ANTIOXIDANT ACTIVITIES OF TILAPIA MUSCLE PROTEIN HYDROLYSATES AND THEIR GLYCATED PRODUCTS AFTER SIMULATED *IN VITRO* GASTROINTESTINAL DIGESTION

Zhang Xiaogang

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กิจกรรมการต้านออกซิเดชันของโปรตีนไฮโดรไลเสทจากกล้ามเนื้อปลานิลและผลิตภัณฑ์ไกลเคทหลังจากการย่อยในระบบย่อยอาหารจำลอง

นายจาง เฉียวกัง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

(Asst. Prof. Dr. Ratchadaporn Oonsivilai)
Chairperson

(Assoc. Prof. Dr. Jirawat Yongsawatdigul)
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(Assoc. Prof. Dr. Jaruwan Siritapetawee)
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จาง เฉียวกัง : กิจกรรมการต้านออกซิเดชันของโปรตีนไฮโดรไลเสทจากกล้ามเนื้อปลานิลและผลิตภัณฑ์ไกลเคทหลังจากการย่อยในระบบย่อยอาหารจำลอง (ANTIOXIDANT ACTIVITIES OF TILAPIA MUSCLE PROTEIN HYDROLYSATES AND THEIR GLYCATED PRODUCTS AFTER SIMULATED *IN VITRO* GASTROINTESTINAL DIGESTION) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 179 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อประเมินความสามารถในการต้านออกซิเดชันทางเคมีและในเซลล์ของโปรตีนไฮโดรไลเสทจากปลานิลที่ผ่านการย่อยด้วยระบบย่อยอาหารจำลอง (*in vitro* gastrointestinal digesta) และผลิตภัณฑ์จากปฏิกิริยาไกลเคชันของไฮโดรไลเสท นอกจากนี้ได้ทำการระบุและจำแนกคุณลักษณะของเพปไทด์ที่มีสมบัติในการต้านออกซิเดชันที่ได้จากไฮโดรไลเสทที่ผ่านการย่อยด้วยระบบย่อยอาหารจำลองที่แสดงกิจกรรมการต้านออกซิเดชันสูงที่สุด

โปรตีนกล้ามเนื้อปลานิลถูกย่อยด้วยเอนไซม์อัลคาเลส (Alcalase) ที่ระดับการย่อย (degree of hydrolysis) แตกต่างกัน โปรตีนและตัวอย่างไฮโดรไลเสททั้งหมดถูกย่อยได้เล็กน้อยด้วยเอนไซม์เพปซิน (pepsin) และการย่อยเพิ่มขึ้นอย่างมากด้วยเอนไซม์แพนครีเอติน (pancreatin) ทั้งไฮโดรไลเสท และไฮโดรไลเสทที่ผ่านการย่อยด้วยระบบย่อยอาหารจำลอง (digesta) สามารถจับอนุมูลอิสระผ่านการถ่ายเทอะตอมของไฮโดรเจน (hydrogen atom transfer) เป็นหลัก กิจกรรมการต้านออกซิเดชันของไฮโดรไลเสมเพิ่มขึ้นเมื่อระดับการย่อยเพิ่มขึ้น และมีค่าสูงสุดที่เวลาการย่อย 16 ชั่วโมง ซึ่งมีค่าระดับการย่อยเท่ากับ 35.36 ± 0.06% อย่างไรก็ตาม หลังผ่านกระบวนการย่อยด้วยระบบย่อยอาหารจำลอง พบว่า ไฮโดรไลเสทที่ผ่านการย่อยเป็นเวลา 10 ชั่วโมง แสดงความสามารถในการจับอนุมูลอิสระทางเคมีและจับสารออกซิเจนที่ไวต่อปฏิกิริยา (reactive oxygen species; ROS) ที่เกิดขึ้นภายในเซลล์ตับ (HepG2 cells) ที่เหนียวนำจาก 2,2'-เอโซบีส-2-เมธิลโพรพิโอนามิดีนไดไฮโดรคลอไรด์ (2,2'- azobis (2-methylpropionamidine) dihydrochloride, AAPH) ได้สูงที่สุด ในขณะที่โปรตีนที่ผ่านการย่อยในระบบทางเดินอาหารมีประ สิทธิภาพน้อยที่สุด กระบวนการไกลเคชั่นของไฮโดรไลเสทเกิดมากที่สุดที่อุณหภูมิ 90 องศาเซลเซียล เป็นเวลา 12 ชั่วโมง โดยเกิดขึ้นพร้อมกับการเพิ่มขึ้นของโมเลกุลขนาดใหญ่ของเมลานอยดิน (melanoidins) กระบวนการไกลเคชั่นมีผลในการเพิ่มประสิทธิภาพของการไล่จับอนุมูลอิสระเป็นอย่างมาก โดยเฉพาะอย่างยิ่งความสามารถในการรีดิวซ์ เมื่อเทียบกับน้ำตาลกลูโคล (glucose) และฟรุคโตส (fructose) น้ำตาลไซโลส (xylose) มีประสิทธิภาพสูงสุดสำหรับปฏิกิริยาไกลเคชั่น ส่งผลให้ได้ผลิตผลที่มีความสามารถในการต้านออกซิเดชันสูงสุด อย่างไรก็ตาม ระดับสูงสุดของการเกิดกระบวนการไกลเคชั่นส่งผลให้ความสามารถในการต้านอนุมูลอิสระในระดับเซลล์และความสามารถในการย่อยได้

เคชั่นส่งผลให้ความสามารถในการต้านอนุมูลอิสระในระดับเซลล์และความสามารถในการย่อยได้ (digestibility) ต่ำสุด กระบวนการย่อยด้วยระบบย่อยอาหารจำลองมีผลลดความสามารถในการรีดิวซ์ของไฮโดรไลเสทที่ผ่านกระบวนการไกลเคชัน ผลิตภัณฑ์ที่ได้จากกระ บวนการย่อยด้วยระบบย่อยอาหารจำลองของไฮโดรไลเสทที่ผ่านกระบวนการไกลเคชันมีประสิทธิภาพในการจับสารออกซิเจนที่ไวต่อปฏิกิริยาในระดับเซลล์ดีกว่าเมื่อเทียบกับผลิตภัณฑ์ที่ได้จากกระบวนการย่อยด้วยระบบย่อยอาหารจำลองของไฮโดรไลเสท กระบวนการไกลเคชั่นมีประสิทธิภาพในการเพิ่มความสามารถจับอนุมูลอิสระทางเคมี แต่ไม่ส่งผลดีต่อการแสดงกิจกรรมในระบบทางชีวภาพ ดังนั้น ความสามารถในการต้านอนุมูลอิสระของผลิตภัณฑ์ที่ผ่านการย่อยในระบบทางเดินอาหารควรเป็นปัจจัยหนึ่งที่ใช้พิจารณาเพื่อกำหนดสภาวะที่เหมาะสมในการผลิตโปรตีนไฮโดรไลเสทและผลิตภัณฑ์จากกระบวนการไกลเคชั่น

เพปไทด์ 9 สายที่ระบุได้จากตัวอย่างไฮโดรไลเสทที่ย่อยเป็นเวลา 10 ชั่วโมงและ เพปไทด์ 6 สายที่เกิดจากกระบวนการย่อยอาหารจำลองโดยใช้ซอฟแวร์คอมพิวเตอร์ (*in silico* GI digestion) ได้ถูกสังเคราะห์ขึ้น ค่าความเข้มข้นที่มีประสิทธิภาพในการกำจัดอนุมูลอิสระ2,2'-เอซิโนบีส (3-เอธิลเบนโซไธเอโซไลน์-6-ซัลโฟนิกแอซิด (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$) ในระดับ 50% (50% effective concentration; EC_{50}) ของเพปไทด์ที่มีซิสเตอีน (C) และ ไทโรซีน (Y) ในโครงสร้างอยู่ในช่วง 2 ถึง 17 ไมโครโมลลาร์ ซึ่งมีประสิทธิภาพมากกว่ากรดแอสคอบิก และเพปไทด์ที่มีไลซีน (K) อยู่ในโครงสร้าง เพปไทด์ LPGYF และ SC เป็นเพปไทด์ตั้งต้นและเพปไทด์ที่เกิดจากการย่อยที่มีประสิทธิภาพดีที่สุดตามลำดับ ซึ่งให้ผลในการจับอนุมูล $ABTS^{\bullet+}$ ได้สูงสุด เพปไทด์จากการย่อยอาหารด้วยคอมพิวเตอร์ที่มี C และ Y ในโครงสร้าง ได้แก่ SC, CH, PGY, และ EVPGY ยังคงแสดงกิจกรรมได้สูงเมื่อเทียบกับเพปไทด์ตั้งต้น เพปไทด์ทั้ง 15 สายแสดงศักยภาพในการไล่จับอนุมูลออกซิเจนที่ไวต่อปฏิกิริยาภายในเซลล์ นอกจากนี้ เพปไทด์ SC, CH, และ PGY เพิ่มการแสดงออกของยีนส์เอนไซม์คาตาเลส (catalase gene; *CAT*) และ ยีนส์เอนไซม์ซุปเปอร์ออกไซด์ดิสมิวเตส (superoxide dismutase gene; *SOD1*) ทั้งใน HepG2 เซลล์และ HepG2เซลล์ที่ถูกกระตุ้นด้วย AAPH แต่ไม่ส่งผลต่อการแสดงออกของยีนส์เอนไซม์กลูตาไธโอนเปอร์ออกซิเดส (glutathione peroxidase gene; *GPx1*) โดยเพปไทด์ PGY มีประสิทธิภาพในการต้านอนุมูลอิสระภายในเซลล์ได้ดีที่สุด ดังนั้น เพปไทด์ที่ได้จากการย่อยด้วยระบบย่อยอาหารจำลองของโปรตีนไฮโดรไลเสทจากเนื้อ ปลานิลมีศักยภาพในการเป็นสารอาหารที่ให้ประโยชน์ต่อสุขภาพในการต้านออกซิเดชัน

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา Xiaogang Zhang

ปีการศึกษา 2562 ลายมือชื่ออาจารย์ที่ปรึกษา

ZHANG XIAOGANG : ANTIOXIDANT ACTIVITIES OF TILAPIA MUSCLE PROTEIN HYDROLYSATES AND THEIR GLYCATED PRODUCTS AFTER SIMULATED *IN VITRO* GASTROINTESTINAL DIGESTION. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 179 PP.

ANTIOXIDANT ACTIVITY/GASTROINTESTINAL DIGESTION/PROTEIN HYDROLYSATE/GLYCATED PRODUCTS/PURIFICATION

This study aimed at systematically assessing the chemical and cellular antioxidant activities of the *in vitro* gastrointestinal (GI) digesta of tilapia protein hydrolysates, and their glycated products. Antioxidant peptides derived from the hydrolysate whose digesta showed the highest activity were also identified and characterized.

Tilapia muscle protein was hydrolyzed by Alcalase to various degree of hydrolysis (DH). Proteins and all hydrolysates were slightly digested by pepsin but hydrolyzed extensively by pancreatin. Both hydrolysate and digesta predominantly scavenged free radicals via hydrogen atom transfer (HAT). The antioxidant activities of the hydrolysates increased with the increasing DH and reached the highest at 16 h of hydrolysis with 35.36 ± 0.06% DH. However, the digesta of 10-h hydrolysate displayed the highest chemical and intracellular reactive oxygen species (ROS) scavenging capacity on 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH)-induced HepG2 cell oxidative stress model, while the protein digesta displayed the lowest. Glycation of hydrolysate was intensified at 90 °C for 12 h, accompanied by increasing larger molecular weight (MW) of melanoidins. Glycation drastically increased free radical scavenging capacity, especially reducing power. Compared with glucose and

fructose, xylose was the most effective sugar for glycation yielding the highest chemical antioxidant activities. However, the highest degree of glycation exhibited the lowest intracellular ROS scavenging capacity and digestibility. GI digestion reduced reducing power of glycated hydrolysates. All digesta of glycated hydrolysates were less effective at intracellular ROS scavenging capacity compared to their respective hydrolysate digesta. Glycation of hydrolysate could be effective to enhance chemical antioxidant activities but not efficiency to improve the activity in biological system. Antioxidant activity of digesta should be considered when optimizing conditions used for production of protein hydrolysate and glycated products.

Nine novel tilapia peptides identified from the 10-h hydrolysate and 6 fragments from their *in silico* GI digesta were synthesized. The 50% effective concentration (EC_{50}) of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$) scavenging capacity of all C- and Y-containing peptides ranged from 2 to 17 μM, which were more effective than that of ascorbic acid and K-containing peptides. LPGYF and SC were the best parent and digested peptides, respectively, that showed the highest $ABTS^{\bullet+}$ scavenging activity. The *in silico* digested C- and Y-containing peptides, namely SC, CH, PGY and EVPGY, remained high activity compared to the parents. Fifteen peptides showed potent intracellular ROS scavengers. In addition, SC, CH and PGY up-regulated expression of *CAT* and *SOD1* on both HepG2 and AAPH-induced HepG2 cell models, but not *GPx1* expression. PGY was the most effective cellular antioxidant. Tilapia hydrolysate digested peptides could be the potent nutraceuticals against oxidative stress.

School of Food Technology Student's Signature Xiaogang zhang

Academic Year 2019 Advisor's Signature

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LIST OF ABBREVIATIONS

h	=	Hour
H_2O_2	=	Hydrogen peroxide
kDa	=	kilodalton
m	=	Meter
M	=	Molar
min	=	Minute
mL	=	Milliliter
NaCl	=	Sodium chloride
KCl	=	Potassium chloride
NaOH	=	Sodium hydroxide
nm	=	Nanometer
ºC	=	Degree centigrade
rpm	=	Revolutions per minute
s	=	Second
v/v	=	volume/volume
w/v	=	Weight per volume
GI	=	Gastrointestinal
$ABTS^{\bullet+}$	=	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation
DPPH•	=	*α*, *α*-diphenyl-*β*-picrylhydrazyl free radical
ROO•	=	Peroxyl radical

LIST OF ABBREVIATIONS (Continued)

•OH	=	Hydroxyl radical
$O^{\bullet-}$	=	Superoxide anion radical
$ONOO^-$	=	Peroxynitrite
LDL	=	Low-density lipoprotein
FCR	=	Folin-Ciocalteu reagent
FRAP	=	Ferric reducing antioxidant power
CUPRAC	=	Cupric reducing antioxidant capacity
ORAC	=	Oxygen radical absorbance capacity
TEAC	=	Trolox equivalent antioxidant capacity
FICC	=	Fe^{2+} chelating capacity
EC_{50}	=	50% effective concentration
ROS	=	Reactive oxygen species
CAA	=	Cellular antioxidant activity
MW	=	Molecular weight
HAT	=	Hydrogen atom transfer
ET	=	Electron transfer
NBT	=	Nitro blue tetrazolium chloride
SEC	=	Size exclusion chromatography
RPC	=	Reversed-phase chromatography
GSH	=	Glutathione
GA	=	Garlic acid
SOD	=	Superoxide dismutase

LIST OF ABBREVIATIONS (Continued)

CAT	=	Catalase
GPx	=	Glutathione peroxidase
DH	=	Degree of hydrolysis
QSAR	=	Quantitative structure activity relationship
DOE	=	Design of experiment
RSM	=	Response surface methodology
UF	=	Ultrafiltration
IEC	=	Ion-exchange chromatography
HPLC	=	High performance liquid chromatography
DCFH-DA	=	2′,7′-dichlorofluorescin di-acetate
DCFH	=	Dichlorofluoorescin
ABAP or AAPH	=	2,2′-azobis (2-amidinopropane) dihydrochloride
DCF	=	Dichlorofluorescein
Keap1	=	Kelch-like ECH associated-protein 1
Nrf2	=	Nuclear factor erythroid 2-related factor 2
ARE	=	Antioxidant response element
PCA	=	Principal component analysis
SIN-1	=	3-morpholinosydnonimine hydrochloride
DHR 123	=	Dihydrorhodamine 123
FL	=	Fluorescein sodium salt
TNBS	=	2,4,6-trinitrobenzenesulfonic acid solution

LIST OF ABBREVIATIONS (Continued)

Hip-His-Leu	=	Hippuryl-His-Leu acetate salt
Ferrozine	=	3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*′-disulfonic acid monosodium salt hydrate
Trolox	=	(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
TPTZ	=	2,4,6-tris(2-pyridyl)-*s*-triazine
MTT	=	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
DMEM	=	Dulbecco's modified eagle medium
FBS	=	Fetal bovine serum
EDTA	=	Ethylenediaminetetraacetic acid
NEAAs	=	Non-essential amino acids
ACN	=	Acetonitrile
TFA	=	Trifluoroacetic acid
P	=	Tilapia protein powder
P_0	=	Heated protein
SGF	=	Simulated gastric fluid
SIF	=	Simulated intestinal fluid
H	=	Hydrolysate
GD	=	Gastric digesta
GID	=	Gastric-intestinal digesta
P-GD	=	Protein gastric digesta

LIST OF ABBREVIATIONS (Continued)

P-GID	=	Protein GI digesta
PD	=	Protein digesta
H-GID	=	Digesta of hydrolysate
Pep_0	=	Thermally inactivated pepsin
Pan_0	=	Inactivated pancreatin
Pep-Pan-I	=	Enzyme blank of GI digestion
A_0	=	Inactivated Alcalase
A_0-*GID*	=	*In vitro* GI digesta of A_0
TE	=	Trolox equivalents
AUC	=	Area under the curve
DMSO	=	Dimethylsulfoxide
V_e	=	Elution volume
AsA	=	Ascorbic acid
GAPDH	=	Gene encoding glyceraldehyde 3-phosphate dehydrogenase
CAT	=	Gene encoding catalase (CAT)
SOD1	=	Gene encoding superoxide dismutase (SOD)
GPx1	=	Gene encoding glutathione peroxidase (GPx)
SF	=	*SEC* fractions
RF	=	*RPC* fractions
BPC	=	Basic peak chromatograms
ACE	=	Angiotensin Ⅰ-converting enzyme
DPP Ⅳ	=	Dipeptidyl peptidase Ⅳ

LIST OF ABBREVIATIONS (Continued)

PEP	=	Prolyl endopeptidase
BDE	=	Bond dissociation energy
IP	=	Ionization potential
P_{app}	=	Apparent permeability coefficient
MRPs	=	Maillard reaction products
E_0	=	Enzyme blank
DNS	=	3,5-Dinitrosalicylic acid
S	=	Sugars
G	=	Glucose
F	=	Fructose
X	=	Xylose
S_tH_n	=	Glycated hydrolysate
S_0H_n	=	Un-glycated hydrolysate
CML	=	*N*-ε-carboxymethyl-lysine
CEL	=	*N*-ε-carboxyethyl-lysine
AGEs	=	Advanced glycation end-products
RAGE	=	Receptor for AGEs
GRS	=	Gene encoding glutathione reductase (GRS)

CHAPTER I

INTRODUCTION

1.1 Background and significance of the study

Oxidative stress is related to many pathophysiological processes such as aging, inflammation, diabetes mellitus, and cancer (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012; Zhichun Chen & Zhong, 2014; Incalza, D'Oria, Natalicchio, Perrini, Laviola, & Giorgino, 2018; Sies & Jones, 2020). Food protein-derived antioxidant peptides have been considered as a natural antioxidant, which have been shown to protect biological systems from oxidative damages (Girgih et al., 2014; Guo et al., 2020; Nwachukwu & Aluko, 2019; Samaranayaka & Li-Chan, 2011). However, when protein hydrolysates are applied as nutraceuticals, the oral administration likely modifies their activities due to hydrolysis occurring during gastrointestinal (GI) digestion by proteases (Ao & Li, 2013; Jang, Liceaga, & Yoon, 2016; Ketnawa, Wickramathilaka, & Liceaga, 2018; Samaranayaka, Kitts, & Li-Chan, 2010). Therefore, the antioxidant activities of protein hydrolysate might not totally correlate with those of the digesta. Process optimization of protein hydrolysate which is based merely on the antioxidant activities of the hydrolysate might not yield the product with the optimal health benefits. However, this approach has been commonly practiced for the production of protein hydrolysate with antioxidant activities (Raghavan, Kristinsson, & Leeuwenburgh, 2008; Yarnpakdee, Benjakul, Kristinsson, & Bakken, 2015; X. Zhang, Cao, Sun, Sun, & Xu, 2019).

Application of peptides as antioxidant for foods or nutraceuticals are still limited (Bhandari, Rafiq, Gat, Gat, Waghmare, & Kumar, 2020; Sohaib et al., 2017). One of the reasons would be the lower antioxidant activity of protein hydrolysates compared to standard antioxidants such as ascorbic acid, quercetin, and glutathione. According to the chemical antioxidant activities, such as 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH•), hydroxyl radical (•OH), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$) scavenging capacities, the 50% effective concentration (EC_{50}) of peptides or protein hydrolysates frequently ranged from 20 µg/mL - 10 mg/mL (Liu, Xing, Fu, Zhou, & Zhang, 2016; Wiriyaphan, Xiao, Decker, & Yongsawatdigul, 2015; X.-R. Yang, Zhang, Ding, Chi, Wang, & Huo, 2019; Yu, He, Tang, Zhang, Zhu, & Sun, 2018). In contrast, quercetin, (+)-catechin, and *L*-ascorbic acid showed EC_{50}<5 µg/mL for DPPH• and $ABTS^{\bullet+}$ scavenging assays (Zheng Chen, Bertin, & Froldi, 2013; Faitanin et al., 2018). Antioxidant peptide/hydrolysate have also shown lower intracellular reactive oxygen species (ROS) scavenging capacity than that of α-tocopherol, trolox, and ascorbic acid (Wiriyaphan, Chitsomboon, & Yongsawadigul, 2012; Wiriyaphan et al., 2015; Yarnpakdee et al., 2015). Therefore, the improvement of antioxidant activity of peptides/protein hydrolysates should be sought to increase its utilization.

Glycation or Maillard reaction is the reaction between primary amino and active carbonyl groups that are initiated spontaneously during thermal processing and food storage**.** Numerous studies indicated that glycation improved the chemical antioxidant activity of proteins, peptides, and amino acids, such as reducing power, DPPH•, $ATBS^{\bullet+}$, and peroxyl radical (ROO•) scavenging capacities (Nooshkam, Varidi, & Bashash, 2019). Maillard reaction products (MRPs) have also extended food shelf-life

and inhibited lipid oxidation (Nooshkam et al., 2019; Sohaib et al., 2017). A few studies have reported that MRPs promoted the cellular antioxidant activity (CAA) to a greater extent than that of the original hydrolysates (Han et al., 2018; Zha, Wei, Chen, Dong, Zeng, & Liu, 2015). Most studies have focused on MRPs alone without comparing with that of the original hydrolysates (He et al., 2019; Karnjanapratum, O'Callaghan, Benjakul, & O'Brien, 2016). In contrast, some reports have shown that MRPs caused oxidative stress of diabetic mice (Yao, Han, Dong, Zeng, & Liu, 2016) and intestinal inflammation in early life in rats (Hillman et al., 2019). Thus, glycation is likely an effective strategy to improve the chemical antioxidant activity of peptides/hydrolysate, but the efficiency to improve the antioxidant activity in biological system still needs further investigation.

Digestibility of glycated products were lower than that of the original proteins/peptides, and peptide profiles in the digesta were also significantly changed (Q. Zhang, Wang, & Fu, 2020; Zhao et al., 2019). The antioxidant activities (DPPH• and/or $ABTS^{\bullet+}$ scavenging capacity and reducing power) of MRPs generated from the grass carp scale hydrolysate-ribose and unicorn leatherjacket skin gelatin hydrolysate-galactose systems were decreased after GI digestion (Chen et al., 2019; Karnjanapratum et al., 2016). The suppression of oxidative stress or/and inflammation have been evaluated for MRPs (Chen, Fang, & Wang, 2020; Karnjanapratum et al., 2016), but their digesta have rarely studied.

Therefore, antioxidant activities of both hydrolysates and glycated hydrolysates did not appear to reflect the activities of the digesta counterpart. Comparison between activities of the hydrolysate/glycated hydrolysate and its digesta has not been

systematically elucidated. Antioxidant activity of digesta of hydrolysates/glycated hydrolysates should be evaluated to reflect the more biological relevant properties.

Evaluation of food digestion in humans is the most reliable technique, but the trials are difficult to control, expensive, and might not all be justifiable on ethical grounds. Compared with the dynamic models, the static *in vitro* digestion method is more convenience and easy to access (Guerra, Etienne-Mesmin, Livrelli, Denis, Blanquet-Diot, & Alric, 2012; Mulet Cabero et al., 2020). A standardized static *in vitro* digestion method has been established by the COST action INFOGEST (Brodkorb et al., 2019; Minekus et al., 2014). Egger et al. (2017) evaluated digestion of milk protein by the INFOGEST static method and found that the endpoint of digestion was comparable to *in vivo* protein digestion. Therefore, the static *in vitro* method has been accepted as a useful technique to assess the endpoints of food digestion. Moreover, the digesta of protein hydrolysate contained peptides and amino acids that resulted from gastric (pepsin) and intestinal (pancreatin) digestion. Nevertheless, peptides/amino acids derived from the proteases used in the analysis could also be generated due to the autolysis and/or hydrolysis (Dave, Hayes, Mora, Montoya, Moughan, & Rutherfurd, 2016; Whitcomb & Lowe, 2007). This could also contribute to the activities of interest. Dave et al. (2016) reported that peptides derived from trypsin by *in vitro* digestion model revealed $ABTS^{\bullet+}$ and DPPH• scavenging capacity. Therefore, the antioxidant activities of the peptides derived from the enzyme blanks of *in vitro* model should be assessed to obtain more biologically relevant antioxidant activity of the digesta. Thus far, the issue of enzyme blanks has not been well characterized in publications.

Peptides responsible for antioxidant activity of digesta of protein hydrolysate, which have shown the highest activity, must be identified and characterized.

Contamination of antioxidant peptides derived from the digestive enzymes, however, poses challenges in the purification and identification of antioxidant peptide from hydrolysate digesta. Bioinformatics tools, such as BIOPEP http://www.uwm.edu.pl/ biochemia/index.php/en/biopep and PeptideCutter https://web.expasy.org/ peptide_ cutter/ are usually applied for *in silico* GI digestion to theoretically predict the potential cleave sites of peptides upon GI digestion (Agyei, Tsopmo, & Udenigwe, 2018; Gallego, Mora, & Toldrá, 2018). It can avoid the interferences of peptides derived from the enzymes. Purification and identification of antioxidant peptides from the protein hydrolysate followed by *in silico* GI digestion could be an alternative for the prediction of antioxidant peptides from hydrolysate digesta. Additionally, the importance of different amino acid residues contributing to the free radical scavenging capacity of peptides can be evaluated based on the activity of synthetic peptides, as well as comparison of antioxidant activity of peptides before and after GI digestion, which are still not clearly understood. Peptides containing C, M, W, Y, F, K, P, E and H residues are usually considered as good at free radical scavenging capacity (Nimalaratne, Bandara, & Wu, 2015; Nwachukwu et al., 2019; Samaranayaka et al., 2011; X.-R. Yang et al., 2019). However, some reports have shown that only Y, W, C and M residues played a dominant role on $ABTS^{\bullet+}$ or peroxyl radical (ROO•) scavenging capacity (Sheih, Wu, & Fang, 2009; Yang, Cai, Yan, Tian, Du, & Wang, 2020; Zheng, Zhao, Dong, Su, & Zhao, 2016).

Many chemical assays with different mechanisms have been conducted to evaluate the antioxidant activity of the interested compounds (López-Alarcón & Denicola, 2013; Nwachukwu et al., 2019). The ORAC assay monitors the peroxyl radical (ROO•) scavenging activity via the hydrogen atom transfer (HAT) mechanism

(Apak, Özyürek, Güçlü, & Çapanoğlu, 2016). The FRAP and and peroxynitrite ($ONOO^-$) scavenging assays are related to the electron transfer (ET) mechanism (Apak et al., 2016; Kooy, Royall, Ischiropoulos, & Beckman, 1994). The TEAC is a mixed-mode (ET/HAT) assay (Apak et al., 2016). Combination of various chemical assays under various mechanisms could be useful to interpret the mechanisms of the antioxidant peptides.

Cellular antioxidant activity (CAA) assay is more biologically relevant as it addresses some issues of cells such as uptake, distribution and metabolism of antioxidant (López-Alarcón et al., 2013; Wolfe & Liu, 2007). It has been widely employed to evaluate the antioxidant activity of peptides in biological system (Du, Esfandi, Willmore, & Tsopmo, 2016; Tonolo et al., 2020; Wu et al., 2018). The free radical scavenging capacity of peptides would be positively correlated to their intracellular reactive oxygen species (ROS) scavenging capacity such as NTVPAKSCQAQPTTM and YLEELHRLNAGY (Homayouni-Tabrizi, Asoodeh, & Soltani, 2017; Tonolo et al., 2020). They have also increased gene expression of antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT). However, peptides, FNDRLRQGQLL and DVNNNANQLEPR, showed lower ROO• scavenging capacity than that of GLVYIL and YHNAPGLVYIL, but they displayed similar intracellular ROS scavenging capacity (Du et al., 2016). The main reason would be they induced the various cellular responses such as modulated the level of glutathione (GSH), increased the activity of SOD, glutathione peroxidase (GPx) and CAT. Thus, CAA assay is not only characterizing the intracellular ROS scavenging capacity, but also investigating the underlying mechanisms acted by peptides such as modulating in gene expression of antioxidant enzymes to enhance intrinsic CAA.

1.2 Research objectives

The objectives of this study were:

1. To assess chemical and cellular antioxidant activity of the *in vitro* GI digesta of tilapia hydrolysate with various DH values.

2. To characterize the modification of tilapia protein hydrolysate under glycation following *in vitro* GI digestion, based on chemical and cellular antioxidant activities.

3. To identify tilapia antioxidant peptides from the hydrolysate whose digesta showed the highest antioxidant activity.

4. To evaluate chemical and cellular antioxidant activity of synthetic antioxidant peptides and their *in silico* GI digested peptides.

1.3 Research hypotheses

Antioxidant activity of tilapia protein hydrolysates would be varied upon GI digestion due to structural modification. Glycation of tilapia protein hydrolysates will modify the chemical and cellular antioxidant activity. Glycated hydrolysates would be further hydrolyzed upon GI digestion, while the glycated digesta would exhibit intracellular ROS scavengers. Antioxidant peptides could be effective at the cellular level as they exhibit ROS scavenging capacity via scavenging free radicals and modulating gene expression of antioxidant enzymes.

1.4 Scope of the study

Defatted tilapia muscle protein was hydrolyzed by Alcalase to various DH values and followed by *in vitro* GI digestion to obtain hydrolysate digesta. The changes

in molecular weight (MW) during hydrolysis and digestion were investigated by size exclusion chromatography (*SEC*). Chemical antioxidant activity, cytoprotection and intracellular ROS scavenging capacity assays based on HepG2 cell lines of hydrolysates and their digesta were also carried out. In addition, tilapia hydrolysates with various DH values were glycated with glucose, fructose and xylose followed by *in vitro* GI digestion. The glycated hydrolysates and their *in vitro* GI digesta were characterized based on UV-vis spectroscopy, MW distribution, chemical and cellular antioxidant activities. The hydrolysate digesta that showed the highest intracellular ROS scavenging capacity was selected for purification of antioxidant peptides by *SEC* and sequential reversed-phase chromatography (*RPC*). Peptides were identified by LC-MS/MS. The identified peptide sequences were further digested by *in silico* GI digestion. All target peptides including parent and their digesta were synthesized and characterized for their $ABTS^{\bullet+}$ scavenging and intracellular ROS scavenging activities, as well as regulation of gene expression of antioxidant enzymes.

1.5 Expected results

Yield of the protein hydrolysate with optimal antioxidant activity under GI digestion would be demonstrated. The efficiency of glycation to improve the antioxidant activity of hydrolysate on chemical and cellular models would be obtained. Modification of glycated hydrolysates upon GI digestion would be exhibited. This research would lead to more understandings of antioxidant peptides presenting in the hydrolysate digesta and their mode of action. Valorization of tilapia can be enhanced via production of protein hydrolysate or glycated hydrolysate with optimal antioxidant activity.

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CHAPTER II

LITERATURE REVIEWS

2.1 Oxidative stress and human diseases

Reactive oxygen species (ROS) is an umbrella term for an array of reactive species, including reactive oxygen, nitrogen and sulfur species etc., that occur as a normal metabolism of aerobic life (Parvez, Long, Poganik, & Aye, 2018; Sies & Jones, 2020). ROS can be divided into two species, non-radical ROS such as hydrogen peroxide (H_2O_2), singlet molecular oxygen ($^1O_2$) and hypochlorous acid, and free radical ROS including hydroxyl radical (•OH), superoxide anion radical ($O^{\bullet-}$), peroxynitrite ($ONOO^-$) and peroxyl radical (ROO•), among others. They are mainly generated by enzymatic catalysis at the biological membranes, mitochondrial, endoplasmic reticulum (ER) and peroxisomal, as well as interconversion of ROS (Parvez et al., 2018). The exogenous stimuli such as UV light, heavy metal ions, ionizing radiation, and xenobiotics can also generate ROS (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012; Parvez et al., 2018).

ROS at physiological levels is important for signaling, which is beneficial for cell proliferation, differentiation and migration (Sies et al., 2020). Normally, the redox homeostasis in cells is maintained by the endogenous non-enzymatic antioxidant and enzymatic antioxidant such as glutathione (GSH), superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), as well as exogenous antioxidants

containing polyphenols, α-tocopherol and ascorbic acid (Birben et al., 2012). However, the dysregulated redox homeostasis with high concentration of intracellular ROS would induce modification of macromolecules leading to dysfunction of proteins, lipids and DNA, and cell growth arrest or death (Birben et al., 2012; Sies et al., 2020). This shift in balance between oxidant and antioxidant in favor of oxidants resulting in harmful effects of ROS, which is termed oxidative stress (Agyei, Danquah, Sarethy, & Pan, 2015; Birben et al., 2012). Oxidative stress is relative to many pathophysiological processes such as aging, diabetes mellitus, cardiovascular and metabolic diseases, and cancer (Agyei et al., 2015; Birben et al., 2012; Zhichun Chen & Zhong, 2014; Incalza, D'Oria, Natalicchio, Perrini, Laviola, & Giorgino, 2018; Sies et al., 2020).

2.2 Antioxidant peptides and human health

Peptides are short-chain protein fragments containing 2 – 20 amino acid residues that are encrypted in the primary sequences of proteins (Li-Chan, 2015; Samaranayaka & Li-Chan, 2011). Antioxidant activity of peptides could be exhibited fully after releasing from the proteins. Amino acids, namely Y, W, C, M, H and K, are potential to scavenge free radicals by either hydrogen atom transfer (HAT) or electron transfer (ET) mechanism (Karel, Tannenbaum, Wallace, & Maloney, 1966; Marcuse, 1960; Zheng, Zhao, Dong, Su, & Zhao, 2016). High content of hydrophobic amino acids, namely A, L, P, F, H, W and Y, and negative amino acids, D and E, found in protein hydrolysates linked with strong free radical scavenging capacity through direct ET (Nwachukwu & Aluko, 2019). The advantage of peptides containing multiple amino acid residues on a single chain could possess superior antioxidant activity to that of single amino acids (Aluko, 2015). Only amino acid C displayed slight reducing power,

but the higher activity was detected for dipeptides, GC and PC (Zheng et al., 2016). GC and PC also exhibited higher 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$) scavenging capacity than that of single amino acid. In addition, the reduced folding of protein during proteolysis propensity ensures the most of reactive residues in peptides are available for antioxidant reactions (Nwachukwu et al., 2019; Raghavan, Kristinsson, & Leeuwenburgh, 2008). Thus, peptides would be more effective to be the antioxidant than amino acids and proteins. Moreover, the most important carboxylic group of D and E residues, and the nitrogen of ε-amino in K, guanidine group from R and imidazole group from H that can be involved in the linkage for establishing a coordinate covalent bond with metal ions (Caetano-Silva, Netto, Bertoldo-Pacheco, Alegria, & Cilla, 2020). Thus, some peptides would be the effective metal ions chelator to inhibit the generation of free radicals (Caetano-Silva et al., 2020; Wu, Yang, Sun, Bao, & Lin, 2020), such as chelating of iron suppresses •OH by inhibition of Fenton reaction. Therefore, antioxidant peptides have been considered as a natural antioxidant that can diminish the oxidative stress for prevention or therapy of aging (Guo et al., 2020), and chronic diseases such as antihypertension (Girgih et al., 2014), antiosteopenic effects (Mada et al., 2017), and anti-fatigue effects (Xu, Li, Regenstein, & Su, 2017).

2.3 Preparation of antioxidant peptides

In addition to some natural antioxidant peptides in foods such as anserine (β-alanyl-1-methylhistidine), carnosine (β-Ala-His), glutathione (γGlu-Cys-Gly), and melatonin (N-acetyl-5-methoxy tryptamine) (Mine, Li-Chan, & Jiang, 2010; Samaranayaka et al., 2011), food protein-derived antioxidant peptides have been

investigated widely in recent decades (Nwachukwu et al., 2019; Samaranayaka et al., 2011; Zheng et al., 2016). Antioxidant peptides would be released during muscle post-mortem and storage period by autolysis (Fu, Young, & Therkildsen, 2017; Liu, Chen, Huang, Huang, & Zhou, 2017), as well as processing (Samaranayaka et al., 2011; Toldrá, Gallego, Reig, Aristoy, & Mora, 2020). Fermentation such as yogurt and fish sauce processing were also the effective approach to gain bioactive peptides (Aloglu & Oner, 2011; Hamzeh, Noisa, & Yongsawatdigul, 2020; Jemil et al., 2017; Padghan, Mann, & Hati, 2017). However, protein hydrolysis by exogenous enzymes was the most effective strategy for production of antioxidant hydrolysates/peptides (Li-Chan, 2015; Nwachukwu et al., 2019; Samaranayaka et al., 2011). Antioxidant peptides can be released from food proteins by a single protease (Ketnawa, Martínez-Alvarez, Benjakul, & Rawdkuen, 2016; Raghavan et al., 2008), the combination of various enzymes (Guo et al., 2020; Yarnpakdee, Benjakul, Kristinsson, & Bakken, 2015), and proteases combined with thermal, ultrasonic or microwave pretreatment etc., (Ikram, Zhang, Ahmed, & Wang, 2020; Noman et al., 2020). Antioxidant peptides have also been obtained from the various food protein resources, including plant (Wong, Xiao, Wang, Ee, & Chai, 2020), animal (Toldra, Gallego, Reig, Aristoy, & Mora, 2020), milk (FitzGerald, Cermeño, Khalesi, Kleekayai, & Amigo-Benavent, 2020), egg (Benede & Molina, 2020), and mushroom proteins (Zhou et al., 2020), and so on. However, the procedure of conventional approaches to obtain the exact antioxidant peptide sequences as shown in Figure 2.1, which could be quite troublesome (FitzGerald et al., 2020; Toldrá et al., 2020). Its limitations such as complex mixtures, unknown sequences, time consuming, costs, and incomplete peptide identification have been listed by FitzGerald and Nongonierma (FitzGerald et al., 2020; Nongonierma & FitzGerald, 2017). The time

and costs can be reduced with a predictive strategy for the peptide sequences and bioactivities based on *in silico* analysis using bioinformatics tools and peptide databases (Toldrá et al., 2020). The diagram was shown in Figure2.1. The commonly used tools, such as PeptideCutter https://web.expasy.org/peptide_cutter/, BIOPEP http://www.uwm.edu.pl/biochemia/index.php/ en/biopep, Peptide ranker http://distilldeep. ucd.ie/ Peptide Ranker/, PepBank http://pepbank.mgh.harvard.edu/, and AutoDock Vina http://vina.scripps.edu/, have become popular as a powerful tool for bioactive peptide discovery including selection of the most appropriate protein substrate and enzymes for generation of peptides, determination of the mechanism of action by peptides, bioactivity prediction, and so on (FitzGerald et al., 2020; García-Vaquero, Mora, & Hayes, 2019; Guo et al., 2020; Ji, Udenigwe, & Agyei, 2019; Toldrá et al., 2020). The more targeted *in silico* approach is more rapid and less chemical-reagent consumption (FitzGerald et al., 2020). The complement of bioinformatics to the classical strategy would be a highly efficient approach for commercialization of antioxidant peptides. However, this is not well established. The prediction of bioactivities and GI stability of identified peptides has been conducted frequently (García-Vaquero et al., 2019; Guo et al., 2020). Flaxseed protein-derived antioxidant peptides have been predicted by various enzymes using BIOPEP (Ji et al., 2019). Plant proteases, papain, ficin, and bromelain, showed superior to release antioxidant peptides. However, there was no confirmation by the actual hydrolysis system.

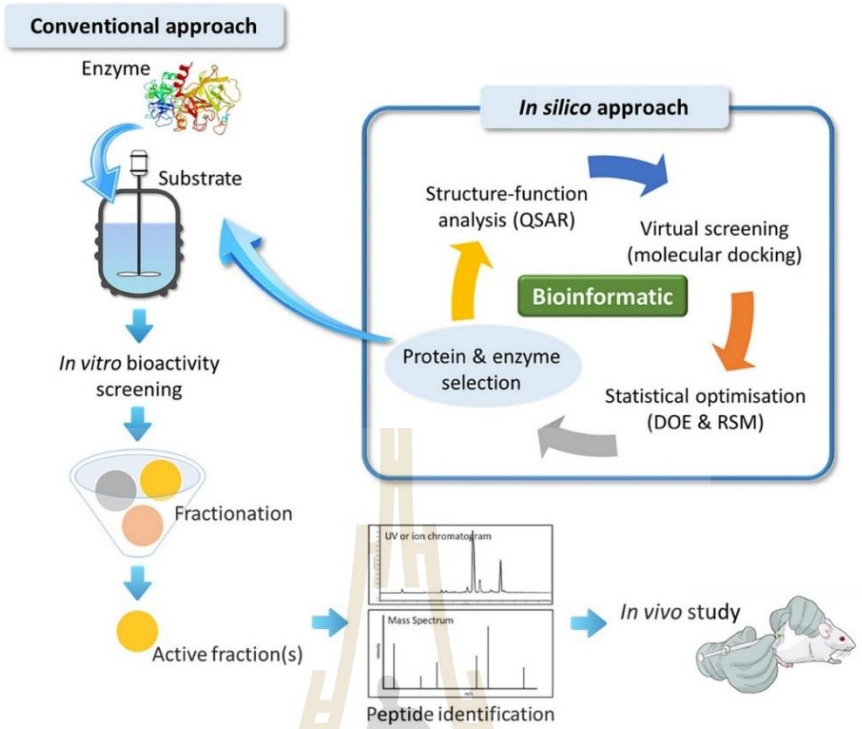


Figure 2.1 Diagrammatic representation of conventional and *in silico* approaches for food-protein derived bioactive peptide discovery.

From FitzGerald et al. (2020). QSAR: quantitative structure activity relationship; DOE: design of experiment; RSM: response surface methodology.

2.4 GI digestion of peptides

2.4.1 Stability of antioxidant peptides upon GI digestion

When protein hydrolysates are applied as functional foods or an ingredient in functional feed, the oral administration likely modifies their activities due to hydrolysis occurring during GI digestion by proteases (Chakrabarti, Guha, & Majumder, 2018; Jang, Liceaga, & Yoon, 2016). *In vitro* GI digestion increased the antioxidant activities of hydrolysates derived from fish muscle protein or skin (Ketnawa et al., 2016; Ketnawa, Wickramathilaka, & Liceaga, 2018; Samaranayaka, Kitts, & Li-

Chan, 2010). Nevertheless, the antioxidant activities of the positively charged fraction of casein hydrolysate and synthetic peptide, ATSHH, decreased after digestion (Ao & Li, 2013; Jang et al., 2016). Peptides were digested slightly by pepsin on the stomach, but significantly degraded by pancreatin digestion on the intestine (Ketnawa et al., 2018; Wiriyaphan, Xiao, Decker, & Yongsawatdigul, 2015). The reasons could be pepsin has strict/narrow specificity resulting a low digestion in peptide bonds. In contrast, pancreatin is a mixture of endopeptidases and exopeptidases, it possesses a broad specificity contributing to its high digestive ability towards hydrolysate/protein (Keil, 1992). In this case, the stability of antioxidative protein hydrolysates/synthetic peptides have generally evaluated to investigate the potential efficacy of peptides after GI digestion (Jang et al., 2016; Ketnawa et al., 2018; Liu et al., 2019; Wiriyaphan et al., 2015). In other studies, the simulated *in vitro* GI digestion approach has been employed directly to evaluate the bioactive peptides with high stability to pass through the GI tract (Feng, Peng, Wang, Li, Lei, & Xu, 2019; Gallego, Mauri, Aristoy, Toldrá, & Mora, 2020; Gong, Wang, Wu, Li, Qin, & Li, 2020).

2.4.2 Simulated *in vitro* GI digestion models

Evaluation of food digestion in humans is the most reliable technique, but the trials are difficult to control, expensive, and might not all be justifiable on ethical grounds. Therefore, the static or dynamic *in vitro* models have been used for many decades to simulate the digestion of food (Brodkorb et al., 2019; Guerra, Etienne-Mesmin, Livrelli, Denis, Blanquet-Diot, & Alric, 2012; Mulet Cabero et al., 2020). Dynamic or semi-dynamic models provide more physiologically relevant data than static counterparts, but they are more time-consuming, expensive and difficult to access (Guerra et al., 2012; Mulet Cabero et al., 2020). For the static model, however, the

different conditions, including the ratio of enzyme to substrate, the digestive pH, fluids, and time, etc., have been conducted for the various samples (Jang et al., 2016; Ketnawa et al., 2018; Liu et al., 2019; Wiriyaphan et al., 2015). A standardized static *in vitro* digestion method has been established by the COST action INFOGEST (Brodkorb et al., 2019; Minekus et al., 2014). Egger et al. (2017) evaluated digestion of milk protein by the INFOGEST static method and found that the endpoint of digestion was comparable to *in vivo* protein hydrolysis. It has been widely used in recent years (Dave, Hayes, Mora, Montoya, Moughan, & Rutherfurd, 2016; Gallego et al., 2020; Gong et al., 2020). Additionally, bioinformatics tools have also applied usually for *in silico* GI digestion to predict the potential cleave sites of peptides under GI tract in theoretically (Agyei, Tsopmo, & Udenigwe, 2018; Gallego, Mora, & Toldrá, 2018). PeptideCutter tool of ExPASy has successfully predicted the decrease in antioxidant activity of peptide, AEEEYPDL, during *in vitro* GI digestion (Gallego et al., 2018).

2.4.3 Interference of peptides from the digestive enzymes

The digesta of protein hydrolysate contained peptides and amino acids that resulted from gastric (pepsin) and intestinal (pancreatin) digestion. Nevertheless, peptides/amino acids derived from the proteases used in the analysis could also be generated due to the autolysis and/or hydrolysis (Dave et al., 2016; Whitcomb & Lowe, 2007). This could also contribute to the activities of interest. Dave et al. (2016) reported that peptides derived from trypsin by *in vitro* digestion revealed the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$) and α, α-diphenyl-β-picrylhydrazyl free radical (DPPH•) scavenging capacity. Therefore, the antioxidant activities of the peptides derived from the enzyme blanks of *in vitro* model should be assessed to obtain more biologically relevant antioxidant activity of the

digesta. Furthermore, when the bioactive peptides are identified from the digesta of protein or its hydrolysate, it should also be concluded carefully. Thus far, the issue of enzyme blanks has not been well characterized in publications.

2.5 Purification of antioxidant peptides

The ultrafiltration (*UF*) by membranes has been considered as a simple, economical and effective approach for the commercial production of antioxidant peptide fractions (Acquah, Chan, Pan, Agyei, & Udenigwe, 2019; Wiriyaphan et al., 2015; Zheng, Su, Ren, Gu, You, & Zhao, 2012). Because the smaller peptides usually showed higher antioxidant activity (Nwachukwu et al., 2019; Raghavan et al., 2008; Wiriyaphan, Chitsomboon, Roytrakul, & Yongsawadigul, 2013; Wiriyaphan et al., 2015). However, the enhancement of peptide activity by *UF* is still insufficient because the small but inactive peptides cannot be removed, which is also restricted by the pore size of membranes or sample concentration (Acquah et al., 2019). The *UF* has been usually used for the first step of purifications (Jang et al., 2016; Zheng et al., 2012). The more rigorous and elaborate chromatography, such as size exclusion chromatography (*SEC*), reversed-phase chromatography (*RPC*), and ion-exchange chromatography (*IEC*) based on the size, hydrophobicity, and charge of peptides, respectively, have generally combined for the purification of antioxidant peptides at analytical level (Acquah et al., 2019; Jang et al., 2016; Yang, Zhang, Ding, Chi, Wang, & Huo, 2019). As discussed previously, peptides are likely modified upon GI tract, and the peptides from the enzymes for simulated *in vitro* GI digestion poses challenges in the purification and identification of antioxidant peptide from hydrolysate digesta. Therefore, until now, purification and identification of antioxidant peptides from the protein hydrolysate, its

digesta shows the highest antioxidant activity, followed by *in silico* GI digestion, which could be an alternative for the prediction of antioxidant peptides from hydrolysate digesta with high biological activity. So far, it has not been well conducted. Antioxidant peptides have been purified normally from protein hydrolysates (Wiriyaphan et al., 2013; Yang et al., 2019; Zheng et al., 2012). Antioxidant peptides have also been identified from the protein hydrolysate, which showed the highest activity, followed by simulated GI digestion to evaluate the peptides of hydrolysate digesta (Gallego et al., 2018; Jang et al., 2016). Antioxidant peptides from the prepared digesta of protein or its hydrolysate have also been identified directly (Cermeño et al., 2019; Feng et al., 2019; Gong et al., 2020; Masoud Homayouni-Tabrizi, Hoda Shabestarin, Ahmad Asoodeh, & Mozhgan Soltani, 2016). Total 48 novel antihypertensive peptides have been purified and identified from the hydrolysate fragments of Green Macroalga *Ulva lactuca*, which displayed a higher angiotensin I converting enzyme inhibitory activity (García-Vaquero et al., 2019). Total 86 peptides were then obtained by *in silico* GI digestion using ExPASy PeptideCutter tool for further evaluations.

2.6 Antioxidant activity assays

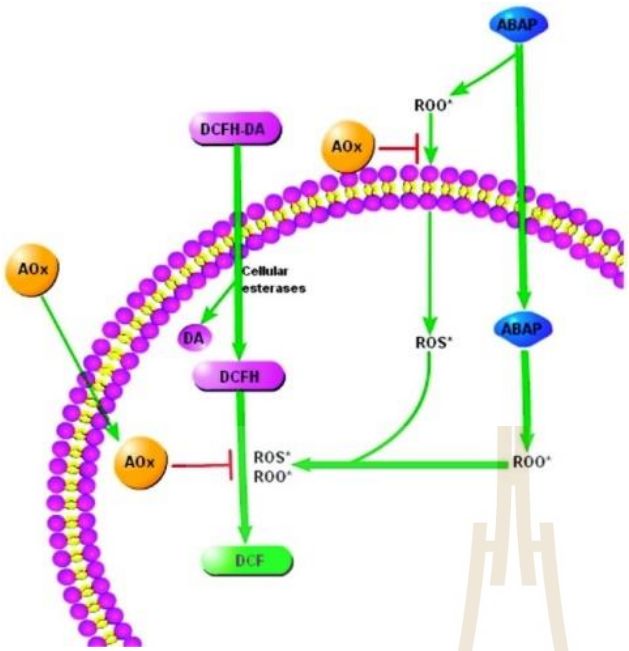
2.6.1 Chemical antioxidant activity assays

There are more than 19 *in vitro* chemical assays have been used for evaluation of antioxidant activity of interested compounds (Alam, Bristi, & Rafiquzzaman, 2013; Apak, Özyürek, Güçlü, & Çapanoğlu, 2016a, 2016b, 2016c). The major assays monitoring the reactions by molecular probes can be roughly divided into two categories based on the reaction mechanisms. HAT-basic assays measure the capacity of an antioxidant to trap free radicals by hydrogen donation (Apak et al., 2016b;

Pisoschi, Pop, Cimpeanu, & Predoi, 2016). The oxidation in these assays, such as oxygen radical absorbance capacity (ORAC), and inhibition of β-carotene/crocin bleaching and low-density lipoprotein (LDL) oxidation, are generally initiated by the ROO• (Pisoschi et al., 2016; Prior, Wu, & Schaich, 2005). ET-based assays are relied on one electron transfer reductive ability of an antioxidant compound versus radical species (Pisoschi et al., 2016), such as total phenols (or phenolic) assay by Folin-Ciocalteu reagent (FCR), ferric reducing antioxidant power (FRAP) or cupric reducing antioxidant capacity (CUPRAC) assay (Apak et al., 2016a; Prior et al., 2005). However, DPPH• and trolox equivalent antioxidant capacity (TEAC) assays have also been demonstrated involving both HAT and ET (Apak et al., 2016b; Jiménez, Selga, Torres, & Julià, 2004; Pisoschi et al., 2016; Prior et al., 2005). Metal ions such as iron and copper can induce the metal-initiated Fenton-type reactions generating reactive species (Apak et al., 2016a). Thus, metal chelators can form coordination complex with metal ions suppressing in generation of ROS or inhibition of lipid oxidations. The assay of ROS which are involved in the biological system should also be employed for assessment of antioxidant activity of interested compounds, including $O_2^{\bullet-}$, H_2O_2, ROO•, •OH, $^1O_2$, and $ONOO^-$ scavenging capacity, etc. (Huang, Ou, & Prior, 2005). Thus, there is no single assay which can provide unequivocal antioxidant capacity of interested compounds. The best evaluation system is using the different methods with various reaction mechanisms to obtain the highly biologically relevant assays for directing the further studies (Carocho & Ferreira, 2013; Huang et al., 2005; Niki, 2010; Zhang et al., 2017). Moreover, the combining chemical assays under various mechanisms could also be useful to interpret the primary mechanisms of the antioxidant.

2.6.2 Cellular antioxidant activity (CAA) assays

The cellular antioxidant activity (CAA) assay is a more biologically relevant technique, as it addresses some issues of cells, such as their uptake, distribution, and ability to metabolize the antioxidant (López-Alarcón & Denicola, 2013; Wolfe & Liu, 2007). The method and proposed principle of CAA assay are shown in Figure 2.2 (Wolfe et al., 2007). Cells are pre-treated with antioxidant and 2′,7′-dichlorofluorescin di-acetate (DCFH-DA). This step can also be divided into that cells were treated by antioxidant followed by DCFH-DA (Mine, Young, & Yang, 2015; Yousr, Aloqbi, Omar, & Howell, 2016). In this case, the antioxidant is binding to the cell membrane and/or passing through the membrane to enter the cells. The DCFH-DA diffuses into the cell where cellular esterases cleave the diacetate moiety to form the more polar dichlorofluoorescin (DCFH), which is trapped within the cells. When the treated cells are incubated with the oxidative stressor, such as 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP or AAPH) which can diffuse into cells and then spontaneously decompose to form ROO•, the intracellular DCFH is oxidized by the ROO• or new generated free radicals to the fluorescent dichlorofluorescein (DCF). The antioxidant scavenges the more ROS, which can prevent the oxidation of DCFH to the lower extent, resulting in less intracellular DCF. Thus, the lower DCF fluorescence indicates the higher CAA.



Cell seeding

↓ 24 h incubation

Washing with PBS

↓

Antioxidants + 25 μM DCFH-DA

↓ 1h incubation

Washing with PBS

↓

600 μM ABAP/AAPH

↓

Recording fluorescence

($\lambda_{Ex/Em}$ = 485/538 nm)

Figure 2.2 Method and proposed principle of the cellular antioxidant activity (CAA) assay.

From Wolfe et al. (2007). Aox, antioxidant; ABAP/AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; DCF, dichlorofluorescein; DCFH, dichlorofluoorescin; DCFH-DA, dichlorofluorescin di-acetate; ROS, reactive oxygen species.

The underlying mechanisms of antioxidant protected the cellular oxidative stress were proposed and described in Figure2.3 (Wen et al., 2016). On one hand, antioxidant as a free radical scavenger which directly scavenges the absolute amount of intracellular free radicals. On the other hand, antioxidant can promote the intrinsic cellular antioxidant activity by improving the level of endogenous antioxidant such as GSH and/or by enhancing the expression of antioxidant enzymes to increase the antioxidant enzyme activity (Birben et al., 2012). The antioxidant enzymes are involving SOD, catalase, GPx, and so on.

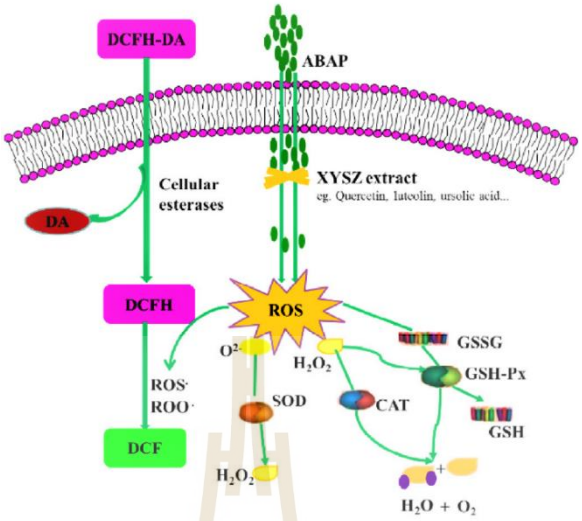


Figure 2.3 Effect of the phytochemical extracts on ABAP-induced oxidative stress in HepG2 cells.

From Wen et al. (2016). Aox, antioxidant; ABAP/AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; DCF, dichlorofluorescein; DCFH, dichlorofluoorescin; DCFH-DA, dichlorofluorescin di-acetate; ROS, reactive oxygen species; GSH, glutathion; GSSG, oxidized glutathion; SOD, Superoxide dismutase; GSH-Px/GPx, glutathione peroxidase; CAT, catalase.

In addition to assessment of the expression level of antioxidant enzymes and enzyme activity, the regulation of upstream gene expression of antioxidant enzymes, and other genes relevant to the antioxidant signaling pathway are also valuable to explore the underlying mechanisms acted by antioxidant (Halliwell, 2012; Sies, 2015). For example, Kelch-like ECH associated-protein 1(Keap1)/nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway plays a

key role in cell response to oxidative stress (De Freitas Silva et al., 2018; Sies et al., 2020; Vriend & Reiter, 2015). Under normal condition, the transcription factor for antioxidant enzymes, Nrf2, is ubiquitinated by the E3 ubiquitin ligase complex, Keap1-Cul3-Rbx1, and degraded by the proteasome in the cytosol. When the electrophiles such as chalcones and peptides (De Freitas Silva et al., 2018; Tonolo et al., 2020) modified Keap1, the Nrf2 was dissociated, then phosphorylated and migrated to the nucleus. The nuclear Nrf2 with Maf (a coactivator for Nrf-2) activates the DNA promoter (ARE) leading to the transcription of antioxidant enzymes. Thus, the dissociation of Keap1/Nrf2 complex in the cytosol before nuclear translocation of Nrf2 is crucial for the activation of Keap1/Nrf2/ARE signaling pathway. The expression level of Keap1 and Nrf2 can be detected to confirm with the expression of antioxidant enzymes reflecting the antioxidant mechanisms action by antioxidant (Tonolo et al., 2020; J. Wu et al., 2018).

2.7 Antioxidant capacity of peptides

Protein hydrolysates except for some pure peptides usually showed a low antioxidant potential when compared with the standard antioxidants. According to the commonly used analyses, such as DPPH•, ROO• and $ABTS^{\bullet+}$ scavenging, the 50% effective concentration (EC_{50}) of peptides/protein hydrolysates was range of 20 µg/mL ~ 10 mg/mL, which have also been expressed as Trolox equivalents as lower than 1500 µmol (375 mg) Trolox/g protein or solid (Cermeño et al., 2019; Liu, Xing, Fu, Zhou, & Zhang, 2016; Wiriyaphan et al., 2015; Yang et al., 2019; Yu, He, Tang, Zhang, Zhu, & Sun, 2018; Zhang, Noisa, & Yongsawatdigul, 2020; Zheng et al., 2016). Some pure cysteine-, tyrosine- or tryptophan-containing peptides displayed high activity as 0.3 –

5 μmol TE/μmol peptide (Cermeño et al., 2019; Zheng et al., 2016). Nevertheless, the EC_{50} of Trolox were lower than 5 μg/mL for DPPH• and $ABTS^{•+}$ assay, respectively (Faitanin et al., 2018). Catechin also showed EC_{50} of 8 μM based on DPPH• assays (Chen, Bertin, & Froldi, 2013). Antioxidant peptides, such as PHP, GLVYIL, YHNAPGLVYIL, LKKISQRYQKF, ALPQYLKTVYQHQK and IQPKTKVIPYVRYL, showed effective intracellular ROS scavenging capacity (Du, Esfandi, Willmore, & Tsopmo, 2016; Tonolo et al., 2020; Wu et al., 2018). PHP, NTVPAKSCQAQPTTM (oxidized M), QGPIVLNPWDQVKR and APSFSDIPNPIGSENSE also up-regulated expression of antioxidant enzymes (Tonolo et al., 2020; Wu et al., 2018). Corn gluten hydrolysate fractions with molecular weight of less than 1 kDa and 1-3 kDa down-regulated gene expression of catalase, but up-regulated GPx (Wang et al., 2018). However, when compared with the standard antioxidants such as *α*-Tocopherol, Trolox and ascorbic acid, antioxidative peptide/hydrolysate have shown lower intracellular ROS scavenging capacity (Wiriyaphan, Chitsomboon, & Yongsawadigul, 2012; Wiriyaphan et al., 2015; Yarnpakdee et al., 2015). The improvement of antioxidant activity of peptide would be an alternative strategy to promote its utility.

2.8 Glycation of peptides

Maillard reaction or glycation between primary amino and active carbonyl groups is initiated spontaneously during thermal processing and food storage. Maillard reaction products (MRPs), therefore, is recognized as the normal, natural and endogenous products (Cui, Hayat, & Zhang, 2019; Nooshkam, Varidi, & Bashash, 2019). Maillard reaction significantly depends on the reaction conditions such as type of reactants and glycation time (Chen et al., 2019; Nooshkam et al., 2019; Vhangani &

Van Wyk, 2013). The extent of Maillard reaction is usually increased with reaction temperature and time (Chen et al., 2019; Nooshkam et al., 2019; Vhangani et al., 2013). Generally, the reactivity of sugars is in the order of aldopentoses > aldohexoses, monosaccharides > di- or oligosaccharides, aldoses > ketoses (Bunn & Higgins, 1981; Chen et al., 2019; Nooshkam et al., 2019; Spark, 1969). Smaller molecular weight (MW) of peptides would possess higher reactivity with sugar than the larger one (Nie, Xu, Zhao, & Meng, 2017; Su, Zheng, Cui, Yang, Ren, & Zhao, 2011; Yu et al., 2018). In contrast, MRPs from fructose-amino acid/protein systems exhibited a higher browning intensity and free radical scavenging capacities than that of glucose despite that fructose generally showed lower reaction activity than did glucose (Ashoor & Zent, 1984; Benjakul, Lertittikul, & Bauer, 2005; Hwang, Kim, Woo, Lee, & Jeong, 2011). Therefore, the reaction mechanisms of Maillard reaction and the generated MRPs might be unique for the different reaction systems (Hemmler, Roullier-Gall, Marshall, Rychlik, Taylor, and Schmitt-Kopplin 2018).

2.9 Characterization of glycated peptides

Maillard reaction is a complex cascade reaction (Nooshkam et al., 2019). Monitoring of the progress of Maillard reaction is beneficial to investigate the extent of reaction and establish the relationship between various stage of MRPs and antioxidant activity. The early fructosamine before the Amadori rearrangement which can be detected by the nitro blue tetrazolium chloride (NBT) (Martinez-Saez, Fernandez-Gomez, Cai, Uribarri, & del Castillo, 2017). The colorless intermediate of Maillard reaction could be evaluated by the absorbance at 294 nm (Nooshkam et al., 2019; Wang, Zhang, Wang, Wang, & Liu, 2019). Fluorescent adducts which might be the precursors

of brown pigments can be monitored at an excitation wavelength of 347 nm and emission wavelength of 415 nm (Martinez-Saez et al., 2017; Morales & Jiménez-Pérez, 2001). For the final stage of Maillard reaction, the condensation reactions formed the browning products, melanoidins, which showed the maximum absorbance at 420 nm (Morales et al., 2001; Nooshkam et al., 2019). Moreover, the absorbance at about 214 nm are usually used to monitor the peptides (Chen et al., 2019; Han et al., 2019; Zhang et al., 2020). In this case, *SEC* can be used to analyze the variation of MW during glycation reflecting the extent of glycation (Chen et al., 2019; Chen, Fang, & Wang, 2020; Han et al., 2019). Meanwhile, it would be also effective to monitor the changes in various stage of MRPs when the three wavelengths as 214, 294 and 420 nm were conducted simultaneously.

2.10 Antioxidant capacity of glycated peptides

Glycation improved the chemical antioxidant activity of proteins, peptides, and amino acids such as reducing power, DPPH•, $ATBS^{\bullet+}$, ROO•, •OH scavenging capacity (Fu, Zhang, Soladoye, & Aluko, 2019; Nooshkam et al., 2019). MRPs have also extended food shelf-life and inhibited lipid oxidation of foods (Nooshkam et al., 2019; Sohaib et al., 2017). The free radical scavenging capacities of glycated amino acids, peptides/hydrolysates were positively correlated with the extent of glycation (Chen et al., 2019; Nooshkam et al., 2019; Vhangani et al., 2013). However, a plateau has also reached for the extended glycation of grass carp (*Ctenopharyngodon idellus*) scale hydrolysate (Chen et al., 2019). The extreme reaction such as ribose-lysine and fructose-lysine were treated at 121 °C for 2 h reduced their activities (Vhangani et al.,

2013). Thus, the free radical scavenging capacity of glycated peptides should be characterized for the different reaction systems.

The efficiency of glycation for the improvement of antioxidant activity in biological system are still rarely elucidated and inconclusive. The MRPs have also been frequently investigated individually but not compared with that of original hydrolysates resulting in the incomparable results. MRPs from the unicorn leatherjacket skin hydrolysate-galactose system were effective to mitigate the H_2O_2-induced oxidative stress and DNA damage (Karnjanapratum, O'Callaghan, Benjakul, & O'Brien, 2016). MRPs induced from the soybean peptides, *L*-cysteine and xylose which also retarded galactose induced aging in ICR mice by increasing in the activities of antioxidant enzymes and total antioxidant activities of serum (He et al., 2019). MRPs from shrimp by-products protein hydrolysate-glucose and scallop mantle hydrolysates-ribose systems have also promoted the higher CAA than the original hydrolysates (Han et al., 2018; Zha, Wei, Chen, Dong, Zeng, & Liu, 2015). In contrast, MRPs caused oxidative stress and intestinal inflammation as well (Hillman et al., 2019; Yao, Han, Dong, Zeng, & Liu, 2016). Glycation is likely an effective strategy to improve the antioxidant activity of peptides/hydrolysate. Nevertheless, the improvement of activity in biological systems should be further evaluated and demonstrated.

2.11 GI digestion of glycated peptides

Protein hydrolysate would be modified upon GI digestion resulting in the changes of antioxidant activity as discussed previously. Glycated hydrolysate could also be digested upon GI digestion. The digestibility of glycated proteins or peptides were lower than that of origins, and the peptide profiles in the digesta were also significantly

changed (Zhang, Wang, & Fu, 2020; Zhao et al., 2019). MRPs from the grass carp scale hydrolysate with ribose and unicorn leatherjacket skin gelatin hydrolysate with galactose systems showed reduced activities in DPPH• and/or $ATBS^{\bullet+}$ scavenging capacity and reducing power upon GI digestion (Chen et al., 2019; Karnjanapratum et al., 2016). The $ATBS^{\bullet+}$ and •OH scavenging capacity of MRPs from the snapper fish scale peptides with xylose did not change by GI digestion (Chen et al., 2020). However, the comparison between the antioxidant activity of MRPs before and after GI digestion is still scarce. Only the effect of MRPs on suppression of oxidative stress or/and inflammation has been studied, but digesta of MRPs has not been widely investigated (Chen et al., 2020; Karnjanapratum et al., 2016). Activity of digesta of glycated hydrolysate would be more biological relevant than that of glycated hydrolysate.

2.12 References

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CHAPTER III

CHEMICAL AND CELLULAR ANTIOXIDANT ACTIVITIES OF *IN VITRO* DIGESTA OF TILAPIA PROTEIN AND ITS HYDROLYSATES

3.1 Abstract

Production of protein hydrolysate as nutraceuticals is typically based on the activity of the hydrolysate, which might not yield the optimal activity under physiological condition due to structural modification of peptides upon gastrointestinal (GI) digestion. This study systematically compared the chemical and cellular antioxidant activities of the *in vitro* digesta of tilapia protein and its hydrolysates prepared with various degree of hydrolysis (DH) by Alcalase. The enzymes used in the *in vitro* GI digestion analysis significantly contributed to the peptide content, Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC). Proteins and all hydrolysates were slightly digested by pepsin but hydrolyzed extensively by pancreatin**.** Both hydrolysate and digesta predominantly scavenged free radicals via hydrogen atom transfer (HAT). The antioxidant activities of the hydrolysates increased with the increasing DH up to 16 h of hydrolysis. However, the digesta of 10-h hydrolysate displayed the highest chemical and HepG2 cellular antioxidant activities, while the protein digesta displayed the lowest. Principal

component analysis (PCA) showed that the TEAC of the digesta was positively correlated with the cellular antioxidant activity (CAA). Therefore, the production of protein hydrolysate should be optimized based on the activity of the hydrolysate digesta rather than that of hydrolysates.

Keywords: antioxidant activity; protein hydrolysate; *in vitro* GI digestion; oxidative stress

3.2 Introduction

Antioxidant peptides derived from food proteins have been widely elucidated and characterized in various food protein sources (Nwachukwu & Aluko, 2019; Saito et al., 2003; Samaranayaka, Kitts, & Li-Chan, 2010; Wiriyaphan, Xiao, Decker, & Yongsawatdigul, 2015). They have been considered as a natural antioxidant, which can retard oxidative deterioration of food during storage (Nwachukwu et al., 2019; J. Wang, Lu, Guo, Li, & Huang, 2019; L. Zhang, Li, Hong, & Luo, 2020). In addition, antioxidant peptides have been shown to protect biological system from oxidative damages which can cause many pathophysiological processes such as aging, inflammation, diabetes and cancer (Nwachukwu et al., 2019; Samaranayaka & Li-Chan, 2011; Sies & Jones, 2020). Inclusion of the protein hydrolysate containing antioxidant peptides in feeds has also ensured desirable rates of growth performance and feed efficiency in pigs, calves, poultry and fish (Hou, Wu, Dai, Wang, & Wu, 2017; K. Zheng, Liang, Yao, Wang, & Chang, 2012; Keke Zheng, Liang, Yao, Wang, & Chang, 2013). The underlying mechanisms are related to improvement in intrinsic total antioxidant capacity, immune response, among others. However, when protein hydrolysates are applied as functional foods or an ingredient in functional feed, the oral administration likely modifies their

activities due to hydrolysis occurring during gastrointestinal (GI) digestion by proteases (Chakrabarti, Guha, & Majumder, 2018; Jang, Liceaga, & Yoon, 2016). *In vitro* GI digestion increased the antioxidant activities of hydrolysates derived from fish muscle protein or skin (Ketnawa, Martínez-Alvarez, Benjakul, & Rawdkuen, 2016; Ketnawa, Wickramathilaka, & Liceaga, 2018; Samaranayaka et al., 2010). Nevertheless, the antioxidant activities of the positively charged fraction of casein hydrolysate and synthetic peptide, ATSHH, decreased after digestion (Ao & Li, 2013; Jang et al., 2016). Therefore, the antioxidant activities of protein hydrolysate might not totally correlate with those of the digesta. Thus, process optimization of protein hydrolysate which is based merely on the antioxidant activities of the hydrolysate might not yield the product with the optimal health benefits. However, this approach has been commonly practiced for the production of protein hydrolysate with bioactivities (Raghavan, Kristinsson, & Leeuwenburgh, 2008; Yarnpakdee, Benjakul, Kristinsson, & Bakken, 2015; X. Zhang, Cao, Sun, Sun, & Xu, 2019). The antioxidant activities of the digesta would be more physiologically relevant and should be considered as one of the criteria for optimizing production of the antioxidant hydrolysate. Comparison between activities of the hydrolysate and its digesta has not been systematically elucidated.

Evaluation of food digestion in humans is the most reliable technique, but the trials are difficult to control, expensive, and might not all be justifiable on ethical grounds. Therefore, the static or dynamic *in vitro* models have been used for many decades to simulate the digestion of food (Brodkorb et al., 2019; Guerra, Etienne-Mesmin, Livrelli, Denis, Blanquet-Diot, & Alric, 2012; Mulet Cabero et al., 2020). Dynamic models provide more physiologically relevant data than static counterparts,

but they are more time-consuming, expensive and difficult to access (Guerra et al., 2012; Mulet Cabero et al., 2020). A standardized static *in vitro* digestion method has been established by the COST action INFOGEST (Brodkorb et al., 2019; Minekus et al., 2014). Egger et al. (2017) evaluated digestion of milk protein by the INFOGEST static method and found that the endpoint of digestion was comparable to *in vivo* protein hydrolysis. Therefore, the *in vitro* static method has been accepted as a useful technique to assess the endpoints of food digestion.

The digesta of protein hydrolysate contained peptides and amino acids that resulted from gastric (pepsin) and intestinal (pancreatin) digestion. Nevertheless, peptides/amino acids derived from the proteases used in the analysis could also be generated due to the autolysis and/or hydrolysis (Dave, Hayes, Mora, Montoya, Moughan, & Rutherfurd, 2016; Whitcomb & Lowe, 2007). This could also contribute to the activities of interest. Dave et al. (2016) reported that peptides derived from trypsin by *in vitro* digestion revealed the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$) and α, α-diphenyl-β-picrylhydrazyl free radical (DPPH•) scavenging capacity. Therefore, the antioxidant activities of the peptides derived from the enzyme blanks of *in vitro* model should be assessed to obtain more biologically relevant antioxidant activity of the digesta. Thus far, the issue of enzyme blanks has not been well characterized in publications.

Many chemical assays with different mechanisms have been conducted to evaluate the antioxidant activity of the interested compounds (López-Alarcón & Denicola, 2013; Nwachukwu et al., 2019). Combining chemical assays under various mechanisms could be useful to interpret the primary mechanisms of the antioxidant peptides. The cellular antioxidant activity (CAA) assay is a more biologically relevant

technique, as it addresses some issues of cells, such as their uptake, distribution and ability to metabolize the antioxidant (López-Alarcón et al., 2013; Wolfe & Liu, 2007). If chemical antioxidant assays could be used to predict the biological activity of peptides, it would greatly facilitate the optimization of antioxidant hydrolysates/ peptides production with more relevant health benefits. However, the relationship between the chemical and cellular antioxidant activities of food peptides has not been well established.

To increase the value and utilization of tilapia, protein hydrolysates with antioxidant activities should be produced under the optimal condition. The objectives of this study were to assess antioxidant activity of the *in vitro* GI digesta of tilapia hydrolysate with various degrees of hydrolysis (DH). Cytoprotection and intracellular ROS scavenging capacity assays based on HepG2 cell lines were also carried out. The relationships between the chemical and cellular antioxidant activities were also established based on principal component analysis (PCA) and Pearson correlation analysis.

3.3 Materials and methods

3.3.1 Chemicals and reagents

Alcalase® 2.4 L FG was purchased from Novozymes (Bagsvaerd, Denmark). Pepsin (from porcine gastric mucosa), pancreatin (from porcine pancreas), 3-morpholinosydnonimine hydrochloride (SIN-1), dihydrorhodamine 123 (DHR 123, ≥ 95.0%), fluorescein sodium salt (FL, BioReagent), 2,4,6-trinitrobenzenesulfonic acid solution (TNBS, 5.0%, w/v, BioReagent), reduced *L*-glutathione (GSH, ≥ 98.0%), cytochrome C, aprotinin, hippuryl-His-Leu acetate salt (Hip-His-Leu), 2,2'-azinobis (3-

ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, ≥ 98.0%), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*′-disulfonic acid monosodium salt hydrate (ferrozine, 97.0%), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97.0%), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ, ≥ 99.0%), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and 2′7′-dichlorodihydro-fluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH, 98.0%) and tryptophan (99.0%) were bought from ACROS Organics™ (Morris Plains, NJ, USA). *L*(+)-ascorbic acid was obtained from CARLO ERBA Reagents S.A.S (Rodano, Italy). The synthetic peptides, AGNQVLNLQADLPK (AK-14) and NTFLFFK (NK-7), were obtained from GL Biochem (Shanghai) Ltd. (Shanghai, China). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone (HyClone, Logan, UT, USA). Trypsin-EDTA (ethylenediaminetetraacetic acid), *L*-Gln, and non-essential amino acids (NEAAs) were obtained from Gibco (Carlsbad, CA, USA) for the cell culture studies**.** Acetonitrile (ACN) and trifluoroacetic acid (TFA) were of HPLC grade. All the other chemicals were of analytical grade.

3.3.2 Preparation of protein hydrolysates

Fresh tilapia (*Oreochromis niloticus*) was bought from a local market in Nakhon Ratchasima, Thailand. Fish were stored in ice and transported to the laboratory at Suranaree University of Technology within 1 h of their purchase. Fish were descaled, gutted, and skinned. Dorsal meat was collected and minced using a mincer (Kenwood A920, Havant, UK). The tilapia mince was defatted two times using isopropanol at a sample to solvent ratio of 1:4 (w/v) at 40 °C for 20 min. Subsequently, the defatted sample was left in the fume hood and dried in a hot air oven at 60 °C for 3 h. The sample

was ground and passed through a 230-mesh sieve to obtain tilapia protein powder (*P*), which was vacuum-packed and stored at -18 °C for further use. The proximate composition of *P* was analyzed according to AOAC (2000).

P containing 2 g of protein was suspended in 20 mL of 0.15 M $NaHCO_3$ to reach a final protein content of 10% (w/v), and the pH was then adjusted to 8.0 ± 0.1 using 10 M NaOH. The protein was hydrolyzed by 5% Alcalase (w/w of protein) in a 160-rpm shaking water bath at 50 °C for various time intervals of 2, 6, 10, and 16 h**.** Subsequently, the samples were heated at 90 °C for 10 min to terminate the enzyme activity and cooled immediately in ice water. The pH of the hydrolysate was adjusted to 7.0 ± 0.1, and the volume was brought to 25 mL. The samples were then centrifuged at 10,000 ×*g* and 4 °C for 20 min, filtered through Whatman 4 filter paper, and denoted as H_2, H_6, H_{10} and H_{16} for hydrolysis times of 2, 6, 10 and 16 h, respectively. The hydrolysates were immediately subjected to simulated *in vitro* GI digestion, as described in 2.3.

Thermally inactivated Alcalase (A_0) was also prepared by heating Alcalase at 90 °C for 10 min. The heated-*P* with inactivated Alcalase, denoted as P_0-A_0, was prepared by adding thermally inactivated Alcalase into the *P*, which was previously heated at 90 °C for 2 min and further heated for 8 min.

3.3.3 Simulated *in vitro* GI digestion

Simulated *in vitro* GI digestion was carried out according to Minekus et al**.** (2014) with slight modifications. The oral phase, *α*-amylase and lipase were ignored as tilapia protein powder and liquid hydrolysates contained mainly proteins/peptides**.** Gastric digestion was prepared by mixing 5 mL of hydrolysate, 3.2 mL of the simulated gastric fluid (*SGF*) stock solution, and 0.075 mM $CaCl_2$ in the final mixture**.** The

mixture was adjusted to a pH of 3.0 ± 0.1 using 6 M HCl and maintained a total volume of 9.2 mL. After pre-incubation in a 37 °C water bath for 15 min, freshly prepared pepsin in an *SGF* stock (0.8 mL) was added to achieve a 2,000 U/mL in the final mixture**.** The mixture was digested for 2 h at 37 °C with 160-rpm shaking. For intestinal digestion, 5.5 mL of the simulated intestinal fluid (*SIF*) stock solution and 0.20 mL of 10 M NaOH were rapidly mixed with 10 mL of gastric chime. The pH was adjusted to a pH > 5.6, and $CaCl_2$ was added to attain a 0.3 mM concentration in the final mixture. The mixture was finally adjusted to a pH of 7.0 ± 0.1 and a total volume of 17.5 mL. After pre-incubation in a 37 °C water-bath for 15 min, freshly prepared pancreatin in an 2.5 mL of *SIF* stock was added to achieve a 100 U/mL, which was determined based on the trypsin activity of the final mixture, and the mixture was digested for a further 2 h at 37 °C.

To terminate the enzyme in either the gastric (*GD*) or gastric-intestinal digesta (*GID*), the samples were heated at 90 °C for 10 min and cooled immediately in ice water. The *GD*s of the hydrolysates were expressed as H_2-*GD*, H_6-*GD*, H_{10}-*GD* and H_{16}-*GD*, while the *GID*s were referred to as H_2-*GID*, H_6-*GID*, H_{10}-*GID* and H_{16}-*GID* for hydrolysates obtained with 2, 6, 10, and 16 h of hydrolysis, respectively. The *GD*s and *GID*s were directly used for the chemical assays. The *GID*s were also lyophilized for the cellular assays.

The protein digesta were also prepared using the same amount of the initial protein and expressed as *P*-*GD* and *P*-*GID* for the gastric and GI digesta, respectively. Several enzyme blanks were prepared during digestion and expressed as thermally inactivated pepsin (Pep_0), inactivated pancreatin (Pan_0), and an enzyme blank of GI digestion (*Pep*-*Pan*-I), which was prepared with the procedure described

for the *in vitro* GI digestion, but without peptide samples. The contribution of Alcalase (A_0) on antioxidant activity was also investigated by incubating the inactivated A_0 following the *in vitro* GI digestion and was denoted as A_0-*GID*.

3.3.4 DH and digestibility

The α-amino group content was analyzed by an TNBS assay (Adler-Nissen, 1979) with slight modifications. A sample diluted in 50 µL of 1% SDS was added 250 µL of 0.2125 M phosphate buffer (pH 8.2) and 250 µL of freshly diluted 0.05% TNBS and incubated at 50 °C for 1 h in the dark. Then, 500 µL of 0.1 M HCl was added to stop the reaction. After incubation at room temperature for 30 min, the absorbance was measured at 420 nm (Stone, Staffordshire, ST15 OSA, UK). *L*-Leu was used as a standard. The total α-amino group content of the sample was measured by hydrolyzing the sample in 6 M HCl at 110 °C for 24 h in a heating block (Boekel Scientific, Feasterville, PA, USA). Subsequently, the pH was adjusted to 7.0 ± 0.1 for the TNBS analysis. The DH was calculated using equation (1) according to Benjakul and Morrissey (1997). The digestibility was calculated using equation (2).

$$\text{DH (\%)} = \left(\frac{L_t - L_0}{L_{total,p} - L_0}\right) \times 100, \tag{3.1}$$

$$\text{Digestibility (\%)} = \left(\frac{L_{ad} - L_{bd}}{L_{total,h} - L_{bd}}\right) \times 100, \tag{3.2}$$

where, L_t, L_0, and $L_{total,p}$ were the α-amino group contents of hydrolysate at time t and 0 h of hydrolysis and the total α-amino group of protein, respectively. L_{bd} and L_{ad} were the α-amino group content before and after *in vitro* digestion, respectively. $L_{total,h}$

was total α-amino group content of the respective hydrolysate. For protein digestibility, $L_{total,p}$ was used instead of $L_{total,h}$. The results were calculated after the subtraction of the respective enzyme blanks.

3.3.5 Molecular weight (MW) distribution

The samples were diluted with the mobile phase (7:3 v/v of 0.1% TFA: ACN), and 100 µL was injected into a Superdex Peptide 10/300 GL column equipped with fast protein liquid chromatography (FPLC) system (ÄKTA purifier, GE Healthcare Bioscience Co., Uppsala, Sweden). The peptides were eluted by the mobile phase at room temperature with a flow rate of 1.0 mL/min and total elution volume of 27 mL, which ensured complete elution of all peptides and column equilibration for the new run. Aprotinin (6,512 Da), AK-14 (1,481 Da), NK-7 (916 Da), Hip-His-Leu (429 Da), and tryptophan (204 Da) were used as the external standards. Cytochrome C (12,400 Da) was used to determine the void volume (V_0). LogMW *vs* K_{av} of the standards was plotted, where K_{av} was defined according to Wu et al. (Wu, Mansy, Wu, Surerus, Foster, & Cowan, 2002).

3.3.6 Chemical antioxidant activity

3.3.6.1 Trolox equivalent antioxidant capacity (TEAC)

The TEAC assay was carried out according to the ABTS radical cation ($ABTS^{\bullet+}$) decolorization method (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999) with slight modifications. The $ABTS^{\bullet+}$ stock containing 7 mM ABTS and 2.45 mM potassium persulfate was prepared and incubated in the dark for 12 – 16 h before use. Phosphate buffered saline (PBS, 10 mM, pH 7.4) containing 0.138 M NaCl and 0.0027 M KCl were used to the dilute $ABTS^{\bullet+}$ stock to obtain a working solution with an absorbance of approximately 0.7 at 734 nm. A 50 µL of

sample was mixed with 950 µL $ABTS^{\bullet+}$ and incubated at room temperature for 15 min, and the absorbance was measured within 30 min. Trolox was used as a standard, and the activity was expressed as Trolox equivalents (TE)/g initial protein.

3.3.6.2 Ferric reducing antioxidant power (FRAP)

The FRAP assay was carried out according to Benzie & Strain (1996) with some modifications. The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer at a pH of 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \bullet 6H_2O$ at ratio of 10:1:1 (v/v), and incubated at 37 °C for 10 min. A 100 µL of sample was incubated with 1000 µL of the FRAP reagent at 37 °C for 10 min in the dark, and the absorbance was measured at 593 nm. The activity was expressed as TE/g initial protein.

3.3.6.3 Fe^{2+} chelating capacity (FICC)

The FICC was assessed according to Decker & Welch (1990) with slight modifications. A 50 µL of sample, 1000 µL of deionized water and 50 µL of 1 mM $FeCl_2$ were mixed and incubated for 5 min in the dark, and then, 50 µL of 5 mM ferrozine was added and incubated for a further 20 min. The absorbance was measured at 562 nm. EDTA was applied as a standard, and the results were expressed as EDTA equivalents/g initial protein.

3.3.6.4 Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed in a black 96-well microplate as described by Dávalos et al., (2004) with slight modifications. Equal volumes (50 µL) of the sample, 150 mM PB (pH 7.4), and 280 nM fluorescein (FL) in 75 mM PB (pH 7.4) were mixed and incubated at 37 °C for 15 min. Subsequently, 50 µL of 48 mM AAPH in 75 mM PB was added, and the fluorescence at $\lambda_{Ex/Em}$ = 485/520 nm was

recorded at 1-min intervals for 110 min (Varioskan LUX Multimode Microplate Reader, Thermo Fisher Scientific, Waltham, MA, USA). Blanks were prepared using DI water instead of the sample. The area under the curve (AUC) was estimated by subtracting the baseline using SkanIt™ software 4.1 (Thermo Fisher Scientific, Waltham, MA, USA). The activity was expressed as TE/g initial protein.

3.3.6.5 Peroxynitrite ($ONOO^-$) scavenging

The peroxynitrite scavenging ability was evaluated according to Kooy et al., (1994) with some modifications. The SIN-1 and DHR 123 stock solutions were prepared in a 50 mM cold deoxygenated PBS solution (pH 7.4) and freshly diluted by 50 mM PBS for further use. Equal volumes (50 µL) of the sample, 100 mM PBS (pH 7.4), 10 µM DHR 123 and 40 µM SIN-1 were placed in a 96-well black microplate at 37 °C, and the fluorescence at $\lambda_{Ex/Em}$ = 500/536 nm was recorded at 2.5-min intervals for 120 min. The blanks were prepared using DI water. After the baseline was subtracted, the integrated area of the fluorescence curve plotted as a function of time was calculated using SkanIt™ Software 4.1. The results were expressed as GSH equivalents/g initial protein.

3.3.7 Cellular antioxidant activities

3.3.7.1 Cell culture

The human hepatocellular carcinoma (HepG2) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in a humidified incubator at 37 °C with 5% CO_2. The culture medium was DMEM containing 10% (v/v) heat-inactivated FBS, 1% (v/v) *L*-Gln, 1% (v/v) NEAAs, and 1% (v/v) penicillin-streptomycin (10,000 µg/mL). The growth medium was replaced by fresh media once every other day. After the HepG2 cells reached 80 – 90%

confluence in the Corning 75-cm² culture flask, the cells were trypsinized using 0.05% trypsin-EDTA.

3.3.7.2 Cytotoxicity and cytoprotection

The cell seeding density was 2×10^4 cells/well in a clear 96-well microplate**.** The cells were incubated and treated for 48 h as follows. The cell viabilities were detected by an MTT assay to evaluate the cytotoxicity and/or cytoprotection. The cytotoxicity of the digesta was detected by incubating the 24-h cultured cells with different concentrations (0 – 15 mg/mL) of the digesta for 24 h. The 50% lethality concentration of AAPH was determined for the cytoprotection assay by incubating the 36-h cultured cells with various concentrations of AAPH (0 – 60 mM) for 12 h. The cytoprotection of the digesta was measured by incubating the 24-h cultured cells with the digesta (0.1 and 2.0 mg/mL) for 12 h. Subsequently, 40 mM AAPH was used to induce cellular oxidative stress for 12 h. The non-treated cells, cells treated by AAPH only (oxidative stress control), and 0.05 mg/mL ascorbic acid positive control were also tested in the same plate as the plate containing the digesta samples. For the MTT assay, the MTT reagent prepared with a concentration of 0**.**5 mg/mL in 1×PBS was incubated with the cells for 2 h in the dark. DMSO was used to dissolve formazan, and the absorbance was detected at 570 nm by a SPECTROstar NANO system (BMG LABTECH GmbH, Ortenberg, Germany).

3.3.7.3 Intracellular ROS scavenging capacity

The intracellular ROS scavenging capacity was assessed as described by Wolfe et al., (2007) with slight modifications. The cells were seeded to achieve 6×10^4 cells/well and cultured on a sterile Corning® 96-well black polystyrene microplate with a flat clear bottom until 100% confluence was reached. The digesta and

25 μM DCFH-DA prepared in the medium without FBS were added to the microplate and incubated for 1 h. Subsequently, the medium was discarded, the cells were washed once with 1×PBS, and 600 μM AAPH in 1×PBS was applied. After 1 h of incubation, the fluorescence intensity at $\lambda_{Ex/Em}$ = 485/538 nm was read at 37 °C. The cells treated by DCFH-DA only **(**cell blank**)**, DCFH-DA followed by APPH (oxidative stress control), and 0.05 mg/mL ascorbic acid (positive control) were also studied in each plate along with the digesta samples. The results were expressed as the cellular antioxidant activity (CAA) unit and calculated according to Kellett et al., (Kellett, Greenspan, & Pegg, 2018) using the following equation (3).

$$\text{CAA unit} = \left(1 - \frac{\text{Fluorescence intensity}_{\text{sample}}}{\text{Fluorescence intensity}_{\text{oxidative stress control}}}\right) \times 100 \qquad (3.3)$$

3.3.8 Statistical analyses

All the tests were carried out in 3 replicates and expressed as the mean ± SD. Comparisons between the two means were performed using independent sample t-tests ($p = 0.05$). When there were more than two treatments, the mean differences were analyzed by ANOVA using Tukey HSD (IBM SPSS Statistics for Windows, version 23.0. Armonk, NY: IBM Corp) (p = 0.05). All the calculations, PCA, and Pearson correlation analysis were conducted with OriginPro 2018 (OriginLab Corporation, Northampton, MA, USA).

3.4 Results and discussion

3.4.1 Antioxidant activities of the enzymes used in hydrolysis and *in vitro* GI digestion

The enzyme blanks of the hydrolytic reaction (Alcalase, A_0) and gastric digestion (pepsin, *Pep*-I) displayed a negligible α-amino group content and antioxidant activities compared with those of the digesta of 10-h hydrolysate (H_{10}-$GID^{\#}$, Table 3.1). However, when pepsin and pancreatin were prepared as the enzyme blank for the GI digestion process (*Pep*-*Pan*-I), the α-amino content and antioxidant activities (TEAC and ORAC) were approximately 15 – 40% of those of H_{10}-$GID^{\#}$. These results suggested that the peptides derived from pancreatin possessed significant levels of α-amino and antioxidant activities. These peptides are probably generated by the autolysis and/or hydrolysis of the digestive enzymes presented (Dave et al., 2016; Whitcomb et al., 2007). Moreover, some peptides might have originally been present in the commercial enzymes, as seen from the MW distribution of the thermally inactivated pancreatin (Pan_0) (Table 3.2). Furthermore, A_0 could be digested to antioxidant peptides, resulting in A_0-*GID* having higher activities than *Pep*-*Pan*-I (Table 3.1). Therefore, the α-amino content and antioxidant activities of the enzyme blanks indicated that the proteases used in the hydrolysate and *in vitro* GI digestion appeared to significantly contribute to the α-amino content and antioxidant activities observed in the digesta**.** Nevertheless, most published studies disregarded or at least did not clarify the contribution of the peptides derived from the digestive enzymes. The antioxidant activities of the hydrolysates derived from the fish muscle protein, skin or surimi byproduct were increased by *in vitro* digestion (Ketnawa et al., 2016; Ketnawa et al., 2018; Samaranayaka et al., 2010; Wiriyaphan et al., 2015; Yarnpakdee, Benjakul,

Kristinsson, & Kishimura, 2015). However, no significant alteration in the antioxidant activities of casein hydrolysate were observed after digestion (Ao et al., 2013). Pancreatin digestion decreased the ORAC of the dipeptide SM, and TEAC of tripeptide NCS (Wang, Li, Wang, & Xie, 2015). Our results revealed that the enzyme blanks prepared for the standard *in vitro* GI analysis are necessary, and readings from blank should be subtracted from the sample to reflect the true antioxidant activity value of the hydrolysate/protein digesta.

Table 3.1 Alpha-amino group content and chemical antioxidant activities of enzyme blanks. [1]

Parameters \ Samples		A_0	*Pep*-I	*Pep-Pan*-I	A_0-*GID*	H_{10}-*GID*[#]
α-Amino	Free	0.02±0.00[a]	0.01±0.00[a]	1.05±0.01[b]	1.02±0.02[b]	2.69±0.09[c]
(mmol *L*-Leu)	Total	–	–	–	2.00±0.15[a]	4.73±0.12[b]
TEAC (μmol Trolox)		12.18±0.98[a]	39.02±0.00[b]	389.54±0.55[c]	487.47±0.65[d]	1004.83 ±7.35[e]
FRAP (μmol Trolox)		0.11±0.08[a]	0.03±0.00[a]	1.94±0.04[c]	1.78±0.04[b]	4.64±0.04[d]
FICC (μmol EDTA)		5.99±0.40[a]	ND	7.33±0.68[ab]	11.59±2.11[c]	8.25±0.35[b]
ORAC (μmol Trolox)		12.39±1.09[a]	0.57±0.03[a]	235.69±58.40[b]	370.63±10.35[c]	984.54±34.18[d]
$ONOO^-$ (μmol GSH)		ND	ND	0.59±0.05[a]	1.06±0.16[b]	3.95±0.19[c]

[1] The results were calculated as the sample that was brought to 25 mL.

[a-e] Different lowercase letters in the same row indicated the differences in mean values ($p < 0.05$).

[#] Without enzyme blank subtraction.

'–', the values were not determined; ND, the values were not detected; A_0, thermally-inactivated Alcalase; *Pep*-I, blank of gastric (pepsin) digestion; *Pep-Pan*-I, blank of GI (pepsin and pancreatin) digestion; A_0-*GID*, GI digesta of A_0; H_{10}-*GID*, GI digesta of 10-h hydrolysate.

Table 3.2 MW distribution of protein hydrolysates, digesta and enzyme blanks. [1]

Sample	Percentage of area under chromatogram			
	> 1,250 Da	1,250 – 330 Da	330 – 220 Da	< 220 Da
Protein hydrolysates				
H_2	12.29±1.31^{c}	77.49±1.67^{b}	10.00±0.36^{a}	0.22±0.12^{a}
H_6	7.60±0.68^{b}	75.64±1.88^{ab}	15.59±0.57^{b}	1.17±0.76^{ab}
H_{10}	4.46±0.30^{a}	75.55±0.92^{ab}	18.73±0.70^{c}	1.25±0.39^{ab}
H_{16}	3.61±0.54^{a}	73.52±0.34^{a}	21.02±0.75^{d}	1.85±0.15^{b}
	Digesta of protein hydrolysates			
P-GID	5.79±0.30^{A}	37.50±0.27^{BCD}	35.27±0.37^{BC}	21.44±0.36^{D}
H_2-*GID*	6.13±0.11^{A}	37.74±0.38^{CD}	35.70±0.32^{BC}	20.43±0.55^{CD}
H_6-*GID*	5.71±0.21^{A}	38.77±0.61^{D}	36.24±0.16^{BC}	19.28±0.27^{ABC}
H_{10}-*GID*	5.76±0.24^{A}	37.59±0.16^{BCD}	36.61±0.30^{C}	20.04±0.27^{BC}
H_{16}-*GID*	6.07±0.45^{A}	37.52±0.26^{BCD}	36.59±0.46^{C}	19.82±0.24^{BC}
Enzyme blanks				
Pan_0	16.47±0.34^{D}	36.57±0.49^{BC}	28.90±0.96^{A}	18.06±0.69^{A}
A_0-Pep_0-Pan_0	14.96±1.08^{C}	36.32±0.11^{B}	29.83±1.40^{A}	18.90±0.42^{AB}
A_0-*GID*	8.99±0.43^{B}	26.87±0.74^{A}	34.57±0.64^{B}	29.56±0.71^{E}

[1] MW at elution volume (V_e) 13.20, 18.10 and 20.77 mL were calculated by formula $\text{LogMW} = -0.649\ln(K_{av}) + 2.1656$ $R^2 = 0.988$, respectively, and used to define the MW distribution of peptides.

[a-d] Different lowercase letters indicate differences in mean values among protein hydrolysates ($p < 0.05$).

[A-D] Different uppercase letters indicate differences in mean values among digesta and enzyme blanks ($p < 0.05$).

A_0, Pep_0, and Pan_0 were referred to as thermally-inactivated Alcalase, pepsin and pancreatin, respectively; A_0-Pep_0-Pan_0, the mixture of A_0, Pep_0 and Pan_0; A_0-*GID*, digesta of A_0.

3.4.2 DH and MW distribution

The crude protein and fat contents of *P* were 83.87 ± 0.29% (d.b.) and 1.91 ± 0.33% (d.b.), respectively. The DH of the *P* hydrolysate increased rapidly within 6 h and then increased slowly afterward until reaching 35.36 ± 0.06% DH at 16 h (Figure 3.1A). Similar profile has been reported by others (Ketnawa et al., 2016; Raghavan et al., 2008). All the hydrolysates showed a similar pattern in *SEC*. Majority of peptides (~75%) in all samples exhibited apparent MW values ranging from 330 – 1250 Da (Table 3.2). This was the net result of the products from hydrolysis of large peptides and the conversion to smaller peptides. As the hydrolysis process was prolonged, the proportion of peptides having an MW < 330 Da increased in concomitant with a decrease of large peptides (MW > 1250). The smaller peptides were generated as DH increased during hydrolysis.

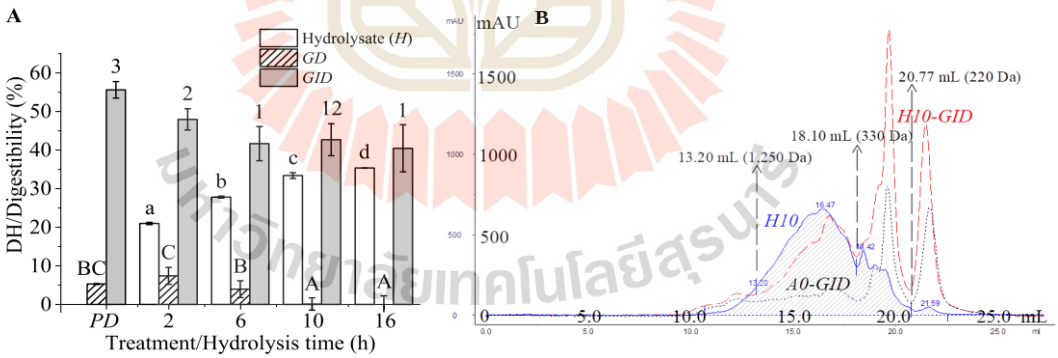

Figure 3.1 DH or digestibility of the protein or its hydrolysates (A) and size exclusion chromatograms (*SEC*s) of the enzyme blank, which was the digesta of thermally inactivated Alcalase (A_0-*GID*), 10-hydrolysate (H_{10}), and its digesta (H_{10}-*GID*) (B).

PD, protein digesta; *GD*, gastric digesta; *GID*s, GI digesta. The different lowercase and uppercase letters and numbers indicated the differences in the mean values of the hydrolysate, *GD*s, and *GID*s, respectively ($p < 0.05$). Elution volume (V_e) at 13.20, 18.10 and 20.77 mL was used to define the M_W distribution of the peptides, as detailed in Table 3.2.

The protein and its hydrolysates were digested slightly by pepsin but significantly degraded by pancreatin (Figure 3.1A). Hydrolysates from threadfin bream (*Nemipterus spp*.) surimi byproduct (Wiriyaphan et al., 2015) and trout frame (Ketnawa et al., 2018) were also largely digested by pancreatin, but slightly by pepsin. Pepsin has a high specificity towards aromatic or Leu residues in both P_1 and P_1' (Keil, 1992), resulting in a relatively low degree of digestion. In contrast, pancreatin is a mixture of endopeptidases and exopeptidases, which possess a broad specificity (Whitcomb et al., 2007). The protein showed the highest digestibility ($p < 0.05$, figure 3.1A), while the hydrolysates showed a lower digestibility; in particular, the hydrolysates hydrolyzed for the extended hydrolysis time (> 6 h) exhibited the lowest digestibility ($p > 0.05$, Figure 3.1A). Larger peptides were digested easily by the digestive enzymes, but shorter peptides are digested to a lesser extent due to the limited peptide bonds. All the digesta exhibited similar *SEC* patterns (Figure 3.1B) and MW distributions ($p > 0.05$, Table 3.2). Approximately 55% of the peptides in the digesta showed an apparent MW < 330 Da. Therefore, the protein and hydrolysates were digested to yield similar sizes of peptides in their digesta, and a majority of peptides exhibiting apparent MW < 330 Da. Thus, protein and its hydrolysate would eventually yield peptides with similar sizes upon GI digestion.

3.4.3 Chemical antioxidant activities

The tilapia protein hydrolysates and their digesta exhibited various chemical antioxidant potentials (Figure 3.2A – E). High antioxidant values were observed for the TEAC and ORAC compared with those of the FRAP and $ONOO^-$ scavenging capacity. The tilapia protein hydrolysates hydrolyzed by Flavourzyme, Protamex and papain (Yarnpakdee, Benjakul, Kristinsson, & Kishimura, 2015), as well as hydrolysates derived from fish skin gelatin and surimi byproduct (Ketnawa, Benjakul, Martínez-Alvarez, & Rawdkuen, 2017; Wiriyaphan et al., 2015), also exhibited a FRAP value that was lower than the TEAC value. The ORAC assay monitors the peroxyl radical (ROO•) scavenging activity via the hydrogen atom transfer (HAT) mechanism (Apak, Özyürek, Güçlü, & Çapanoğlu, 2016). The FRAP and $ONOO^-$ scavenging assays are related to the electron transfer (ET) mechanism (Apak et al., 2016; Kooy et al., 1994). The TEAC is a mixed-mode (ET/HAT) assay (Apak et al., 2016). Therefore, the high TEAC and ORAC in the tilapia protein hydrolysate and digesta indicated that HAT might be the predominant mechanism responsible for the ability of tilapia protein-derived peptides to scavenge free radicals. The majority of amino acid residues are protonated at pH values of 3.6 (FRAP assay) and 7.4 (TEAC, ORAC and $ONOO^-$ assays). These protonated residues have the potential to donate a hydrogen atom and transfer the excess electron (Aluko, 2015). Therefore, it is likely that HAT contributed to the free radical scavenging capacity of the peptides.

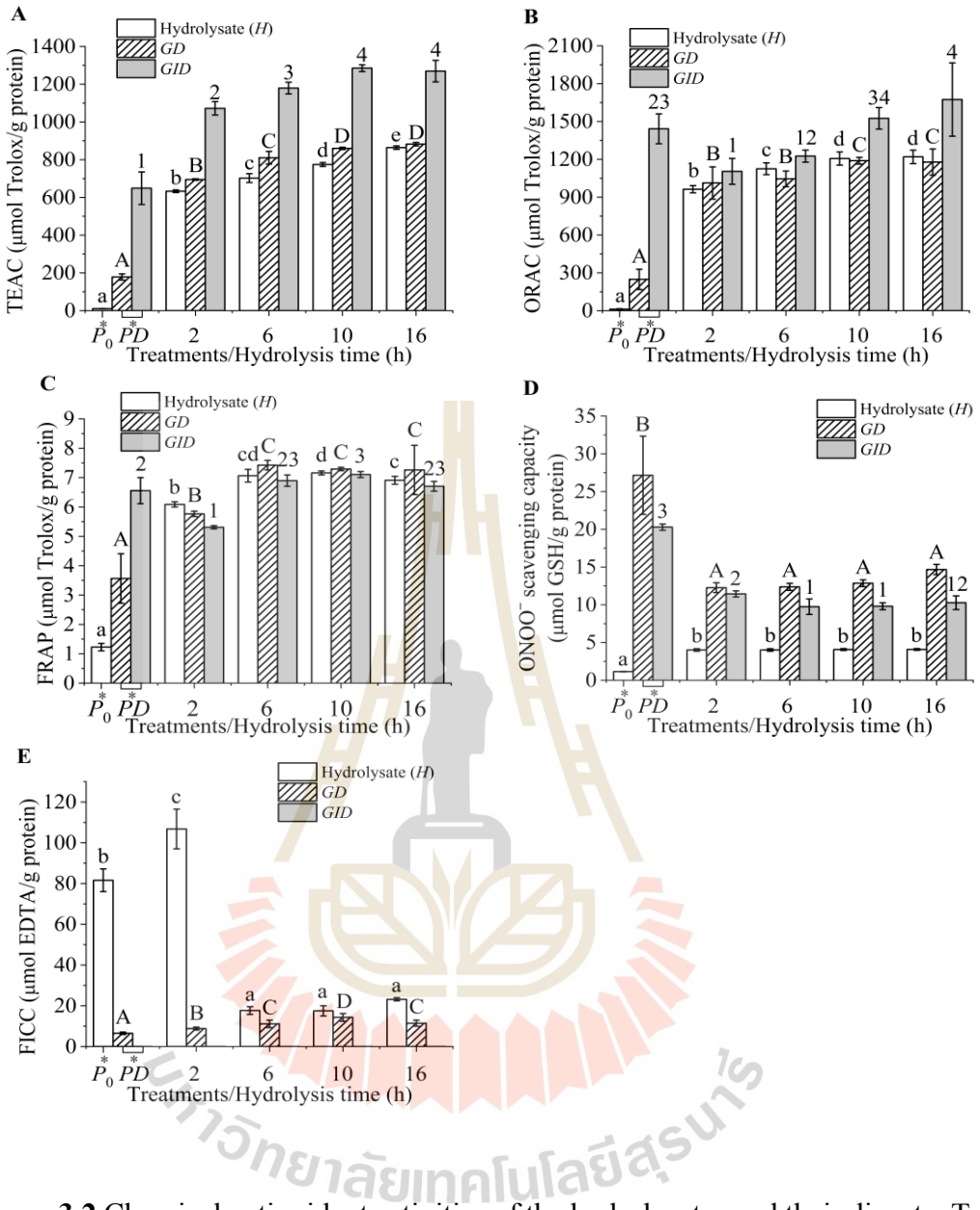

Figure 3.2 Chemical antioxidant activities of the hydrolysates and their digesta, Trolox equivalent antioxidant capacity (TEAC) (A), oxygen radical absorbance capacity (ORAC) (B), Fe^{2+} reducing antioxidant power (FRAP) (C), peroxynitrite $ONOO^-$ scavenging capacity (D), and FICC (E).

P_0, heated protein; *PD*, protein digesta; *GD*, gastric digesta; *GID*, GI digesta**.** The different lowercase and uppercase letters and numbers indicated the

differences in the mean values of the hydrolysate, *GD*s, and *GID*s, respectively ($p < 0.05$).

In addition, thiol containing compounds such as Cys, GSH and Cys-containing tripeptides were the effective $ONOO^-$ scavengers (Balavoine & Geletii, 1999; Kooy et al., 1994; Saito et al., 2003), but tripeptides containing His and Tyr showed a weak $ONOO^-$ scavenging activity (Saito et al., 2003). Meanwhile, a lack of Cys (0.95% or less) has been reported in tilapia proteins (Adeyeye, 2009; Yarnpakdee, Benjakul, Kristinsson, & Kishimura, 2015). Therefore, the weak $ONOO^-$ scavenging ability of tilapia protein hydrolysates could also be attributed to a lack of Cys (< 30 μmol GSH equivalents/g protein).

The H_2 exhibiting a DH of 20.97% showed a Fe^{2+} chelating capacity (FICC) of 106.77 μmol EDTA equivalents/g protein, but low values (< 30 μmol) were observed for the hydrolysates at extended hydrolysis times (> 2 h) and all the digesta (Figure 3.2E). The tilapia protein hydrolysates with DH values ranging from 10% to 40% also showed a negligible FICC (< 3 μmol EDTA equivalents/g solid) (Yarnpakdee, Benjakul, Kristinsson, & Kishimura, 2015). The gelatin hydrolysates obtained from farmed Giant catfish skin (~10% DH) exhibited approximately 170 μmol EDTA equivalents/g sample (Ketnawa et al., 2016). Hydrolysates with higher DH values and their digesta did not possess a good metal chelating capacity because they contained shorter peptides with few negatively charged amino acid residues. Therefore, our results implied that HAT might be a predominant mechanism of peptides in hydrolysates and their digesta, which contributed to the antioxidant activity, while electron transfer and metal chelating were less important. The TEAC and ORAC appeared to be important

assays for monitoring the antioxidant activities of the peptides derived from tilapia protein.

The TEAC and ORAC of the hydrolysate increased with increasing the hydrolysis time (Figure 2A – B). In addition, these values drastically increased after pancreatin digestion ($p < 0.05$). Smaller peptides possessed a better free radical scavenging capacity, and similar results have also been reported by others (Nwachukwu et al., 2019; Raghavan et al., 2008). The shorter peptide is preferred to react with water-soluble $ABTS^{\bullet+}$ and ROO• radicals, leading to higher TEAC and ORAC values, respectively (You, Zhao, Regenstein, & Ren, 2010). The lower scavenging power of longer peptides is attributed to an increased repulsion of bulky peptides (Nwachukwu et al., 2019). Moreover, amino acid residues with an antioxidative potential might be hidden inside steric structures as inactive forms. These hidden amino acid residues are exposed during hydrolysis, leading to an improvement in the activity (Mirzaei, Aminlari, & Hosseini, 2016). Thus, the TEAC of the hydrolysate increased continually and reached the highest in the H_{16} sample and further increased after GI digestion. After all the active residues were exposed upon proteolysis, the antioxidant activities became constant, as observed in the digesta of H_{10} and H_{16}. It should be noted that the activities of the H_{10} and H_{16} digesta were comparable, although the parent hydrolysates of the latter showed higher activities ($p < 0.05$). Therefore, it might not be necessary to search for the hydrolysate with the highest antioxidant activity, but rather the activities of the digesta should be considered when optimizing the production of antioxidative hydrolysates. In addition, the protein digesta showed lower TEAC and ORAC values than did the hydrolysate digesta (Figure 3.2A-B). Since both digesta appeared to have

similar sizes of peptides (Table 3.1), such differences in antioxidant activity could be mainly attributed from varied peptide sequences.

Pearson correlation analysis showed that the TEAC and ORAC of the hydrolysate were significantly correlated with the DH ($r = 0.95$ and 0.91, respectively). In addition, the TEAC and ORAC of the digesta were less correlated with the values obtained from the hydrolysates ($r = 0.80 – 0.85$). Our results indicated that the chemical antioxidant activities of the hydrolysate could not be used to predict the activities of its digesta. This is likely due to the differences in the size and sequence of antioxidant peptides of the hydrolysates and their digesta. Hydrolysis time of 10 h (H_{10}) rather than 16 h (H_{16}) would be the optimal as it yielded hydrolysate with the highest antioxidant activity upon GI digestion) Thus, the activity of the hydrolysate digesta should be considered as one of the criteria to optimize production of antioxidative protein hydrolysate.

3.4.4 Cellular antioxidant activities

All the digesta were noncytotoxic up to 5 mg/mL and exhibited a dose-dependent cytoprotection behavior and intracellular ROS scavenging activity, except for the protein digesta (*PD*), whose CAA value decreased as the concentration increased (Figure 3A – B). All the digesta with concentrations of 0.1 mg/mL exhibited a cytoprotection capability that was comparable to that of 0.05 mg/mL ascorbic acid ($p > 0.05$), while the digesta of H_2 was the most effective ($p < 0.05$, Figure **3**.3A). However, the cytoprotection of all the digesta with concentrations of 2.0 mg/mL was comparable ($p > 0.05$). Based on the ROS scavenging activity, the digesta of H_{10} with a 0.5 mg/mL concentration revealed he highest activity comparable to the 0.05 mg/mL ascorbic acid ($p > 0.05$). In addition, the digesta of H_{10} showed the highest CAA unit at a

concentration of 5 mg/mL (Figure **3**.3B). Note that the protein digesta showed the lowest CAA unit ($p < 0.05$). These results indicated that the digesta of the protein and its hydrolysates possessed similar cytoprotective capacities, but the H_{10} digesta was the most effective ROS scavenger. It can be seen that the cytoprotective results did not agree with the intracellular ROS scavenging activity. This discrepancy has been previously reported (Du, Esfandi, Willmore, & Tsopmo, 2016; Yarnpakdee, Benjakul, Kristinsson, & Bakken, 2015). The intracellular ROS scavenging capacity assay monitors the ability of the compound to remove intracellular ROS, while cytoprotection measured the cell viability, which is related to the ROS-induced oxidative stress that causes membrane damage and biomolecular dysfunction, as well as the mitochondrial dysfunction and/or DNA damage that causes apoptosis (Du et al., 2016), and so on. Therefore, the same peptides might display various cytoprotection and ROS scavenging activity responses. The low ROS scavenging activity of the protein digesta, corresponding to the lowest TEAC and ORAC (Figure 3.2A – B). These results implied that protein digesta contained peptides with lower cellular antioxidant activity than did hydrolysate digesta. Different peptide sequences in the protein and hydrolysate digesta should be the main reasons contributing to the varied cellular antioxidant activities. Our results demonstrated that production of protein hydrolysate is an effective means to improve the antioxidant activity of tilapia proteins, but excessive hydrolysis (16 h) resulted in a reduction of CAA of hydrolysate digesta.

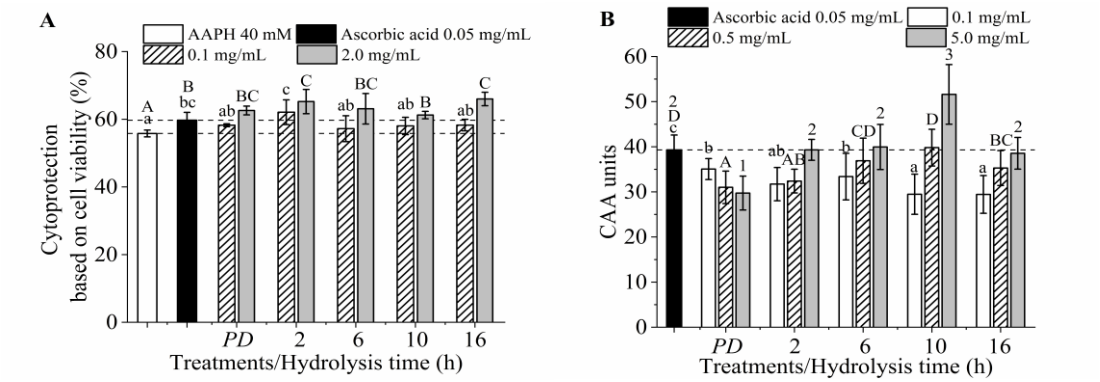

Figure 3.3 Cytoprotection at 0.1 – 2.0 mg/mL (A) and intracellular ROS scavenging capacity at 0.1 – 5.0 mg/mL (B) for all the digesta.

PD, protein digesta. The different lowercase and uppercase letters and numbers indicated the differences in the mean values of the digesta at each concentration ($p < 0.05$). AAPH, 2,2′-Azobis (2-methylpropionamidine) dihydrochloride.

3.4.5 PCA

According to the chemical and cellular antioxidant activities of the digesta of the protein and its hydrolysates, almost 66% of the total variance can be explained by PC1 and PC2 (Figure **3**.4). The protein digesta was clearly separated from the hydrolysate digesta with a high correlation to the $ONOO^-$ scavenging activity. The digesta of H_{10} exhibited distinct characteristics of the TEAC and CAA (at 0.5 and 5 mg/mL). The tilapia protein hydrolyzed by Alcalase for 10 h appeared to show the optimal antioxidant activities upon *in vitro* GI digestion.

The loadings of the variables showed that PC1 was positively correlated with the TEAC and CAA (0.5 and 5.0 mg/mL) of the digesta. PC2 was positively correlated with the cytoprotection (at 0.1 and 2 mg/mL) but negatively correlated with the ORAC, FRAP and α-amino group content of the digesta. The Pearson correlation showed that the TEAC of the digesta was positively correlated with its CAA at both 0.5 and 5.0 mg/mL, where $r = 0.70$ and 0.74, respectively. It was suggested that the TEAC could be used to predict the intracellular ROS scavenging capacity of peptides in the digesta. In contrast, the TEAC of the hydrolysates showed a lower Pearson correlation with the CAA (0.5 and 5.0 mg/mL) of the digesta, where $r = 0.62$ and 0.70, respectively. The chemical antioxidant activities of the hydrolysates also showed a weak correlation with those of their respective digesta, as discussed in section 3.3. Therefore, TEAC of hydrolysate digesta showing the highest correlation with the CAA of digesta could be used to screen the antioxidant peptides derived from tilapia muscle proteins. Protein digesta was distinctively partitioned from the hydrolysate digesta with lower values of TEAC and CAA. Digesta of H_{10} showed high correlation with TEAC and CAA. Thus, the excessive hydrolysis (16 h) generated hydrolysate showing the highest antioxidant potential but did not yield the maximum capacity upon digestion. As activities of digesta are more closely related to health, the static *in vitro* GI digestion of hydrolysate should be used as one of the criteria for optimizing production of antioxidative protein hydrolysate.

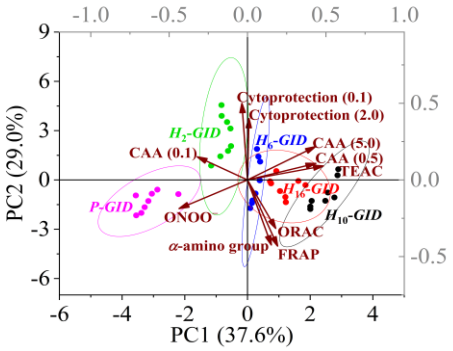

Figure 3.4 PCA biplots (PC1 *versus* PC2) of the digesta of tilapia protein and its hydrolysates based on their α-amino group content and chemical and cellular antioxidant activities.

Cytoprotection (0.1 or 2.0) and CAA (0.1, 0.5 or 5.0) indicated the activities of the digesta at concentrations specified in parentheses in mg/mL.

3.5 Conclusions

The antioxidant activities of peptides derived from proteases used in the standard INFOGEST *in vitro* GI digestion should be systematically analyzed and subtracted from those of samples to better evaluate the true activities of the digesta. The antioxidant activities of the hydrolysate did not appear to reflect the activities of the digesta. The digesta of H_{16} exhibited lower antioxidant activities than did the H_{10} digesta although the hydrolysate of the former possessed higher activities. The protein digesta also showed the least antioxidant activities. Our results demonstrated that excessive hydrolysis of 16 h might not necessarily yield peptides with the highest activities upon *in vitro* GI digestion. The antioxidant activity of the hydrolysate digesta should be considered when optimizing the condition used for production of the protein hydrolysate with an antioxidant activity.

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CHAPTER IV
CHARACTERISTICS AND ANTIOXIDANT ACTIVITIES OF GLYCATED TILAPIA PROTEIN HYDROLYSATES UPON *IN VITRO* GASTROINTESTINAL DIGESTION

4.1 Abstract

Glycation, or called Maillard browning reaction has been shown to improve chemical antioxidant activity of food-derived peptides, while its effect on cellular antioxidant activity (CAA) remains unclear. Moreover, the effect of gastrointestinal (GI) digestion on antioxidant activity of glycated peptides is rarely elucidated. This study investigated the changes of antioxidant activity of tilapia hydrolysates upon glycation and following *in vitro* GI digestion. The glycation of hydrolysate was carried out at 90 °C within 12 h. Larger molecular weight melanoidins increased with the extended reaction time. The length of peptides presented in the hydrolysate prepared at 2 and 10 h had slight effect on the glycation of hydrolysates toward each sugar. Glycation drastically increased 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$) and peroxynitrite ($ONOO^-$) scavenging activities ($p < 0.05$), as well as ferric reducing antioxidant power (FRAP). Compared to glucose and fructose, xylose was the most effective sugar, yielding the highest chemical antioxidant activities ($p < 0.05$). However, the highest degree of reaction

exhibited the lowest intracellular ROS scavenging capacity in HepG2 cell model. The extended reaction of hydrolysates resulted in lower digestibility. GI digestion reduced the FRAP of glycated hydrolysates, and all digesta were less effective in CAA than that of hydrolysate counterpart. Glycation of protein hydrolysate only improved chemical antioxidant activities but decreased biological antioxidant activity.

Keywords: Maillard reaction products (MRPs), gastrointestinal (GI) digestion, reducing power, molecular weight (MW), cellular antioxidant activity (CAA)

4.2 Introduction

Food protein-derived antioxidant peptides possess the potential to retard food oxidation and mitigate oxidative damage in biological system (Nwachukwu & Aluko, 2019; Sohaib et al., 2017). However, the applications of peptides as antioxidant for foods or nutraceuticals are still limited (Bhandari, Rafiq, Gat, Gat, Waghmare, & Kumar, 2020; Sohaib et al., 2017). One of the reasons would be the lower antioxidant activity of protein hydrolysates compared to common antioxidants such as ascorbic acid or tocopherol. According to the chemical antioxidant activities such as 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•), hydroxyl radical (•OH), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$) scavenging capacities, the 50% effective concentration (EC_{50}) of peptides or protein hydrolysates ranged of 20 μg/mL ~ 10 mg/mL (Liu, Xing, Fu, Zhou, & Zhang, 2016; Wiriyaphan, Xiao, Decker, & Yongsawatdigul, 2015; Yang, Zhang, Ding, Chi, Wang, & Huo, 2019; Yu, He, Tang, Zhang, Zhu, & Sun, 2018). Nevertheless, trolox, quercetin, **(+)**-catechin, and *L*-ascorbic acid showed EC_{50} values lower than 5 μg/mL for DPPH•

or $ABTS^{\bullet+}$ scavenging assays (Z. Chen, Bertin, & Froldi, 2013; Faitanin et al., 2018). Additionally, antioxidant peptides/hydrolysates have also shown lower intracellular reactive oxygen species (ROS) scavenging capacity than that of *α*-tocopherol, trolox, and ascorbic acid (Wiriyaphan, Chitsomboon, & Yongsawadigul, 2012; Wiriyaphan et al., 2015; Yarnpakdee, Benjakul, Kristinsson, & Bakken, 2015). Therefore, the improvement of antioxidant activity of peptides/protein hydrolysates should be sought to increase its utilization.

Maillard reaction between primary amino and active carbonyl groups is initiated spontaneously during thermal processing and food storage. Numerous studies indicated that Maillard browning reaction improved the chemical antioxidant activity of proteins, peptides, and amino acids, such as reducing power, DPPH•, $ATBS^{\bullet+}$, and peroxyl radical (ROO•) scavenging capacities (Nooshkam, Varidi, & Bashash, 2019). Maillard reaction products (MRPs) has also extended food shelf-life and inhibited lipid oxidation (Nooshkam et al., 2019; Sohaib et al., 2017). While a few study has reported that MRPs promoted the cellular antioxidant activity (CAA) to a greater extent than that of the original hydrolysates (Han et al., 2018; Zha, Wei, Chen, Dong, Zeng, & Liu, 2015), and most reports have been frequently investigated MRPs alone, without comparing with the original hydrolysates. MRPs from the unicorn leatherjacket skin hydrolysate-galactose system were effective to mitigate the H_2O_2-induced oxidative stress in human histiocytic lymphoma cells (U937 cells) and DNA damage (Karnjanapratum, O'Callaghan, Benjakul, & O'Brien, 2016). MRPs induced from soybean peptides, *L*-cysteine and xylose have also retarded galactose induced aging in institute of cancer research (ICR) mice by increasing in the activities of antioxidant enzymes and total antioxidant activities of serum (Shudong He et al., 2019). Nevertheless, some reports

showed that MRPs caused oxidative stress in both diabetic mice (Yao, Han, Dong, Zeng, & Liu, 2016) and intestinal inflammation in early life in rats (Hillman et al., 2019). Therefore, glycation is likely an effective strategy to improve the chemical antioxidant activity of peptides/hydrolysate, but the efficiency to improve the antioxidant activity of such compounds in biological system is still rarely elucidated and inconclusive. The glycation of hydrolysate enhancing in both chemical and cellular antioxidant needs to be systematically evaluated.

The effect of gastrointestinal digestion on antioxidant activity of MRPs appeared to vary with samples (Q. Zhang, Wang, & Fu, 2020; D. Zhao et al., 2019). The antioxidant activities (DPPH• and/or $ABTS^{\bullet+}$ scavenging capacity, and reducing power) of MRPs generated from the grass carp scale hydrolysate-ribose and unicorn leatherjacket skin gelatin hydrolysate-galactose systems decreased after GI digestion (K. Chen et al., 2019; Karnjanapratum et al., 2016). In contrast, the $ABTS^{\bullet+}$ and •OH scavenging capacities of MRPs from the snapper fish scale peptides-xylose were not changed upon the GI digestion (X. Chen, Fang, & Wang, 2020). The suppression of oxidative stress of MRPs on alcohol-induced liver damage in mice has been evaluated (X. Chen et al., 2020), but the effect of GI-digested MRPs has not been throughly studied. Therefore, MRPs might be degraded upon GI digestion by protease leading to its structural modification resulting in change of antioxidant capacity of glycated hydrolysate. The antioxidant activity of digesta of glycated hydrolysates should be evaluated to reflect the more biological relevant properties. While the changes in both chemical and cellular antioxidant activity of glycated hydrolysate upon GI digestion is rarely investigated.

In this study, tilapia protein hydrolysates with various degree of hydrolysis (DH) values were reacted with different sugars, namely glucose, fructose, or xylose. The reactivity of each reaction, and spectroscopic properties as early colorless and browning MRPs were characterized upon glycation and following GI digestion, as well as the changes in MW, chemical and cellular antioxidant activities.

4.3 Materials and methods

Nitro blue tetrazolium chloride (NBT), quinine hemisulfate salt monohydrate (99.0-101.0%), and 3,5-Dinitrosalicylic acid (DNS, 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Preparation of tilapia muscle protein powder and its hydrolysates was followed X. Zhang, Noisa, and Yongsawatdigul (2020). Defatted tilapia protein powder was added 0.15 M $NaHCO_3$ to contain 10% crud protein (w/v) and pH was adjusted to pH 8.0 and hydrolyzed by 5% Alcalase (based on crude protein, w/w) at 50 °C for 2 and 10 h. After centrifugation and filtration, supernatants were collected and stored at – 80 °C. The 2- and 10-h hydrolysates were referred to as H_2 and H_{10}, respectively.

4.3.1 Glycation of hydrolysates

For glycation, a 150 mM of each reactant, namely sugars (glucose, fructose or xylose) and hydrolysates (H_2 or H_{10}, based on α-amino group content, *L*-Leu equivalents) were mixed in a total volume of 3 mL. The reactions were carried out in a 20-mL Pyrex screw cap tube and placed in a 90 °C water-bath for 0, 2, 4, 6, 8, 10 and 12 h, and subsequently cooled down in ice-water. Final volume of glycated hydrolysate was brought to 5 mL with DI and stored at – 80 °C for further analyses. The glycated hydrolysate was named as S_tH_n. Where, the *S* stands for sugars including

G = glucose, *F* = fructose, and *X* = xylose; the t was the glycation time as 0, 2, 4, 6, 8, 10, and 12 h; H_n was the 2- or 10-h hydrolysate. For example, G_2H_2 was the glycated H_2 with glucose for 2 h. The G_0H_2 represents the mixture of H_2 and glucose without glycation.

4.3.2 Simulated *in vitro* GI digestion

The simulated *in vitro* GI digestion was conducted according to the INFOGEST static method with slight modifications as described by X. Zhang et al. (2020). In the oral phase, *α*-amylase and lipase were ignored as the glycated tilapia protein hydrolysates contained mainly monosaccharide, peptides and MRPs. The *GID* following the sample was used to indicate the GI digesta of samples. The enzyme blank (E_0) during GI digestion was prepared without samples. All results were reported after subtraction of the corresponding enzyme blank. Digestibility of hydrolysate was calculated as described by X. Zhang et al. (2020). Digestibility of glycated hydrolysate was calculated as equation:

$$\text{Digestibility (\%)} = \left[\frac{L_{ad,\,g} - L_{bd,\,g}}{L_{total,h} - (L_{bd,\,h} - L_{bd,\,g}) - L_{bd,\,g}}\right] \times 100 = \frac{L_{ad,\,g} - L_{bd,\,g}}{L_{total,h} - L_{bd,\,h}} \times 100$$

where, $L_{bd,\,g}$ and $L_{ad,\,g}$ were the *α*-amino group content of glycated hydrolysate before and after *in vitro* digestion, respectively. $L_{total,h}$ was total *α*-amino group content of the respective hydrolysate. $L_{bd,\,h}$ was the *α*-amino group content of respective hydrolysate before *in vitro* digestion. The results were calculated after the subtraction of the respective enzyme blanks.

4.3.3 Alpha-amino group content

Alpha-amino group content of samples were measured using 2,4,6-trinitrobenzenesulfonic acid solution (TNBS) assay modified from Adler-Nissen (1979) as described by X. Zhang et al. (2020). Absorbance was measured at 420 nm by GENESYS™ 10S UV-Vis Spectrophotometer (Thermo Scientific™, Rochester, New York, USA). *L*-Leu was used as a standard. Results were expressed as *L*-Leu equivalents.

4.3.4 Reducing sugar

Reducing sugar was detected by DNS method (Miller, 1959) with slight modifications. DNS reagent was prepared to contain 0.4 M NaOH, 1.06 M sodium potassium tartrate and 48 mM DNS. A 200 µL of diluted sample and DNS reagent (1:1) were incubated at 95 °C in a water-bath for 15 min, and then cooled down in ice-water. The mixture was then diluted by 1000 µL of DI water and the absorbance at 540 nm was measured. The corresponding sugars including glucose, fructose or xylose was employed as the standard for the different glycation systems. Residual concentration of each sugar was reported.

4.3.5 Characteristic of glycated hydrolysates

4.3.5.1 Fructosamine

Fructosamine (1-amino-1-deoxy-D-fructose) content was monitored by NBT assay (Martinez-Saez, Fernandez-Gomez, Cai, Uribarri, & del Castillo, 2017) with slight modifications. NBT at 0.23 mM was prepared weekly in 100 mM sodium carbonate buffer (pH 10.8) and stored at 4 °C. All samples were diluted 20 times, and then 50 µL of each sample was incubated with 950 µL of NBT at 37 °C for 20 min. DI water was used as a blank. All results were reported as the absorbance at 530 nm.

4.3.5.2 Absorbances at 294 and 420 nm

All samples were diluted for 100 or 10 times for the measurement of absorbance at 294 and 420 nm to monitor the colorless and browning MRPs, respectively.

4.3.5.3 Fluorescent intensity

The glycated hydrolysates and their digesta were diluted for 600 and 200 times, respectively, to avoid the fluorescent quenching effects and nonlinear responses. Fluorescence was measured using a JASCO FP-8300 spectrofluorometer (Tokyo, Japan) at $\lambda_{Ex/Em}$ = 347/415 nm with 5 nm bandwidth (Morales & Jiménez-Pérez, 2001). Quinine hemisulfate salt, which was dissolved in 0.1 M H_2SO_4, was used as a standard, and the results were expressed as quinine hemisulfate equivalents (μM).

4.3.5.4 Size exclusion chromatography (*SEC*)

Molecular weight (MW) distribution was detected as described by X. Zhang et al. (2020) with slight modifications. A Superdex Peptide 10/300 GL column was equipped with fast protein liquid chromatography (FPLC; ÄKTA pure 25, Version 1.8.0.6, GE Healthcare Bioscience Co., Uppsala, Sweden). A 100 μL of diluted sample was loaded onto the column. The eluted compounds were monitored at 214, 294, and 420 nm.

4.3.6 Chemical antioxidant activities

4.3.6.1 $ABTS^{\bullet+}$ scavenging capacity

$ABTS^{\bullet+}$ scavenging capacity was conducted based on Re, Pellegrini, Proteggente, Pannala, Yang, and Rice-Evans (1999) with slight modifications X. Zhang et al. (2020). Absorbance of $ABTS^{\bullet+}$ working solution was adjusted to 0.7 ± 0.01 at 734 nm. All samples were diluted for 300 times, and a 50 μL

of each sample was incubated with 950 μL of $ABTS^{\bullet+}$ working solution for 15 min. Absorbance was measured within 30 min. DI water was used as a blank. The $ABTS^{\bullet+}$ scavenging capacity was calculated as the following equation:

$$ABTS^{\bullet+}\text{scavenging capacity (\%)} = \frac{A_{blank}-A_{sample}}{A_{blank}} \times 100\%$$

Where, A_{blank} and A_{sample} were referred to as the absorbance of blank and sample, respectively.

4.3.6.2 Ferric reducing antioxidant power (FRAP)

FRAP assay was modified from Benzie and Strain (1996) as shown in X. Zhang et al. (2020). All samples were diluted for 100 times, and a 100 μL of each sample was incubated with 1000 μL of FRAP reagent at 37 °C for 10 min in the dark, and the absorbance was measured at 593 nm. DI water was used as the blank.

4.3.6.3 Peroxynitrite ($ONOO^-$) scavenging capacity

Peroxynitrite scavenging capacity was conducted according to Kooy, Royall, Ischiropoulos, and Beckman (1994) as described by X. Zhang et al. (2020). Results were expressed as glutathione equivalents (μmol GSH) based on the final volume of glycation (5 mL) or the *in vitro* GI digestion (25 mL).

4.3.7 Cellular antioxidant activity

Intracellular ROS scavenging capacity was evaluated according to Wolfe and Liu (2007) with slight modification as described by X. Zhang et al. (2020). Oxidative stress in cells induced by 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), while cells treated by the sample only were used as a control.

4.3.8 Statistical analyses

All tests were carried out in 3 independent replicates and expressed as the mean ± SD**.** Comparison between two means was performed using independent sample t-tests (p = 0.05). When there were more than two treatments, the mean differences were analyzed by ANOVA using Tukey HSD (IBM SPSS Statistics for Windows, version 23.0. Armonk, NY: IBM Corp) (p = 0.05).

4.4 Results and discussion

4.4.1 Characteristic of glycated hydrolysates

Concentration of α-amino groups and reducing sugars were reduced along with glycation time (p < 0.05, Figure 4.1A-B). While the early and advanced MRPs were increased (p < 0.05, Figure 4.1D-F) except for fructosamine which decreased in X_tH_2 and X_tH_{10}, when the reaction time was greater than 2 h (Figure 4.1C). When H_2 and H_{10} were glycated with the same sugar, the S_tH_2 and S_tH_{10} showed the same trends and approximate levels on consuming in reactants (Figure 4.1A-B) and accumulation of MRPs (Figure 4.1C-F). It is suggested that the peptide size between H_2 and H_{10} was not the main factor affecting on their glycation. When peanut hydrolysate was fractionated by 1000, 3000, 5000 and 10000 Da of MW cut-off membranes, the smaller MW fraction preferably reacted with glucose compared to the larger ones (Su, Zheng, Cui, Yang, Ren, & Zhao, 2011). Fraction with MW < 3000 Da from chicken bone protein hydrolysate showed higher reactivity towards galactose than that with MW > 3000 Da (Nie, Xu, Zhao, & Meng, 2017). In this study, the larger peptides were available in H_2 compared to H_{10} (Figure 4.2A-B), while the majority of peptides (> 85%) in both H_2 and H_{10} were lower than 1000 Da (Figure 4.2A), which could partly explain similar results observed in both samples.

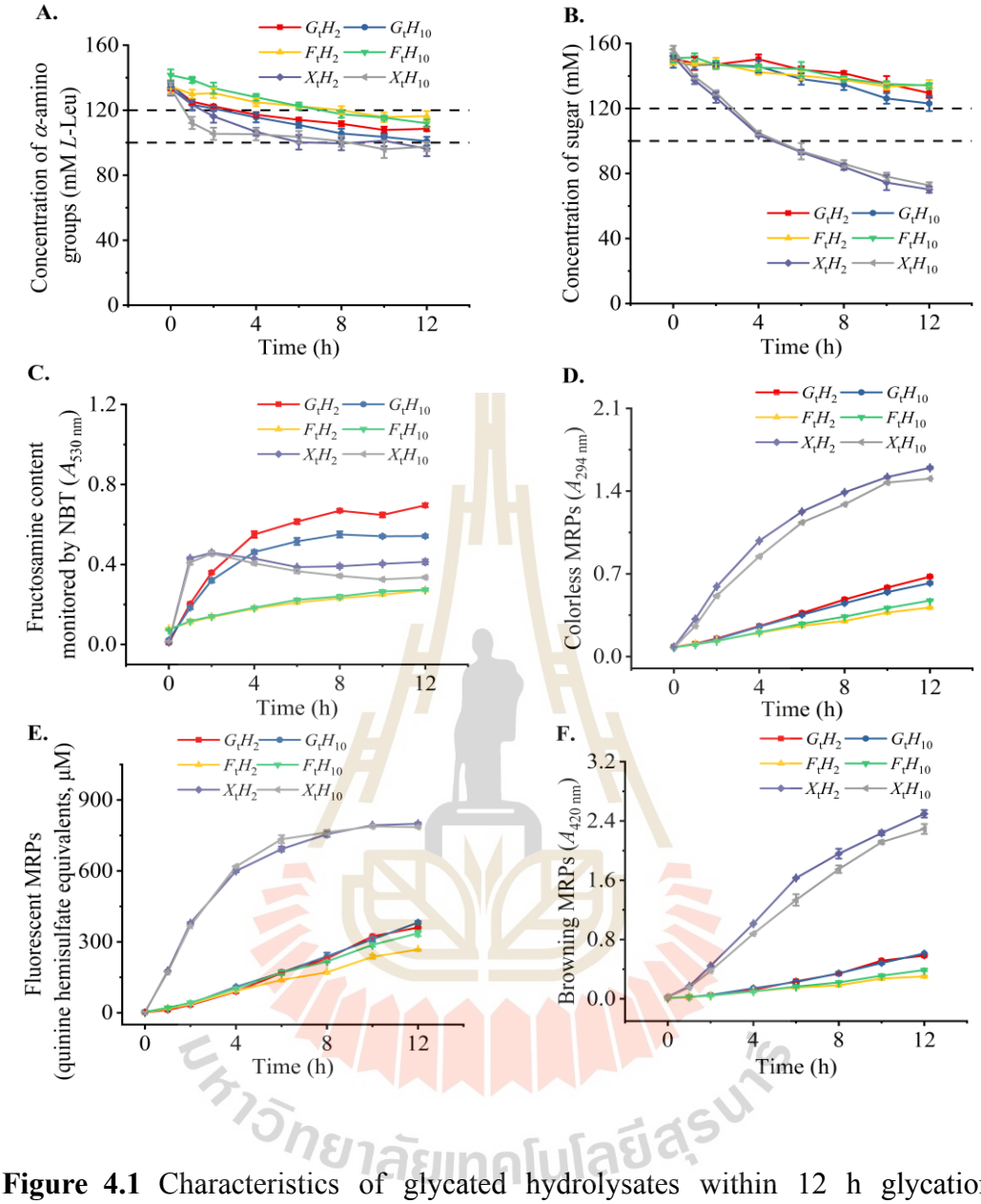

Figure 4.1 Characteristics of glycated hydrolysates within 12 h glycation, the concentration of α-amino groups (A), concentration of reducing sugars (B), fructosamine (C), colorless MRPs (D), fluorescent MRPs (E), and browning MRPs (F).

The glycated hydrolysate was named as S_tH_n. *S* stands for sugars including *G* = glucose, *F* = fructose, and *X* = xylose; the t was the glycation time as 0, 2, 4, 6, 8, 10, and 12 h; H_2 and H_{10} were the 2- and 10-h hydrolysates, respectively.

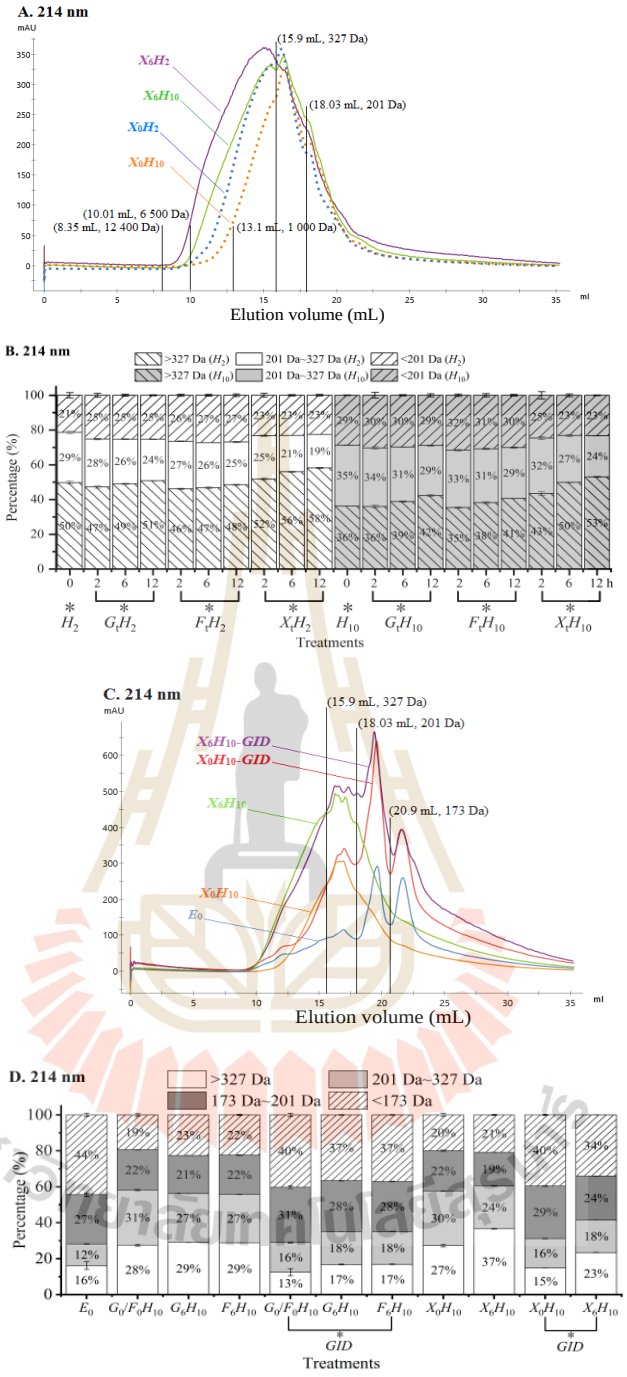

Figure 4.2 Size exclusion chromatography (*SEC*) monitored at 214 nm and corresponding MW distribution, the typical chromatograms (A) and MW distribution charts (B) of un-glycated hydrolysate and glycated hydrolysate, the typical chromatogram (C) and MW distribution charts (D) of un-glycated hydrolysate and glycated hydrolysate upon GI digestion.

MW at elution volume (V_e) 8.35, 10.01, 13.1, 15.9, 18.03 and 20.9 mL were calculated by formula log MW =3.7209K_{av}^2-5.6405K_{av}+4.3691, R^2=0.9996, respectively, and used to define the MW distribution of compounds. The glycated hydrolysate was named as S_tH_n. *S* stands for sugars including *G* = glucose, *F* = fructose, and *X* = xylose; the t was the glycation time as 0, 2, 4, 6, 8, 10, and 12 h; H_2 and H_{10} were the 2- and 10-h hydrolysates, respectively. The *GID* following the sample name was used to indicate the GI digesta of samples. E_0 was the enzyme blank of GI digestion.

According to the change on peptides and sugars (Figure 4.1A-B), as well as MRPs formation (Figure 4.1C-F), xylose revealed the highest reactivity. It was in agreement with those reported previously that reactivity of sugar towards glycation was aldopentoses > aldohexoses, aldose > ketose (Bunn & Higgins, 1981; Laroque, Inisan, Berger, Vouland, Dufossé, & Guérard, 2008; Nooshkam et al., 2019). This could be associated to the lowest steric hindrance of accessible carbonyl group of aldopentoses (Laroque et al., 2008; Nooshkam et al., 2019). In addition, structure of sugars in open (carbonyl) structure showed higher reactivity than those in the ring (hemiacetal or hemiketal) structure. Pentoses are less stable than hexoses and represent higher proportion of open chain than hexoses (Bunn et al., 1981; Laroque et al., 2008). Between aldose and ketose, aldehyde carbonyl group in aldose possesses higher electrophilicity than that of ketone group leaded to its high reactivity (Laroque et al., 2008). The interconversion of ketoses through their cyclic furanose form to the acyclic form is more complex than that of aldoses through their cyclic pyranose form (simple mutarotation) (Laroque et al., 2008), resulted in their lower reactivity.

4.4.2 MW distribution of glycated hydrolysates

All un-glycated hydrolysates, glycated hydrolysates and their digesta displayed strong absorption signal at 214 nm (Figure 4.2A & C), but hydrolysates and their digesta were lesser sensitive at 294 nm (Figure A.1A & C), and insensitive at 420 nm (Figure A.1B & D). The same trends have also been observed in other reports (Han et al., 2019; Han et al., 2018). Therefore, the apparent MW distribution of glycated hydrolysates and their digesta were discussed mainly based on the data from 214 nm (Figure 4.2).

Peptides in both H_2 and H_{10} showed MW < 6500 Da (Figure 4.2A), and the majority of peptides (> 85%) were lower than 1000 Da. In comparison with H_{10}, the lesser proteolysis of H_2 contained larger peptides with MW > 327 Da but less peptides with MW < 327 Da ($p < 0.05$, Figure 4.2B). All glycated hydrolysates displayed MW < 12400 Da (Figure 4.2A). The S_tH_2 contained larger molecules (> 327 Da) than S_tH_{10} ($p < 0.05$, Figure 4.2B), which is due to the fact of larger peptides presented in H_2. For each reaction system, compounds with MW > 327 Da were increased ($p < 0.05$, Figure 4.2B) with extended reaction time. Fractions with MW ranged of 327 ~ 201 Da reduced with reaction time ($p < 0.05$, Figure 4.2B). Molecules with the MW < 201 Da did not change in all glycated products. This indicated that the smaller peptides with the MW of 327 ~ 201 Da would be involved in reaction to form larger molecules by condensation**.** Larger molecules have also been generated through Maillard browning of scallop female gonad hydrolysates with ribose (Han et al., 2019), in which, the molecules with MW > 1000 Da was gradually increased, in concomitant with a decrease of compounds with MW of 200 ~ 1000 Da. The X_tH_2 and X_tH_{10}, showed the greatest changes in MW distribution ($p < 0.05$, Figure 4.2B), while the changes for fructose was

the least. This was significantly correlated with the reactivity of sugars as xylose > glucose > fructose. Gelatin hydrolysates from grass carp (*Ctenopharyngodon idellus*) scales were glycated, in which ribose was the most effective sugar to form MRPs with larger MW compared with xylose and glucose (K. Chen et al., 2019). Therefore, the accumulation of large MW glycated products occurred for the larger peptides and the higher intensity of reaction (Nooshkam et al., 2019).

The MW distribution of glycated hydrolysates was assessed by *SEC*, by which the peptides, intermediate and browning MRPs were monitored at 214, 294 and 420 nm, respectively. The intermediate products were observed throughout the elution time (Figure A.1A), indicating that these products can be formed in a wide range of MW. However, melanoidins were eluted out early (Figure A.1B), suggesting that the browning MRPs were the large MW compounds.

4.4.3 Chemical antioxidant activity of glycated hydrolysates

All S_0H_2 and S_0H_{10} showed significant $ABTS^{\bullet+}$ scavenging capacity, but negligible FRAP and $ONOO^-$ scavenging activity (Figure 4.3). This revealed that the reducing sugars would not significantly contribute to the free radical scavening capacity of samples. Tilapia peptides would mainly scavenge free radicals by HAT mechanism rather than through ET (X. Zhang et al., 2020). Glycation increased chemical antioxidant activities in a time-dependent manner for all sugars, especially for their reducing power as measured by FRAP and $ONOO^-$ scavenging assays ($p < 0.05$, Figure 4.3B-C). MRPs possessed high reducing power, which was in agreement with previous studies (K. Chen et al., 2019; Karnjanapratum, Benjakul, & O'Brien, 2017). This could be related to the formation of hydroxyl groups provided by conjugation and strecker degradation of MRPs (Nooshkam et al., 2019; Yoshimura, Iijima, Watanabe, &

Nakazawa, 1997). The reaction intermediate compounds such as heterocyclic pyrrole might donate hydrogen atom or electron so that could exert antioxidant activities (Dittrich et al., 2009; Hwang, Kim, Woo, Lee, & Jeong, 2011; Nooshkam et al., 2019; W.-q. Wang, Bao, & Chen, 2013). In addition, other MRPs such as kojic acid, furfural, 5-HMF, and maltol, were reported to inhibit soybean oil oxidation (Yi & Kim, 1982). 5-HMF showed $ABTS^{\bullet+}$, DPPH• scavenging activities and inhibited peroxyl radical-induced hemolysis (L. Zhao et al., 2013). The volatile phenolic compounds generated during Maillard reaction might also contribute to free radical scavenging capacity of MRPs (Shan He et al., 2020; Qinzhu, Yan-ling, Dong-xiao, Tian, Yang, & Shan, 2018; W. Wang, Zhang, Wang, Wang, & Liu, 2019). Therefore, hydroxyl, heterocyclic, phenolic compounds formation in glycation of hydrolysate would involve in the improvement of chemical antioxidant activity.

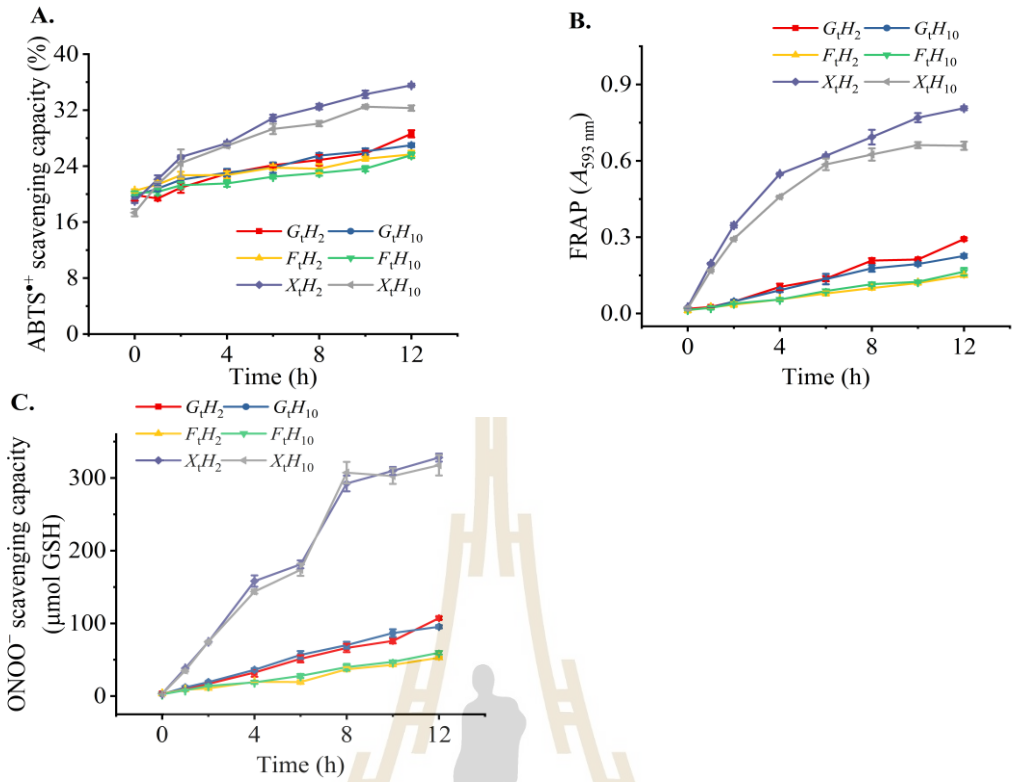

Figure 4.3 Chemical antioxidant activities of glycated products formed at various reaction periods, $ABTS^{\bullet+}$ scavenging capacity (A), ferric reducing antioxidant power (FRAP) (B), and peroxynitrite ($ONOO^-$) scavenging capacity (C).

The glycated hydrolysate was named as S_tH_n. *S* stands for sugars including *G* = glucose, *F* = fructose, and *X* = xylose; the t was the glycation time as 0, 2, 4, 6, 8, 10, and 12 h; H_2 and H_{10} were the 2- and 10-h hydrolysates, respectively.

Xylose-glycated hydrolysates (X_tH_n) showed the highest antioxidant activity. This was due to the high reactivity of xylose generating more MRPs (Figure 4.1D-F). Antioxidant activities of glycated hydrolysates increased with the accumulation of colorless and fluorescent products, which increased with the reaction

time (Figure 4.1D-E). It should be noted that changes of antioxidant activities did not absolutely correlate with formations of browning MPRs in X_tH_n (Figure 4.1F). It has been shown that free radical scavenging capacity of MRPs might be significantly correlated with the low MW colorless intermediates or fluorescent adducts (Chuyen, Ijichi, Umetsu, & Moteki, 1998; Lee et al., 2009; Morales et al., 2001). The final melanoidins did not significantly contribute to the free radical scavenging capacity of MRPs (K. Chen et al., 2019; Lertittikul, Benjakul, & Tanaka, 2007; Morales et al., 2001). Vhangani and Van Wyk (2013) also reported that the extreme glycation reduced their ROO• and •OH scavenging capacities, and reducing power. Rufián-Henares and Morales (2007) found that the color in amino acid-glucose model systems were not correlated with its antioxidant activities, while it might be attributed by the compounds with MW < 10000 Da. A quantity of 26 MRPs with MW < 500 Da were identified in processed aged garlic extract (Wakamatsu, Stark, & Hofmann, 2019). Therefore, improvement of antioxidant activity of glycated hydrolysate would be mainly contributed to the colorless, fluorescent and smaller MRPs. The extreme glycation of tilapia hydrolysate to form numerous melanoidins would be less important for its free radical scavenging capacity.

4.4.4 Characteristic and chemical antioxidant activity of glycated hydrolysate digesta

The level of biological advanced glycation end products (AGEs) is positively correlated to the daily intake of AGEs, and the excess biological AGEs could contribute to the pathogenesis of various chronic diseases (Nowotny, Schröter, Schreiner, & Grune, 2018; Q. Zhang et al., 2020). In this study, a middle content of AGEs at 6-h glycation (Figure 4.1F) was conducted to investigate the modification of

glycated hydrolysate upon *in vitro* GI digestion.

Compared with the digestibility of un-glycated hydrolysate (S_0H_{10}), digestibility of F_6H_{10} was found to be the highest, while X_6H_{10} was the lowest (Figure 4.4A). This was negatively correlated with the extent of glycation (Figure 4.1). Reduction in digestibility of protein upon glycation has also been observed (Poulsen et al., 2013; Q. Zhang et al., 2020). One reason could be the condensation of produced compounds during reaction, leading to generation of larger molecules such as melanoidins. These larger MW melanoidins are inaccessible by the digestive enzymes, and they would hardly be digested resulted the extended glycation showed the lower digestibility (Poulsen et al., 2013; Q. Zhang et al., 2020). Moreover, the addition of sugar to the N-terminus or epsilon-amino groups of peptides also affected the accessibility of digestive enzymes toward peptide bonds, and the aminopeptidases could not cleave the peptides from the terminus. GI digestion also decreased the content of fructosamine, colorless MRPs, and fluorescent adducts ($p < 0.05$, Figure 4.4B-D), while the browning MRPs was increased ($p < 0.05$, Figure 4.4E), while the X_6H_{10} with the highest extent of glycation also showed the most variations.

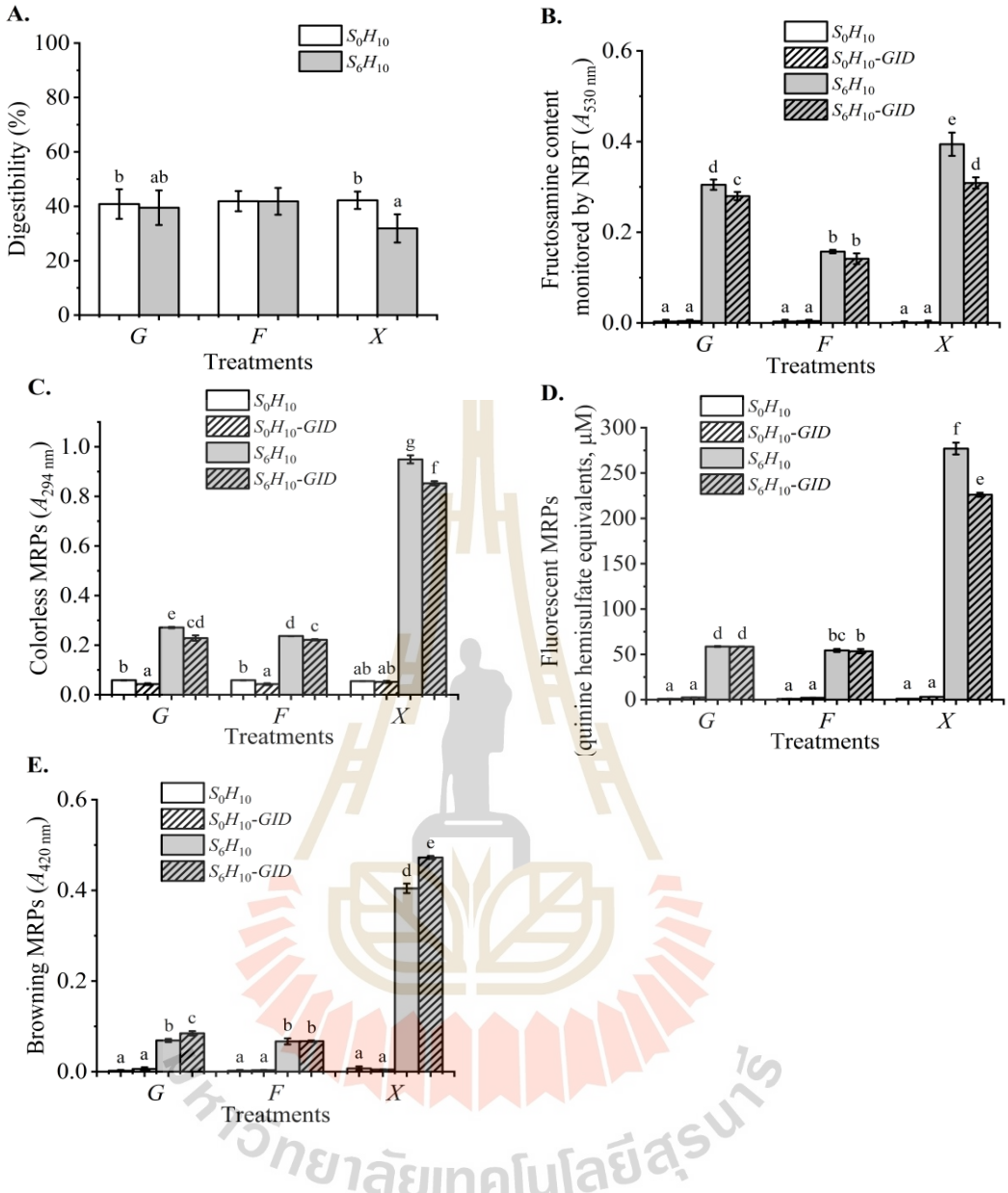

Figure 4.4 Digestibility and characteristics of un-glycated hydrolysates and glycated hydrolysate upon GI digestion, digestibility (A), fructosamine (B), colorless MRPs (C), fluorescent MRPs (D), and browning MRPs (E).

The S_tH_{10} was the glycated 10-h hydrolysate, where, *S* is the abbr. of sugar type, and *G* = glucose, *F* = fructose, and *X* = xylose; t is the glycation time as 0 and 6 h; H_{10} was the 10-h hydrolysate. The *GID* following the sample name was used to

indicate the GI digesta of samples. The different lowercase indicated the differences in the mean values within respective treatment ($p < 0.05$).

SEC analysis revealed that enzyme blank for *in vitro* GI digestion showed significant level of small peptides (Figure 4.2C-D) which might be from the autolysis and/or hydrolysis of the presented digestive enzymes (pepsin and pancreatin) (Dave, Hayes, Mora, Montoya, Moughan, & Rutherfurd, 2016; Whitcomb & Lowe, 2007). It was also observed in our previous study (X. Zhang et al., 2020). MW distribution showed that all samples were digested upon *in vitro* GI digestion, and smaller peptides especially for fraction of MW < 201 Da were increased, and the fraction of MW > 201 Da was decreased ($p < 0.05$, Figure 4.2D). However, the digesta of glycated H_{10}, namely S_6H_{10}-*GID*, showed higher proportion of large molecules (MW > 201 Da) and less small molecules (MW < 201 Da) ($p < 0.05$) than respective S_0H_{10}-*GID*. X_6H_{10}-*GID* showed the highest proportion of large molecules of MW > 327 Da ($p < 0.05$) but the least small molecules of MW < 201 Da ($p < 0.05$). Thus, lower digestibility was evident in the glycated hydrolysate especially for sample with higher glycation.

The digesta of un-glycated hydrolysates (S_0H_{10}-*GID*) showed higher $ABTS^{\bullet+}$ scavenging capacity than that of original hydrolysates (Figure 4.5A), which is likely relative to the smaller peptides formed upon GI digestion (X. Zhang et al., 2020). The $ABTS^{\bullet+}$ scavenging capacity of G_6H_{10} and F_6H_{10} with low extent of glycation were also increased after GI digestion as shown in G_6H_{10}-*GID* and F_6H_{10}-*GID*. This could be also due to the generated smaller peptides as their high digestibility (Figure 4.4A). In contrast, the activity of X_6H_{10}-*GID* did not change compared to its glycated hydrolysate (X_6H_{10}) which had high extent of glycation. One reason could be because the glycated

hydrolysate with high extent of glycation was digested to a lesser extent, forming lesser smaller peptides, and/or degraded MRPs. All un-glycated hydrolysate and their digesta also showed negligible reducing power (Figure 4.5B-C). Glycation also significantly increased the reducing power of hydrolysate (Figure 4.3B-C). However, GI digestion significantly decreased the FRAP of all glycated hydrolysates ($p < 0.05$, Figure 4.5B). These changes were the same as the variation of fructosamine, colorless MRPs and fluorescent adducts upon digestion of glycated hydrolysates (Figure 4.4B-D). GI digestion did not affect the $ONOO^-$ scavenging capacity of all glycated hydrolysates (Figure 4.5C).

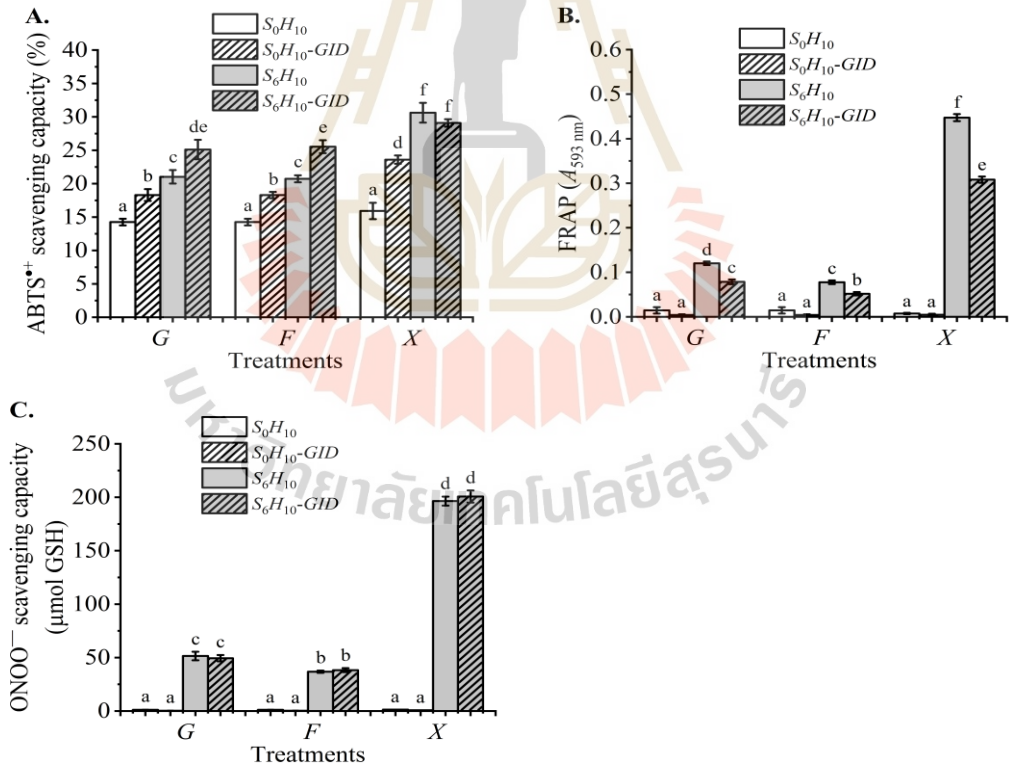

Figure 4.5 Chemical antioxidant activities of un-glycated hydrolysates and glycated hydrolysates upon GI digestion, $ABTS^{\bullet+}$ scavenging capacity (A), ferric reducing antioxidant power (FRAP) (B), and $ONOO^-$ scavenging capacity (C).

The S_tH_{10} was the glycated 10-h hydrolysate, where, *S* was the abbr. of type of sugars, and *G* = glucose, *F* = fructose, and *X* = xylose; t was the glycation time as 0 and 6 h; H_{10} was the 10-h hydrolysate. The *GID* following the sample name was used to indicate the GI digesta of samples. The different lowercase indicated the differences in the mean values within respective treatment ($p < 0.05$).

Therefore, all glycated hydrolysates would be digested upon GI digestion. Hydrolysate glycated to higher extent significantly decreased its digestibility, and its chemical antioxidant activities were reduced more upon GI digestion, especially for the reducing power which would be changed as the variations of fructosamine, colorless MRPs and fluorescent adducts, but not the browning MRPs.

4.4.5 Cellular antioxidant activity

All glycated hydrolysates and their digesta were nontoxic on HepG2 cell model up to 3.5 mg/mL. Intracellular ROS scavenging capacity of all samples was concentration-dependent (Figure 4.6A). All un-glycated hydrolysates, S_0H_{10}, showed similar CAA at 0.02 ~ 2.0 mg/mL (total solid basis), indicating that the different sugars presenting in the hydrolysate did not significantly affected on the CAA. The CAA of the glycated products prepared from any sugar for 6 h were not higher than those of S_0H_{10}, despite of the fact that glycation leding to the higher chemical antioxidant activities ($p < 0.05$, Figure 4.3). The X_6H_{10} showed the highest extent of browning and chemical antioxidant activity ($p < 0.05$, Figure 4.1 & 3), but exerted the lowest CAA ($p < 0.05$, Figure 4.6A). The results suggested that the glycation of tilapia hydrolysate by glucose, fructose and xylose did not enhance the cellular antioxidant activity, and the glycated products with the highest extent of reaction exhibited the lowest CAA. High level of advanced glycated end-products (AGEs) could aggravate the oxidative stress

in diabetic mice, healthy individuals and patients with chronic diseases (Yao et al., 2016; Q. Zhang et al., 2020). However, the X_6H_{10} up to 2.0 mg/mL did not show the pro-oxidation compared with the control (Figure 4.6B, $p > 0.05$). MRPs from shrimp (*Litopenaeus vannamei*) by-products formed by glucose showed stronger inhibition on oxidative stress of HepG2 cells than the original hydrolysate (Zha et al., 2015). MRPs from scallop (*Chlamys farreri*) mantle hydrolysates-ribose promoted the survival and reduced the DNA damage of HepG2 cells (Han et al., 2018). Thus the various MRPs revealed different effects on the oxidative stress. Kitts, Chen, and Jing (2012) reported that low MW MRPs from sugar-amino acid systems were more effective than the high MW species in protecting Caco-2 cells against oxidation. This is likely due to the easily absorption of low MW MRPs and subsequently well distribution in extracellular and intracellular compartments (Poulsen et al., 2013). An extended glycation generates large MW MRPs which are hardly absorbed. In this study, xylose-glycated hydrolysate showed a higher extent than those prepared by glucose and fructose. Then, the lower CAA of X_6H_{10} could be due to the generation of larger MW MRPs such as melanoidins.

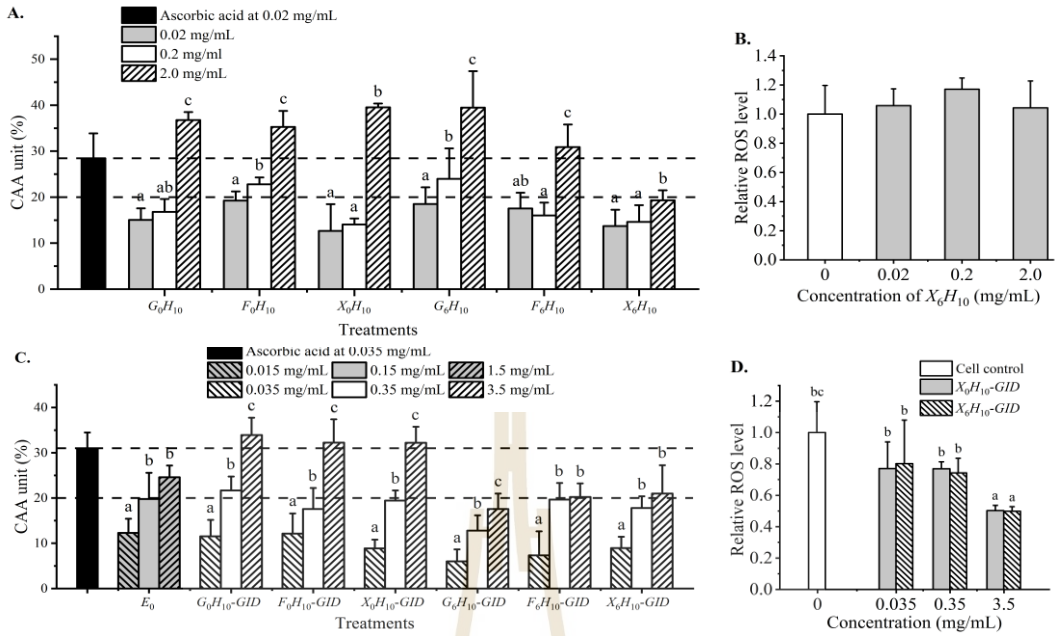

Figure 4.6 Cellular antioxidant activity of the un-glycated and glycated hydrolysates (A), relative reactive oxygen species (ROS) of xylose-glycated product (B), Cellular antioxidant activity of digesta of the un-glycated and glycated hydrolysates (C), relative ROS of the digesta of the mixture of xylose and hydrolysate and xylose-glycated product (D).

The S_tH_{10} was the glycated 10-h hydrolysate, where, *S* was the abbr. of type of sugars, and *G* = glucose, *F* = fructose, and *X* = xylose; t was the glycation time as 0 and 6 h; H_{10} was the 10-h hydrolysate. The *GID* following the sample name was used to indicate the GI digesta of samples. E_0 was the enzyme blank of GI digestion. The different lowercase indicated the differences in the mean values within each sample ($p < 0.05$).

Peptides from the enzyme blank (E_0) for GI digestion also showed CAA in a concentration-dependent manner up to 1.5 mg/mL (Figure 4.6C). To compare the CAA between the un-glycated hydrolysates, glycated hydrolysates and their digesta, the non-toxic concentrations ranged from 0.035 to 3.5 mg/mL were applied for all digesta. Digesta prepared from the un-glycated hydrolysates, (S_0H_{10}-*GID*), showed similar CAA (Figure 4.6C). But they displayed slightly lower CAA than the respective hydrolysate sample (S_0H_{10}, Figure 4.6A) even though they contained smaller and more peptides from E_0, which also contributed to CAA to a certain extent (Figure 4.6C). The smaller peptides could preferentially penetrate through cell membranes (Fernández-Musoles, Salom, Castelló-Ruiz, Contreras, Recio, & Manzanares, 2013; B. Wang & Li, 2017), but the amino acid compositions would also significantly affect the absorption rate of peptides by cells. The apparent permeability coefficient (P_{app}) of peptides, namely IQW, RWQ, WQ and YFCLT, across the Caco2 cell monolayer were obviously lower than that of LKP, RVPSL, QIGLF and GLLLPH (Ding, Wang, Zhang, Yu, & Liu, 2018; Ding, Wang, Zhang, & Liu, 2015; Ding, Zhang, Jiang, Wang, Liu, & Liu, 2014; Fernández-Musoles et al., 2013; Xu, Fan, Yu, Hong, & Wu, 2017). Therefore, significant difference in CAA between the un-glycated sample and its digesta could be because tilapia peptides were likely digested by pepsin and pancreatin to form various antioxidant peptide sequences.

Moreover, all digesta obtained from various glycated samples did not show higher CAA than the respective digesta of un-glycated hydrolysate (Figure 4.6C). The X_0H_{10}-*GID* and X_6H_{10}-*GID* did not show the pro-oxidation as well (Figure 4.6D). The lower MW of MRPs generated by GI digestion could be more effectively absorbed and distributed inside cells (Poulsen et al., 2013). However, the absorption of MRPs

would also be correlated with their exact structures. Free AGEs such as *N*-ε-carboxymethyl-lysine (CML) and *N*-ε-carboxyethyl-lysine (CEL) are more likely to be absorbed by simple diffusion across the epithelium, while the peptide bonded AGEs would be mainly carried out by the peptide transporters (Poulsen et al., 2013; Q. Zhang et al., 2020; D. Zhao et al., 2019). Therefore, the various MRPs in the different glycated hydrolysates and their GI digesta would be absorbed at different rates by cells resulting in various CAA. Furthermore, the AGEs could induce the expression of receptor for AGEs (RAGE) in both HepG2 cells and other animal models (H. Chen et al., 2017; Q. Zhang et al., 2020). The interactions between AGEs and RAGE might influence further absorption of other MRPs and peptides. But the binding affinities of food AGEs with RAGE are still unknow (Q. Zhang et al., 2020). X_6H_{10}-*GID* contained more and larger MW MRPs than that of G_6H_{10}-*GID* and F_6H_{10}-*GID* (Figure 4.2D), while they showed comparable CAA. AGEs presented in the G_6H_{10}-*GID* and F_6H_{10}-*GID* might preferably interact with the RAGE when compared with that of X_6H_{10}-*GID*. The digesta of glycated hydrolysates did not show effective CAA. The underlying mechanisms such as absorption of MRPs by cells and interaction of MRPs with RAGE should be further explored. The chemical and cellular antioxidant activity of hydrolysate and glycated hydrolysate would be modified upon GI digestion (Figure 4.5-4.6). Therefore, as the production of protein hydrolysate (X. Zhang et al., 2020), glycated hydrolysate should be also optimized based on the activity of its digesta rather than that of glycated hydrolysates.

4.5 Conclusions

Glycation of hydrolysate improved chemical antioxidant activity, especially for the reducing power, and it was likely related to the early colorless and fluorescent MRPs rather than the browning melanoidins. However, the xylose-glycated hydrolysate with extended glycation showed lower digestibility. GI digestion decreased chemical antioxidant activities of glycated hydrolysate especially those subjected to extended browning reaction. All digesta of glycated hydrolysates were lesser effective intracellular ROS scavengers than those digesta of unglcated hydrolysates. Therefore, glycation of tilapia hydrolysate would be effective to enhance chemical antioxidant activity but did not improve cellular antioxidant activity. The underlying mechanisms of glycated product showed lower CAA than un-glycated ones such as cell absorption and interaction to RAGE of cell membranes should be further investigated.

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CHAPTER V

IDENTIFICATION OF TILAPIA ANTIOXIDANT PEPTIDES AND THEIR *IN SILICO* GASTROINTESTINAL DIGESTED PEPTIDES PROTECTED AAPH-INDUCED HEPG2 CELL OXIDATIVE STRESS

5.1 Abstract

Peptides from tilapia muscle protein hydrolysate were isolated by size exclusion chromatography (*SEC*) and sequential reversed-phase chromatography (*RPC*). The lesser polar fractions displayed potent 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$) scavenging capacity and were selected for peptide identification by LC-MS/MS. Nine novel peptides, namely EKL, EKP, HKPA, ELSC, ALSC, ASLCH, SLCH, LPGYF and LEVPGY, and 6 fragments from *in silico* gastrointestinal digestion of identified peptides, including EK, KPA, SC, CH, PGY and EVPGY were synthesized. The 50% effective concentration (EC_{50}) of $ABTS^{\bullet+}$ scavenging capacity of the most effective parent (LPGYF) and digested peptide (SC) were 3.91±0.02 μM and 2.11±0.03 μM, respectively. All C- and Y-containing peptides showed EC_{50} of 2~17 μM, which were more effective than ascorbic acid (AsA). In contrast, K-containing peptides exhibited less antioxidant activity with EC_{50} values

greater than 250 μM. The *in silico* digested peptides containing C- and Y residues, SC, CH, PGY and EVPGY, remained high activity. All 15 peptides showed potent intracellular reactive oxygen species (ROS) scavengers on the 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH)-induced HepG2 cell oxidative stress model. In addition, digested peptides, SC, CH and PGY, up-regulated expression of *CAT* and *SOD1* on both HepG2 and AAPH-induced HepG2 cell oxidative stress models. PGY was the most effective cellular antioxidant. All peptides did not up-regulated expression of *GPx1*. Peptides derived from tilapia muscle protein could be the potent nutraceuticals against oxidative stress.

Keywords: peptide, antioxidant, *in silico* digestion, reactive oxygen species (ROS), gene expression

5.2 Introduction

Oxidative stress is related to many pathophysiological processes, such as ageing, inflammation, diabetes mellitus and cancers (Nwachukwu & Aluko, 2019; Sies & Jones, 2020). Food protein-derived antioxidant peptides have been considered as nutraceutical to diminish the oxidative stress and investigated widely in recent decades (Nwachukwu et al., 2019; Samaranayaka & Li-Chan, 2011; Zheng, Zhao, Dong, Su, & Zhao, 2016). Peptides serving on the antioxidant activity of hydrolysates have been identified from various sources (Fan, He, Zhuang, & Sun, 2012; Huang, Lin, & Chang, 2015; Kangsanant, Thongraung, Jansakul, Murkovic, & Seechamnanturakit, 2015). However, it has been

demonstrated that antioxidant peptides are likely modified under the gastrointestinal (GI) tract due to the action of digestive proteases (Gallego, Mora, & Toldrá, 2018; Ren, Liang, Zhang, Hou, Li, & Ma, 2018; Zhang, Noisa, & Yongsawatdigul, 2020). Identification of digested peptides would be more relevant to health. But pancreatin used for *in vitro* GI digestion also contained a certain amount of peptides, which have been demonstrated showing significant antioxidant activity (Zhang et al., 2020). Contamination of antioxidant peptides derived from the digestive enzymes presented by autolysis or/and hydrolysis (Dave, Hayes, Mora, Montoya, Moughan, & Rutherfurd, 2016; Whitcomb & Lowe, 2007), thus, poses challenges in the purification and identification of antioxidant peptide from hydrolysate digesta**.** Bioinformatics tools, such as BIOPEP http**:**//www**.**uwm**.**edu**.**pl/biochemia/ index**.**php/en/ biopep and PeptideCutter https**:**//web**.**expasy**.**org/peptide_cutter/ are usually applied for *in silico* GI digestion to predict the potential cleave sites of peptides under GI digestion (Agyei, Tsopmo, & Udenigwe, 2018; Gallego et al., 2018). It can avoid the interferences of peptides derived from the enzymes. Therefore, *in silico* GI digestion could be a means to obtain a pooled of digested peptides of protein hydrolysate, which could be used for identification of antioxidant peptides. In addition, it can also evaluate the variation of antioxidant activity of peptides upon GI digestion.

Type of amino acids and their sequence on the peptide greatly contribute to free radical scavenging capacity of the peptide. Peptides containing C, M, W, Y, F, K, P, E and H residues are usually considered as good free radical scavenger (Nimalaratne,

Bandara, & Wu, 2015; Nwachukwu et al., 2019; Samaranayaka et al., 2011; Yang, Zhang, Ding, Chi, Wang, & Huo, 2019). However, some reports have shown that only Y, W, C and M residues played a dominant role at $ABTS^{\bullet+}$ or peroxyl radical (ROO•) scavenging capacity (Sheih, Wu, & Fang, 2009; Q. Yang, Cai, Yan, Tian, Du, & Wang, 2020; Zheng, Zhao, Dong, et al., 2016). The important role of various amino acid residues contributing to the free radical scavenging capacity of peptide is still inconclusive. Cellular antioxidant activity (CAA) assay is more biologically relevant as it addresses some issues of cells such as uptake, distribution and metabolism of antioxidant (López-Alarcón & Denicola, 2013; Wolfe & Liu, 2007). It has been widely employed to evaluate the antioxidant activity of peptides in biological system (Du, Esfandi, Willmore, & Tsopmo, 2016; Tonolo et al., 2020; Wu et al., 2018). The $ABTS^{\bullet+}$ scavenging capacity of peptides, namely NTVPAKSCQAQPTTM, FLKKISQRYQKF, ALPQYLKTVYQHQK and IQPKTKVIPYVRYL, were positively correlated to their intracellular reactive oxygen species (ROS) scavenging capacity (Tonolo et al., 2020). NTVPAKSCQAQPTTM has also increased gene expression of superoxide dismutase (SOD). YLEELHRLNAGY has showed effective effects on both free radical scavenging capacities and increasing gene expression of SOD and catalase (CAT) (Homayouni-Tabrizi, Asoodeh, & Soltani, 2017). However, peptides, FNDRLRQGQLL and DVNNNANQLEPR, showed lower ROO• scavenging capacity than that of GLVYIL and YHNAPGLVYIL, but they displayed similar intracellular ROS scavenging capacity (Du et al., 2016). The main reason would be they induced the various cellular

responses such as modulated the level of glutathione (GSH), increased the activity of SOD, glutathione peroxidase (GPx) and CAT. Thus, CAA assay is not only characterizing the intracellular ROS scavenging capacity, but also investigating the underlying mechanisms acted by peptides such as modulating in gene expression of antioxidant enzymes to enhance intrinsic CAA (Homayouni-Tabrizi et al., 2017; Tonolo et al., 2020; Wang et al., 2018).

Therefore, in this study, tilapia muscle protein was hydrolyzed by Alcalase to 35% degree of hydrolysis (DH) and then purified by size exclusion chromatography (*SEC*) and sequential reversed-phase chromatography (*RPC*). The fractions displayed higher $ABTS^{\bullet+}$ scavenging capacity were selected for peptide identification by LC-MS/MS. The identified peptide sequences were further digested by *in silico* GI digestion. All target peptides including parent and their digesta were synthesized and characterized for their $ABTS^{\bullet+}$ scavenging activity and intracellular reactive oxygen species (ROS) scavenging capacity on 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH)-induced HepG2 cell oxidative stress model. The role of amino acid residues on the peptides toward $ABTS^{\bullet+}$ and ROS scavenging was also elucidated. The digested peptides showing high ROS scavenging capacity were characterized for their effect on gene expression of catalase (*CAT*), superoxide dismutase (*SOD1*) and glutathione peroxidase (*GPx1*).

5.3 Materials and methods

Tilapia muscle protein powder and protein hydrolysate were prepared according to Zhang et al. (2020). Defatted tilapia protein powder of 10% in 0.15 M $NaHCO_3$ (w/v) was adjusted to pH 8.0 and then hydrolyzed by 5% Alcalase (based on crude protein, w/w) at 50 °C for 10 h. After centrifugation, supernatants were collected and stored at – 80 °C and used within a week.

5.3.1 Purification and identification of antioxidant peptides

5.3.1.1 *SEC*

A 100 µL of diluted hydrolysate was injected into a Superdex Peptide 10/300 GL column (GE Healthcare Bioscience Co., Uppsala, Sweden) equipped with fast protein liquid chromatography (FPLC; ÄKTA pure 25, Version 1.8.0.6, GE Healthcare Bioscience Co., Uppsala, Sweden). Peptides were eluted out and fractionated at ambient temperature (25 – 28 °C). The elution program with mobile phase A (DI water) and mobile phase B (30% ACN containing 0.1% TFA, v/v) was shown in Figure 5.1A1 and detailed on Table A1. Peptides were detected at 214 nm and 280 nm. *SEC* fractions (*SF*) were collected, lyophilized and resuspended in 2% CAN (v/v) containing 0.1% TFA (v/v) for further use. $ABTS^{\bullet+}$ scavenging capacity of peptide fractions at 10 µM (*L*-Leu equivalents) was evaluated. Compared with the initial hydrolysate (*H*), peptide yield (%) and relative antioxidant activities were calculated.

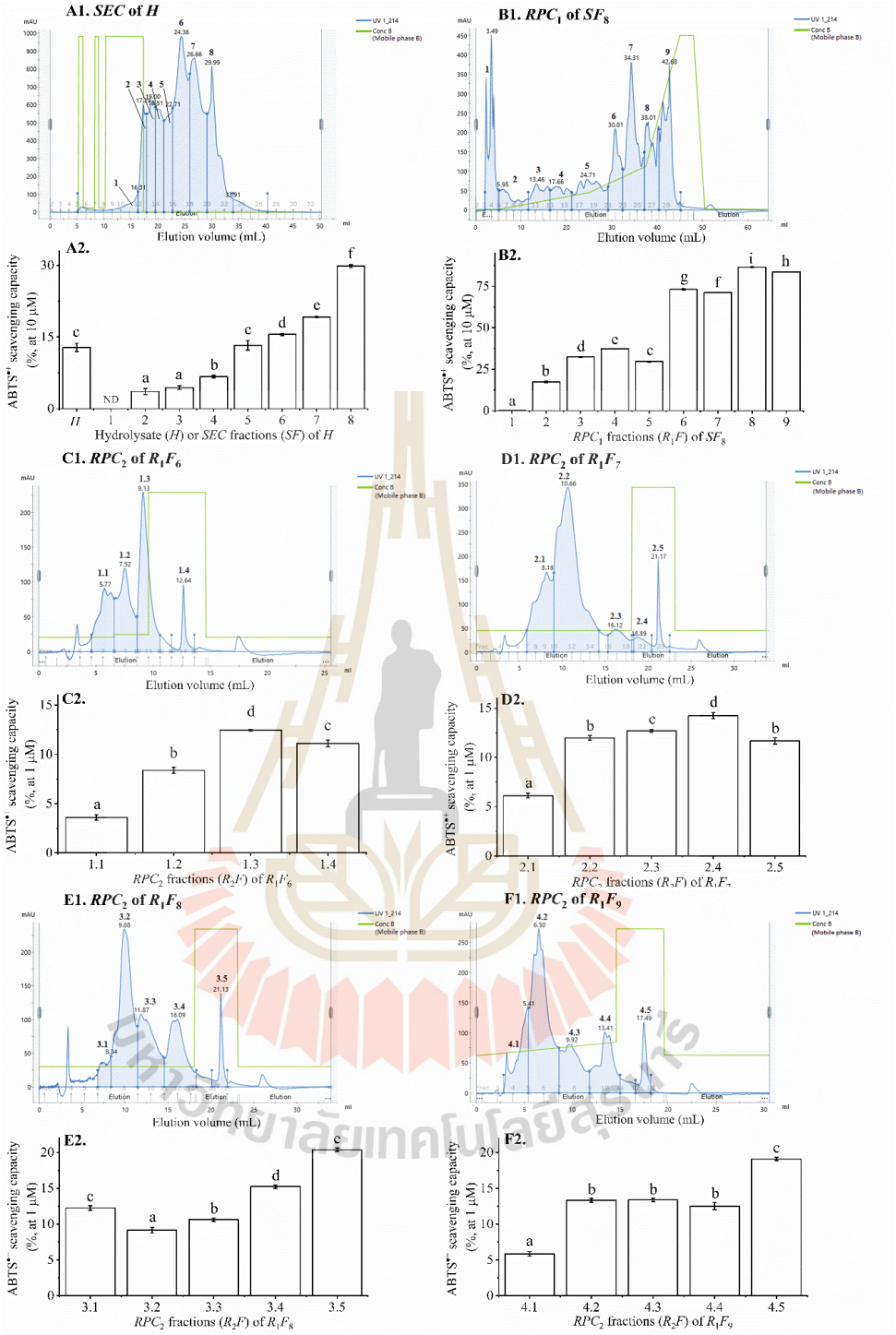

Figure 5.1 Chromatograms of tilapia protein hydrolysate by *SEC* (A1), sequential *RPC* (B1, and C1, D1, E1 and F1) and $ABTS^{\bullet+}$ scavenging capacity of fractions from *SEC* (A2), and sequential *RPC* (B2, and C2, D2, E2 and F2).

Different lowercase letters indicated the differences in the mean values of $ABTS^{\bullet+}$ scavenging capacity of fractions (p<0.05). *SEC*, size exclusion chromatography; *RPC*, reversed-phase chromatography.

5.3.1.2 *RPC*

Fraction from *SEC* exhibiting the highest $ABTS^{\bullet+}$ scavenging capacity, namely SF_8, were injected into a SOURCE™ 5RPC ST 4.6/150 column (GE Healthcare Bioscience Co., Uppsala, Sweden). Peptides were eluted by mobile phase A (0.1% TFA in DI water, v/v) and mobile phase B (0.1% TFA in ACN v/v). Elution program was shown in Figure 5.1B1 and detailed on Table A1. Four fractions from the *RPC*, namely $R_1F_{6\text{-}9}$, which showed higher $ABTS^{\bullet+}$ scavenging capacity at 10 μM than others, were collected and individually rechromatogramed by *RPC* using the same mobile phase. Elution programs were shown in Figure 5.1C1-F1 and detailed on Table A1. Finally, 16 fractions from the second *RPC* displaying higher $ABTS^{\bullet+}$ scavenging capacity at 1 μM than others were selected for peptide identification.

5.3.1.3 LC-MS/MS

Dionex UltiMate 3000 UHPLC+ focused Systems (Thermo Fisher Scientific Inc., Waltham, MA, U.S.) coupled with a micrOTOF Q II mass spectrometer (Bruker Daltonics, Bremen, Germany) was applied for LC-MS/MS. Peptide separation was achieved using a Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7-Micron column (Agilent Technologies, Santa Clara, CA, U.S.). Mobile phases were A: 0.1% TFA in DI water and B: 0.1% TFA in CAN. Flow rate was 0.3 mL/min. Column

temperature was 27 °C. The different elution programs were employed for the various fractions as shown in Table A2. The micrOTOF Q II was calibrated using sodium formate (10 mM NaOH and 0.2% formic acid in isopropanol) and it was also used as the internal mass calibrant included in all samples. Mass spectra were acquired in positive ion mode and scans were performed for Auto MS/MS between 50 and 1500 m/z. The analysis was performed under the collision energy of 8.0 eV, nebulizer pressure of 2.0 bar, drying gas flow rate of 8.0 L/min, and the dry heater temperature was 180 °C. The compass DataAnalysis software was used to analyze the mass spectra. The original MS/MS spectrum file (.mgf) were exported for the following *de novo* sequencing by PepNovo (v1.2.5) (https://proteomics.ucsd.edu/ProteoSAFe/index.jsp). It was conducted on May 17th, 2019. Cysteine protecting group and protease were none. Parent mass tolerance was 2 Da. Ion tolerance was 0.5 Da. The homologous proteins of identified peptide sequences were searched by NCBI standard protein BLAST (BLASTP 2.9.0+ https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) and/or UniProtKB (peptide search https://www.uniprot.org/peptidesearch/) on May 17th – 25th, 2019. The organism or taxon optional was *Oreochromis niloticus* (Nile tilapia, taxid: 8128). Query cover and percent identity are 100%. The identified peptide sequences, which showed higher signal intensity in basic peak chromatograms (BPC) of MS spectrum and contained likely antioxidative amino acid residues such as W, Y, K, C, P and H, were screened for further *in silico* simulated GI digestion.

5.3.1.4 *In silico* GI digestion

The *in silico* GI digestion was carried out using algorithm developed by the PeptideCutter (https://web.expasy.org/peptide_cutter/). It was conducted on May 25th, 2019. Pepsin (pH 1.3 and pH > 2), trypsin and chymotrypsin-low specificity (C-term to [FYWML], not before P) were employed. The identified parent peptide sequences and peptide digesta were also compared against the previous reports and BIOPEP database (Minkiewicz, Iwaniak, & Darewicz, 2019) (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) on June 6th, 2019. Finally, 15 peptides including 9 identified parent peptides and 6 digested peptides were synthesized by GL Biochem (Shanghai) Ltd. (Shanghai, China) for further evaluations.

5.3.2 Peptide content

Peptide content was analyzed by TNBS assay as described by Zhang et al. (2020) and expressed as *L*-Leu equivalents. The absorbance was measured at 420 nm by GENESYS 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific, Madison, WI 53711, USA).

5.3.3 $ABTS^{\bullet+}$ scavenging capacity

$ABTS^{\bullet+}$ decolorization was conducted as described by Zhang et al. (2020) with slight modifications. Absorbance of $ABTS^{\bullet+}$ working solution was adjusted to 0.7 ± 0.01 at 734 nm. A 50 µL of sample was mixed with 950 µL of $ABTS^{\bullet+}$ and incubated at room temperature for 15 min. Absorbance was measured within 30

min. DI was used as a blank. $ABTS^{\bullet+}$ scavenging capacity was calculated using the equation:

$$ABTS^{\bullet+} \text{ scavenging capacity (\%)} = \frac{A_{blank}-A_{sample}}{A_{blank}} \times 100\%$$

Where, A_{blank} and A_{sample} are referred to as the absorbance of blank and sample, respectively.

5.3.4 Cellular antioxidant activities

HepG2 cells were cultured in a humidified incubator at 37 °C with 5% CO_2 as described by Zhang et al. (2020). For cytotoxicity, the cell seeding density was 2×10^4 cells/well in a clear 96-well microplate with 100 µL of complete medium, dulbecco's modified eagle medium (DMEM) containing 10% (v/v) heat-inactivated FBS, 1% (v/v) *L*-Gln, 1% (v/v) non-essential amino acids, and 1% (v/v) penicillin-streptomycin (10,000 µg/mL). After 24-h incubation, the medium was replaced by the complete medium containing peptide (10 – 100 µM) and incubated for 24 h. Then MTT assay was employed to measure the cell viability.

For intracellular ROS scavenging capacity, cells were seeded to achieve 6×10^4 cells/well and cultured on a sterile Corning® 96-well black polystyrene microplate with a flat clear bottom until 100% confluence was reached. The peptide and 25 µM DCFH-DA prepared in the complete medium without FBS were added to the microplate and incubated for 1 h. Subsequently, the medium was discarded, cells

were washed once with 1×PBS, and 600 μM AAPH in 1×PBS was added. After 1 h of incubation, the fluorescence intensity at $\lambda_{Ex/Em}$ = 485/538 nm was read at 37 °C. Ascorbic acid at 0.05 mg/mL was used as the positive control in each plate. Results were expressed as CAA unit using the equation:

$$\text{CAA unit} = \left(1 - \frac{\text{Fluorescence intensity}_{\text{sample}}}{\text{Fluorescence intensity}_{\text{oxidative stress}}}\right) \times 100$$

Where, oxidative stress group is referred to the cells treated by DCFH-DA followed by APPH, without peptide pre-treatment.

5.3.5 Gene expression

A 1 mL of complete medium containing 6×10^5 cells was seeded in a 6-well plate and incubated for 24 h. Two models were carried out. The first model was prepared by treating cells by peptide alone (peptide only) at varied concentrations of 10 – 100 μM for 4 h. The second model was prepared by incubating cells and peptides for 4 h, followed by replacement of 1 mL of the complete medium containing 600 μM AAPH, and further incubated for 4 h, which was referred to peptide + AAPH. All treated cells were washed with 1×PBS and harvested by 0.05% trypsin-EDTA. Total RNA was isolated immediately using a NucleoSpin® RNA Plus kit (MACHEREY-NAGEL GmbH & Co. KG., Düren, Germany) according to the manufacturer's instructions. Concentration of total RNA was detected using LVis plate by the

SPECTROstar NANO system (BMG Labtech GmbH, Ortenberg, Baden-Württemberg, Germany). The total RNA (1 µg) was converted into complementary DNA (cDNA) using a ReverTra Ace® qPCR RT Master Mix with gDNA Remover (FSQ-301, Toyobo Co., Ltd., Osaka, Japan) following the manufacturer's protocol. Thereafter, cDNA was amplified by mixing 0.5 µL of cDNA with 12 µL of freshly prepared reagent containing 5.25 µL of RNase-free DI, 0.5 µL of 100 µM primer, and 6.25 µL of Vivantis 2×Taq Master Mix (PLMM01, Vivantis Technologies Sdn. Bhd., Selangor Darul Ehsan, Malaysia). Glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) was used as housekeeping gene. Expression of *CAT*, *SOD1*, and *GPx1* were detected. The RT-PCR product size of *GAPDH* and target genes, forward and reverse primers were as follows: *GAPDH* (247 bp), forward: 5′-ACC TGA CCT GCC GTC TAG AA-3′, reverse: 5′-TCC ACC CTG TTG CTG TA-3′; *CAT* (380 bp), forward: 5′-TCC GGG ATC TTT TTA ACG CCA TTG-3′, reverse: 5′-TCG AGC ACG GTA GGG ACA GTT CAC-3′; *SOD1* (272 bp), forward: 5′-CTA GCG AGT TAT GGC GAC-3′, reverse: 5′-CAT TGC CCA AGT CTC CAA C-3′; *GPx1* (224 bp), forward: 5′-CGC CAA GAA CGA AGA GAT TC-3′, reverse: 5′-CAA CAT CGT TGC GAC ACA C-3′. Agarose gel electrophoresis was conducted by 1.5% agarose stained with ethidium bromide. VC 100 bp Plus DNA Ladder was used as the marker (0.1 µg/µL, NL1407, Vivantis Technologies Sdn. Bhd., Selangor Darul Ehsan, Malaysia). The expression level of antioxidant enzyme genes was calculated relative to the housekeeping gene *GAPDH*.

5.3.6 Statistical analyses

All tests were carried out in 3 replicates and expressed as the mean ± SD. Comparisons between the two means were performed using independent sample t-tests ($p = 0.05$). When there were more than two treatments, the mean differences were analyzed by ANOVA using Tukey HSD (IBM SPSS Statistics for Windows, version 23.0. Armonk, NY: IBM Corp) ($p = 0.05$).

5.4 Results and discussion

5.4.1 Purification of antioxidant peptides

The protein hydrolysate (*H*) with 35% DH was prepared as its GI digesta showed the highest chemical and cellular antioxidant activities (Zhang et al., 2020). $ABTS^{\bullet+}$ scavenging capacity of hydrolysate fractions obtained from *SEC* ($SF_{1\text{-}8}$) increased with elution time ($p < 0.05$, Figure 5.1A1-2). As a stepwise gradient was applied (Figure 5.1A1 & Table A1), peptides eluted later under less polar mobile phase could be presumed to be smaller with higher hydrophobicity. Numerous studies have demonstrated that smaller peptides and peptides containing hydrophobic amino acid residues possessed better antioxidant activities (Nimalaratne et al., 2015; Nwachukwu et al., 2019). Compared with the original *H*, SF_8 showed activity about 2.3 times higher with about 12.4% peptide yield (Table 5.1).

Table 5.1 Yields and antioxidant activity of tilapia protein hydrolysate fractions during purification by *SEC*, sequential *RPC* [1]

Fractions		Peptide content (μmol *L*-Leu) *	Peptide yield (%) #	Relative activity
H		187.87 ± 6.55	100.00 ± 3.49	1.00 ± 0.07 †, a
SF_8		23.21 ± 0.98	12.35 ± 0.52	2.32 ± 0.02 †, b
R_1F_6		0.74 ± 0.028	0.40 ± 0.015	5.69 ± 0.03 †, d
R_1F_7		0.68 ± 0.012	0.36 ± 0.007	5.55 ± 0.01 †, d
R_1F_8		0.74 ± 0.005	0.40 ± 0.002	6.73 ± 0.01 †, ef
R_1F_9		1.43 ± 0.052	0.76 ± 0.027	6.51 ± 0.01 †, e
$R_2F_{1.1}$	From R_1F_6	0.24 ± 0.020	0.13 ± 0.010	2.80 ± 0.21 ‡, b
$R_2F_{1.2}$		0.24 ± 0.011	0.13 ± 0.006	6.52 ± 0.24 ‡, e
$R_2F_{1.3}$		0.18 ± 0.001	0.01 ± 0.001	9.68 ± 0.07 ‡, j
$R_2F_{1.4}$		0.01 ± 0.000	0.01 ± 0.000	8.64 ± 0.25 ‡, gh
$R_2F_{2.1}$	From R_1F_7	0.22±0.006	0.12 ± 0.003	4.76 ± 0.18 ‡, c
$R_2F_{2.2}$		0.29 ± 0.001	0.16 ± 0.001	9.32 ± 0.18 ‡, ij
$R_2F_{2.3}$		0.04 ± 0.003	0.02 ± 0.002	9.88 ± 0.12 ‡, jk
$R_2F_{2.4}$		0.01 ± 0.000	0.004 ± 0.000	11.08 ± 0.24 ‡, l
$R_2F_{2.5}$		0.01 ± 0.001	0.004 ± 0.000	9.08 ± 0.25 ‡, hi
$R_2F_{3.1}$	From R_1F_8	0.04 ± 0.003	0.02 ± 0.002	9.54 ± 0.24 ‡, ij
$R_2F_{3.2}$		0.50 ± 0.003	0.27 ± 0.002	7.11 ± 0.30 ‡, f
$R_2F_{3.3}$		0.14 ± 0.009	0.08 ± 0.005	8.27 ± 0.18 ‡, g
$R_2F_{3.4}$		0.06 ± 0.004	0.03 ± 0.002	11.86 ± 0.18 ‡, m
$R_2F_{3.5}$		0.01 ± 0.002	0.01 ± 0.001	15.85 ± 0.18 ‡, o
$R_2F_{4.1}$	From R_1F_9	0.28 ± 0.014	0.15 ± 0.007	4.55 ± 0.25 ‡, c
$R_2F_{4.2}$		0.58 ± 0.009	0.31 ± 0.005	10.38 ± 0.21 ‡, k
$R_2F_{4.3}$		0.21 ± 0.017	0.11 ± 0.009	10.42 ± 0.18 ‡, k
$R_2F_{4.4}$		0.08 ± 0.014	0.05 ± 0.007	9.74 ± 0.39 ‡, j
$R_2F_{4.5}$		0.01 ± 0.003	0.004 ± 0.002	14.85 ± 0.18 ‡, n

[1] For fractions possessing relatively high $ABTS^{\bullet+}$ scavenging capacity. *SEC*,

size exclusion chromatography; *RPC*, reversed-phase chromatography; *H*, original hydrolysate.

* Peptide content (μmol *L*-Leu) was calculated back based on 1 mL original hydrolysate.

[#] Yields were calculated, based on the peptide content relative to *H*.

[†]and [‡] Relative activities were calculated relative to activity of *H* at 1 and 10 μM, respectively.

[a – o] The different lowercase letters indicated the differences in the mean values ($p < 0.05$).

SF_8 was selected for further *RPC* purification by a gradient elution program (Figure 5.1B1 & Table A1). The most polar fraction (R_1F_1) showed negligible $ABTS^{\bullet+}$ scavenging capacity (Figure 5.1B1-2). Antioxidant activity increased with decreasing polarity of mobile phase, and the lesser polar fractions (R_1F_{6-9}) displayed 5.6 – 6.7 times higher activity than the original *H* ($p < 0.05$, Table 5.1). These results suggested that tilapia antioxidant peptides are likely less polar molecules. In the second RPC of R_1F_{6-9}, the same trend of less polar peptides with higher antioxidant activities was still observed (Figure 5.1C-F & Table A1). Similar trend has been reported by others (Sangtitanu, Sangtanoo, Srimongkol, Saisavoey, Reamtong, & Karnchanatat, 2020; Wen, Zhang, Feng, Duan, Ma, & Zhang, 2020). The non-polar or polar uncharged amino acid residues such as C, Y, W, M, P, and H, have been reported to present in

peptides contributing to their antioxidant activities (Nimalaratne et al., 2015; Nwachukwu et al., 2019; Samaranayaka et al., 2011).

Antioxidant activity increased with purification steps (Table 5.1). The most effective fractions, namely $R_2F_{3.5}$ and $R_2F_{4.5}$, displayed 15.9- and 14.9-times higher activity than the original *H*, respectively. Total 16 fractions of $R_2F_{1.2\text{-}1.4}$, $R_2F_{2.2\text{-}2.5}$, $R_2F_{3.1\text{-}3.5}$, and $R_2F_{4.2\text{-}4.5}$ showing relatively high activity were selected for the further peptide identification by LC-MS/MS.

5.4.2 Identification and *in silico* digestion of peptides

Total 9 peptides were identified from tilapia protein hydrolysate (Figure 5.2). They contained potential antioxidant amino acid residues as K, P, H, C, or Y (Nimalaratne et al., 2015; Nwachukwu et al., 2019; Samaranayaka et al., 2011). These peptides were derived from the tilapia proteins including myosin, actin-related proteins, collagen, zinc finger protein, plexin or cathepsin (Table 5.2). All peptides were novel. Based on the *in silico* GI digestion, all peptides except for EKP were digested. Total 7 peptide fragments were generated from *in silico* GI digestion of 8 parent peptides (Table 5.2). All *in silico* GI digested peptides except for EVPGY were the small di-/tripeptides. Escudero et al., (Escudero, Mora, & Toldrá, 2014) reported that pentapeptides, IAGRP and PTPVP, were resistant to the *in vitro* GI digestion. Small peptides such as HL, IY, TY, PW, YPQ, PHL, VPW, PIKK, SGGY and AHSVGP have been identified from the *in vitro* or *ex vivo* GI digesta of protein/hydrolysates (Borawska, Darewicz, Pliszka, &

Vegarud, 2016; Feng, Peng, Wang, Li, Lei, & Xu, 2019; Ren et al., 2018). Proteases involving in GI digestion are the mixture of endopeptidases and exopeptidases, and possess a broad specificity, resulting in degradation of ingested peptides (Fernández-Musoles, Salom, Castelló-Ruiz, Contreras, Recio, & Manzanares, 2013; B. Wang & Li, 2017; Whitcomb et al., 2007). In addition, *in silico* GI digestion by PeptideCutter tool of ExPASy has successfully predicted the decrease in antioxidant activity of peptide, AEEEYPDL, during *in vitro* GI digestion (Gallego et al., 2018). The *in silico* GI digestion seems like effective for the prediction of peptide degradation upon GI tract. The dipeptide, AS, was considered as the lowest potent antioxidant. Amino acid residues, A and S, have been reported to exhibit less free radical scavenging potential (Zheng, Zhao, Dong, et al., 2016). Total 15 peptides including 9 parent and 6 digested peptides were synthesized. These peptides were classified into three groups as K-containing peptides, EKL, EK, EKP, HKPA and KPA, C-containing peptides, ELSC, ALSC, SC, ASLCH, SLCH and CH, and Y-containing peptides, LPGYF, PGY, LEVPGY and EVPGY.

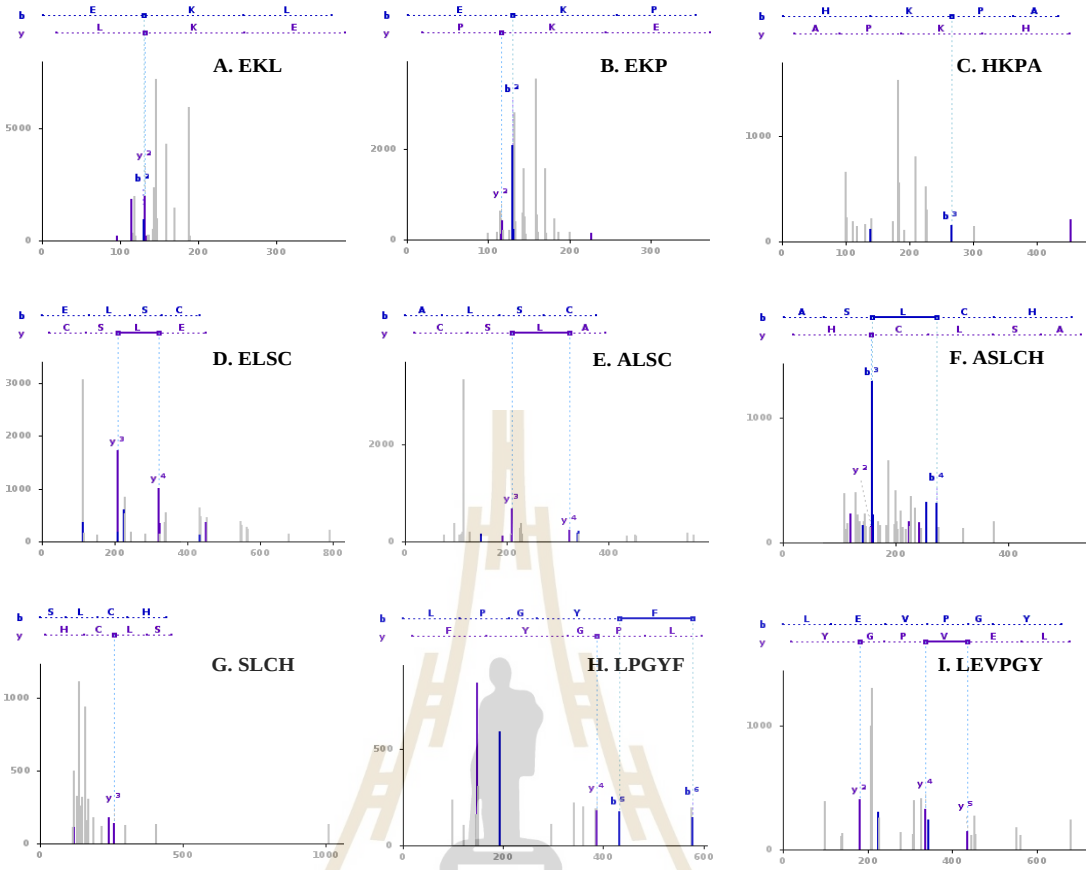

Figure 5.2 *de novo* sequencing MS/MS spectrum of identified peptides, EKL (A), EKP (B), HKPA (C), ELSC (D), ALSC (E), ASLCH (F), SLCH (G), LPGYF (H), and LEVPGY (I).

Table 5.2 The fraction, protein source of identified antioxidant peptides and their *in silico* GI digestion cleave sites [1]

No.	Source (fraction, MS compound)	Parent peptides	*In silico* GI digestion *	Target peptides	Protein database	Accession sequence ID (sequence range)	Homologous protein	Previously reported bioactive peptides (from Biopep peptide database)			
								Antioxidative	ACE inhibitor	DPP IV inhibitor	PEP inhibitor
1	$R_2F_{3.3,}$ Cmpd 24	EKL	**EK-**L	**K-containing peptides:** EKL, EK, EKP, HKPA, KPA	UniProtKB	I3KR57(478-480, 907-909)	Myosin Via	KP, LKP, VKP, YAKPA and YAKP; HK (Zheng, Zhao, Dong, et al., 2016); KS, KA, AKG, TKA, VK, MK and IKG (X.-R. Yang et al., 2019)	EK, LEK, EKGP, IIAEK, KL, LKLP, KP, IKP, GKP, LKP, MKP, VIKP, YAKP, YAKPA, VLKP, VKP, KPVAAP	EK, KP	KPPI, KPPV
						I3K775(402-404)	Myosin IHa				
						I3J6R8(1720-1722, 1773-1775)	Zinc finger protein 292a				
2	$R_2F_{2.3}$, Cmpd 9	EKP	**EKP**		UniProtKB	I3KVR4(537-539)	Myosin phosphatase Rho interacting protein				
						I3J596(178-180)	Actin-related protein 2/3 complex subunit				
3	$R_2F_{3.5}$, Cmpd 4	HKPA	H-**KPA**		UniProtKB	I3KFA1(204-207),	Zinc finger, MYM-type 2,				
						I3K7A1(412-415)	Zinc finger and BTB domain containing 12, tandem duplicate 1				
4	$R_2F_{2.3}$, Cmpd 36, 41, 45, $R_2F_{2.4}$, Cmpd 34	ELSC	E-L-**SC**	**C-containing peptides:** ELSC, ALSC, SC	UniProtKB	I3JST4(10211024, 1472-1475)	Obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	EL, ELLI, ISELGW, GFGPEL, ALSAF, VCSV and CSQAPLA; CS (Zheng, Zhao, Dong, et al., 2016)	AEL, VIPEL, FAL, ALEP	AL, ALAV, ALP, RALP	
						I3K427(528-531)	Tyrosine-protein kinase				
						I3JJE4(1358-1361)	Zinc finger, SWIM-type containing 8				
						I3J8Q3(708-711)	Aminopeptidase				
.5	$R_2F_{4.2}$, Cmpd 13	ALSC	A-L-**SC**		UniProtKB	XP_003445054.1(1354-1359)	plexin-A4 isoform X2				
						XP_005449468.1(512-517)	lon protease homolog 2, peroxisomal				

Table 5.2 The fraction, protein source of identified antioxidant peptides and their *in silico* GI digestion cleave sites [1] (Continued)

No.	Source (fraction, MS compound)	Parent peptides	*In silico* GI digestion *	Target peptides	Protein database	Accession sequence ID (sequence range)	Homologous protein	Previously reported bioactive peptides (from Biopep peptide database)			
								Antioxidative	ACE inhibitor	DPP Ⅳ inhibitor	PEP inhibitor
6	$R_2F_{1.2}$, Cmpd 28	ASLCH	AS-L-**CH**	**C-containing peptides:** ASLCH, SLCH, CH	NCBI Blastp	XP_019222017.2(116-120)	zinc finger protein 318	CHI; CG, CQ, CE, GC and PC (Zheng, Zhao, Dong, et al., 2016)	ASL	AS, SL	
						XP_019211841.1(249-253)	RING finger protein 145 isoform X1				
7	$R_2F_{1.3}$, Cmpd 10	SLCH	S-L-**CH**			XP_025763933.1(434-437)	zinc finger protein 638				
						XP_013129112.2(37-40)	collagen alpha-5(Ⅳ) chain				
8	$R_2F_{4.4}$, Cmpd 39	LPGYF	L-**PGY**-F	**Y-containing peptides:** LPGYF, PGY, LEVPGY, EVPGY	NCBI Blastp	XP_003450194.1(182-186)	mitochondrial ornithine transporter 1 isoform	YG (Zheng, Zhao, Dong, et al., 2016); GY (Liang et al., 2020); TY and SGGY (Feng et al., 2019); YGS (L. Zheng, Su, Ren, Gu, You, & Zhao, 2012); YPG (Ren et al., 2018); GYP (Ohata, Uchida, Zhou, & Arihara, 2016); GFGPGL and GPGGFI	PGL, LPG, PG, IAPG, KVLPG	PPG, LAPPG, GPAG, APG, PPG, GPGA, GPGE, PG	PG
9	$R_2F_{4.3}$, Cmpd 6	LEVPGY	L-**EVPGY**		NCBI Blastp	XP_005451705.1(1393-1398)	plexin-A4 isoform X1				

[1] Excepted for the peptides was noted by the references, the previously reported near antioxidant, angiotensin Ⅰ-converting enzyme (ACE), dipeptidyl peptidase Ⅳ (DPP Ⅳ), and prolyl endopeptidase (PEP) inhibitory peptide sequences, which were searched by BIOPEP database on June 6th, 2019.

* the '-' among the peptide sequences indicated the cleavable sites upon in silico GI digestion.

5.4.3 Antioxidant activities of peptides

5.4.3.1 $ABTS^{\bullet+}$ scavenging capacity

K-containing peptides appeared to be less effective antioxidant as compared to the C- and Y-containing peptides (Figure 5.3A-B). The 50% effective concentration (EC_{50}) of C- and Y-containing peptides ranged from 2 to 17 μM, which were higher than that of ascorbic acid ($p < 0.05$, Figure 5.3B). They were also comparable with other antioxidants including Trolox, glutathione (GSH), or gallic acid. Previous reports have shown that Y- or C-containing peptides, YG, YS, YQ, CG, CS, CQ, and PC, possessed the $ABTS^{\bullet+}$ scavenging capacity in the range of 0.50 – 5.00 μmol Trolox equivalents/μmol peptide (Zheng, Zhao, Dong, et al., 2016). Peptides containing C and Y, VECYGPNRPQF, also showed high $ABTS^{\bullet+}$ scavenging capacity with EC_{50} of 9.8 ± 0.5 μM (Sheih et al., 2009). However, K-containing peptides, namely HK, VELLVPK, AGNQVLNLQADLPK, SVPQPK and EVPKA, showed almost no $ABTS^{\bullet+}$ scavenging capacity (Hernández-Ledesma, Quirós, Amigo, & Recio, 2007; Wiriyaphan, Chitsomboon, Roytrakul, & Yongsawadigul, 2013; Zheng, Zhao, Dong, et al., 2016). The EC_{50} of some other K-containing peptides, including KS, KA, AKG, TKA, VK, MK and IKG, ranged from 1 to 30 mM (Yang et al., 2019). Therefore, peptides containing C or Y amino acid residues would be more effective than those containing K residue towards $ABTS^{\bullet+}$ scavenging capacity.

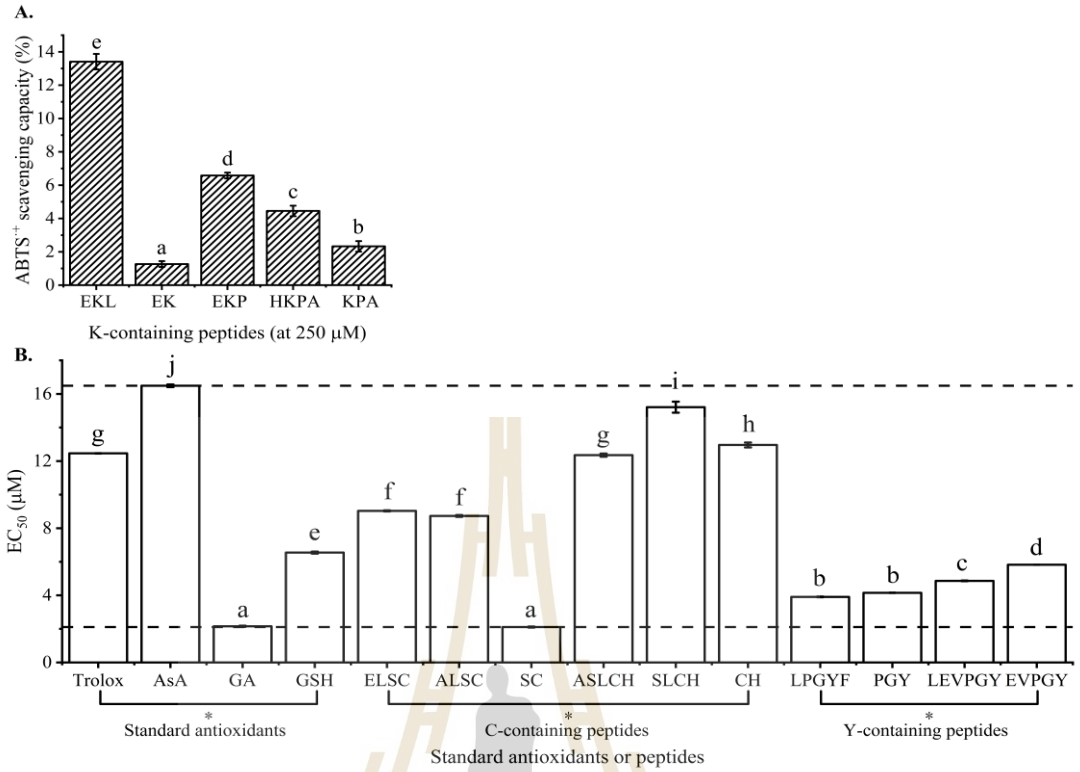

Figure 5.3 ABTS$^{•+}$ scavenging capacity of synthetic K-containing peptides at 250 μM (A), 50% effective concentration (EC_{50}) of standard antioxidants (Trolox, AsA, GA, GSH), synthetic C- and Y-containing peptides (B).

Different lowercase letters indicated the differences in the mean values of the ABTS$^{•+}$ scavenging capacity of synthetic peptides or standard antioxidants ($p < 0.05$). AsA, ascorbic acid; GA, garlic acid; GSH, glutathione.

Additionally, peptides EKL, HKPA, LEVPGY and ASLCH were likely to be digested under GI tract (Table 5.2), and the less active peptides, EK, KPA, EVPGY, and CH, would be generated (Figure 5.3A-B, $p < 0.05$). However, activity of LPGYF was comparable to its digested product, PGY ($p > 0.05$). Peptides ELSC and ALSC, and SLCH were digested to SC and CH, respectively, which showed higher activity

than its parent peptides ($p < 0.05$). The antioxidant activity of tilapia peptides displayed variations after GI digestion. It was worth noting that when amino acid residues of L, P, H, F, A, V, S, or E were cleaved from the C- or N-terminus of parent peptides, activity of digested peptides did not change tremendously. The K-containing peptides upon digestion (EK and KPA) still showed weak $ABTS^{\bullet+}$ scavenging capacity of < 4% at 250 μM (Figure 5.3A). The C- and Y-containing digested peptides, SC, CH, PGY or EVPGY, remained strong activity with EC_{50} < 14 μM (Figure 5.3B). Zheng, Zhao, Dong, et al. (2016) reported that free amino acids, namely Y and C, showed effective $ABTS^{\bullet+}$ scavenging capacity as 1 – 6 μmol TE/μmol amino acid, and Y- and C-containing dipeptides also displayed outstanding activity ranged of 0.5 – 5 μmol TE/μmol peptide. Other free amino acids such as H, P, and K showed negligible $ABTS^{\bullet+}$ scavenging capacity, and the H-, P-, and/or K-containing dipeptides also revealed weak activity. Yang et al. (2020) confirmed that Y amino acid residue in tetrapeptide, ATVY, was the crucial residue contributing to $ABTS^{\bullet+}$ scavenging capacity. These results indicated that the requirement of peptides with high free radical scavenging capacity is containing amino acid residues possessing high antioxidant potential such as C and Y. Thus, food proteins rich in C and Y amino acids could be vital sources for production of high antioxidant peptides.

$ABTS^{\bullet+}$ scavenging capacity of EKP was 2 times higher than that of KPA (Figure 5.3A). It could be due to the present of E which readily donates electrons (Nwachukwu et al., 2019). The H residue has been credited as powerful free radical scavenger because the imidazole ring can participate in hydrogen atom or electron transfer (HAT/ET) (Nwachukwu et al., 2019). However, activity of CH was 5 times lower than that of SC (Figure 5.3B). The imidazole-imidazole hydrogen bonding has

been proposed between two side chains of H residues at pH 7.8 (Movellan, Wegstroth, Overkamp, Leonov, Becker, & Andreas, 2020). Under the testing pH 7.4 of $ABTS^{\bullet+}$ scavenging assay, the imidazole side chain is partially protonated. Therefore, the formation of strong intramolecular hydrogen bond between imidazole ring and sulfydryl group (SH) of C residue could be presumed, resulting in low hydrogen atom donating ability. When A, S, or/and L were located at the N-terminus of CH-containing peptides, $ABTS^{\bullet+}$ scavenging capacity decreased by 20% of CH (Figure 5.3B). A slight decrease in antioxidant capacity was also observed when L, F, E, and/or V were located at N-terminus of PGY-containing peptides. However, added P or L to the C-terminus of EK, or H located at the N-terminus of KPA, which increased $ABTS^{\bullet+}$ scavenging capacity in the range of 1 – 10 times (Figure 5.3A). Addition of EL or AL to the N-terminus of SC decreased its activity about 3 times (Figure 5.3B). SC and K-containing peptides were varied remarkably by addition of these amino acid residues. Thus, the presence of A, L, P, F, and H in peptides does not necessarily contribute to strong ET capacity, as typically reported (Nwachukwu et al., 2019). Free radical scavenging activity also correlated with the bond dissociation energy (BDE) and ionization potential (IP) of the active group such as thiol group in C-containing peptide (Leung, Venus, Zeng, & Tsopmo, 2018). BDE and IP of peptide vary with the peptide sequences. CQV had the lowest BDE and IP than that of QVC and QCV, while it showed the highest hydroxyl radical scavenging capacity (Leung et al., 2018).

5.4.3.2 Intracellular ROS scavenging

All peptides were non-toxic up to 100 μM. Among all C-and Y-containing peptides at 10 μM, LPGYF showed the highest intracellular ROS scavenging capacity ($p < 0.05$), and SLCH exhibited the lowest ($p < 0.05$, Figure 5.4).

However, the activity of other C- and Y-containing peptides were comparable ($p > 0.05$), and they were slightly higher than that of K-containing peptides ($p < 0.05$). Intracellular ROS scavenging capacity of peptides increased with concentration ($p < 0.05$) and reached plateau at concentrations > 50 µM (Figure 5.4). At 50 µM, SC, CH, LPGYF and PGY displayed higher activity than 50 µM ascorbic acid ($p < 0.05$), while other peptides showed comparable activity except for EKL exhibiting lower activity ($p < 0.05$). Thus, peptides, SC, CH, and PGY, derived from the *in silico* GI digestion and the parent peptide, LPGYF, were the potent antioxidant peptides diminishing the excessive intracellular ROS.

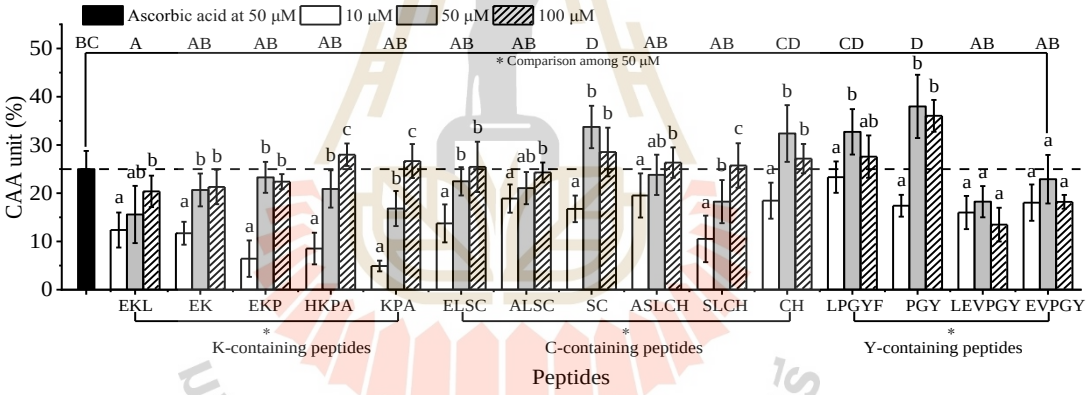

Figure 5.4 Intracellular ROS scavenging capacity (CAA unit) of ascorbic acid and synthetic peptides. Different lowercase letters indicate differences in the mean values of the CAA of each peptide at 10, 50, 100 µM ($p < 0.05$).

Different uppercase letters indicate differences in the mean values of the CAA among ascorbic acid and all peptides at 50 µM ($p < 0.05$). CAA, cellular antioxidant activity.

Our results also indicated that intracellular ROS scavenging capacity of peptides did not necessarily correlate with their $ABTS^{\bullet+}$ scavenging (Figure 5.3 & Figure 5.4). This was due to different mechanism underlying these assays. The $ABTS^{\bullet+}$ assay monitors the reactivity of peptide towards $ABTS^{\bullet+}$ by HAT and/or ET (Apak, Özyürek, Güçlü, & Çapanoğlu, 2016). The reaction is in the aqueous solution and reactivity of the peptide would depend on BDE and IP (Leung et al., 2018; Zheng, Zhao, Xiao, Zhao, & Su, 2016), diffusion limit (Galano & Alvarez-Idaboy, 2011), and steric hindrance (Tian & Schaich, 2013). The $ABTS^{\bullet+}$ scavenging capacity of ALSC, ELSC, SC, LPGYF and PGY, were positively correlated to their intracellular ROS scavenging capacity (Figure 5.3B & Figure 5.4). This positive correlation has also been observed in other peptides, including NTVPAKSCQAQPTTM, FLKKISQRYQKF, ALPQYLKTVYQHQK and IQPKTKVIPYVRYL (Tonolo et al., 2020). In contrast, LEVPGY and EVPGY with excellent free radical scavenging activity, but showed low intracellular ROS scavenging capacity (Figure 5.3 & Figure 5.4). Peptides GLVYIL and YHNAPGLVYIL also showed higher ROO• scavenging capacity than FNDRLRQGQLL and DVNNNANQLEPR, but they showed similar intracellular ROS scavenging capacity (Du et al., 2016). In addition, the digested peptide, CH, displayed higher intracellular ROS scavenging capacity than its parents, ASLCH and SLCH, despite of its moderate free radical scavenging capacity (Figure 5.3B & Figure 5.4). Smaller peptides can preferentially penetrate through cell membranes (Fernández-Musoles et al., 2013; B. Wang et al., 2017; L. Wang et al., 2018), contributing to high intracellular ROS scavenging capacity. Nevertheless, a larger peptide, DVNNNANQLEPR, displayed similar intracellular ROS scavenging capacity to a smaller peptide, GLVYIL

(Du et al., 2016). Thus, size of peptide is not the only factor governing intracellular ROS scavenging capacity.

The amino acid compositions would significantly affect the absorption rate of peptides by cells. According to the Caco2 cell monolayer transportation model, the apparent permeability coefficient (P_{app}) values of GLLLPH was 2 times that of YFCLT (Ding, Wang, Zhang, Yu, & Liu, 2018). RVPSL showed much higher P_{app} value than the smaller WQ, RWQ, LKP and IQW (Ding, Wang, Zhang, & Liu, 2015; Fernández-Musoles et al., 2013; Xu, Fan, Yu, Hong, & Wu, 2017). In addition, peptides containing C, Y or W seemed to have lower absorption rate (Ding et al., 2018; Ding et al., 2015; Ding, Zhang, Jiang, Wang, Liu, & Liu, 2014; Fernández-Musoles et al., 2013; Xu et al., 2017). Peptides, namely DKTEIPTINTIASGEPT, EKDDTGTPITKIELVPSH QGPIVLNPWDQVKR, FNDRLRQGQLL, DVNNNANQLEPR and RDPEER (Du et al., 2016; Tonolo et al., 2020; Wen et al., 2020), which did not contain highly antioxidant potential amino acid residues such as C, Y or W (Zheng, Zhao, Dong, et al., 2016), showed low free radical scavenging capacity, but their effective intracellular ROS scavenging capacity were detected. This was similar trend to K-containing peptides observed in this study. This could be attributed to high absorption rate of K-containing peptides. The cellular uptake rate should be one of the reasons affecting the intracellular ROS scavenging capacity of peptides.

Additionally, the intracellular ROS scavenging capacity of peptide might be varied with its terminal residues, secondary structure (Wong, Xiao, Wang, Ee, & Chai, 2020), peptide metabolites in cells (Du et al., 2016; Wong et al., 2020; Xu et al., 2017), distribution of peptide in cells as cytoplasm or mitochondria (Mirończuk-Chodakowska, Witkowska, & Zujko, 2018), and the diffusion limit of intracellular ROS

generated (Parvez, Long, Poganik, & Aye, 2018; Sies et al., 2020). All novel antioxidant peptides from the tilapia protein hydrolysate and their *in silico* GI digestion displayed effective intracellular ROS scavenging capacity regardless of $ABTS^{\bullet+}$ scavenging capacity. Peptides, SC, CH and PGY, from the *in silico* GI digestion revealed the highest intracellular ROS scavenging capacity and excellent free radical scavenging activity. They were selected to evaluate the effect on gene expression of antioxidant enzymes.

5.4.4 Regulation of gene expression of antioxidant enzymes

In addition to ROS scavenging capacity, peptides might be able to modulate expression of genes encoding antioxidant enzymes (Homayouni-Tabrizi et al., 2017; Tonolo et al., 2020; Wang et al., 2018; Wu et al., 2018). Three selected peptides, SC, CH and PGY, regulate expression of *CAT*, *SOD1* and *GPx1* in both models tested (Figure 5.5), peptide only and peptide+AAPH group. Up-regulation of *CAT* and *SOD1* decreased at 100 μM ($p < 0.05$, Figure 5.5F-G). Expression of *GPx1* was suppressed by all peptides ($p < 0.05$, Figure 5.5H). SOD catalyzes the superoxide anion ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2), which is further decomposed to H_2O and O_2 by catalase (Mirończuk-Chodakowska et al., 2018). H_2O_2 is also reduced by GSH in the reaction catalyzed by GPx. Therefore, 3 peptides induce the expression of *CAT* and *SOD1* enhancing decomposition of intracellular $O_2^{\bullet-}$ and H_2O_2. All peptides were unlikely to increase the CAA through *GPx1* up-regulation. Peptides at 100 μM showed relatively low up-regulation of *CAT* and *SOD1*. This high concentration of peptide showed less effective effect on regulation of antioxidant enzymes which could be one of the reasons explaining comparable intracellular ROS scavenging capacity between 50 and 100 μM ($p > 0.05$, Figure 5.4).

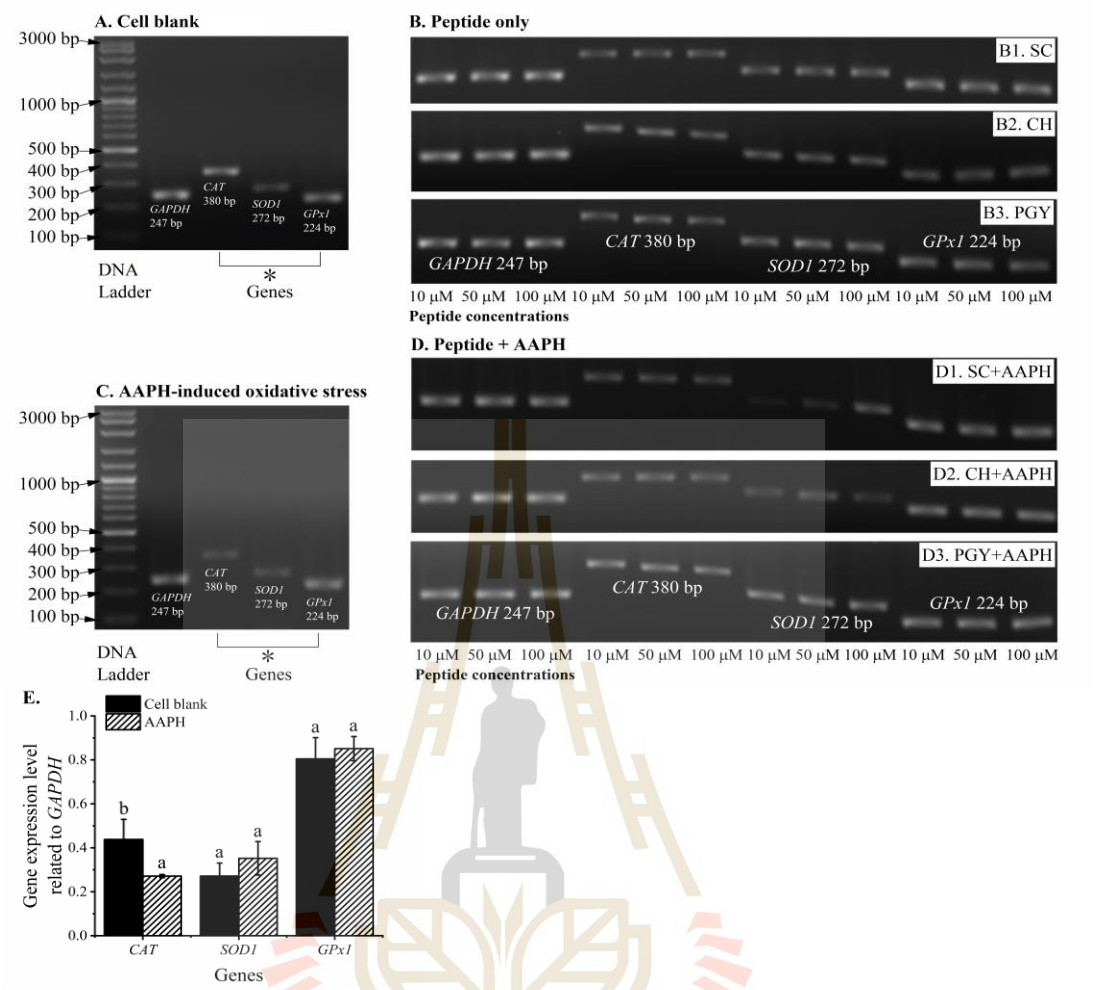

Figure 5.5 Representative 1.5% agarose gel electrophoresis of *CAT*, *SOD1* and *GPx1* for normally grown cells (A); cells were treated by peptide only (B), namely SC (B1), CH (B2), PGY (B3); cells were treated by AAPH (C); and cells were treated by peptide followed by AAPH (D), namely SC+AAPH (D1), CH+AAPH (D2), PGY+AAPH (D3); expression level of *CAT*, *SOD1*, and *GPx1* for cell blank and AAPH-induced oxidative stress group (E); expression level of *CAT* (F), *SOD1* (G), and *GPx1* (H) for cell blank, peptide only and peptide+AAPH group, respectively.

GAPDH, gene encoding glyceraldehyde 3-phosphate dehydrogenase, housekeeping gene; *CAT*, gene encoding catalase; *SOD1*, gene encoding SOD; *GPx1*, gene encoding GPx. The number following the gene of encoding antioxidant enzymes in A-D indicated the RT-PCR product size of genes**.** The different lowercase letters in F-H indicated the differences in the mean values of the gene expression level at respective treatment ($p < 0.05$). SOD, superoxide dismutase; GPx, glutathione peroxidase.

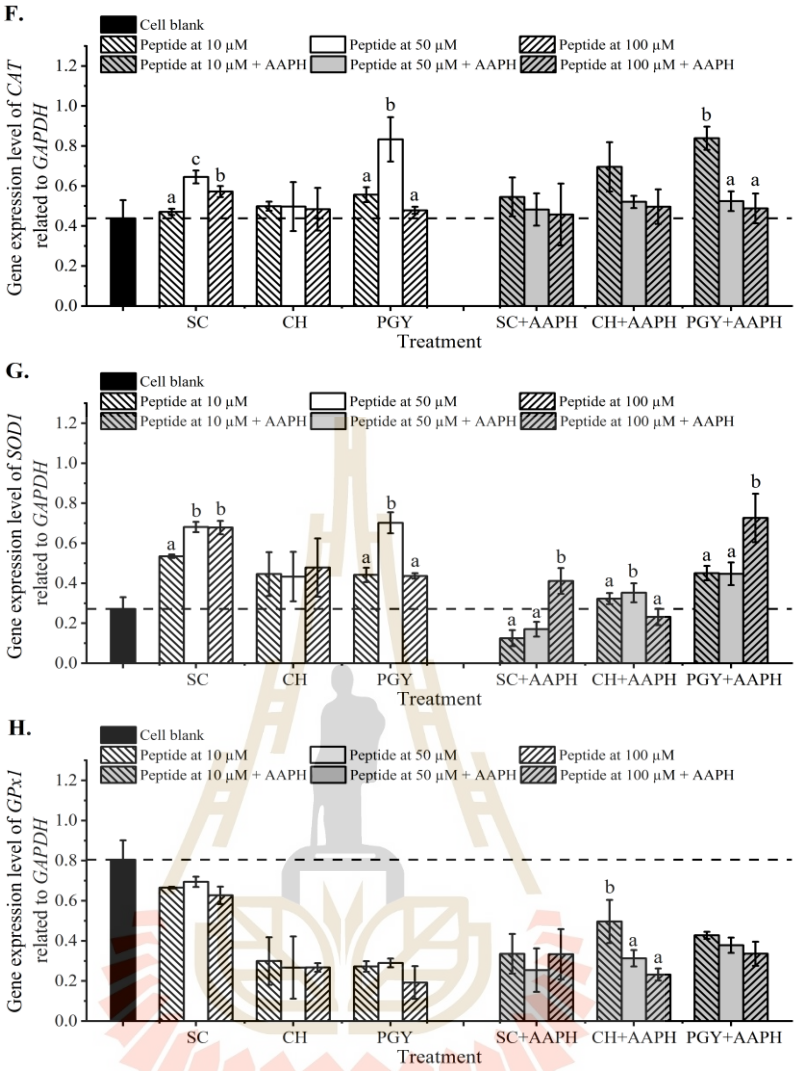

Figure 5.5 Representative 1.5% agarose gel electrophoresis of *CAT*, *SOD1* and *GPx1* for normally grown cells (A); cells were treated by peptide only (B), namely SC (B1), CH (B2), PGY (B3); cells were treated by AAPH (C); and cells were treated by peptide followed by AAPH (D), namely SC+AAPH (D1), CH+AAPH (D2), PGY+AAPH (D3); expression level of *CAT*, *SOD1*, and *GPx1* for cell blank and AAPH-induced oxidative stress group (E); expression level of *CAT* (F), *SOD1* (G), and *GPx1* (H) for cell blank, peptide only and peptide+AAPH group, respectively. (continued)

In peptide only group, the effect of peptide modulating the cellular antioxidant response under normal cell was demonstrated. Expression of *CAT* did not change by CH ($p > 0.05$, Figure 5.5F). It was up-regulated by SC and PGY, and PGY was the best ($p < 0.05$). Expression of *SOD1* was up-regulated by 3 peptides ($p < 0.05$, Figure 5.5G) with SC and PGY being the most effective. Expression of *GPx1* was down-regulated by all peptides ($p < 0.05$, Figure 5.5H). Therefore, PGY was the most potent peptide inducing the expression of *CAT* and *SOD1*, which would enhance activity of catalase and SOD. The peptide, YLEELHRLNAGY, significantly increased the gene expressions of SOD and catalase of HepG2 cells (Homayouni-Tabrizi et al., 2017). Peptides, NTVPAKSCQAQPTTM (oxidized M), QGPIVLNPWDQVKR and APSFSDIPNPIGSENSE, also upregulated expression of glutathione reductase gene (*GRS*) and *SOD1* (Tonolo et al., 2020). Corn gluten hydrolysate fractions with molecular weight of less than 1 kDa and 1-3 kDa down-regulated expression of *CAT*, but up-regulated *GPx1* (Wang et al., 2018). Gene expression of various antioxidant enzymes was inconsistent when the cells were treated by the different peptides. This was due to the various mechanisms involved. Kelch-like ECH associated-protein 1 (Keap1)/nuclear factor erythroid 2–related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway plays a key role in cell response to oxidative stress (De Freitas Silva et al., 2018; Sies et al., 2020; Vriend & Reiter, 2015). Under normal condition, the transcription factor for antioxidant enzymes, Nrf2, is ubiquitinated by the E3 ubiquitin ligase complex, Keap1-Cul3-Rbx1, and degraded by the proteasome in the cytosol. When the electrophiles such as chalcones and peptides (De Freitas Silva et al., 2018; Tonolo et al., 2020) modified Keap1, the Nrf2 was dissociated in the cytosol. Nrf2 was then phosphorylated and migrated to the nucleus. The nuclear Nrf2 with Maf

(a coactivator for Nrf-2) activates the DNA promoter (ARE) leading to the transcription of phase Ⅱ antioxidant enzymes. Thus, the dissociation of Keap1/Nrf2 complex in the cytosol before nuclear translocation of Nrf2 is crucial for the activation of Keap1/Nrf2/ARE signaling pathway. The C amino acid residues in Keap1 could be modified via Michael reaction leading to conformational changes and the subsequent release of Nrf2 (De Freitas Silva et al., 2018). Antioxidant peptides, NTVPAKSCQAQPTTM, QGPIVLNPWDQVKR and APSFSDIPNPIGSENSE, would also bind to the Keap1 and thereby free Nrf2 (Tonolo et al., 2020). In addition to the modification of Keap1, melatonin as a proteasome inhibitor induced accumulation of Nrf2 in nucleus, stimulating the transcription of antioxidant enzymes (Vriend et al., 2015). The tested peptides, SC and CH, might involve in modification of the C residues in Keap1 via formation of disulfide bond induced by ROS. PGY might be the potentially proteasome inhibitor as it could inhibit angiotensin Ⅰ-converting enzyme (ACE) and dipeptidyl peptidase IV (DPP-IV) according to BIOPEP. This might explain why PGY up-regulated the expression of *CAT* and *SOD1*. In addition, K-containing peptides would be the effective ACE and DPP-IV inhibitor according to BIOPEP, which might also be the proteasome inhibitor that can stimulate the transcription of antioxidant enzymes. This could be another reason explaining why K-containing peptides showed weak free radical scavenging capacity but effective intracellular ROS scavengers.

The dissociation of Keap1/Nrf2 complex is also induced by the ROS (Sies et al., 2020; Vriend et al., 2015). The adaptive oxidative stress would induce the cellular stress responses to increase the intrinsic antioxidant activity by up-regulation the expression of antioxidant enzymes (Sies et al., 2020). However, the excessive ROS

would induce cellular oxidative damage resulting in down-regulation of gene expression of antioxidant enzymes. The free radical generator, AAPH, down-regulated expression of *CAT* compared with the control ($p < 0.05$, Figure 5.5E), but had no effect on expression of *SOD1* and *GPx1* ($p > 0.05$). Expression of *CAT* and *SOD1* was also down-regulated by H_2O_2-induced HepG2 cells but up-regulated the expression of *GPx1* (L. Wang et al., 2018). Thus, in addition to the peptide directly stimulates the gene expression of antioxidant enzymes as shown in the peptide only group, the regulation of the gene expression of antioxidant enzymes were also evaluated under peptides protected cell against oxidative stress as the peptide+AAPH group (Figure 5.5).

When cells were protected by peptides before addition of AAPH, expression of *CAT* was up-regulated by 10 μM PGY ($p < 0.05$, Figure 5.5E & F), but not at higher concentrations. In contrast, expression of *SOD1* was increased to the highest by 100 μM PGY ($p < 0.05$, Figure 5.5G). Expression of *GPx1* was still suppressed in the peptide+AAPH group (Figure 5.5H). Therefore, the 3 peptides up-regulated the expression of *CAT* and *SOD1*, leading to enhance catalase and SOD activities to improve the intrinsic CAA against the AAPH-induced oxidative stress. Peptides could directly scavenge the intracellular ROS, which would reduce to the level of cellular stress responses (Sies et al., 2020). Thus, cells under protection of peptides also up-regulated the expression of *CAT* and *SOD1*. However, expression of *CAT* and/or *SOD1* decreased at higher concentrations (Figure 5.5F-G). It might be because of the pro-oxidation of peptides at higher concentrations. Melatonin at ＞1 μM increased the intracellular ROS level (Bejarano, Espino, Barriga, Reiter, Pariente, & Rodriguez, 2011). Additionally, the activation of kinases phosphorylate Nrf2 also leads to nuclear translocation of Nrf2, up-regulating transcription of antioxidant enzymes (De Freitas

Silva et al., 2018; Joo, Kim, Lee, Kim, Koo, & Kim, 2016; Minelli, Conte, Grottelli, Bellezza, Cacciatore, & Bolaños, 2009). Dipeptide, cyclo(H-P), would activate p38 mitogen-activated protein kinase (MAPK), leading to Nrf2-mediated up-regulation of ARE-related genes and protected cells against H_2O_2-mediated apoptotic on PC12 cells (Minelli et al., 2009). Tripeptide, PHP, decreased the affinity of Nrf2 for Keap1 on AAPH-induced HepG2 cell oxidative stress, resulting in nuclear translocation of Nrf2 thereby up-regulating expression of catalase, SOD and GPx (Wu et al., 2018).

Our results indicated that antioxidant peptides, SC, CH and PGY, effectively scavenge intracellular ROS. They would enhance intrinsic cellular antioxidant activity for prevention of oxidative stress by stimulating the expression of *CAT* and *SOD1*, which would, in turn, enhance catalase and SOD activity. Under the actual oxidative stress, peptides also protect the cells against oxidative stress by up-regulate the expression of *CAT* and *SOD1*. PGY was the best peptides from both models tested, and CH was the least effective. Mechanisms involved in cellular antioxidant activities of these peptides should be further investigated.

5.5 Conclusion

Tilapia peptides with high $ABTS^{\bullet+}$ scavenging capacity were less polar molecules containing amino acid residues such as C and Y. All novel C-, Y- and K-containing peptides displayed effective intracellular ROS scavenging capacity regardless of their $ABTS^{\bullet+}$ scavenging capacity. LPGYF was the best parent peptide to scavenge $ABTS^{\bullet+}$ and intracellular ROS. The *in silico* GI digested peptides, SC, CH, and PGY, showed high $ABTS^{\bullet+}$ and intracellular ROS scavenging capacity. They also up-regulated expression of *CAT* and *SOD1*. PGY was the best in all peptides based on

cellular assays. Thus, peptides did not only scavenge intracellular ROS, but also stimulate expression of *CAT* and *SOD1* to improve the intrinsic CAA. Tilapia protein-derived antioxidant peptides could be the potent nutraceuticals.

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CHAPTER VI

SUMMARY

Antioxidant activities of both hydrolysates and glycated hydrolysates did not appear to reflect the activities of the digesta counterparts. Digesta of hydrolysate at 16 h of hydrolysis exhibited lower antioxidant activities than did the 10-h hydrolysate digesta although the hydrolysate of the former possessed higher activities. The protein digesta also showed the least antioxidant activities. Thus, the excessive hydrolysis of 16 h might not necessarily yield peptides with the highest activities upon *in vitro* GI digestion. Additionally, glycation of hydrolysates improved the chemical antioxidant activities, especially the reducing power. Glycated hydrolysate would be useful to retard oxidative deteriorations in food system. However, the higher extent of glycation led to lower digestibility and intracellular ROS scavenging capacity upon *in vitro* GI digestion. All digested products of glycated hydrolysates were not effective at intracellular ROS scavenging capacity. Glycation of tilapia hydrolysate would not be effective to improve antioxidant activity in biological system. Antioxidant activity of digesta should be considered when optimizing the condition for production of protein hydrolysate or its derivatives for nutraceutical products.

Tilapia peptides with high $ABTS^{\bullet+}$ scavenging capacity were less polar molecules containing amino acid residues, such as C and Y**.** All novel C-, Y- and K-containing peptides displayed effective intracellular ROS scavenging capacity

regardless of their $ABTS^{\bullet+}$ scavenging capacity**.** The *in silico* GI digested tilapia peptides, SC, CH, and PGY, showed high $ABTS^{\bullet+}$ and intracellular ROS scavenging capacity. They also stimulate expression of *CAT* and *SOD1* to improve the intrinsic CAA. GI digested peptide, PGY, was the best in all peptides based on cellular assays. Tilapia protein-derived antioxidant peptides could be the potent nutraceuticals. But antioxidant peptides from the digesta of hydrolysate should be further characterized to understand the mechanisms involved in cellular antioxidant activities.

APPENDIX

Table A.1 Elution programs for peptide purifications

Segments	Target %B	Elution volume (mL)	Flow rate (mL/min)	**Segments**	Target %B	Elution volume (mL)	Flow rate (mL/min)
SEC* of *H							
Sampling	0.00	0.30	0.80	**7**	100.00	0.70	0.80
1	0.00	5.00	0.80	**8**	2.50	0.00	0.80
2	100.00	0.00	0.80	**9**	2.50	1.30	0.80
3	100.00	0.80	0.80	**10**	100.00	0.00	0.80
4	2.50	0.00	0.80	**11**	100.00	7.00	0.80
5	2.50	2.20	0.80	**12**	0.00	0.00	0.80
6	100.00	0.00	0.80	**13***	0.00	33.00	0.80
***RPC*$_1$ of *SF*$_8$**							
Sampling	2.00	0.30	0.20	**5**	85.00	3.00	0.40
1	2.00	4.70	0.20	**6**	2.00	2.50	1.00
2	10.00	20.00	0.40	**7***	2.00	13.00	1.00
3	22.50	12.50	0.40	**8***	2.00	1.00	0.20
4	85.00	7.50	0.40				
***RPC*$_2$ of *R*$_1$*F*$_6$**							
Sampling	12.00	0.60	0.20	**5**	90.00	5.00	0.20
1	12.00	6.00	0.20	**6**	12.00	0.00	1.00
2	13.50	0.00	0.20	**7***	12.00	10.00	1.00
3	13.50	3.00	0.20	**8***	12.00	1.00	0.20
4	90.00	0.00	0.20				

Table A.1 Elution programs for peptide purifications (Continued)

Segments	Target %B	Elution volume (mL)	Flow rate (mL/min)	Segments	Target %B	Elution volume (mL)	Flow rate (mL/min)
***RPC*$_2$ of R_1F_7**							
Sampling	13.00	0.60	0.20	**4**	13.00	0.00	1.00
1	13.00	17.50	0.20	**5***	13.00	10.00	1.00
2	90.00	0.00	1.00	**6***	13.00	1.00	0.20
3	90.00	5.00	1.00				
***RPC*$_2$ of R_1F_8**							
Sampling	16.00	0.60	0.20	**4**	16.00	0.00	1.00
1	16.00	17.50	0.20	**5***	16.00	10.00	1.00
2	90.00	0.00	1.00	**6***	16.00	1.00	0.20
3	90.00	5.00	1.00				
***RPC*$_2$ of R_1F_9**							
Sampling	22.00	0.60	0.20	**4**	22.00	0.00	1.00
1	29.00	14.00	0.30	**5***	22.00	10.00	1.00
2	90.00	0.00	0.30	**6***	22.00	1.00	0.20
3	90.00	5.00	0.30				

* for column equilibration.

Table A.2 Elution programs for peptide identifications

Segments	Retention time (min)	Target %B	**Segments**	Retention time (min)	Target %B
***R*$_2$*F*$_{1.2}$ and *R*$_2$*F*$_{2.2-2.5}$**					
1	0	5	**6**	50	80
2	0	5	**7**	55	80
3	5	13	**8**	57	5
4	40	30	**9**	60	5
5	45	40			
***R*$_2$*F*$_{1.3-1.4}$**					
1	0	5	**5**	45	80
2	0	5	**6**	55	80
3	5	14	**7**	57	5
4	40	25	**8**	60	5
***R*$_2$*F*$_{3.1-3.5}$**					
1	0	5	**6**	45	80
2	0	5	**7**	56	80
3	3	16	**8**	57	5
4	38	28	**9**	60	5
5	43	55			
***R*$_2$*F*$_{4.2}$**					
1	0	5	**6**	45	80
2	0	5	**7**	56	80
3	3	20	**8**	57	5
4	38	25	**9**	60	5
5	43	55			
***R*$_2$*F*$_{4.3-4.5}$**					
1	0	5	**6**	45	80
2	0	5	**7**	56	80
3	3	20	**8**	57	5
4	38	32	**9**	60	5
5	43	55			

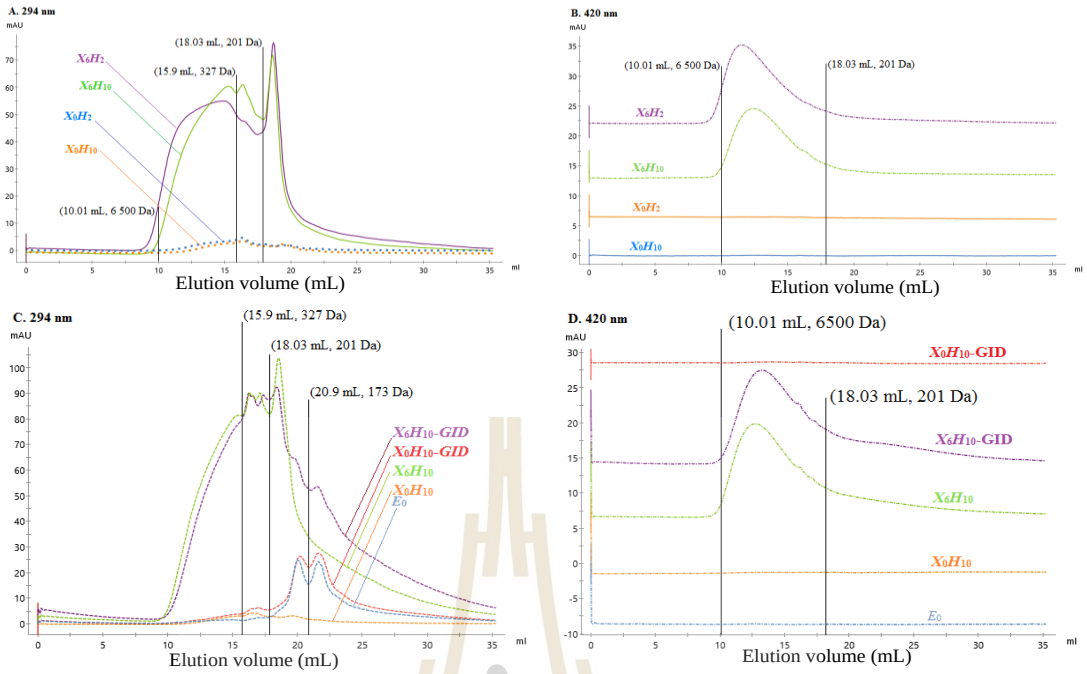

Figure A.1 Size exclusion chromatography (*SEC*), the typical chromatograms of un-glycated hydrolysates and glycated hydrolysates at 294 nm (A) and 420 nm (B), and the typical chromatograms of digesta of un-glycated hydrolysate and glycated hydrolysate at 294 nm (C) and 420 nm (D).

The S_tH_n was the glycated hydrolysate, where, *S* was the abbr. of type of sugars, and *G* = glucose, *F* = fructose, and *X* = xylose; t was the glycation time as 0, 2, 6, and 12 h; H_2 and H_{10} were the 2- and 10-h hydrolysates, respectively. The *GID* following the sample name was used to indicate the GI digesta of samples. E_0 was the enzyme blank of GI digestion.

BIOGRAPHY

Xiaogang Zhang was born in January 26th, 1986 in Guang'an city, Sichuan province, China. He studied for high school at Guang'an Daishi Middle School of Sichuan Province (1999-2005). In 2009, he received the degree of Bachelor of Engineering in Food Science and Engineering from Xichang College, Xichang city, Sichuan province, China. In 2012, he received the degree of Master of Agriculture in Food Science from Guizhou University, Guiyang city, Guizhou province, China. In 2015, he received the letter of admission to study for the degree of Doctor of Philosophy (Food Technology) at Suranaree University of Technology. In 2016, he received the One Research One Grant (OROG) Scholarship from Suranaree University of Technology. During his graduate study, he obtained opportunities to present his research works at the Food Innovation Asia Conference (FIAC) from 2018 to 2020, BITEC, Bangkok, Thailand. He awarded “First Place for the poster presentation” in FIAC2018. He also published his researched work under the title “Chemical and cellular antioxidant activities of *in vitro* digesta of tilapia protein and its hydrolysates” in Foods (volume 9, issue 6, page 833) in 2020.