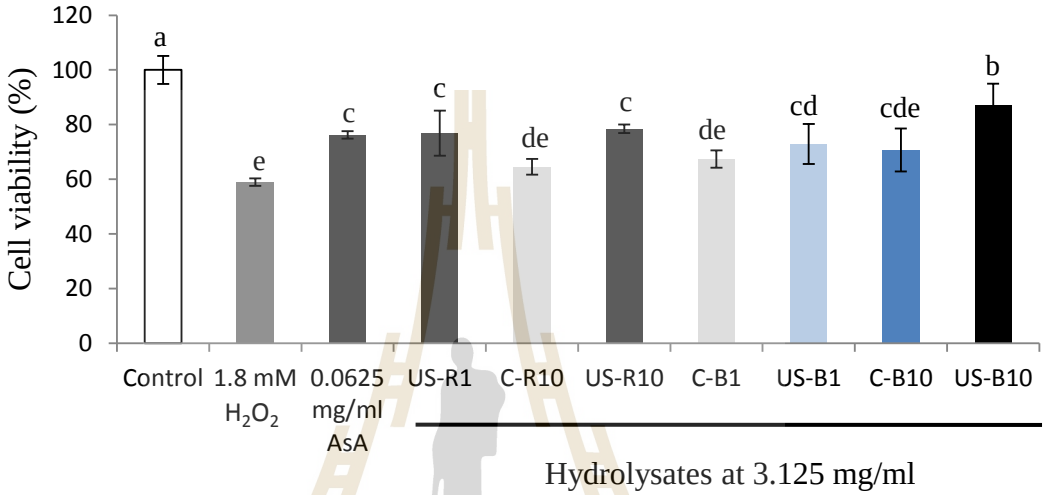

Figure 4.5 Cytoprotection of egg white hydrolysates at 3.125 mg/ml. Results corresponded to mean±standard deviation (n=3). Bars with different letters indicated significant differences (p<0.05)

The result of this study was in accordance with other studies where soybean peptides at concentration of 0.25-1.00 mg/ml showed protective effect at 30 min post-H_2O_2 stimulation (Yi et al., 2020). Hamzeh et al. (2020) also reported that peptides obtained from 6 and 12 months fermented fish sauce at concentration of 50 µg/ml showed cytoprotective effect similarly to 500 µM AsA. It can be concluded that peptides derived from various natural sources exhibited cytoprotection against H_2O_2-mediated oxidative stress at relatively low concentration.

It was apparent from Figure 4.5, oxidative damage occurred when cells were treated by 1.8 mM H_2O_2 for 3 h. Meanwhile the addition of 0.0625 mg/ml AsA resulted in cytoprotection (Figure 4.5). Hydrolysates of US-R1% and US-R10% showed comparable cytoprotective effect with 0.0625 mg/ml AsA (p<0.05) and slightly higher than US-B1% and CB10% (p<0.05; Figure 4.5). With the pretreatment of ultrasound, hydrolysates yielded higher cytoprotective effect both in boiled and raw egg white regardless of the applied amount of enzyme.

Hydrolysates from CB1% and C-R10% exhibited the least cytoprotective effect (p<0.05) with approximately 65% cell viability. Both samples contained the similar size of peptides (Table 4.2), which presumably resulted in the comparable cellular antioxidant activity. In addition, hydrolysate of US-B10% possessed the most effective cytoprotection about 87.052% cell viability (p<0.05). Ultrasound pretreatment of boiled egg white improved cellular antioxidant capacity. Noted that optimum hydrolysis condition by the help of ultrasound was crucial to determine antioxidant activity, in which the effect of its highest DH (Table 3.1) and strongest metal chelating ability (Table 4.3) of US-B10% was recently confirmed in cellular level.

DCFH-DA is a common non-fluorescent probe for ROS. In cytosol, esterase starts to cleave DCFH-DA to DCFH product. The more free radicals generated in cell, the more intense oxidation DFCH to DCF occurs, indicating high fluorescence intensity. Therefore, fluorescence intensity is mostly proportionate with the amount of ROS productions (Bao et al., 2017).

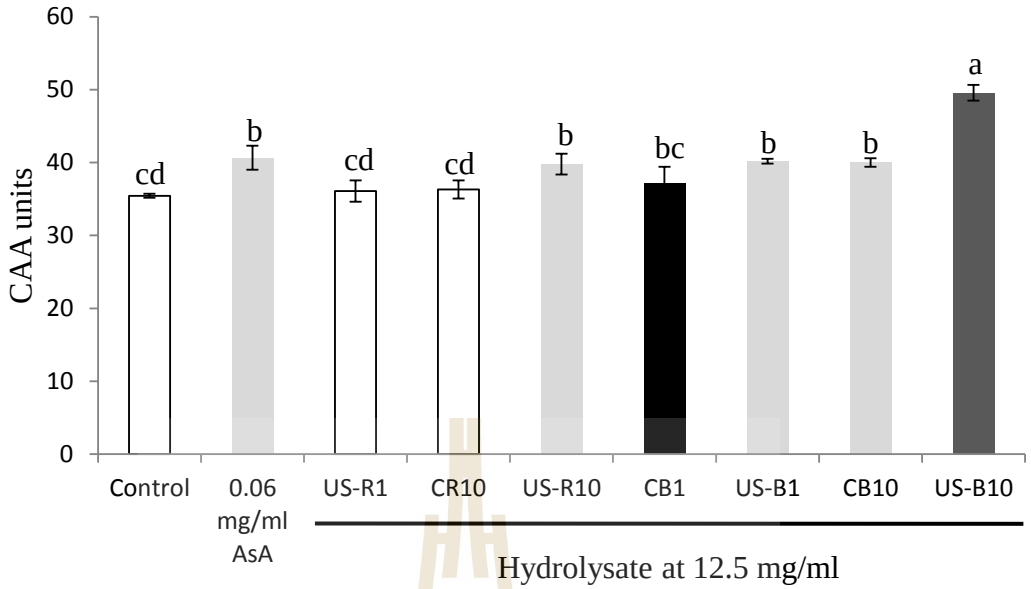

Figure 4.6 Effect of egg white hydrolysates on intracellular ROS scavenging activity of H_2O_2-induced HepG2 cells. Different letters on bars indicated significant differences. Mean±standard deviation (n=3) values were presented.

Majority of hydrolysates showed similar CAA as 0.06 mg/ml AsA, except for US-R1% and CR10%, whose CAA unit was comparable to that of control (p<0.05, Figure 4.6). Most of all, US-B10% showed the most effective ROS intracellular scavenger (p<0.05). It should be noted that intracellular ROS scavenging property of US-B10% was well correlated with its metal chelating ability. The chelation power was an essential property to counteract the oxidative stress accumulated in cells (Adjimani & Asare, 2015). More importantly, majority of low molecular weight peptides also determined its antioxidant activity (Tintore et al., 2015). Important to note that the highest intracellular ROS scavenging activity of US-B10% was in agreement with the highest cytoprotective result. It signified that US-

B10% was not only capable to remove intracellular ROS, but also to maintain cell viability under H_2O_2-induced excessive ROS production. Our findings suggested that ultrasound played a key role in the enhancement of cytoprotective and intracellular antioxidant properties which was dominantly achieved by the excellence metal ion chelating ability of its hydrolysate.

4.4.7 FT-IR

FT-IR was applied to elucidate structural changes of egg white hydrolysates samples. The wavenumbers ranging from 1600-1700 cm^{-1} indicated amide I region containing α-helix in 1650-1660 cm^{-1}, β-sheet in 1665-1680 cm^{-1} and 1614-1637 cm^{-1}, β-turn in 1680-1690 cm^{-1} and random coil in 1640-1642 cm^{-1}, while wavenumbers 1500-1600 cm^{-1} corresponded to amide II region (Pao et al., 2021; Uysal & Boyaci, 2020). The wavenumbers ranging from 1400-1700 cm^{-1} were major bands observed from FT-IR spectra. Second derivatives of FT-IR spectra of all samples revealed that raw egg white predominantly contained β-sheet structure at 1633 cm^{-1} (Figure 4.7a). When egg white was cooked, the major bands shifted to 1625 cm^{-1}. The shifting to a lower wavenumber indicated an increase in intermolecular hydrogen-bonded β-sheet components (Uygun-Saribay et al., 2016). The pronounced changes of hydrolysates were observed at 1641 cm^{-1} (Figure 4.7a), corresponding to a decrease of amide I region from 4.50 to approximately 1.5-2.27, depending on the hydrolysis condition (Table 4.4). These results indicated the disruption of hydrogen bonds and peptide bonds cleavage during hydrolysis, promoting the transition of α-helical structure to random coils (Uygun-Saribay et al., 2016). The significant reduction of amide I region was obvious on hydrolysates when combination of ultrasound pretreatment and 10% Alcalase was applied (Table 4.4), indicating that the transformation

of random coils was more profound than that of the control without ultrasound.

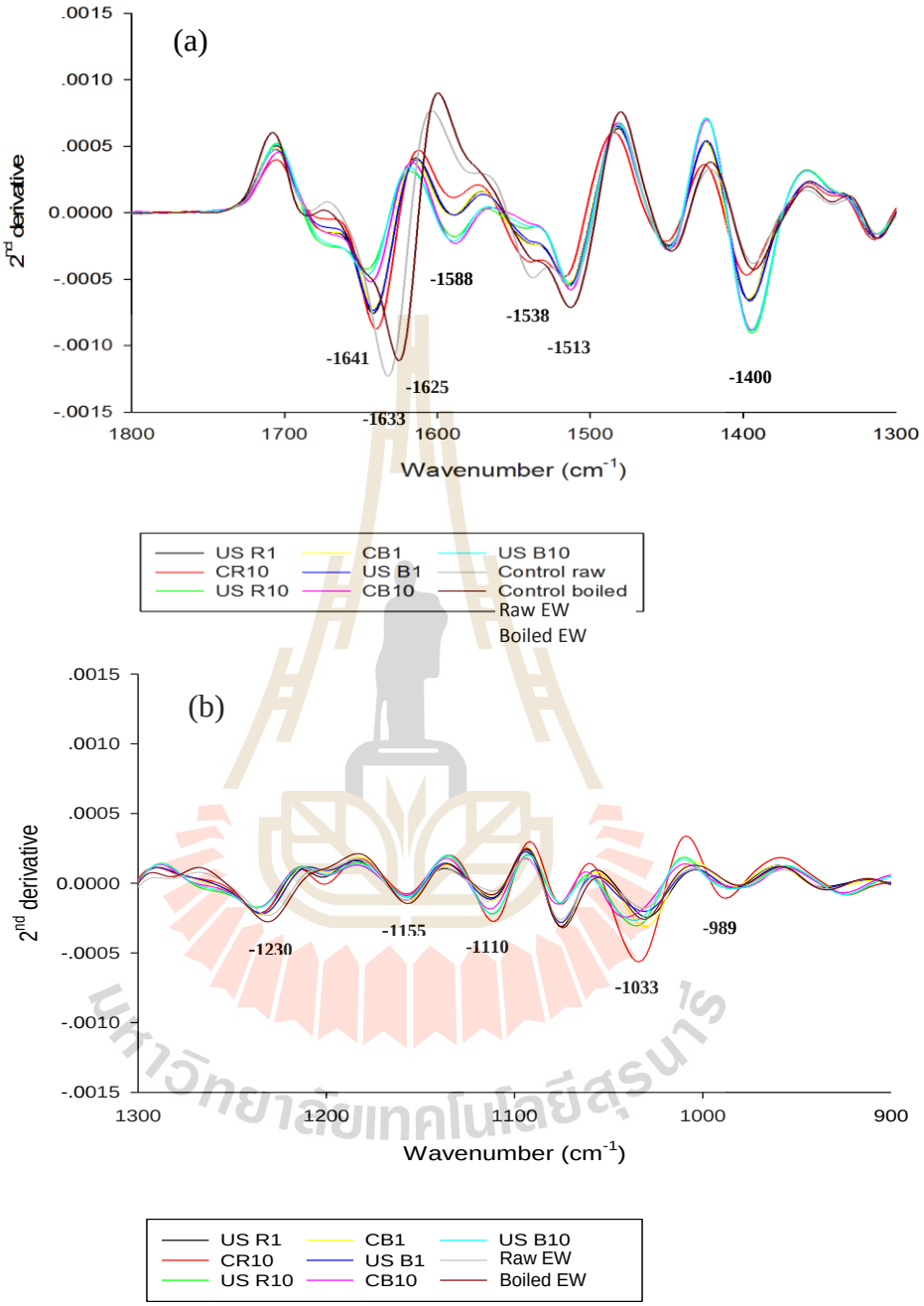

Figure 4.7 Second derivatives of FT-IR spectra of egg white proteins and hydrolysates samples at wavenumbers of (a) 1300-1800 and (b) 900-1300 cm^{-1}.

Hydrolytic reaction usually led to the breakdown of peptide bonds along with the formation of N-terminus (NH_3^+) at 1500 cm^{-1} and C-terminus (COO^-) at 1400 cm^{-1} (Wubshet et al., 2017). As seen in Figure 3.7a, intact egg white protein showed low integral area at amide II region as only peak appeared at 1538 and 1513 cm^{-1} (Table 4.4). Unlike egg white proteins, the amide II peaks of hydrolysates were observed in wavenumbers ranging from 1513-1588 cm^{-1} (Figure 4.7a). Oligopeptides in hydrolysates existed in amide II, which was mostly associated with the more vibration of N-H groups bending and C-H groups stretching (Kobayashi et al., 2017). Amide II containing peak at the wavenumber around 1513 cm^{-1} was assigned to NH_3^+ (scissoring) groups of N-terminal end (Wubshet et al., 2017). The result indicated that hydrolysis was responsible for a complete destruction of secondary structure of egg white proteins. Therefore, as compared to egg white proteins, hydrolysates showed a notable increase of amide II region ($p<0.05$, Table 4.4).

The intense peak at 1400 cm^{-1} (Figure 4.7a) originated from stretching of carboxylic groups (COO^-) indicating an increase of C-terminus (Wubshet et al., 2017). Intact protein of both raw and cooked form showed the lowest integral area at this region (Table 4.4). In contrast, all hydrolysates showed higher integral area ($p<0.05$, Table 4.4) with the hydrolysate US-B10% showed the highest. The result was consistent with the highest DH (Table 4.1) as a result of excellent hydrolysis. This was attributed to combination between boiling process and US pretreatment. In addition, the symmetric and/or asymmetric stretching of carboxylic group frequently indicated the exposure of aliphatic moieties of amino acid side chains upon peptide hydrolysis (Maqsoudlou et al., 2020). Boiling egg white followed by ultrasound pretreatment prior to hydrolysis at high strength of enzyme (10% Alcalase) resulted in

hydrolysate with large proportion of peptides containing aliphatic groups. It was concluded that short peptides of US-B10% consisted of hydrophobic amino acids, which further referred to the highest metal chelation ability (Table 4.3), cytoprotective effect (Figure 4.5) and intracellular ROS scavenging activity (Figure 4.6).

Table 4.4 Integration area of second derivatization of control and ultrasound-treated egg white and their respective hydrolysates.

Wavenumber (cm^{-1})	**1600-1700**	**1500-1600**	**1400**	**900-1300**
Band assignments	**Amide I**	**Amide II**	**C-terminal**	**CO,CC-bending**
Raw EW	4.50±0.09 ^{a}	0.32±0.10 ^{g}	1.07±0.03 ^{g}	0.51±0.00 ^{c}
Boiled EW	4.55±0.05 ^{a}	0.69±0.08 ^{f}	1.30±0.02 ^{f}	0.64±0.02 ^{a}
US-R1%	1.39±0.18 ^{c}	3.23±0.25 ^{b}	2.33±0.14 ^{e}	0.41±0.01 ^{e}
CB1%	0.31±0.08 ^{g}	2.81±0.05 ^{c}	2.32±0.11 ^{e}	0.51±0.02 ^{c}
US-B1%	1.15±0.32 ^{e}	1.48±0.09 ^{e}	2.35±0.04 ^{de}	0.47±0.03 ^{d}
CR10%	2.27±0.19 ^{b}	3.55±0.19 ^{a}	2.38±0.02 ^{d}	0.66±0.01 ^{a}
US-R10%	1.17±0.08 ^{e}	2.99±0.20 ^{c}	3.10±0.21 ^{c}	0.55±0.03 ^{b}
CB10%	1.29±0.08 ^{d}	2.38±0.30 ^{d}	3.42±0.12 ^{b}	0.49±0.02 ^{cd}
US-B10%	0.73±0.01 ^{f}	1.54±0.02 ^{e}	3.80±0.00 ^{a}	0.51±0.02 ^{cd}

* Different superscript in each column indicated significant difference (p<0.05). Mean ± standard deviation (n=3) values were presented.

The wavenumber in region between 900-1300 cm^{-1} corresponded with CO,CC-bending vibrations, which was mainly correlated with the absorption of sugar moieties (Lewis et al., 2013). The hydrolysate CR10% showed a pronounced peak near 1033 cm^{-1} as a fingerprint of glycoproteins/glycopeptides (Khajehpour et al., 2006). However, boiled egg white and the CR10% showed the highest peak area in this region (Table 4.4). The result demonstrated that there was a strong absorption of glycosylated protein on their peptide backbone. Meanwhile, less intensity in the area of hydrolysates counterparts (Table 4.4) indicated a less degree of glycosylation upon hydrolysis. Zhang et al. (2018) reported that antioxidant activity was mediated by glycosylation reaction however the excessive glycosylation could reduce the antioxidant activity. Hydrolysis could be one possible process which led to disruption of glycosylation, resulting in lower CAA of the CR10%.

This study illustrated that N-terminal ends contained in amide II region and C-terminal ends were characteristic FT-IR band of hydrolysates. Ultrasound seemed to involve in the significant increase of amide II and C-terminal vibration with concomitant decrease of amide I region, which resulted in high antioxidant activity of hydrolysates containing hydrophobic amino acids.

Principal component analysis (PCA) was used to evaluate the correlation between antioxidant activity and FT-IR spectra of the hydrolysates. Biplot was constructed by PC-1 and PC-2 which explained 49% and 28% of the variance respectively. Egg white hydrolyzed by 1% and 10% enzymes was clearly separated by PC-1 (Figure 4.8). PCA revealed that enzyme concentration used for hydrolyze egg white contributes on overall protein structure of the hydrolysates.

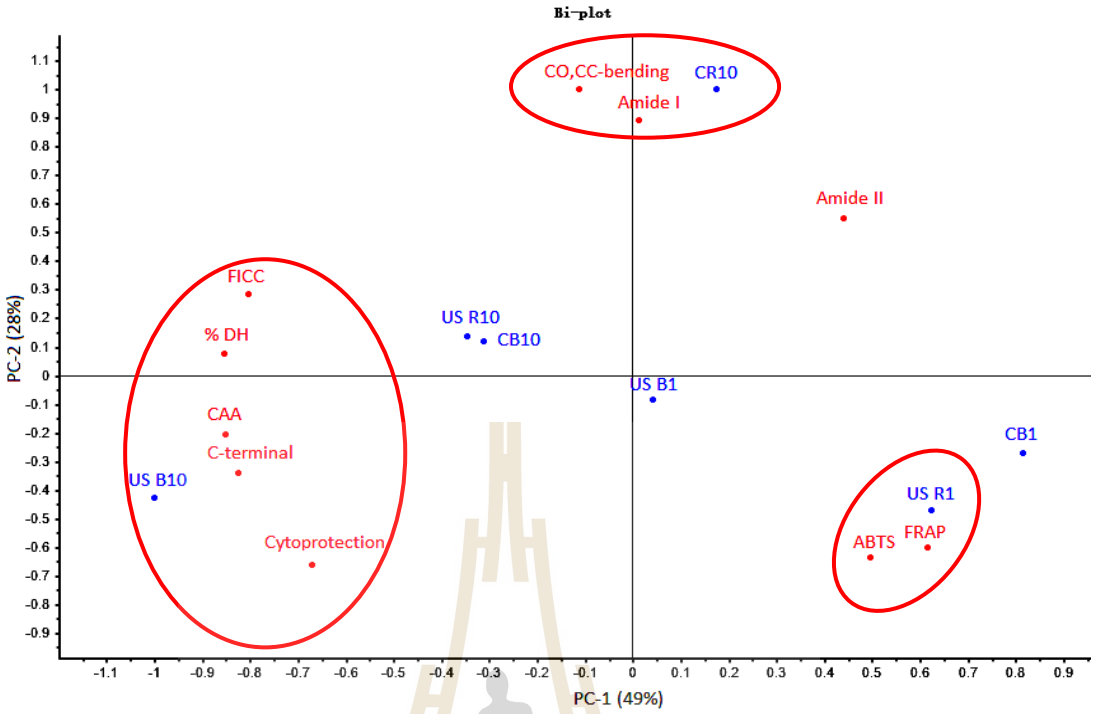

Figure 4.8 PCA Biplot between egg white hydrolysates and measured parameters. The grouping was constructed depending on non significant different among variables.

As seen in Figure 4.8, protein structure of other hydrolysate counterparts were not significant difference. Majority of measured parameter was in same effect as protein structure. The result showed that only amide I and CO,CC-bending vibrations involved in spectra of CR10% as explained in Table 4.4. The hydrolysate US-R1% showed a good correlation with ABTS and FRAP with the opposite region of US-B10% (Figure 4.8). This study indicated that ABTS and FRAP appeared to have limited correlation with cytoprotective effect and ROS level of HepG2 cells.

FT-IR spectra of hydrolysates from CB10% and US-B10% were aligned in opposite quadrant by PC-2 (Figure 4.8), indicating that ultrasound affected the structural changes on boiled egg white. In addition, C-terminal was a dominant feature monitoring DH of hydrolysate US-B10%. This showed a positive correlation with high chelating ability, as confirmed in cytoprotection and CAA. ROS level of HepG2-cells treated by US-B10% was the lowest in significant manner with other hydrolysates. By the help of boiling and ultrasound on egg white, the metal chelation ability of the hydrolysate was predominant to perform cytoprotection and intracellular ROS inhibition in HepG2 cells.

4.5 Conclusions

Influence of ultrasound in antioxidant activity of egg white hydrolysis was clearly revealed. Ultrasound pretreatment on boiled egg white yielded hydrolysate (US-B10%) with the highest DH containing peptides with molecular weight <200 Da and showed the strongest metal chelation ability. The most effective hepatoprotective and intracellular ROS inhibition properties were also addressed to US-B10% at concentration of 3.125 mg/ml and 12.5 mg/ml, respectively. This study suggested that boiling process on egg white promoted hydrolytic reaction. Finally, the combination of ultrasound and boiled egg white appeared to enhance antioxidant activity of the hydrolysate. An obvious evident was studied from the increase of amide II and C-terminal region along with the decrease in amide I region, which predominantly determined high antioxidant activity of US-B10%.

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CHAPTER V

COMPARISON OF MICROWAVE IRRADIATION AND STEAM STERILIZATION PRETREATMENTS ON ANTIOXIDANT ACTIVITY AND STRUCTURAL CONFORMATIONS OF EGG WHITE HYDROLYSATES

5.1 Abstract

Antioxidant activity of hydrolysates obtained from microwave irradiation (MW) and steam sterilization (ST) pretreatments of egg white was investigated in the conjunction with conformational changes. The boiled egg white hydrolysate without pretreatment (CB) possessed the highest degree of hydrolysis (DH) of 43.09% (p<0.05) and exposure of C-terminal region (1400 cm^{-1}), which mainly attributed to the strongest HepG2 cells protection and cellular antioxidant activity (CAA). All pretreatment techniques reduced DH (p<0.05). However, the hydrolysate prepared from 5-min steam sterilization (ST-R5) showed the strongest ABTS radical scavenger (63.55%±0.21), whereas that prepared from 30-min steam sterilization (ST-R30) exhibited the most potent ferric reducing power (FRAP) ability. The highest ferrous ion chelation (FICC) agent was observed in the raw egg white hydrolysate without pretreatment (CR). Antioxidant activity of hydrolysates made from microwave pretreatment was less effective. Fourier transform infrared (FT-IR) spectroscopy

demonstrated that high absorption at amide I (1600-1700 cm^{-1}) and amide II (1500-1600 cm^{-1}) region of hydrolysates prepared from sterilization pretreatment corresponded to high ABTS and FRAP. Principal component analysis (PCA) demonstrated that DH and C-terminal region were in positive correlation with cytoprotection and CAA. Our findings suggested that the production of peptides containing anti oxidative effect should be embarked based on the optimum hydrolytic reaction.

Keywords: microwave, steam sterilization, DH, antioxidant activity, FT-IR.

5.2 Introduction

Protein hydrolysis has been intensively developed to produce peptides containing antioxidant, antihypertensive, anti-inflammatory and antibacterial activities (Mann, et al., 2015). Antioxidant activity is considered as a basis mechanism to protect cell systems against potentially harmful metabolism processes or foreign objects, leading to an extreme oxidation. Oxidative stress occurs when the amount of reactive oxygen species (ROS) is exceeding body defense antioxidant mechanism. ROS is a major cause of several chronic diseases and antioxidants become the most potential remedy to reduce excessive ROS production. Thus production of hydrolysates containing anti oxidative peptides has been widely explored (Nimalaratne et al, 2015).

Chicken egg white has been extensively studied as an excellent quality source of bioactive peptides (Nimalaratne et al, 2015). Previous publications demonstrated that pretreatment of substrate is one of an important means to increase yield and degree of hydrolysis (DH). Several pretreatment technologies have been applied to

egg white, including high-intensity pulsed electric field (PEF) (Lin et al, 2013), high hydrostatic pressure (HHP) (Marciniak et al, 2018), and ultrasound (Stefanovic et al, 2014). The main purpose was to accelerate hydrolytic reaction through unfolding and exposure of enzyme cleaving sites of the protein substrate.

There is much scope of opportunity developing other technologies to prepare egg white as a substrate for enzymatic hydrolysis. Microwave irradiation (MW) and steam sterilization (ST) are drawn attention, regarding to the limited studies concerning the use of these techniques. Microwave is electromagnetic waves in wavelength range of 1 mm–1 m and frequencies range of 0.3-300 GHz. MW enables the disruption of protein aggregation by the quick temperature rising (Lidstrom et al, 2001). Steam sterilization is a chamber that produces steam working by the combination reaction of temperature and pressure. The effect is expected to unfold protein, leading to the exposure of hydrophobic surface (Sezdi & Yoleri, 2014). Along with the modification of protein molecules, susceptibility to enzyme starts to increase, resulting in optimal hydrolytic reaction.

The study aims to elucidate the effect of egg white pretreatments on antioxidant activity of the hydrolysates. Cytoprotection and inhibition of intracellular ROS in HepG2 cells line were also determined. Conformational changes of peptides as a result of various pretreatment were evaluated by Fourier-transform infrared (FT-IR) spectroscopy. The correlation between measured parameters was established by principal component analysis (PCA).

5.3 Materials and methods

5.3.1 Materials

Chicken eggs were purchased from Suranaree University of Technology Farm. Separation of egg white from the yolk was manually carried out. Liquid egg white was referred to as control raw egg white (CR). Some portions of liquid egg white were heated at 95˚C for 30 min and designed as control boiled egg white (CB).

5.3.2 Pretreatments and hydrolysis

Both raw and cooked sample were added deionized water to achieve 5% (w/w) protein concentration. The mixtures were homogenized using a homogenizer (T25 digital ULTRA-TURRAX, IKA Works Inc., NC, USA) and irradiated in microwave digestion unit (Ethos One, Milestone Srl, Sorisole (BG), Italy) at 1400 W to reach various set temperatures; 85, 95, 110, and 121˚C. Time to reach the set temperature was recorded. For steam sterilization, the mixtures were steamed at 121˚C using a steam sterilizer (Tomy SX-700, Tomy Digital Biology Co., Ltd., Tokyo, Japan) for 5, 15, and 30 min. The control and treated egg whites were hydrolyzed using 10% (w/w protein) Alcalase for 4 h at 60˚C and pH of 8.5-9. Supernatant was collected after centrifugation at 10,000 x g for 10 min at 4^{o}C and was adjusted to pH 7. Degree of hydrolysis (DH) was determined by TNBS method (Adler-Nissen, 1979).

5.3.3 Protein recovery

Protein content of egg white hydrolysates were determined by Kjeldahl method (AOAC, 2000). Conversion factor of 5.74 was used to calculate for protein content (Mariotti et al, 2008).

5.3.4 Cellular-based antioxidant activity assays

Human liver hepatoma (HepG2) cell line (American Type Culture Collection, ATCC, Manassas, VA, USA) was cultured in Dulbecco's modified eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and supplemented by 1% (v/v) L-glutamine, 1% (v/v) antibiotics of penicillin-streptomycin, and 1% (v/v) non-essential amino acids at 37˚C in humidified incubator containing 5% CO_2. HepG2 cells were prepared to measure cytotoxicity, cytoprotective, and cellular antioxidant activity assay.

Cells were sub-cultured by trypsinization using 0.05% trypsin-EDTA in 1x PBS and cultivated at density of $2x10^4$ cells/well in a clear 96-well micro-plate (Corning, Jiangsu, China) for 24 h to achieve 80% confluence. Cells were subsequently incubated with various concentrations of hydrolysates; 1.5625–100 mg/ml, ascorbic acid (AsA); 0.015624-0.06 mg/ml, and hydrogen peroxide (H_2O_2); 0.125-4.8 mM. Cytotoxicity was monitored by measuring cell viability after 24 h exposing to hydrolysates, followed by mitochondrial-based MTT assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution at concentration of 0.5 mg/ml in 1x PBS was added to treated cells for 4 h, followed by replacement of DMSO solution to dissolve formazan crystal produced by viable cells. Absorbance reading was recorded using a microplate reader (SPECTROstar NANO system, BMG Labtech GmbH, Ortenberg, Germany) at wavelength of 570 nm. The percentage of viable cells was calculated as follows.

$$\text{Cell viability (\%)} = \frac{\text{Abs}_{\text{treated cells}}}{\text{Abs}_{\text{control cell}}} \text{ x } 100$$

Cytoprotective effect was studied to reveal ability of hydrolysates to protect cell damage due to radical exposure. In brief, HepG2 cells were cultured in a clear 96-well micro-plate (Corning, Jiangsu, China) following protocols explained in chapter IV. Various doses of hydrolysates were applied; 3.125-12.5 mg/ml and incubated overnight. DMEM medium without FBS containing 1.8 mM H_2O_2 was added to induce radical in cell system for another 3 h. The viable cells were determined using MTT assay which reflected cytoprotective ability of hydrolysates.

Intracellular reactive oxygen species (ROS) level in HepG2 cells was monitored by 2',7'-dichlorofluorescein-diacetate (DCFH-DA) non-fluorescent probe. Initially cells were grown in a 96-wells black plate (Corning, NY, USA) at the same protocol as mentioned above. In order to remove any residual compounds, cells were rinsed three times using 1x PBS. Subsequently, cells were added 3.125 mM H_2O_2 in DMEM and incubated for 3 h. Hydrolysates and AsA at concentration of 12.5 mg/ml and 0.06 mg/ml, respectively, were added to cells and further incubated for another 3 h, prior to exposure of 25 μM DCFH-DA solution for 1 h. Fluorescence intensity was read at Ex/Em = 490/535 nm using CLARIOstar microplate reader (BMG Labtech, GmbH, Ortenberg, Germany). The intracellular ROS scavenging activity was expressed as CAA unit using the following equation.

$$\text{CAA unit} = (1 - \frac{\text{Fluorescence intensity of sample}}{\text{Fluorescence intensity of ROS}})\ x\ 100$$

5.3.5 Fourier-transform infrared (FT-IR) spectroscopy

FT-IR spectra of raw and boiled egg white, CR, CB and those obtained from microwave and steam sterilization pretreatments equipped by an attenuated total reflectance (ATR) diamond crystal accessory (Bruker Tensor-27, Karlsruhe,

Germany) was used. Samples were scanned 64 times in range of 400-4000 cm^{-1} at resolution of 4 cm^{-1}. Integration area of second derivative of the average spectra was analyzed by OPUS 7.5 software (Bruker Optics GmbH, Ettlingen, Germany).

Principal component analysis (PCA) was processed by UnscramblerX version 10.5 software (Camo Analytics AS, Oslo, Norway) and analyzed by the Savitzky-Golay algorithm with smoothing filters of seventeen points. Spectra normalization was carried out according to the extended multiplicative signal correlation (EMSC). The score plot was used to distinguish DH and antioxidant activity between raw and boiled egg white, and hydrolysates prepared from pretreatments. Imaging spectra was established by Sigmaplot software version 13.0 (Systat Software Inc., UK).

5.4 Results and Discussion

5.4.1 Degree of hydrolysis and protein recovery

Degree of hydrolysis (DH) is a crucial indicator of the extent of proteolysis reaction, exhibiting the amount of peptide bonds being cleaved by proteases (Nchienzia et al, 2010). DH values of hydrolysates prepared from pre-treated egg white and the correlation with protein recovery were summarized in Table 5.1. The highest DH was achieved from control hydrolysate of boiled egg white (CB) for approximately 43.09% (p<0.05). Protein recovery was congruent with the extent of DH, yielding 77.45% (p<0.05, Table 5.1). In contrast, raw egg white without pretreatment (CR) resulted in hydrolysate with markedly low DH of 15.28% (p<0.05) and 48.67% protein succeeded to be recovered from the substrate.

Table 5.1 Degree of hydrolysis and protein recovery of egg white hydrolysates.

Pretreatments		Hydrolysates	DH (%)	Protein recovery (%)
-		Control raw (CR)	15.28±0.36 [j]	48.67±0.00 [e]
-		Control boiled (CB)	43.09±0.00 [a]	77.45±0.70 [a]
Steam sterilization (ST)	5 min	ST-Raw (ST-R5)	13.49±0.00 [k]	39.55±0.78 [f]
		ST-Boiled (ST-B5)	16.72±0.01 [i]	35.31±0.78 [g]
	15 min	ST-Raw (ST-R15)	16.76±0.01 [i]	48.66±0.53 [e]
		ST-Boiled (ST-B15)	28.61±0.01 [g]	45.73±0.00 [e]
	30 min	ST-Raw (ST-R30)	10.04±0.01 [l]	17.94±0.72 [h]
		ST-Boiled (ST-B30)	15.29±0.05 [j]	19.79±0.21 [h]
Microwave (MW)	85˚C	MW-Raw (MW-R85)	34.96±0.55 [e]	57.09±0.63 [d]
		MW-Boiled (MW-B85)	40.69±0.02 [c]	68.44±2.71 [c]
	95˚C	MW-Raw (MW-R95)	28.17±0.16 [g]	66.46±0.00 [c]
		MW-Boiled (MW-B95)	41.30±0.06 [b]	73.30±0.63 [b]
	110˚C	MW-Raw (MW-R110)	23.71±0.03 [h]	59.43±6.27 [d]
		MW-Boiled (MW-B110)	39.97±0.02 [d]	59.66±0.58 [d]
	121˚C	MW-Raw (MW-R121)	31.80±0.02 [f]	69.34±0.72 [c]
		MW-Boiled (MW-B121)	41.86±1.02 [b]	73.21±0.66 [b]

***** Raw (R) and boiled (B) egg whites were pre-treated by steam sterilization (ST) at 121˚C and microwave irradiation (MW) at power of 1400 W, further hydrolyzed by 10% (w/w protein) Alcalase. Results were presented as mean ± standard deviation values (n=3). Different superscripts in each column indicated significant differences (p<0.05).

A possible explanation regarding to the different behavior was the effect of boiling on egg white which caused protein denaturation and inactivation of protease inhibitors, especially ovomucoid, which exhibited denaturation temperature at 79.75˚C (Julia et al, 2007; Plancken et al, 2004). Cooking at 95˚C applied on egg white increased the ability of enzyme to reach the reactive sites of the unfolded proteins.

It was apparent from Table 3.1 that DH values and protein recovery of hydrolysates prepared from pre-treated egg whites was different from those of the control samples (p<0.05). There was no significant difference between CR and ST-B30 (Table 5.1). The less susceptibility of egg white to enzymatic hydrolysis was a main cause of low DH. This condition occurred because of protein aggregation formed at extreme condition of prolong steaming at 121˚C for 30 min, hampering enzyme accessibility (Salazar-Villanea et al, 2016). However, protein recovery of CR was much higher than ST-B30 (p<0.05, Table 5.1), indicating that control was more efficient to perform hydrolysis (Bernardi et al, 2016).

Moreover, ST-R5 hydrolysate showed relatively low DH of 13.49% (p<0.05, Table 5.1). The exposure of high temperature and pressure of ST in short period of 5 min was possible for a gel network formation. As a consequence of gelation, pre-treated raw egg white turned into insoluble mass and potentially inhibited proteolysis (Juliano et al, 2012). As compared to ST-B5, DH was slightly higher than that of the raw sample (ST-R5) (p<0.05, Table 5.1). Apparently, boiling played a role on providing more catalytic sites for the protease.

When the sterilization time increased to 15 min, DH of hydrolysates increased (Table 5.1), especially for boiled egg white (ST-B15, p<0.05). It was also

found that protein recovery of ST-R15 and ST-B15 was higher than that of ST-R5 and ST-B5 (p<0.05, Table 5.1). At certain time of Salazar-Villanea et al (2016) reported that adequate solubility could eventually promote hydrolytic reaction. Sterilization might induce more soluble aggregate that promoted enzyme accessibility.

However, at 30-min ST treatment on both raw and boiled egg white, lowering DH and protein recovery of the hydrolysates was clearly observed (p<0.05, Table 5.1). Prolonging sterilization time likely induced more aggregation of egg white proteins, hampering enzymatic hydrolysis. Thus, optimal sterilization time was required to obtain optimal hydrolysis.

At the same temperature of 121˚C, hydrolysates prepared from both MW-treated raw and boiled egg white yielded higher DH and protein recovery than those obtained from ST pretreatment (p<0.05, Table 5.1). Rapid heating of MW induced rapid denaturation, leading to the overwhelmed exposure of hydrophobic sites. It was likely preferable for the hydrolytic reaction enhancement (Yang & Lesnierowski, 2020). It could be mentioned that MW pretreatment offered more efficiency in hydrolysis better than ST.

The MW-B121 hydrolysate showed comparable DH and protein recovery with MW-B95 hydrolysate (Table 5.1). The result suggested that exposure of the active sites of boiled egg white was uniform for both 95˚C and 121˚C, resulting in similar DH values and protein recovery. In contrast, MW-R95 hydrolysate showed slightly lower DH than MW-R121 (p<0.05, Table 5.1). MW at temperature of 95˚C seemed incapable to inhibit protein coagulation of raw egg white. Baldwin et al (1967) stated that high viscosity due to coagulation could impede enzyme to attack on the active sites. However, insignificant difference of protein recovery between MW-

R95 and MW-R121 was observed (Table 5.1), indicating similar ability in recovering protein during hydrolysis. In addition, the effect of boiling was obvious in DH and protein recovery between pre-treated raw and boiled egg white (MW-R95 vs. MW-B95; MW-R121 vs. MW-B121) (Table 5.1). Aside from partial denaturation due to boiling process, MW pretreatment, either at 95˚C or 121˚C, on raw egg white likely induced gel network formation which was tightly folded and highly resistant to denaturation. Thus, hydrolysis was found minimum, yielding low DH and protein recovery.

At temperature of 85˚C, DH and protein recovery of the boiled egg white (MW-B85) was slightly lower than those at 95˚C and 121˚C (p<0.05, Table 5.1). Incomplete denaturation was developed as minor protease inhibitors, named ovoglobulin G2 and G3, were reported to denature at 92.5˚C. It was crucial affecting structural of the egg white protein (Guha et al, 2018). MW pretreatment on boiled egg white exploded coagulated protein into small particles however, less explosion likely occurred at 85˚C. Note that larger protein particles tended to minimize enzyme accessibility, arising lower DH and protein recovery as consequences. On the contrary, MW-treatment on raw egg white (MW-R85) resulted in DH value higher than those of other irradiation temperatures. The denaturation effect caused by MW led to variability of viscosity (Baldwin et al, 1967). MW at 85˚C possibly retarded coagulation of raw egg white whose the viscosity was reduced. It was beneficial to facilitate protease breaking during hydrolysis.

Among MW pretreatment, temperature of 110˚C resulted in the lowest DH and protein recovery (p<0.05, Table 5.1). MW at certain temperature promoted the formation of dense structure of unfolded protein. It likely turned into insoluble

fraction which was hardly hydrolyzed by the enzyme, suggesting that pretreatment was somewhat not beneficial to recover the protein. Gladovic et al (2020) stated that insufficient cleaving area was possible to lower protease mobility, reducing DH value. The result demonstrated that the appropriate pretreatment condition determined the course of hydrolysis.

A conclusion could be drawn from this study was the significant effect of boiling due to partial denaturation. Hydrolysates obtained from pretreatment of boiled egg white possessed higher DH than those of raw egg white (p<0.05, Table 5.1). However, DH of hydrolysates obtained from pre-treated egg white was significantly lower than the control (without pretreatments), especially the boiled egg white. Our findings revealed the limitation of pretreatments in producing egg white hydrolysates with elevated DH values. It might because of undesirable changes as a consequence of pretreatments.

5.4.2 Antioxidant activity

Different mechanism of ABTS, FRAP, and FICC assays dictated the diverse on antioxidant activities of the hydrolysates from both controls and pre-treated egg white (Table 5.2). ABTS assay evaluated the ability of hydrolysate to scavenge radical via hydrogen and/or electron donor. FRAP assay measured antioxidant compound to simply reduce Fe^{3+}-TPTZ complex based on electron transfer. The chelating property of hydrolysate for ferrous ion was determined by FICC assay (Shahidi & Zhong, 2015). It should be noted that in the same amino acid sequence and composition, it was possible for hydrolysates to contain variation values of antioxidant activity.

Table 5.2 Chemical antioxidant activity of egg white hydrolysates prepared from various pretreatments of egg white before hydrolysis.

	ABTS scavenging activity (%)	**FRAP Trolox equivalence (μM)**	**FICC EDTA equivalence (μM)**
CR	53.20±0.00 [j]	4.21±1.18 [g]	1385.10±39.24 [a]
CB	55.09±0.00 [h]	1.07±0.02 [j]	1234.35±3.59 [b]
ST R5	63.55±0.21 [a]	13.26±0.13 [c]	883.99±0.66 [h]
ST B5	58.15±0.05 [e]	10.41±0.03 [e]	921.05±0.53 [g]
ST R15	59.18±0.21 [d]	10.29±0.09 [e]	508.16±0.32 [l]
ST B15	58.45±0.09 [e]	12.46±0.43 [d]	340.97±0.32 [n]
ST R30	60.51±0.42 [b]	21.48±0.42 [a]	373.04±0.08 [m]
ST B30	57.50±0.05 [f]	19.39±0.34 [b]	617.80±0.86 [k]
MW R85	36.40±0.35 [l]	0.81±0.01 [j]	682.18±1.86 [j]
MW B85	54.19±0.16 [i]	2.28±0.14 [i]	1085.61±5.77 [d]
MW R95	58.40±0.17 [e]	2.50±0.01 [i]	1116.82±1.99 [c]
MR B95	57.23±0.23 [fg]	3.39±0.08 [h]	852.39±1.30 [i]
MW R110	45.62±0.28 [k]	3.68±0.03 [gh]	942.13±1.66 [f]
MW B110	52.84±0.09 [j]	6.08±0.06 [f]	894.07±0.87 [h]
MW R121	57.07±0.13 [g]	0.83±0.05 [j]	1382.48±6.62 [a]
MW B121	59. 62±0.46 [c]	4.05±0.08 [g]	960.02±0.91 [e]

* Results were expressed as mean±standard deviation values (n=3). ABTS, FRAP, and FICC were carried out at peptide concentration of 10, 91, 50 μg Leucine/ml, respectively. Different superscripts in each column indicated significant differences (p<0.05)

Antioxidant activity of ABTS and FRAP (Table 5.2) were negatively correlated with DH value (Table 5.1). Low DH of 13.49% obtained from ST-R5 showed the strongest ABTS scavenging activity (p<0.05, Table 5.2). Long peptide chain of ST-R5 was governed as the most effective hydrogen or electron donor to stabilize radicals. Hydrolysates prepared from MW pretreatment, with majority of high DH (Table 5.1), exhibited lower scavenging property than those of ST pretreatment (p<0.05, Table 5.2). Zhao et al (2020) stated that long peptide chain was competent to maintain its secondary structure which mainly related to antioxidant activity. Sterilization appeared as such a promising pretreatment to yield hydrolysates with potential scavenging activity.

In contrast, hydrolysate of control boiled egg white possessing the highest DH of 43.09% was found to contain relatively low scavenging activity of 55.09% (p<0.05). It was in agreement with scavenging activity behavior of whey protein hydrolysate (Dryakova et al, 2010) and purple wheat bran hydrolysate (Zhao et al, 2020). The results suggested that longer peptide chain length (low DH) showed the strongest ABTS scavenging activity.

Similar trends were observed in reducing power (FRAP). Sterilization resulted in hydrolysates with substantially higher reducing power (p<0.05, Table 5.2). The ST-R30 hydrolysate with the lowest DH (p<0.05, Table 5.1) showed the highest reducing power of 21.48 μM Trolox equivalence. The lowest reducing power was observed on the CB at DH of 43.09% with only 1.07 μM Trolox equivalence. Apparently, DH value was not the only attribute governing reducing power. Specific amino acid residues in peptides as electron donor, such as tryptophan and glutamate, were served as potential reducing power. Cheung et al (2012) reported that ferric reducing power was correlated with sulfur-containing acidic and hydrophobic amino acids, whereas

contribution to peptide chain length was negligible. The results emphasized the efficacy of sterilization as pretreatment to obtain hydrolysates containing reducing power ability.

Hydrolysates of the CR and MW-R121 with DH of 15.28% and 31.80%, respectively, contained peptides with the most chelating ability, accounting for 1380 μM EDTA equivalence. The comparable ion chelating ability was observed in a wide range of DH values, confirming that specific amino acid residues (e.g. Lys, Arg, His, Asp, Gln) might be responsible for the activity. Zhang et al (2009) reviewed that peptides with histidine at N-terminal and acidic and/or basic amino acids was critical components for metal chelation.

The CB hydrolysate with the highest DH (p<0.05, Table 5.1) showed slightly lower chelating capacity than those of CR and MW-R121 (p<0.05, Table 5.2). The results showed that peptide length was not important for ferrous ion chelation, amino acid compositions could be one of the determinants (e.g. Cys, His). Bamdad et al (2011) reported that metal chelation was performed through electrostatic interaction competent to form complex of charged amino acids with ferrous ion.

It was also interesting to note that hydrolysates prepared from MW pretreatment of raw egg white with notably low DH (p<0.05, Table 5.1), exhibited excellence chelating ability as compared to those of MW and ST pretreatment samples (p<0.05, Table 5.2). Lower DH indicated larger molecular weight with possibility to develop a strong structure of peptides. Bamdad et al (2011) stated one of the ion chelation was determined by the peptide structure. It offered a concept of ‘cage’ to entrap metal ion, which was possibly achieved by large molecular weight of peptides. Thus, low DH hydrolysates prepared from MW pretreatment of raw egg white was a great ferrous chelator. Our findings revealed that ferrous ion chelator activity

of hydrolysates were accelerated by microwave pretreatment.

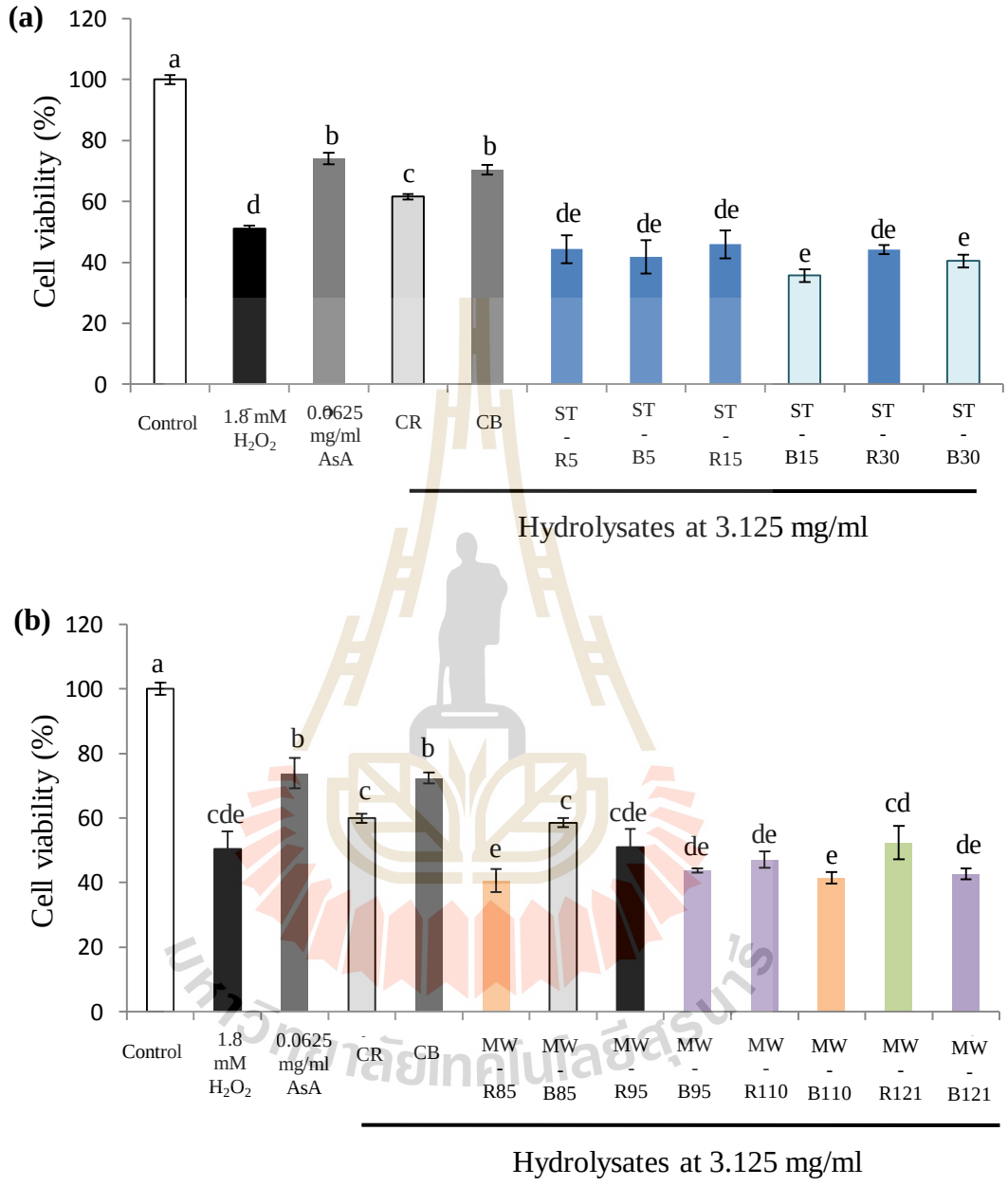


Figure 5.1 Cytoprotective of hydrolysates obtained from (a) steam sterilization, (b) microwave pretreatments of egg white. Data were expressed as mean± standard deviation of triplicates. Different letters on bars indicated significant difference (p<0.05)

Our preliminary study showed that 1.8 mM H_2O_2 started to cause cell lethality by 48.03%±4.19. In contrast, ascorbic acid (AsA) witnessed maintaining cell survival of 74.08%±1.89 at concentration of 62.5 µg/ml. All hydrolysates samples were tested at range of concentrations resulting 3.125 mg/ml. According to Figure 5.1, the CB hydrolysate showed the highest cytoprotective effect of 70.39%±1.63. The result demonstrated that CB was capable to inhibit H_2O_2-induced cell lethality with viability rate similar to that of AsA (p<0.05). It was an evident that small peptides of CB showed a promising protection mechanism over excessive stimulation of H_2O_2.

Tendency of cytoprotection of hydrolysates prepared from steam sterilization and microwave pre-treatements were much lower with approximately 30-40% (p<*0.05*) viable cells, this could be due to excessive heating of pretreatments, enabling secondary structure modifications. Literature stated that antioxidant activity might be relied on secondary structure (Korczek et al, 2019). Thus, the extreme denaturation, which promoted aggregation formation, was apparent to lower the released of such amount of amino acids during hydrolysis which possibly possessed cytoprotection.

The CB hydrolysate showed the highest CAA unit (p<0.05, Figure 5.2), corresponding to the highest DH value (p<0.05, Table 5.1). It indicated that small peptides contributed to cellular antioxidant activity. Bao et al (2017) reported thst smaller peptides could penetrate cell membranes more efficiently and exert their ROS scavenging activity. It was crucial to understand that CB possessed double indirect antioxidant activities as cytoprotection and ROS scavenger. Zhang et al. (2020) reported that cytoprotection was correlated to ability of peptides in maintaining cell viability after H_2O_2-stimulated oxidative stress, while ROS scavenging activity evaluated peptides that could inhibit and encounter intracellular ROS.

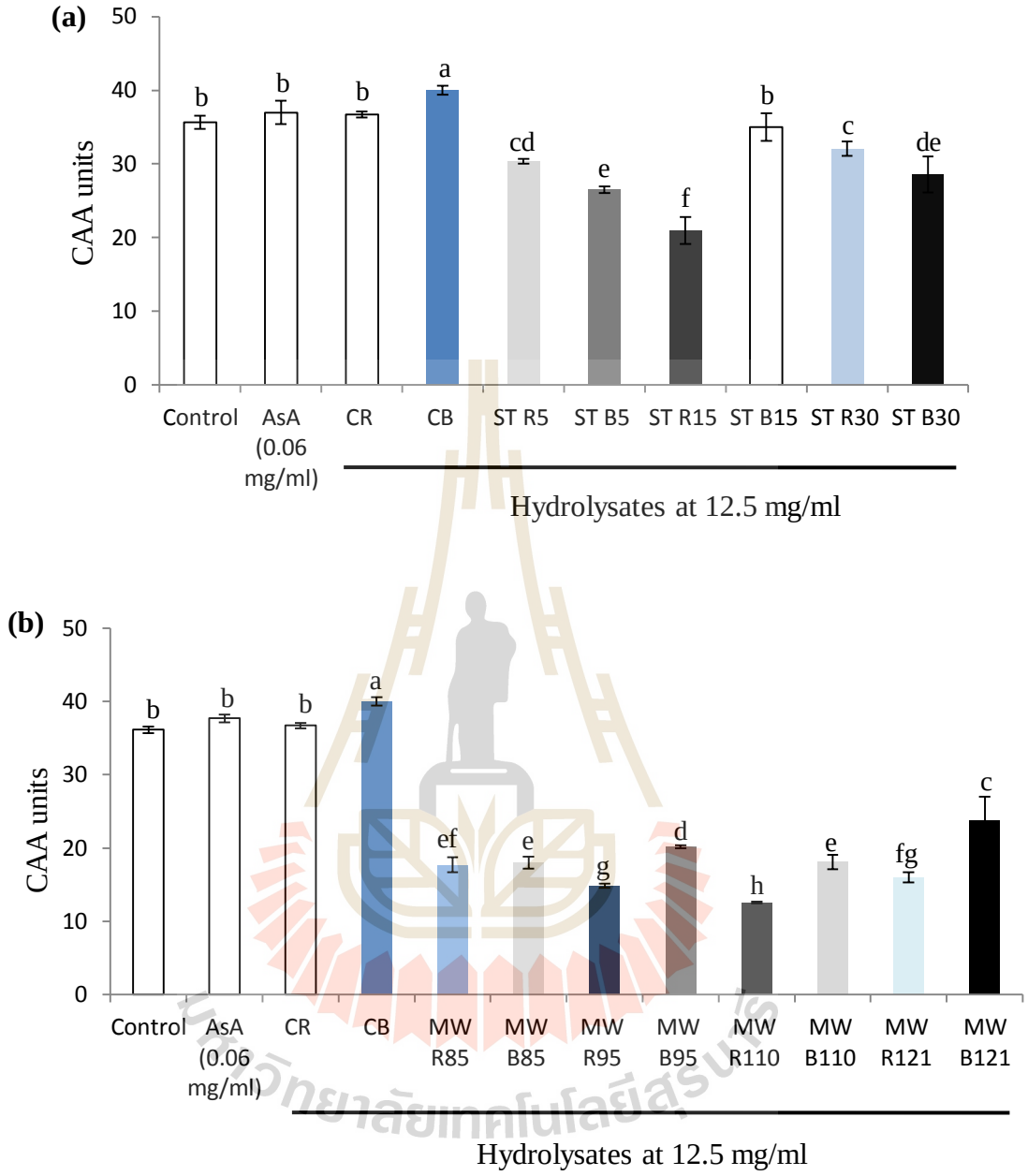


Figure 5.2 Intracellular ROS scavenging activity of hydrolysates at 12.5 mg/ml prepared from (a) steam sterilization and (b) microwave pretreatments. Different letters indicated significant difference (p<0.05). Mean± standard deviation (n=3) values were presented.

The CR hydrolysate showed comparable intracellular scavenging activity to AsA, the control cell, and ST-B15 hydrolysate (p<0.05, Figure 5a). The CR and ST-B15 possessed the highest DH among those of ST-treated egg white (Table 5.1). Note that cooked egg white followed by 15-min sterilization resulted in hydrolysate with an effective ROS scavenger which showed similar ability to AsA. Meanwhile, other hydrolysates prepared from ST-treated egg white showed a comparable activity in scavenging intracellular ROS of approximately 20-32 CAA units, corresponding to high ABTS and FRAP values (Table 5.2). The results suggested that chemical antioxidant activity assays could be a first screening to estimate the antioxidant activity of the hydrolysates, further corroborating in cellular studies.

Hydrolysate of MW-R110 showed the lowest CAA units (Figure 5.2b), correlating to its lowest DH (p<0.05, Table 5.1). Mitochondria was one of ROS producers, however it should be noted that mitochondria was also a target of damaged cell injured by ROS (Jiang et al, 2014). Long peptide size of MW-R110 seemed incompetent to fight against excessive ROS, nor mitochondrial-mediated apoptosis could be stemmed. It might because of lack ability in entering gastrointestinal membrane and liberating its antioxidant activity. Although microwave pretreatment on egg white resulted in hydrolysates with majority comprising high metal chelation ability, the CAA units was much lower than those of control samples (Figure 5.2b). Different amino acid sequences containing anti oxidative peptides could be the major contributor. Our findings demonstrated that pretreatment could have a significant effect in antioxidant activity enhancement of egg white hydrolysates, but the complexity of cellular mechanism was unpredictable by which various activities might occur.

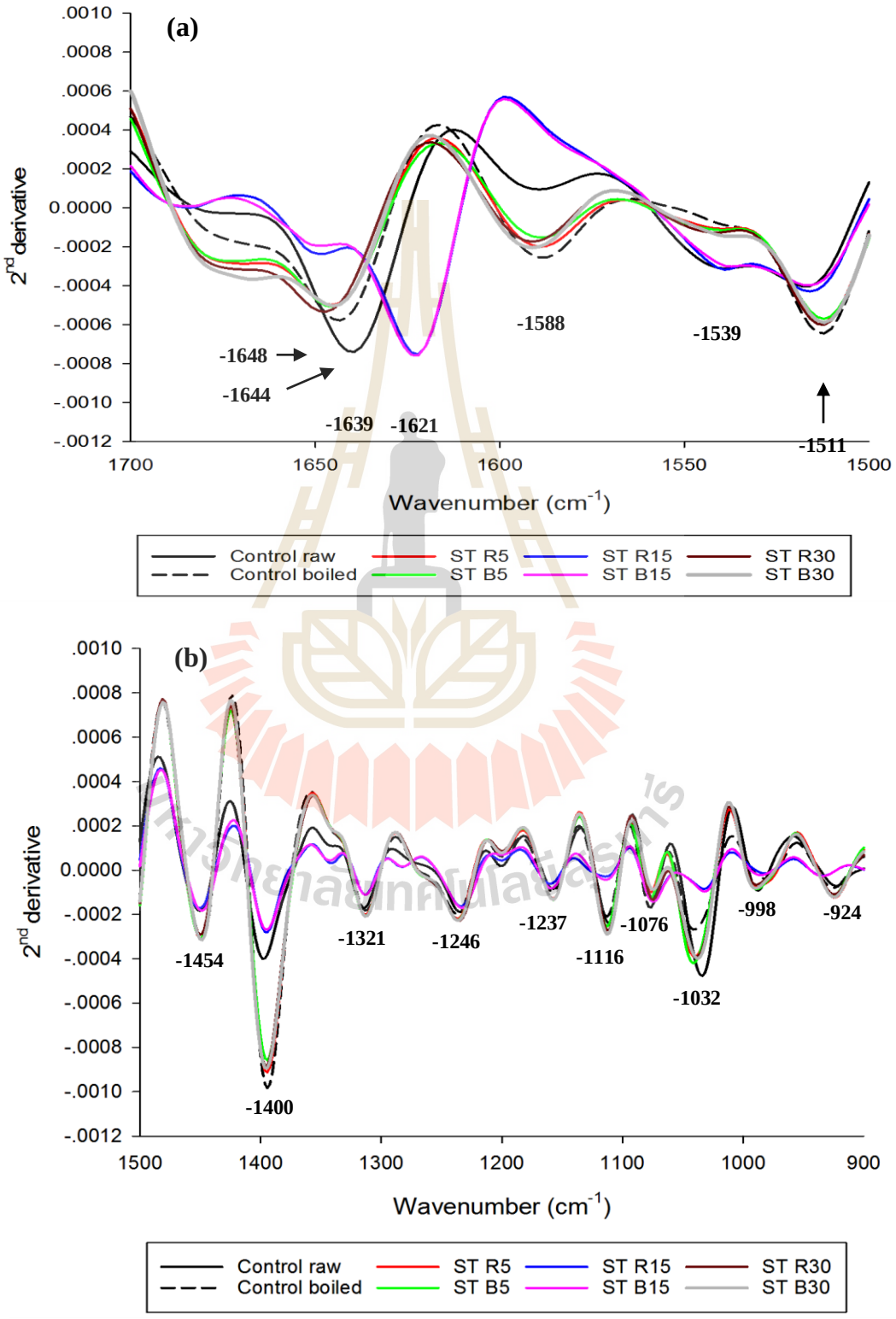
5.4.3 FT-IR

Pretreatment methods on egg white seemed to contribute in structural changes of the hydrolysates (Figure 5.3). The wavenumber ranging from 1600-1700 cm^{-1} was assigned as amide I region containing α-helix at 1642-1660 cm^{-1}, β-sheet at 1665-1680 cm^{-1} and 1614-1637 cm^{-1}, β-turn at 1680-1690 cm^{-1}, and random coils at 1640-1642 cm^{-1}, while the bands at 1500-1600 cm^{-1} was explained as amide II region (Cando et al, 2016; Kristoffersen et al, 2020; Pao et al, 2021).

FT-IR was highly sensitive to have strong absorption in broad range of wavenumber governed by protein; 1500-1700 cm^{-1} (Arunkumar et al, 2019). The intense peak at 1639 cm^{-1} of hydrolysate of CR indicated high β-sheet content (Figure 5.3). Meanwhile hydrolysate of CB showed a shifted peak positioned to 1642-1644 cm^{-1} (Figure 5.3), correlating to the α-helical structure which showed a lower amide I region absorption as compared to the CR ($p<0.05$, Table 5.3). Partial denaturation of cooking egg white was found to have a role in structural transition of the hydrolysates.

Hydrolysates of 15-min ST-treated egg white showed a pronounced peak at 1621 cm^{-1} (Figure 5.3a), resulting high absorbance at amide I region (Table 5.3). The different intensity between hydrolysates prepared from 15-min ST-treated raw and boiled egg white was likely caused by cooking process on egg white. Uygun-Saribay et al (2016) reported that the increased absorption at amide I was corresponded to the increased of intermolecular hydrogen-bonded β-sheet. In contrast, 5-min and 30-min ST-treated egg whites resulted in hydrolysates with a peak centered at 1648 cm^{-1}, corresponding to the absorption of random coils in amide I region. Sterilization appeared to promote the transition of α-helix to random coils. Literatures

reported that thermal denaturation frequently caused aggregation which enhanced the formation of disordered structures (Long et al, 2015; Uygun-Saribay et al, 2016).



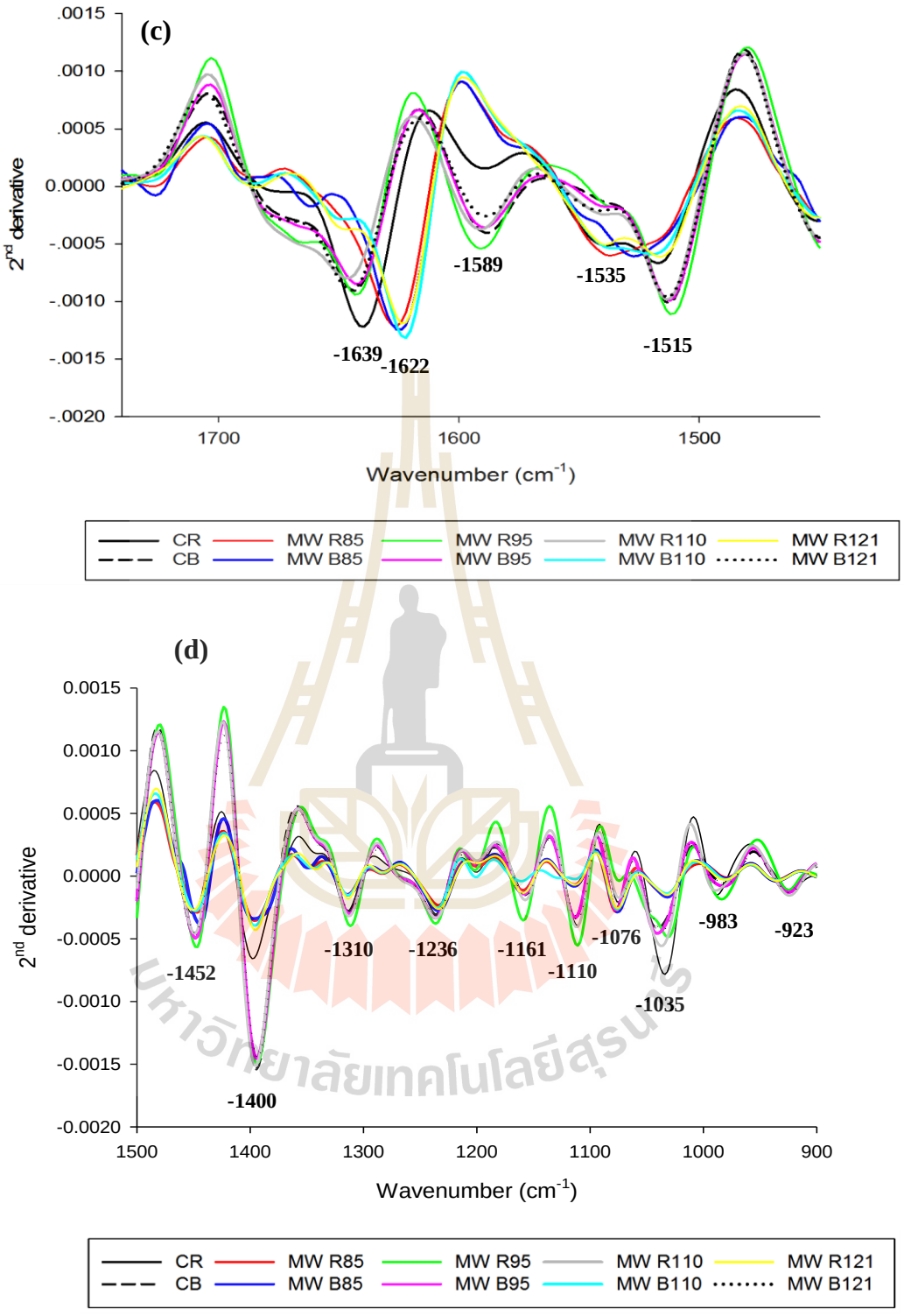


Figure 5.3 Second derivatization of hydrolysates prepared from steam sterilization- (a and b) and microwave-treated (c and d) egg whites at wavenumber of 1500-1800 cm^{-1} and 900-1500 cm^{-1}.

The 15-min ST-treated and 85˚C MW-treated raw egg white resulted in hydrolysates with the highest absorption in amide I region at 1621-1622 cm^{-1} (Figure 5.3b, Table 5.3). Although similar peak was observed at 1622 cm^{-1} (Figure 5.3b), hydrolysates of MW-B110 and MW-R121 showed a much lower amide I region absorption (p<0.05, Table 5.3). High temperature of microwave pretreatment could be the main reason on lowering hydrogen-bonds strength. Long et al (2015) stated that low intensity at amide I region was an actual feature of hydrogen-bonds changes.

Unlike the controls, hydrolysates prepared from MW-B85, MW-R95, and MW R110 showed a developed peak at 1668-1672 cm^{-1} (Figure 5.3b). Noted that a number of β-sheet was observed in high intensity from the hydrolysates of MW-treated. Microwave pretreatment at certain temperature produced hydrolysates with high proportion of β-sheet, further induced formation of firm structure. Yu (2005) reported that β-sheet components partly lowered gastrointestinal digestive enzyme accessibility. It was likely consistent with DH of MW-R95 and MW-R110, accounting for 23.71% and 28.17%, respectively. In contrast, MW-B85 with high DH of 40.69% showed high absorption in amide I region (Table 5.3). The results revealed that the proportion of β-sheet was not the only component determining amide I absorption. Microwave pretreatment lowered amide I by which was likely concomitant with the increased of β-sheet content, mainly corresponding to the low cellular antioxidant activity (Figure 5.2b). Yu (2005) stated that high proportion of β-sheet correlated with low nutritional values of compound, further assumed to contain weak bioactive activity.

Amide II component of hydrolysates prepared from ST-treated egg whites (Figure 5.3a) showed similar peaks to those of hydrolysates of MW-treated

egg whites (Figure 5.3c). However, integrated area under the peak showed that proportion of amide II of majority hydrolysates of ST-treated egg whites was higher than those of majority MW-treated (p<0.05, Table 5.3). Amide II region was mostly associated with N-H bending with C-N stretching vibrations of the primary amine group, occasionally, functional group of aromatic rings could be involved (Halim & Sarbon, 2019; Liu et al, 2019). The results suggested that aromatic rings might construct the structure of hydrolysates prepared from ST-treated egg whites, leading to high CAA (Figure 5.2a).

Although amide II was found in slightly higher absorption (Table 5.3), hydrolysate of control boiled egg white showed similar peak to those of hydrolysates obtained from ST- and MW-treated egg white. In the mean time, hydrolysate of control raw egg white was observed a reduction peak at 1588 cm^{-1} (Figure 5.3) with an increased of absorbance units (Table 5.3). Yu (2005) reported that amide II region mainly consisted of numerous functional groups vibrations, rising strong absorption in this region. These functional groups likely involved in similar CAA of CR to AsA (Figure 5.2).

The appearance of N-terminal and C-terminal on hydrolysates was frequently used to monitor proteolytic reaction. N-terminal region was associated to a characteristic peak at wavenumber near 1500 cm^{-1} and near 1400 cm^{-1} was assigned to C-terminal region (Kristoffersen et al., 2020; Wubshet et al., 2017). As presented in Figure 5.3a, hydrolysate of control boiled showed the most pronounced N-terminal at 1511 cm^{-1}, meanwhile slightly lower absorption was clearly observed as compared to those of hydrolysates prepared from MW-treated egg white at 1515 cm^{-1} (Figure 5.3c). In addition, hydrolysate of control boiled egg white showed the highest

absorption in C-terminal region (Table 5.3). The results revealed that hydrolysis of boiled egg white without pretreatments was optimum, in which the highest DH would be a significant effect (Table 5.1). Noticed that the exposure of free carboxylic groups could be also attributed to asymmetric bending of hydrophobic amino acids (Maqsoudlou et al, 2020) which was important for antioxidant activity. The CAA of hydrolysates prepared from ST-treated surpassed to those of MW-treated and hydrolysate of CB possessed the highest CAA (Figure 5.2). It can be concluded that an increase in C-terminal region determined the CAA of the hydrolysates.

In region between 900-1300 cm^{-1} was sensitive fingerprint of glycosylated protein originated from CO,CC-bending vibrations (Khajehpour et al, 2006; Lewis et al, 2013). As presented in Table 5.3, majority of hydrolysates of ST-treated egg white and only some of hydrolysates from MW-treated egg white showed relatively high absorption in this region (p<0.05). However, those were not as high as hydrolysate of control raw egg white which comprised the highest intensity (p<0.05, Table 5.3). Glycosylation of protein showed an important role in the increased of antioxidant activity through several mechanisms, including hydrogen and/or elctron donor, free radical chain breaking, metal ion chelation and scavenging activity (Zhang et al, 2018). Apparently, glycosylation reaction seemed to influence the potency of radical scavenging and ferric ion reduction power of hydrolysates prepared from ST-treated egg white (Table 5.2). Moreover, high glycosylated protein showed a significant effect on the highest metal chelation ability of hydrolysates of control raw egg white (p<0.05, Table 5.2).

Table 5.3 Integral area ($x10^{-2}$) of second derivatization of FT-IR spectra obtained from egg white hydrolysates.

Wavenumber (cm^{-1})	**1610-1700**	**1500-1600**	**1400**	**900-1300**
Band assignments	**Amide I**	**Amide II**	**C-terminal**	**CO,CC-bending**
Control raw	227.36 ^{bc}	355.04 ^{a}	215.21 ^{d}	66.41 ^{a}
Control boiled	129.49 ^{g}	238.73 ^{cd}	310.83 ^{a}	49.31 ^{b}
ST R5	114.79 ^{g}	193.63 ^{de}	103.67 ^{j}	40.63 ^{c}
ST B5	113.45 ^{g}	130.47 ^{fg}	151.89 ^{e}	53.65 ^{b}
ST R15	189.18 ^{e}	384.09 ^{a}	121.21 ^{h}	35.80 ^{d}
ST B15	190.44 ^{e}	389.83 ^{a}	296.32 ^{b}	37.93 ^{cd}
ST R30	163.91 ^{f}	235.74 ^{cd}	118.52 ^{hi}	52.05 ^{b}
ST B30	52.17 ^{i}	153.38 ^{ef}	134.32 ^{f}	25.68 ^{g}
MW R85	282.15 ^{a}	219.92 ^{d}	115.54 ^{i}	27.71 ^{g}
MW B85	204.17 ^{de}	268.25 ^{bc}	129.71 ^{g}	29.88 ^{f}
MW R95	88.71 ^{h}	116.08 ^{g}	293.76 ^{b}	39.21 ^{cd}
MR B95	73.23 ^{hi}	214.73 ^{d}	274.04 ^{c}	40.09 ^{c}
MW R110	75.09 ^{h}	119.35 ^{g}	126.17 ^{g}	51.69 ^{b}
MW B110	112.97 ^{g}	16885 ^{ef}	170.37 ^{d}	52.03 ^{b}
MW R121	215.72 ^{cd}	273.74 ^{bc}	114.16 ^{i}	35.70 ^{d}
MW B121	248.69 ^{b}	296.30 ^{b}	111.52 ^{i}	31.71 ^{e}

* Results were expressed as mean ± standard deviation values (n=3). Means in each column with different letter indicated significant differences (p<0.05).

Principal component analysis (PCA) can be used to establish correlation between antioxidant activity and structural changes of hydrolysates. PCA score plot was constructed by PC-1 (88%) and PC-2 (6%) to explain the variance.

Hydrolysates prepared by pretreatments of microwave and steam sterilization of egg white were clearly separated by PC-1 (Figure 5.4a) with positive spectra of loading plot at1601 cm^{-1} (Figure 5.4b). Moreover, hydrolysates of controls and pre-treated egg whites were explicitly distinguished by PC-2 (Figure 5.4a) equipped with a great positive spectra of loading plot at 1645 cm^{-1} (Figure 5.4b). The result demonstrated that amide I was the major contribution of data variation of hydrolysates prepared from different pretreatments.

Pretreatments promoted the variation of structural alteration of the hydrolysates, which was verified in loading plot spectra at 1577-1589 cm^{-1} indicating the changes in amide II bands and at 1400 cm^{-1} indicating the influence of C-terminal. In addition, the difference spectra of hydrolysates prepared from controls and pre-treated egg white was evidenced by the minor contribution of CO,CC-bending groups at wavenumber of 982-1075 cm^{-1} (Figure 5.4b).

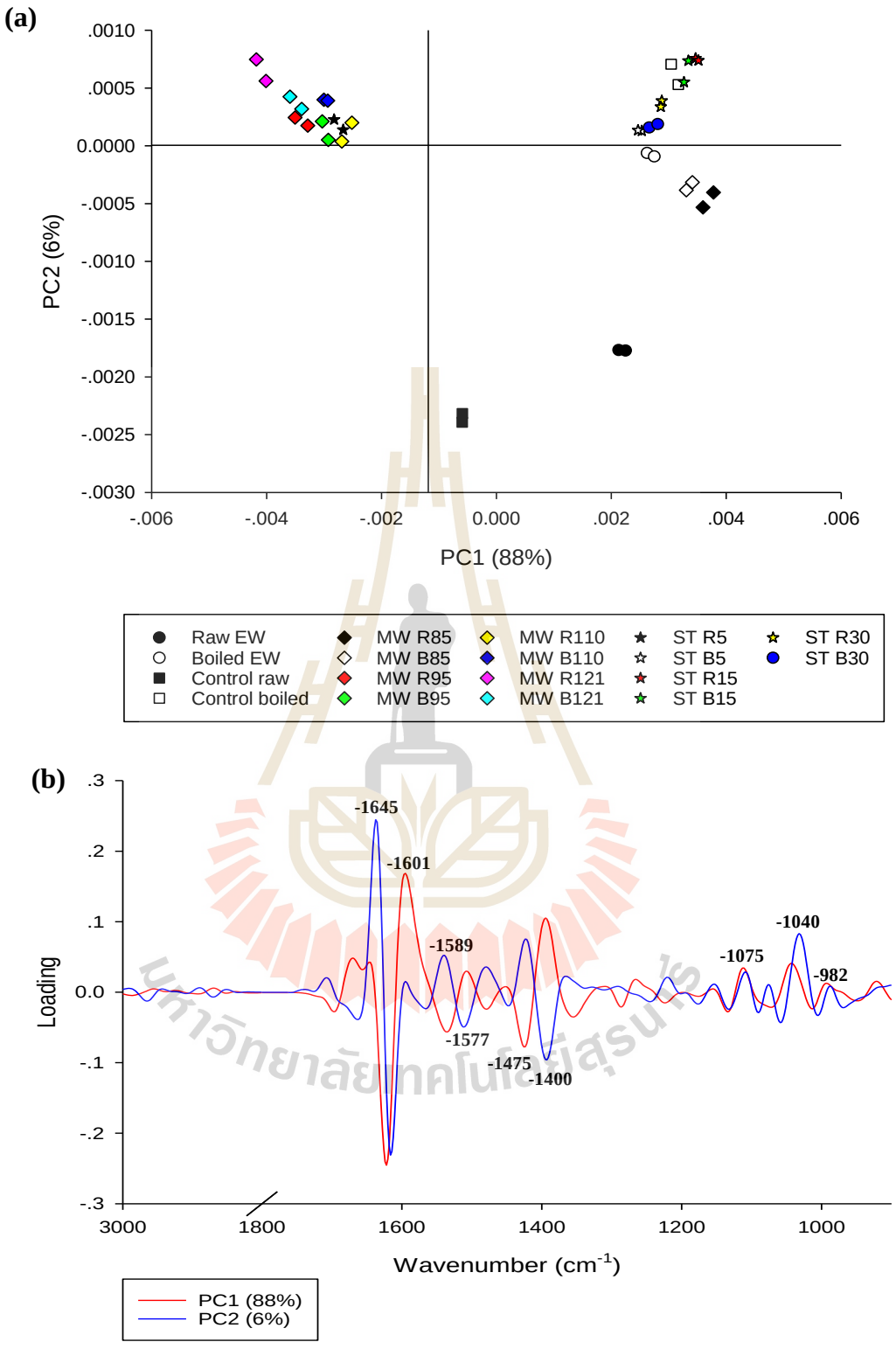


Figure 5.4 Principle component analysis (PCA) score (a) and loading (b) plot of the FT-IR spectra of egg white hydrolysates prepared by various factors.

The biplot result could be a tangible evident to distinguish DH values, antioxidant activity, and structural changes of hydrolysates prepared from controls and pre-treated egg whites with 51% variance was explained by PC-1 and PC-2 (Figure 5.5a). Majority of hydrolysates prepared from ST-treated egg white showed high correlation with FRAP values but yielded weak correlation with structural spectra (Figure 5.5a), indicating a minimum contribution of the structural changes on reducing power activity.

The PC-2 showed positive correlation between DH, C-terminal, cytoprotection and CAA, and negative correlation with ABTS and FRAP (Figure 5.5b). It suggested that DH and C-terminal were useful tools to predict antioxidant activity of hydrolysates, as confirmed in cellular studies. Spectra in same quadrant with control boiled egg white hydrolysate differentiated by PC-1 (Figure 5.5a) tended to possess closely similar antioxidant activity as the changes in structural level were similarly alike. It was likely a reason of higher CAA of hydrolysates prepared from ST-treated than those of MW-treated egg whites (Figure 5.5a).

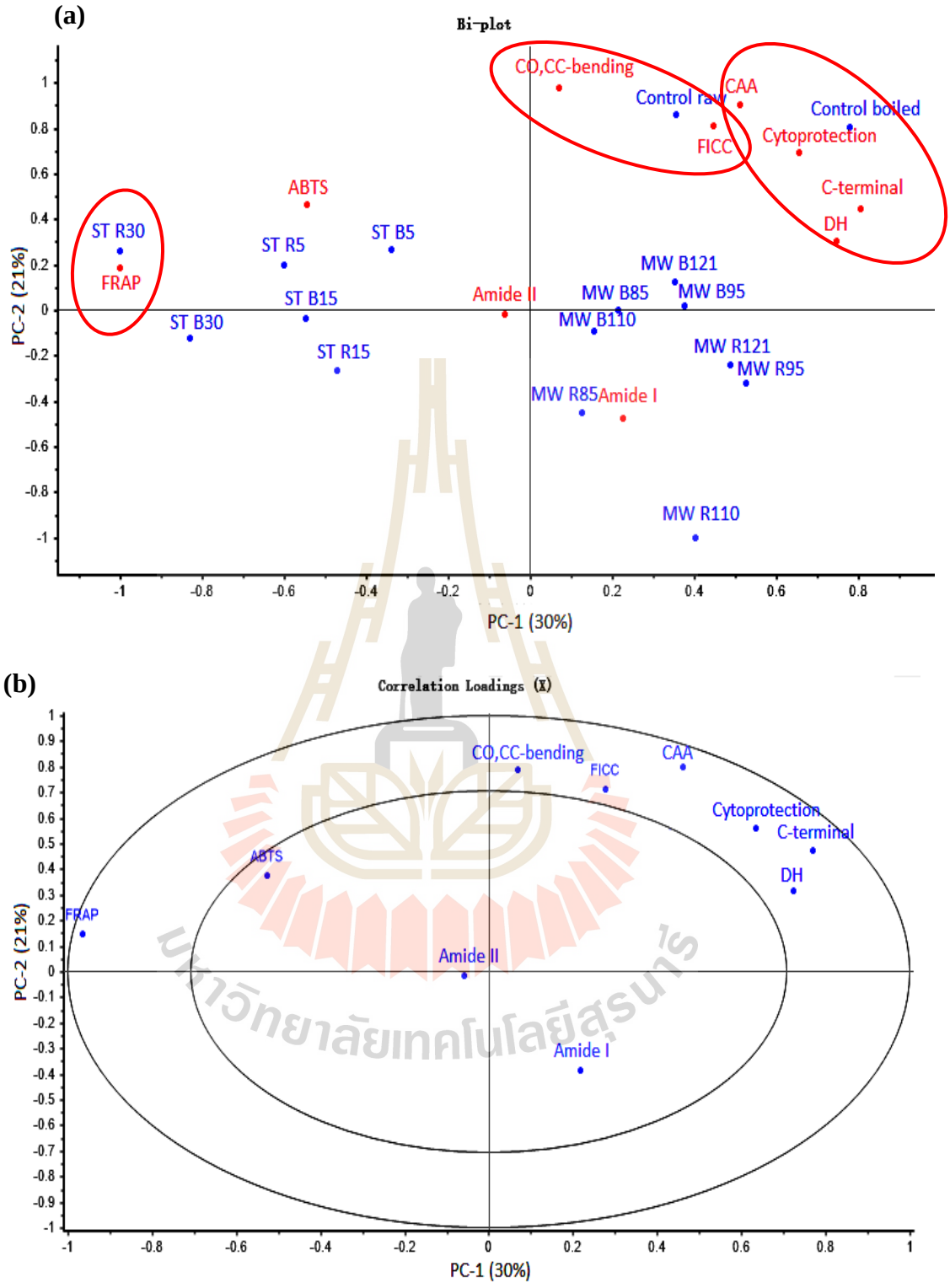


Figure 5.5 PCA biplot (a) and correlation loading plot (b) of hydrolysates obtained from egg white without pretreatment and subjected to microwave (MW) and steam sterilization (ST) pretreatments.

Control raw hydrolysate showed highest content of CO,CC-bending and the strongest chelator of ferrous ion among hydrolysates counterparts (Figure 5.5b). Raw egg white presumed to contain several glycoproteins, generating high content of CO,CC-bending vibrations (Julia et al, 2007). The existence of glycoprotein in raw egg white was frequently associated to the bioactivities (Geng et al, 2017). This might explain the highest metal chelating activity of control raw egg white hydrolysate (p<0.05, Table 5.2). In addition, pretreatments on egg white affected the antioxidant activity of hydrolysates as various changes in CO,CC-bending vibration appeared (Table 5.3). Our findings suggested that the measurement of structural spectra was a potential approach to estimate antioxidant activity of the hydrolysates.

5.5 Conclusions

Pretreatments applied on egg white affected DH, antioxidant activity and the structure of peptides in hydrolysates. Microwave could be suitable to produce hydrolysates with high DH and protein recovery. In contrast, steam sterilization tended to generate hydrolysates with ABTS (ST-R5) and FRAP (ST-R30) ability, which was positively correlated to high absorption in amide I and amide II region. However, boiled egg white hydrolysate without pretreatments possessed the highest DH of 43.09% positively correlated to the highest exposure of C-terminal region, which subsequently determining the highest cytoprotection and CAA. The result suggested that the production of hydrolysate with antioxidant activity should consider DH and C-terminal as the important factors.

5.6 References

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BIOGRAPHY

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