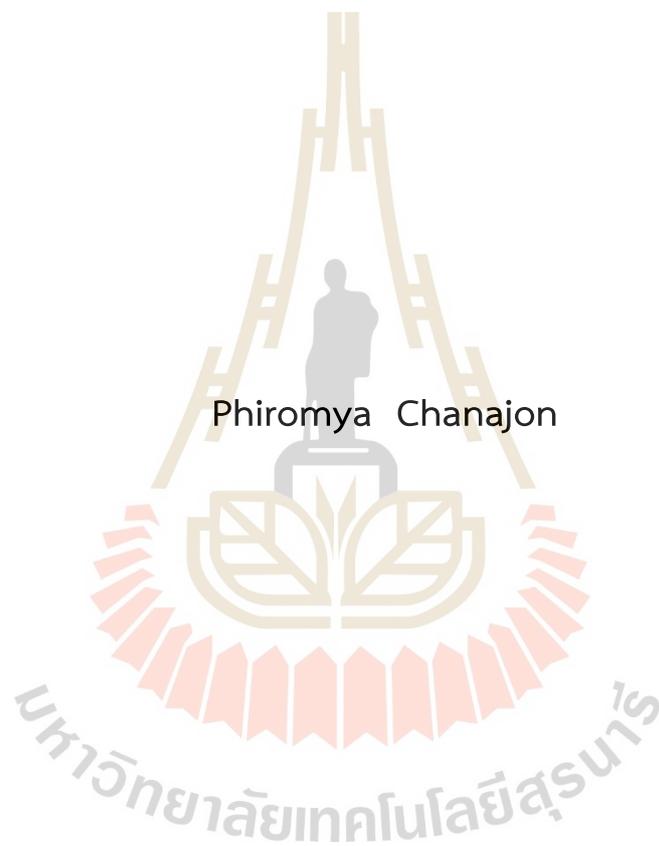# MULTI-BIOACTIVE FUNCTIONS OF ANTIOXIDANT, PROLYL OLIGOPEPTIDASE AND ANGIOTENSIN-I-CONVERTING ENZYME INHIBITORY ACTIVITIES OF CORN GLUTEN MEAL HYDROLYSATE

Phiromya Chanajon

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Food Technology

Suranaree University of Technology

Academic Year 2022

คุณสมบัติเชิงหน้าที่ทางชีวภาพของการต้านอนุมูลอิสระ การยับยั้งเอนไซม์โพรลิลโอลิโกเพปทิเดส และแองจิโอเทนซิน-คอนเวอร์ติงเอนไซม์ของไฮโดรไลเสทจากกากโปรตีนข้าวโพด

นางสาวภิรมณ์ยา ชนะจน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปรัชญาดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2565

MULTI-BIOACTIVE FUNCTIONS OF ANTIOXIDANT, PROLYL OLIGOPEPTIDASE AND ANGIOTENSIN-I-CONVERTING ENZYME INHIBITORY ACTIVITIES OF CORN GLUTEN MEAL HYDROLYSATE

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

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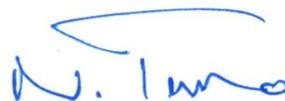_____
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ภิรมณ์ยา ชนะจน : คุณสมบัติเชิงหน้าที่ทางชีวภาพของการต้านอนุมูลอิสระ การยับยั้ง
เอนไซม์โพรลิลโอลิโกเพปทิเดส และแองจิโอเทนซิน-คอนเวอร์ติงเอนไซม์ของไฮโดรไลเสทจาก
กากโปรตีนข้าวโพด (MULTI-BIOACTIVE FUNCTIONS OF ANTIOXIDANT, PROLYL
OLIGOPEPTIDASE AND ANGIOTENSIN-I-CONVERTING ENZYME INHIBITORY
ACTIVITIES OF CORN GLUTEN MEAL HYDROLYSATE) อาจารย์ที่ปรึกษา :
รองศาสตราจารย์ ดร. จิรวัฒน์ ยงสวัสดิกุล, 116 หน้า.

คำสำคัญ: เพปไทด์ยับยั้งโพรลิลโอลิโกเปปทิเดส/เพปไทด์ยับยั้งแองจิโอเทนซิน-คอนเวอร์ติง
เอนไซม์/กิจกรรมต้านอนุมูลอิสระ/ไฮโดรไลเสทจากกากโปรตีนข้าวโพด

การศึกษานี้มีวัตถุประสงค์เพื่อประเมินความสามารถในการต้านอนุมูลอิสระ และการยับยั้งกิจกรรม
ของเอนไซม์โพรลิลโอลิโกเพปทิเดส (Prolyl oligopeptidase; POP) และแองจิโอเทนซิน-คอนเวอร์ติง
เอนไซม์ (Angiotensin-I-converting enzyme; ACE) ของไฮโดรไลเสทจากกากโปรตีนข้าวโพด ศึกษา
กิจกรรมการต้านอนุมูลอิสระและผลต่อการลดการรวมตัวของโปรตีนแอลฟ่าซินิวคลีอิน (α-synuclein)
ในเซลล์ประสาท SH-SY5Y ของเพปไทด์ที่มีฤทธิ์ในการยับยั้งเอนไซม์โพรลิลโอลิโกเพปทิเดส นอกจากนี้
ประเมินความสามารถในการยับยั้งกิจกรรมของแองจิโอเทนซิน-คอนเวอร์ติงเอนไซม์   รวมทั้งฤทธิ์การลด
ความดันโลหิตของเพปไทด์ และเพปไทด์ที่เกิดจากการการย่อยด้วยระบบย่อยอาหารจำลองทาง
คอมพิวเตอร์   กากโปรตีนข้าวโพดที่ถูกย่อยด้วยเพปซินมีความสามารถในการยับยั้งเอนไซม์โพรลิลโอลิโก
เพปทิเดส และกิจกรรมการต้านอนุมูลอิสระ อย่างไรก็ตามกิจกรรมเหล่านี้มีค่าลดลงหลังจากผ่านการย่อย
ด้วยระบบย่อยอาหารในหลอดทดลอง   นอกจากนี้ไฮโดรไลเสทจากกากโปรตีนข้าวโพดมีความสามารถใน
การปกป้องเซลล์จากสภาวะเครียดออกซิเดชัน (oxidative stress) ในเซลล์ที่กระตุ้นด้วยไฮโดรเจนเปอร์
ออกไซด์ (H_2O_2) แต่สมบัตินี้ลดลงหลังผ่านกระบวนการย่อยอาหาร อย่างไรก็ตาม ทั้งไฮโดรไลเสทและ
ไฮโดรไลเสทที่ผ่านการย่อยด้วยระบบย่อยอาหารจำลองยังคงมีศักยภาพในการลดภาวะเครียดออกซิ
เดชั่นใน SH-SY5Y cells โดยการกำจัดอนุมูลอิสระออกซิเจนที่ไวต่อปฏิกิริยา (reactive oxygen
species; ROS) และเพิ่มการแสดงออกของยีนส์เอนไซม์คาตาเลส

เพปไทด์ 3 สายที่แสดงกิจกรรมการยับยั้งเอนไซม์โพรลิลโอลิโกเพปทิเดสถูกแยกได้จาก
ไฮโดรไลเสทของกากโปรตีนข้าวโพด โดยที่เพปไทด์ ALLTLSPLGPA แสดงกิจกรรมการยับยั้งได้ดีที่สุด
อย่างไรก็ตาม เพปไทด์นี้ถูกย่อยด้วยเอนไซม์ในระบบย่อยอาหารทางคอมพิวเตอร์ ทำให้ได้ชิ้นส่วนเพป
ไทด์เป็น SPLGAP ซึ่งแสดงกิจกรรมในการยับยั้งลดลง แต่แสดงความสามารถในการลดกิจกรรมของ
เอนไซม์โพรลิลโอลิโกเพปทิเดสในระดับเซลล์ ในขณะที่เพปไทด์ ALLTLSPLGPA แสดงการลดการ
รวมตัวของโปรตีน α-synuclein ในเซลล์ประสาท SH-SY5Y นอกจากนี้ สามารถระบุเพปไทด์ 7 สาย
ที่มีสมบัติยับยั้งแองจิโอเทนซิน- คอนเวอร์ติงเอนไซม์ โดยเพปไทด์ KQLLGY แสดงฤทธิ์การยับยั้งได้

สูงสุดที่ความเข้มข้น 0.08±0.01 มก. โปรตีน/มล. สำหรับการยับยั้งปฏิกิริยาครึ่งหนึ่ง (50% inhibitory concentration; IC_{50}) การย่อยเพปไทด์ด้วยเอนไซม์ในระบบย่อยอาหารทางคอมพิวเตอร์ ส่งผลให้ได้เพปไทด์ที่แสดงฤทธิ์การยับยั้งแองจิโอเทนซิน- คอนเวอร์ติงเอนไซม์ ทั้งสูงขึ้นและต่ำลง อย่างไรก็ตาม เพปไทด์ KQL ที่ได้จากการย่อยเพปไทด์ KQLLGY แสดงความสามารถในการลดความ ดันโลหิตในหนูทดลองหลังจาก 8 ชั่วโมงที่ให้กินเพปไทด์ในปริมาณ 30 มก/กก น้ำหนักตัว นอกจากนี้ ไฮโดรไลเสทจากกากโปรตีนข้าวโพดมีความสามารถในการลดความดันโลหิตได้เช่นเดียวกัน โดยที่ ความดันโลหิตในการบีบตัว (systolic blood pressure; SBP) ลดลงหลังจาก 2 ชั่วโมงที่ได้รับ ไฮโดรไลเสทที่ปริมาณ 100 มก./กก. น้ำหนักตัว และลดลงต่ำสุดที่ค่า 24.21±4.34 mmHg ที่ 24 ชั่วโมง ยิ่งไปกว่านั้น การศึกษานี้แสดงให้เห็นว่า ค่าพลังงานในการจับกับเอนไซม์ ค่าคงที่การยับยั้ง เอนไซม์ (K_i) และค่าการยับยั้งแองจิโอเทนซิน- คอนเวอร์ติงเอนไซม์ ไม่สามารถนำมาใช้เพื่อบ่งบอก ถึงความสามารถในการลดความดันโลหิตของเพปไทด์หรือโปรตีนไฮโดรไลเสทได้เสมอไป ซึ่งผล การศึกษานี้ชี้ให้เห็นว่าเพปไทด์ที่ได้จากไฮโดรไลเสทจากกากโปรตีนข้าวโพดอาจนำมาใช้เพื่อพัฒนา อาหารฟังก์ชั่นที่มีฤทธิ์ต่อสมองและเสถียรค่าความดันโลหิต ซึ่งจะนำไปสู่การใช้ประโยชน์ของโปรตีน กากข้าวโพด

สาขาวิชาเทคโนโลยีอาหาร   ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2565   ลายมือชื่ออาจารย์ที่ปรึกษา_____

PHIROMYA CHANAJON : MULTI-BIOACTIVE FUNCTIONS OF ANTIOXIDANT,
PROLYL OLIGOPEPTIDASE AND ANGIOTENSIN-I-CONVERTING ENZYME INHIBITORY
ACTIVITIES OF CORN GLUTEN MEAL HYDROLYSATE, THESIS ADVISOR :
ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 116 PP.

Keyword: PROLYL OLIGOPEPTIDASE INHIBITORY PEPTIDE/ACE-INHIBITORY
PEPTIDE/ANTIOXIDANT ACTIVITY/CORN GLUTEN MEAL HYDROLYSAT

Objectives of this study were to evaluate the antioxidant, prolyl oligopeptidase
(POP) inhibitory, and angiotensin-I-converting enzyme (ACE) inhibitory activities of
corn gluten meal hydrolysate (CGM-H). The cellular antioxidant activity and reducing
α-synuclein aggregation in neuroblastoma SH-SY5Y cells of POP inhibitory peptides
were elucidated. In addition, *in vitro* ACE inhibitory activity and antihypertensive
effect of peptides and their *in silico* gastrointestinal (GI)-digested fragments were
determined. CGM was hydrolyzed by 1% pepsin (w/w), resulting in the sample with
POP inhibitory and antioxidant activities. CGM-H protected SH-SY5Y cells against
hydrogen peroxide (H_2O_2) induced oxidative stress, but this ability decreased upon
simulated GI digestion. CGM-H and CGM-H digesta showed potential to reduce
oxidative stress in SH-SY5Y cells by scavenging ROS and up-regulated the expression
of catalase (*CAT*) gene.

Three novel POP inhibitory peptides were isolated from CGM-H. Peptide
ALLTLSPLGPA showed the most effective inhibition. However, it degraded into
SPLGPA upon *in silico* GI digestion, resulting in a decrease in POP inhibitory activity.
However, SPLGPA was able to inhibit POP in SH-SY5Y cells. Meanwhile, peptide
ALLTLSPLGPA reduced the aggregation of α-synuclein protein in SH-SY5Y cells.
Moreover, seven novel ACE inhibitory peptides were isolated from CGM-H. Among
them, KQLLGY showed the lowest IC_{50} value at 0.08±0.01 mg protein/mL. The *in-
silico* GI digestion of identified peptides resulted in either an increase or a decrease in
ACE inhibitory activity. The binding energy from molecular docking and K_i values of
parent peptides were lower than their GI-digested counterparts, which was related to
their ACE inhibition. Peptide KQL degraded from KQLLGY showed a slight reduction of
systolic blood pressure (SBP) of -7.83 mmHg after 8 h oral administration of

spontaneously hypertensive rats (SHRs) at 30 mg/kg BW as compared to saline group, although it showed lower ACE inhibitory activity than other peptides. Thus, *in vitro* ACE inhibition were not related with the *in vivo* antihypertensive effect. In addition, CGM-H also exerted greater extent of antihypertensive ability after 2 h with SBP reduction of -21.30 mmHg after oral administration at dose of 100 mg/kg BW. These results revealed the greater extent of antihypertensive activity of CGM-H as compared to GI-digested peptide. Therefore, CGM-H and its peptides would be further developed to nutraceutical products targeting brain function and blood pressure stabilization, which would ultimately lead to valorization of CGM.
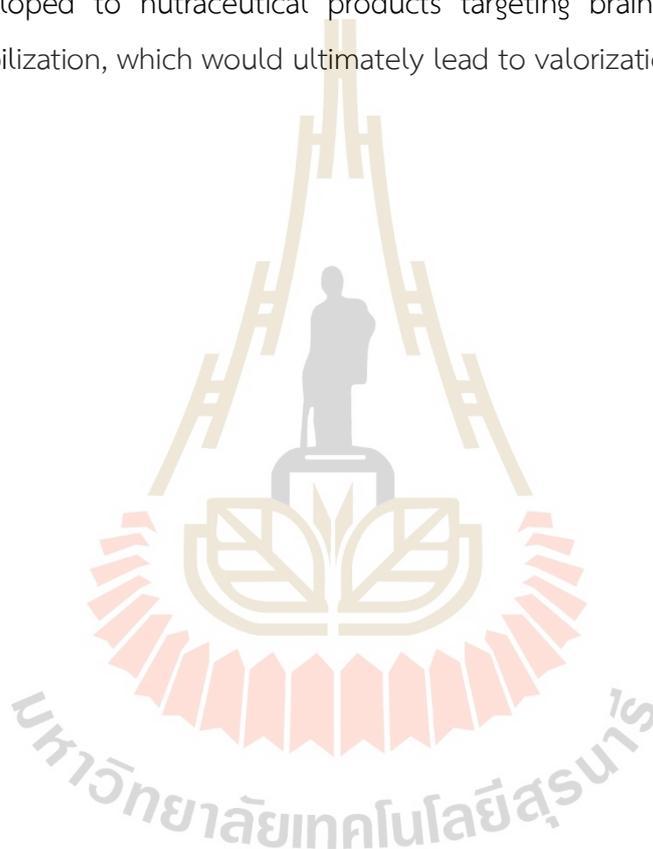
School of Food Technology

Academic Year 2022

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ACKNOWLEDGEMENTS

Phiromya Chanajon

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LIST OF ABBREVIATIONS

CGM	=	Corn gluten meal
CGM-H	=	Corn gluten meal hydrolysate
w/w	=	Weight per weight
IC_{50}	=	Half maximal inhibitory concentration
mg	=	Milligram
mL	=	Milliliter
ROS	=	Reactive oxygen species
MW	=	Molecular weight
kDa	=	Kilodalton
EC_{50}	=	Half maximum effective concentration
ACE	=	Angiotensin-I-converting enzyme
POP	=	Prolyl oligopeptidase
µL	=	Microliter
WHO	=	World Health Organization
DNA	=	Deoxy ribonucleic acid
AVP	=	Arginine-vasopressin
SP	=	Substance P
TRH	=	Thyrotropin-releasing hormone
AD	=	Alzheimer's disease
PD	=	Parkinson's disease
DPPH	=	α, α-diphenyl-β-picrylhydrazyl free radical
H_2O_2	=	Hydrogen peroxide
Kg	=	Kilogram
SBP	=	Systolic blood pressure
DBP	=	Diastolic blood pressure
SHRs	=	Spontaneously hypertensive rats
BW	=	Body weight
h	=	hour

LIST OF ABBREVIATIONS (Continued)

mmHg	=	millimeter mercury
NaOH	=	Sodium hydroxide
ADH	=	Alcohol dehydrogenase
ALDH	=	Aldehyde dehydrogenase
ALS	=	Amyotrophic lateral sclerosis
RAAS	=	Renin-angiotensin-aldosterone system
Ang I	=	Angiotensin I
Ang II	=	Angiotensin II
AT1	=	Angiotensin II type 1
AT 2	=	Angiotensin II type 2
ACE-2	=	Angiotensin converting enzyme 2
Ang 1-7	=	Angiotensin 1-7
B1	=	Bradykinin type 1
B2	=	Bradykinin type 2
NO	=	Nitric oxide
CNS	=	Central nervous system
$^{\circ}$C	=	Degree Celsius
min	=	Minute
AA	=	Amino acid
mm	=	Millimeter
mM	=	Millimolar
g	=	gram
SGF	=	Gastric fluid
KCl	=	Potassium chloride
KH_2PO_4	=	Potassium dihydrogen phosphate
$NaHCO_3$	=	Sodium bicarbonate
NaCl	=	Sodium chloride
$MgCl_2(H_2O)_6$	=	Magnesium chloride hexahydrate
$(NH_4)_2CO_3$	=	Ammonium carbonate

LIST OF ABBREVIATIONS (Continued)

$CaCl_2$	=	Calcium chloride
U	=	Unit
SIF	=	Intestinal fluid
Abs	=	Absorbance
TCA	=	Trichloroacetic acid
TBA	=	Thiobarbituric acid
SEC	=	Size exclusion chromatography
RPC	=	Reverse-phase chromatography
v/v	=	volume per volume
CO_2	=	Carbon dioxide
MTT	=	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
FBS	=	Fetal bovine serum
DMEM	=	dulbecco's modified eagle medium
DMSO	=	Dimethyl sulfoxide
DCFH-DA	=	2,7-dichloro-dihydrofluorescin diacetate
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
CAT	=	Catalase
SOD	=	Superoxide dismutase
GPx	=	Glutathione peroxidase
DH	=	Degree of hydrolysis

CHAPTER I

INTRODUCTION

1.1 Background and significant of the study

In 2021, corn production was approximately 1.2 billion metric tons worldwide and the United States is the largest producer followed by China and Brazil (Souza et al., 2022). Corn is the third important crop of the world after wheat and rice. In 2011-2021, corn production in Thailand was approximately 5 million tons per year, and it has been mainly used for feed production (Moungsree, Neamhom, Polprasert, & Patthanaissaranukool, 2022). Corn gluten meal (CGM) is the main by-product of wet-milling corn starch production, which composes of endosperm and accounts for approximately 5% of dry weight. CGM contains 67-71% protein, 21-26% carbohydrate (w/w), 3-7% fat, 1-2 % fiber ,(w/w), and 1-2% ash, based on dry basis (Li et al., 2019). Typically, CGM has been used in animal feed production due to its low water solubility (Li et al., 2019). Recently, corn peptides prepared from enzymatic hydrolysis have shown various bioactive properties, such as antioxidant activity, antihypertensive activity, immunomodulatory of tumor, and protection of alcohol-induced liver injury (Hu, Chen, & Li, 2020; Hu et al., 2022; Kopparapu et al., 2022; Li et al., 2019; Liu et al., 2020). The peptide CSQAPLA derived from CGM hydrolysate using Alcalase and Flavourzyme was reported to exhibit a reducing power and scavenging capacity on DPPH and superoxide anion radical with IC_{50} values of 0.116 and 0.39 mg/ml, respectively (Jin, Liu, Zheng, Wang, & He, 2016). The partially-purified CGM peptides from Alcalase hydrolysis with molecular weight (MW) less than 1 and those with 1-3 kDa exhibited cellular antioxidant activities with EC_{50} values of 2.85±0.19 mg/ml and 5.05±0.32 mg/ml, respectively. Both peptides also showed cytoprotective effects and intracellular reactive oxygen species (ROS) scavenging activities in HepG2 cells induced oxidative stress by hydrogen peroxide (H_2O_2) (Wang et al., 2016). In addition, peptides from CGM hydrolysate by Alcalase exerted high angiotensin converting enzyme (ACE) inhibitory activity with IC_{50} value of 0.27±0.05 mg/ml and was identified

to be SAP, NAP, AYLQQQ, VNAP, and LNSPAY (Liu et al., 2020). Moreover, CGM hydrolysate fraction could inhibit HepG2 cell growth at 50 and 200 μg/mL after 6 days of treatment (Hu et al., 2022). Based on these previous studies, production of protein hydrolysate with bioactivity would be one means to increase value and utilization of CGM for human consumption.

Dementia is referred to neurocognitive disorder that is a group of symptoms caused by brain disorders that more commonly happened in the elderly. People living with dementia has been estimated more than 55 million and there are nearly 10 million new cases every year worldwide (World Health Organization (WHO), 2021). People having a dementia may lose short-term or long-term memory, not be able to think well, as well as mood disorders, leading to inability to solve problems or control emotion. In addition, dementia has physical, psychological, social and economic impacts. There are many different forms of dementia, which are Alzheimer' disease, vascular dementia, dementia with Lewy bodies, and frontotemporal dementia (https://www.who.int/news-room/fact-sheets/detail/dementia). Dementia involves damage of nerve cells in central nervous system in several areas of the brain. Several extrinsic and intrinsic factors could be a cause of nerve cell damage. ROS such as hydrogen peroxide, superoxide anion, singlet oxygen, hydroxyl radical, and other free radicals are generated through physiological mechanism within the body, which involved in cell signaling and homeostasis (Wang et al., 2016). However, overexpression of free radical and/ or ROS led to oxidative stress within the cells that can cause oxidative damage to cell membranes, lipids, protein, and DNA, which result in aging and further initiate development of chronic diseases, such as cardiovascular disease, diabetes, cancer, atherosclerosis, rheumatoid arthritis, and neurodegenerative disorder (Jin et al., 2016; Wang et al., 2016). Antioxidant molecules can stabilize free radicals, reducing cell damages. Moreover, prolyl oligopeptidase (POP) also known as prolyl endopeptidase (PEP) or post-proline cleaving enzyme (EC 3.4.21.26) has believed to involve in neurodegenerative disorders (Svarcbahs et al., 2019). POP plays a role in the metabolism of proline-containing neuropeptides, such as arginine-vasopressin (AVP), substance P (SP), neurotensin, oxytocin, and thyrotropin-releasing hormone (TRH). These neuropeptides have been reported to improve learning ability and memory in animals (Toide, Shinoda, Fujiwara, & Iwamoto, 1997). POP has been

claimed to be related with neurological disorders, such as Alzheimer's disease (AD), amnesia, depression, schizophrenia, Parkinson's disease (PD) and Huntington disease (HD) (Svarcbahs et al., 2019). In addition, POP has been believed to induce the accumulation of **α**-synuclein protein in the brain (Savolainen, Yan, Myöhänen, & Huttunen, 2015). The **α**-synuclein oligomerization has been shown to be a main component of Lewy bodies, leading to dopamine neuron death in the brain which is a cause of Parkinson's disease (Savolainen et al., 2015; Cui et al., 2021). Brandt et al., (2008) demonstrated that POP did not cleave full-length of **α**-synuclein *in vitro*, but it can accelerate accumulation of **α**-synuclein proteins by protein-protein interaction. Moreover, POP inhibitors have been reported to reduce **α**-synuclein oligomers in SH-SY5Y cells (Kumar et al., 2017; Rostami et al., 2020). Although biological function of POP is still not clear, POP inhibition is considered to be an alternative way for neurodegenerative disorder improvement. Almost POP inhibitors are chemically synthesized based on the *N*-acyl-L-prolyl-pyrrolidine structure (Wilson, Hayes, & Carney, 2011). Some of them could be derived from nature as phenolic compounds (Kobayashi et al., 2002; Wilson et al., 2011). Recently, POP inhibitory peptides from animal and plant sources have been reported. POP inhibitory peptides from Carbernet Sauvignon wine were identified as VGIPG and YPIPF could inhibit degradation of neuropeptides, such as substance P, vasopressin, and neurotensin more than 80% (Yanai, Suzuki, & Sato, 2003). In addition, peptides derived from *in silico* digestion of meat proteins, including serum albumin, collagen, and myosin (PPL, APPH, IPP, and PPG) have also been reported to inhibit POP activity (Lafarga, O'Connor, & Hayes, 2015). Peptide PIHNSLPQNIPPLTQTVP derived from **β**-casein showed POP inhibitory activity with IC_{50} value of 29.8 μM (Hsieh et al., 2016). Moreover, R-phycoerythrin hydrolysate of *Porphyra haitanensis* showed POP inhibition with IC_{50} value of 136.35 μg/mL (Xie et al., 2019) . Thus far, the study of POP inhibitor peptides is still limited, particularly those derived from CGM.

Application of peptides as a functional food for brain health, focusing on peptides digestion and bioactivity changes after gastrointestinal (GI) tract should be evaluated. The *in vitro* and *in silico* models have been used for many decades to evaluate the change of peptide structure and their bioactivity upon GI digestion. A general standard and practical static *in vitro* GI digestion based on physiologically

relevant conditions that can be applied for various endpoint has been developed by the INFOGEST (Minekus et al., 2014). Additionally, the bioinformatics tools based on computer-aided method have also been used for *in silico* GI digestion to predict peptide fragments and cleaving sites of peptides under GI tract by pepsin, trypsin, and/or chymotrypsin (Sayd et al., 2018). Using *in silico* method is more efficient to predict digested peptides (Kęska and Stadnik, 2016).

To assess bioactivities of peptides, *in vivo* study is more relevant, but it is not always technically, ethically, and financially possible. Cellular assay can be used as alternatives testing method that more is physiologically and biologically relevant to *in vivo* than chemical-based assays (López-Alarcón & Denicola, 2013). Primary neuron isolated from brains of mice are used in neuroscience research, such as PC12, BV-2, and SH-SY5Y cells (Wang et al, 2021). SH-SY5Y neuroblastoma cell line was originally derived from a metastatic bone tumor biopsy cell that was widely used for neurodegenerative disorder pathogenesis and mechanism underlying drugs in the nervous system because of its human origin, catecholaminergic, neuronal properties, and ease of maintenance (Kovalevich & Langford, 2013; Şahin et al., 2021; Xicoy, Wieringa, & Martens, 2017; Xiong et al., 2021).Peptide ECH exerted higher DPPH radical scavenging activity than YECG, but the latter showed the highest protection for PC12 cells due to its oxygen radical absorption property (Gu et al., 2012). Peptides fraction with MW<3 kDa from beef hydrolysate prevented apoptosis caused by H_2O_2 induced oxidative stress in SH-SY5Y cells which was related to its antioxidant activities (Lee & Hur, 2019). Cellular studies of food POP inhibitory peptides are still limited. This approach could be used for screening peptides with brain functions.

Hypertension has been reported to be a risk of stoke that is one of causes of dementia (Sierra, 2020). The common cause of stroke is the blockage and bursting of the artery in the brain that would induce brain cell death which lead to the development of stroke-related or post-stroke vascular dementia (Sayed et al., 2020).Hypertension is a chronic increase in arterial blood pressure, associated with various risk factors, for example obesity, diabetes, age, lack of exercise, and etc. In 2021, adult people ages 30–79 years having hypertension e is approximately 1.28 billion worldwide (WHO, 2021).Various allopathic medicines are available which include diuretics, calcium channel blockers, α-blockers, β-blockers, vasodilators,

central sympatholytics, and angiotensin converting enzyme (ACE; EC 3.4.15.1)-inhibitors (Wajngarten & Silva, 2019). ACE is a key enzyme involved with blood pressure regulation by converting angiotensin I to angiotensin II, which is an active vasoconstrictor. Therefore, ACE indirectly increases blood pressure by causing blood vessel to constrict. Many ACE inhibitors, such as benazepril, captopril, enalapril, fosinopril, lisinopril, moexipril, perindopril, quinapril, ramipril, and trandolapril have been used to improve cardiovascular disease (Kumar et al., 2010). However, side effects of these ACE inhibitors include cough, elevated blood potassium levels, dizziness, headache, drowsiness, chest pain, sun sensitivity, rash, increase uric acid levels, weakness, and increase creatin levels have been reported (Kumar et al., 2010). Alternatively, ACE inhibitory peptides derived from animals or/and plants have been reported to exhibit no negative side effects. There are many studies focusing on ACE inhibitory peptides (Abachi, Bazinet, & Beaulieu, 2019; Guo et al., 2020; Pei et al., 2021; R. Wang et al., 2020; Xu et al., 2021). For instance, the CGM hydrolysate showed inhibition against ACE with IC_{50} value of 0.27 mg/mL (Liu et al., 2020). Furthermore, corn germ peptide at dose of 1000 mg/kg body weight reduced the systolic blood pressure (SBP) in acute and long-term (35 days) intragastric administration to spontaneous hypertensive rats (SHRs) (Guo et al., 2020). However, the greatest challenges of bioactive peptides are its stability in GI tract because digestive enzymes in GI tract can degrade these peptides into free amino acid, tri- or di-peptides. The effect of GI digestion on bioactivities of peptides has been investigated (Udenigwe et al., 2021; Zhang, Noisa, & Yongsawatdigul, 2020; Zhao et al., 2019).Changes of peptide structure affect biological activities. The ACE inhibitory activity and antihypertensive effect of peptides from plants proteins have been published. The ACE inhibitory activity of quinoa protein hydrolysate by pepsin-pancreatin sequential digestion with IC_{50} value of 0.36 mg protein/mL decreased systolic blood pressure (SBP) of -33.1 mmHg and diastolic blood pressure (DBP) of -35.7 mmHg after 6 h oral administration at dosage of 400 mg/kg body weight (BW) (Guo et al., 2020). Peptide RGQVIYVL isolated from quinoa bran hydrolyzed by Alcalase and trypsin showed ACE inhibitory activity with IC_{50} value of 38.16 µM and decreased SBP by -25 mmHg of SHRs at concentration of 100 mg/kg BW after 5 weeks oral administration (Zheng et al., 2019). However, ACE inhibitory activity of peptides

or hydrolysate *in vitro* might not always be related to antihypertensive effect *in vivo*. Peptide YLVP with ACE inhibitory activity did not show antihypertensive effect for SHRs (Jao et al., 2012). There are two ways for ACE inhibitory peptides to exert their activity *in vivo*; (1) peptides must be intact form and (2) active digested fragments after GI tract must be absorbed and reach to target sites (Jao et al., 2012). Peptide YAEERYPIL derived from ovalbumin showed ACE inhibition with IC_{50} of 5.4 µg/mL and it was digested into YPI with IC_{50} value of >1000 µg/mL. However, peptide YPI decreased SBP of SHRs at maximum value of -35 mmHg after 2 h, while the parent peptide reduced SBP at -31.6 mmHg after 6 h oral administration of 2 mg/kg BW (Miguel et al., 2006). Therefore, the study of antihypertensive effect of GI-digested peptides might be critical.

1.2 Research objectives

1.2.1 To determine antioxidant properties, POP, and ACE inhibitory activity of CGM hydrolysates and after *in vitro* stimulated GI digestion.

1.2.2 To identify POP and ACE inhibitory peptides from CGM hydrolysate and evaluate their stability under *in silico* GI digestion.

1.2.3 To study the effect of POP inhibitory peptides on α-synuclein aggregation in SH-SY5Y cells.

1.2.4 To evaluate the antihypertensive effect of ACE inhibitory peptides and their *in silico* simulated GI digested fragments.

1.3 Research hypotheses

Protein hydrolysate from CGM shows multi-bioactive functions, including antioxidant, POP inhibitory, and ACE inhibitory activity. CGM hydrolysate exhibited anti-oxidative stress in neuron cells. In addition, peptides derived from CGM hydrolysate potentially reduce aggregation of α-synuclein protein in neuron cells and exhibit antihypertensive effect in SHRs. Moreover, digested peptides obtained from *in silico* digestion show potency in inhibiting POP and ACE as well as decrease blood pressure in SHRs

1.4 Scope of the study

Protein hydrolysate of CGM was prepared using pepsin at the optimum condition. Antioxidant activities, POP, and ACE inhibitory activity of the hydrolysate and *in vitro* GI digested sample were determined. CGM hydrolysate and digested sample were applied on neuroblastoma SH-SY5Y cells to determine the anti-oxidative stress functions. POP and ACE inhibitory peptides were isolated using ultrafiltration, size exclusion, and reverse phase chromatography. Potent POP and ACE inhibitor peptides were identified. Structural change of selected peptides upon *in silico* GI digestion were investigated. In addition, inhibitory potency toward POP and ACE of plausible digested peptides was elucidated. The affinity of peptides binding to POP and ACE was elucidated using molecular docking analysis. Furthermore, the effect of potent POP inhibitory peptides on α-synuclein aggregation in SH-SY5Y cells were determined. Moreover, antihypertensive effects of ACE inhibitory peptides derived from CGM and its digested peptides were evaluated using SHRs.

1.5 Expected results

The bioactive peptides including antioxidant, POP and ACE inhibitory peptides would be obtained from CGM hydrolysate. Stability of POP and ACE inhibitory peptides during *in vitro* GI digestion is revealed. Digested peptides obtained from *in silico* digestion, exhibiting POP and ACE inhibitory activity would be obtained. Moreover, CGM peptides and their digested fragments could exert antihypertensive property in SHRs. In addition, POP inhibitory peptides exerting neuroprotective effect and/or anti-oxidative stress, and reducing α-synuclein aggregation in SH-SY5Y cells would be identified. Using the ACE inhibition property of peptides from *in vitro*, and molecular docking could imply with the antihypertensive effect of peptides. This research would provide a theoretical basis for further development of peptide-based drug and/or functional foods to improve central nervous system (CNS) disorders and hypertension.

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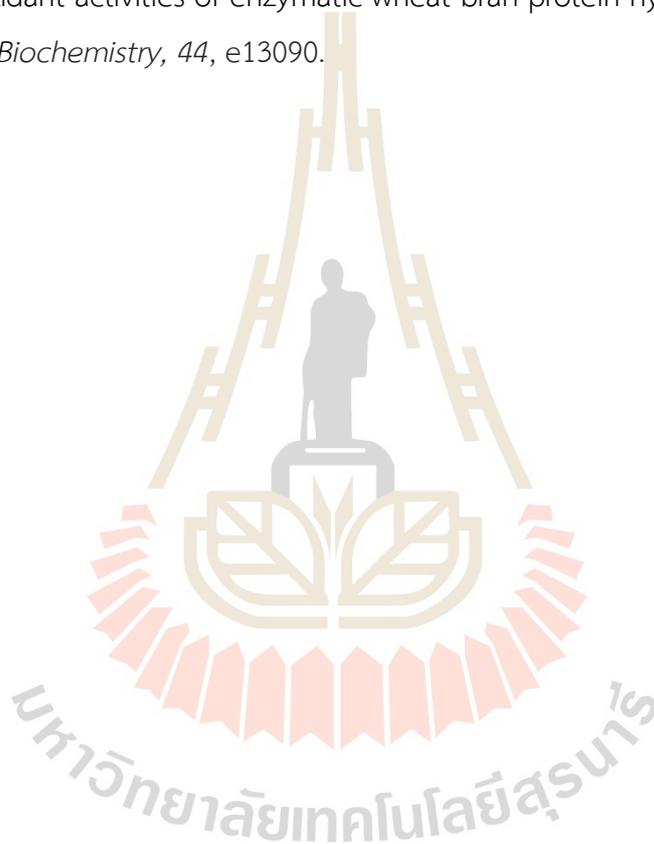
CHAPTER II

LITERATURE REVIEWS

2.1 Corn gluten meal (CGM) and bioactivity of peptides from CGM

A main protein by-product generated from corn starch production is CGM. CGM contains protein of approximately as 60% (w/w) proteins, 21-26% (w/w) carbohydrates, 3–7% (w/w) oil, 1–2% (w/w) fiber, and 1-2% (w/w) ash based on dry basis (Li et al., 2019). In addition, CGM is also rich in carotenes (49-73 mg/kg dry basis) and xanthophylls (224-550 mg/kg dry basis) (Loy & Lundy, 2019). The major proteins present in CGM are about 68% zein which is a major storage protein and 28% glutelin and other protein fractions, including glutelin-1, zein-like, C-zein, g-Zein, Zein-2, D-zein, alcohol-soluble reduced glutelin, and reduced soluble protein (Li et al., 2019). CGM is rich in A, L, P, and Q and lacks W (Di Gioia et al., 1999). In addition, it also contains Y and H, which are present in higher amounts in glutelin than zein (Di Guoia et al., 1999). Zein could be categorized into two main types, which are α-zein and β-zein. The former is soluble in 95% aqueous alcohol or 85% isopropanol, while the latter is soluble in 60% aqueous ethanol (Larkins, 2019). Zein structure is presented in the helical wheel model which is composed of nine homologs repeating units that are arranged by hydrogen bonds to form an anti-parallel structure (Shukla & Cheryan, 2001). The abundant non-polar amino acid residues and the bulky structure of zein are reasons for its poor water solubility, leading to limited utilization in the food industry.

CGM is mostly used in animal feed production or discarded as waste (Li et al., 2019). To increase its value and applications, modification of protein in CGM has been developed. High-pressure micro-fluidization has been used to improve emulsifying properties of CGM (Ozturk & Mert, 2019). In addition, the deamination of zein by 0.5 N sodium hydroxide (NaOH) could also improve its emulsifying properties (Cabra et al., 2007). Enzymatic hydrolysis has been reported to improve its solubility and bioactive activities (Kopparapu et al., 2022). CGM hydrolyzed by papain, ficin, and bromelain

generated corn peptides with antioxidant activity (Hu et al., 2020). The hydrolysate from corn glutelin using Protamex showed radical scavenging and chelating ability (Zheng et al., 2015). In addition, the antioxidant peptides isolated from CGM hydrolyzed by Alcalase included FPLEMMPF and QPQPW (Zheng et al., 2006; Wang et al., 2014). Peptides CSQAPLA, YPKLAPNE, and YPQLLPNE derived from Alcalase- and Flavourzyme-hydrolyzed CGM exhibited radical scavenging activity (Jin et al., 2016). There are suggested that amino acid Y, H, L, P, or C residues were likely to contribute to such activity. CGM contains high levels of L and P that might be a good source for antioxidant peptide production. Moreover, peptides derived from CGM by enzymatic hydrolysis showed an *in vitro* angiotensin-I-converting enzyme (ACE) inhibitory activity and *in vivo* blood pressure lowering effect in spontaneously hypertensive rats (SHRs). Tripeptides LRP, LSP, and LQP were isolated from α-zein hydrolyzed by thermolysin and showed a potent ACE inhibition. In addition, peptide LRP reduced the systolic blood pressure of -15 mmHg at a dose of 30 mg/kg body weight (BW) after intravenous injection (Miyoshi et al., 1991). Peptide PSGQYY derived from CGM hydrolysate prepared by Pescalase showed ACE inhibitory activity (Suh et al., 1999). In addition, ultra-high pressure improved the ACE inhibitory activity of Alcalase-hydrolyzed CGM by up to 74% (Liu Ziyi et al., 2020). Furthermore, peptide QLLPF isolated from Alcalase-hydrolyzed CGM increased alcohol metabolism in the liver (Ma et al., 2016). Additionally, the glycosylation of zein peptide protected alcohol-induced liver injury by increasing the level of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in the liver (Wang et al., 2020). Recently, zein hydrolysate using thermolysin generated peptide FLPFNQL with immunomodulatory activity (Liu et al., 2020). These previous studies demonstrated that CGM could be a potential source of bioactive peptides with health benefits.

2.2 Dementia and hypertension

Dementia is defined by the progressive loss of specific neuronal cell populations, which is associated with protein aggregations in the brain. They are characterized by their clinical symptoms with cognitive or behavioral disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). The World Health Organization (WHO) reported that over 55 million

people worldwide suffer from dementia in 2021 and the estimated number might reach 78 million in 2030 (Shin, 2022). Dementia occurs when nerve cells in the brain and/or peripheral nervous system are damaged and lose their functions over time. Typical symptoms of dementia are memory and learning ability loss, emotional and behavior changes, difficulties with executive and visuospatial functions, and movement disorder (Hansson, 2021; Lamprini, 2022). Therefore, dementia has a physical, psychological, social, and economic impact on patients as well as on the quality of life, such as careers, family, and society. The greatest risk factor of dementia is age increasing and certain genetic polymorphisms as well as other possible causes such as gender, oxidative stress, inflammation, hypertension, stroke, diabetes, smoking, depression, tumors, and chemical exposure (Brown et al., 2005; Checkoway et al., 2011). The hallmark pathologies of AD and PD are an accumulation of protein β-amyloid and α-synuclein, respectively (Association, 2019). Lewy bodies are one type of neuropathological characteristic of Parkinson's disease and dementia that is formed by the aggregation of α-synuclein, leading to damage of nerve cells in the brain (Spillantini et al., 1998). At present, using acetylcholinesterase inhibitors can improve cognitive function, but it doesn't halt the underlying disease progression (Hansson, 2021). In recent years, scientists have particularly improved their understanding of the causes of dementia and developed new approaches for treatment and prevention. The therapeutic options for neurodegenerative diseases have been studied, including (1) antioxidant compounds that can react with reactive oxygen species to prevent oxidative stress in the cell, (2) inhibition of prolyl oligopeptidase (POP) stabilized the neuropeptides level, (3) inhibition of β-amyloid and α-synuclein accumulation (Barnham et al., 2004; Murali Doraiswamy, 2002; Wilson et al., 2011).

Hypertension is defined as systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg which increases the risk of cardiovascular disease (Mills et al., 2020). In 2021, 1.28 billion people worldwide aged group between 30 to 79 years have hypertension as reported by WHO. Most people living with hypertension have no warning signs or symptoms. The only way to detect hypertension is a measurement of blood pressure, therefore hypertension is called a silent killer that is a major cause of premature death worldwide. In addition,

hypertension is also a major risk of stroke that may contribute to the development of dementia (Takeda et al., 2008). There are many risk factors for hypertension such as unhealthy diets (i.e., high sodium and low potassium consumption, high saturated fat, and trans fats intake), high levels of cholesterol, impaired glucose tolerance, high fibrinogen, electrocardiographic abnormalities, stress, being overweight, and smoking (Kannel, 1989; Mills et al., 2020). Reduction of these risk factors is recommended for the prevention and control of hypertension. However, reducing risk factors might not be enough for lowering the blood pressure of patients, they also need medical treatment including thiazide-type diuretics or calcium-channel blockers, inhibitors of the renin-angiotensin-aldosterone system (RAAS) (i.e., angiotensin-converting enzyme (ACE) inhibitor) or angiotensin II type-1 (AT1) receptor blocker (Messerli et al., 2007). RAAS is one of the important targets for the protection of hypertension for several decades. Renin converts angiotensinogen to angiotensin I (Ang I), then Ang I is converted to angiotensin II (Ang II) (vasoconstrictor) by ACE (Schmieder et al., 2007). Ang II interacts with both AT1 and Ang II type 2 (AT2) receptors. In the RAAS system, the AT1 receptor plays a role in the development of vasoconstriction in vascular smooth muscle cells occurs through the interaction between the AT1 receptor (Ghatage et al., 2021) and Ang II. In addition, Ang II could further be degraded by angiotensin-converting enzyme 2 (ACE-2) to generate angiotensin 1-7 (Ang 1-7) which could react with G-protein-coupled-reactor-Mas to induce vasodilation. Furthermore, Ang II could stimulate the aldosterone releasing, increasing water and salt retention in the kidney (Majumder et al., 2014). Additionally, ACE could inactivate bradykinin which is a vasodilator. Bradykinin could bind with bradykinin type 1 (B1) and type 2 (B2) which both receptors could induce nitric oxide (NO) in endothelial cells to maintain vasodilation. Therefore, the inactivation of enzymes in RAS has been proven to be a therapeutic approach for hypertension.

2.3 Role of prolyl oligopeptidase (POP) on central nervous disorder

POP also known as prolyl endopeptidase or post-proline cleaving enzyme (EC 3.4.21.26) was first discovered as an oxytocin-cleaving enzyme that was classified as a serine protease and highly expressed in the brain (Gass and Khosla, 2007; Li et al.,

2010; Männistö and Garcia-Horsman, 2017). POP consists of two domains; (1) the catalytic domain with an **α/β** hydrolase fold and (2) the propeller domain. The catalytic domain includes the canonical catalytic triad that contains Ser554, His680, and Asp641 and it is connected to the propeller domain by a covalent bond (Gass and Khosla, 2007).

POP has endopeptidase activity that cleaves short peptides (approximately ≤ 30 amino acid residues) at the C-terminal side of proline residues of peptides (Gass and Khosla, 2007). The precise physiological function of POP is still unclear. However, POP has been reported to play a role in the metabolism of proline-containing neuropeptides (Toide et al., 1995). This enzyme might degrade proline-containing neuropeptides such as arginine-vasopressin (AVP), substance P, neurotensin, oxytocin, and thyrotropin-releasing hormone (TRH) (Gass and Khosla, 2007). These neuropeptides have been shown to improve learning ability and memory in animals (Toide et al., 1997). Therefore, POP might be a cause of memory loss by reducing bioactive neuropeptides. In addition, the accumulation of **α**-synuclein protein in the brain by POP is the hallmark of Lewy body dementia that is a characteristic of PD (Savolainen et al., 2015). Brandt et al.,(2008) demonstrated that POP did not cleave the full length of **α**-synuclein *in vitro*, but POP can accelerate the accumulation of **α**-synuclein proteins by protein-protein interaction (Brandt et al., 2008). Moreover, Savolainen et al., (2015) reported that POP induced aggregation of **α**-synuclein by protein-protein interaction and enhanced its dimerization. When POP binds with **α**-synuclein, dimers will be formed, which will further aggregate and cause dopamine neuron death in the brain. POP inhibitors have been shown to reduce the dimerization of **α**-synuclein by increasing the formation of autophagosomes (Savolainen et al., 2015). Therefore, POP might play a role in neurocognitive disorder and central nervous system (CNS) diseases. For this reason, the POP inhibitor may be used to prevent neuropeptide degradation and **α**-synuclein aggregation which are a cause of cell communication reduction and dopamine cell death in the brain.

2.3.1 POP inhibitory peptides

Nowadays, POP inhibitors have been developed and almost POP inhibitors are commonly synthesized from chemical compounds based on the *N-*

acyl-L-prolyl-pyrrolidine structure (Wilson et al., 2011). However, some POP inhibitors are derived from natural compounds like phenolic compounds containing a catechol or pyrogallol group (Kobayashi et al., 2002). Recently, POP inhibitory peptides have been reported. POP inhibitory peptides isolated from Carbernet Sauvignon wine, have been identified to be VEIPE and YPIPF (Yanai et al., 2003). These peptides showed inhibition of neuropeptide degradation, such as substance P, vasopressin, and neurotensin more than 80%. Most neuropeptides consist of proline in their sequence, thus peptides containing Pro residues might act as a competitive inhibitor. In addition, POP inhibitory peptides could be derived from fish and cheese by enzymatic digestion (Sørensen et al., 2004). Hsieh et al., (2016) showed that POP inhibitory peptides were found in various proline-rich proteins, such as wheat gluten, soy protein isolate, sodium caseinate, fish skin gelatin, porcine skin gelatin, and tuna cooking juice. They found that the highest POP inhibitory activity (86.8%) peptides were derived from sodium caseinate hydrolyzed by bromelain. In addition, peptides AVPYPQRDMPIQAFLLY, PIHNSLPQNIPPLTQTPV, and HPHPHLSFMAIPP derived from sodium caseinate hydrolysate showed the highest POP inhibitory activity with IC_{50} values below 50 μM. They consisted of 13 -20 amino acid residues and were composed of at least one and up to four internal proline residues. POP inhibitory activity of these peptides was not correlated to their molecular mass and the number of proline residues, but it appeared to depend on adjacent amino acid residue binding to proline (Hsieh et al., 2016). The peptide YPIPF from red wine, having PI and PF, has been reported to show POP inhibitory activity with an IC_{50} value of 87.8 μM (Yanai et al., 2003). Similarly, three peptides, AVPYPQRDMPIQAFLLY, PIHNSLPQNIPPLTQTPV, and HPHPHLSFMAIPP, from sodium caseinate hydrolysate have been shown to contain PY, PQ, PI, PP, PV, and PH in their sequences. Therefore, the adjacent amino acids Y, Q, I, P, V, and H, might be favorable for an inhibitory activity for POP. In addition, POP cleaved peptide bonds at the carboxyl side of proline residues in protein containing the recognition sequence X-Pro-Y; where X is a peptide or protected amino acid and Y is either an amide, a peptide, an amino acid, an aromatic amine, or an alcohol (Hayes, 2014). Therefore, the amino acid sequence of the peptide chain and the location of proline residue within the peptide sequence

are important for enzyme inhibition. The use of *in silico* method showed to be efficient to predict the release of bioactive peptides from known proteins. Peptides PPL and PPG derived from bovine albumin and collagen, respectively, showed the highest POP inhibitory activity at IC_{50} values of 2.86 mM and 2.70 mM, respectively (Lafarga et al., 2015). Inhibition of POP was believed to improve memory and learning disorders by decreasing the metabolism of endogenous neuropeptides. Moreover, the inhibition of POP would be able to reduce α-synuclein aggregation in several models. The POP inhibitor, KYP-2047 (4-phenylbutanoyl-L-prolyl-2[S]-cyanopyrrolidine), could induce autophagy in OLN-AS7 cells (acute brain slices cells), decreasing α-synuclein aggregation (Cui et al., 2021). In addition, KYP-2047 reduced the amount of α-synuclein oligomers in A30P transgenic mouse (Savolainen et al., 2014) and incubation of SH-SY5Y overexpressed cells with KYP-2047 could also reduce α-synuclein oligomers (Dokleja et al., 2014). Unfortunately, the reduction of α-synuclein aggregation by POP inhibitory peptides has not been elucidated yet. Therefore, the study of bioactive peptides to inhibit POP and the ability of peptides to decrease α-synuclein aggregation would be of interest for functional food development.

2.4 Roles of Angiotensin-I-converting enzyme (ACE) on hypertension

ACE (EC 3.4.15.1) is a zinc- and chloride-dependent metallopeptidase that was first identified as a key component of the renin-angiotensin system for blood pressure regulation. It is located on the vascular endothelial cells in the brain, lungs, liver, intestine, pancreas, spleen, skeletal muscle, adrenal gland, and placenta (Shamloo et al., 2015). ACE has been found in two isoforms that are encoded by the same gene in a tissue-specific manner. The transcript 1 is called somatic ACE (sACE), which can exert into two forms: tissue ACE and plasma ACE. They consisted of two homologous catalytically active site domains (N and C domain), each of which contains a conserved HEXXH zinc-binding motif (Natesh et al., 2003; Zhao and Xu, 2008). Transcript 2 is called testis ACE (tACE) which is identical to the C-terminal half of sACE (Natesh et al., 2003). The structure of ACE consists of α-helices that contain zinc ion, localized in the center of the molecule, and two chloride atoms which are activators of both ACE domains (Shamloo et al., 2015). In RAAS, renin hydrolyzes the

prohormone angiotensinogen to release inactive decapeptide Ang I. Then, ACE cleaves the peptide bond of F-H of Ang I to release octapeptide Ang II and C-terminal dipeptide H-L in the presence of Zn^{2+} (Brown and Vaughan, 1998; Zhao and Xu, 2008). Ang II is the potent vasoconstrictor that stimulates the secretion of aldosterone and Na^+, K^+-reabsorption in the kidney, then induces water-sodium retention and increase of blood volume, increasing blood pressure (Coates, 2003; Zhao and Xu, 2008). The inhibition of ACE is considered to be an important target for a therapeutic approach in the treatment of hypertension. Recently, ACE inhibitors have been widely used in the treatment of cardiovascular diseases, such as captopril, a prototype of the sulfhydryl-containing ACE inhibitor, fentiapril, pivalopril, zofenopril, and alacepril (Brown and Vaughan, 1998). However, these inhibitors have some side effects, such as taste disturbances, dry cough, and skin rashes from long-term usage (Daskaya-Dikmen et al., 2017). Therefore, natural compounds with ACE inhibition have been widely studied to overcome this issue. A large number of potent natural ACE inhibitor peptides have been sought. (Li et al., 2004).

2.4.1 ACE inhibitory peptides derived from cereal proteins

The ACE inhibitory peptides were firstly isolated from snake venom that contained 5-13 amino acid residues and most of them had AP or PP at the C-terminal region (Kato and Suzuki, 1971). Over the past decade, ACE inhibitory peptides have been discovered in different food proteins via enzymatic hydrolysate. Protein sources of ACE inhibitory peptides included whey protein, casein, porcine and chicken muscle, fish protein, blood plasma, egg, gelatin, wheat, corn gluten, soybean, rapeseed, and algae (Arihara et al., 2001; Marczak et al., 2003; Matsui et al., 1999; Sangsawad et al., 2017; Sheih et al., 2009; Shin et al., 1995; Wongngam et al., 2022; Wu and Ding, 2001; Yang et al., 2007). With the demand growing for natural health products, there is a need to consider other possible sources that can respond to consumers' needs, especially plant proteins. Rice bran protein hydrolysate using Protease G6 showed ACE inhibitory activity with an IC_{50} value of 0.0156 mg/mL (Suwanapan et al., 2020). ACE inhibitory peptide, ITL, isolated from rice bran protein hydrolysate using alkaline protease showed ACE inhibition with an IC_{50} value of 0.0118 mg/mL (Dou et al., 2020). In addition, peptide SSYYPFK derived from oat

hydrolysate using Alcalase, Flavourzyme, and trypsin exerted ACE inhibitory activity with an IC_{50} value of 81.8 µg/mL with a competitive inhibition mode (Zheng et al., 2020). Several corn peptides with ACE inhibitory activity, including SAP, NAP, AYLQQQ, VNAP, and LNSPAY, were released by Alcalase-hydrolyzed CGM (Liu et al., 2020). In addition, peptides SAGGYIW and APATPSFW were identified from wheat gluten hydrolysate prepared by protease of *Pseudomonas aeruginosa* showed ACE inhibitory activity with IC_{50} values of 2 and 36 µg/mL, respectively (Zhang et al., 2020). Therefore, cereal protein has been proven as a source of ACE inhibitory peptides. The ACE inhibitory peptides can be classified into 3 groups; (1) inhibitor type, the IC_{50} value of this type is not affected by preincubation of the peptide with ACE,; (2) prodrug type inhibitor, this type of peptide could exert its inhibitory activity after gastrointestinal proteases digestion, such as peptide IVGRPRHQG does not exert its antihypertensive activity after administrated intravenously, however, they exert activity after oral administration that was hydrolyzed by trypsin into HQG and IVGRPR,; (3) substrate type, these peptides were hydrolyzed by ACE to release new peptides with weak or no activity (Li et al., 2004). The progressive current research proved that ACE inhibitory peptides reduced blood pressure in animal and clinical trials. Peptide IQW and LKP exerted antihypertensive effects on SHRs and reduced plasma Ang II levels (Majumder et al., 2015). The arginine-rich cationic peptide fraction from flaxseed hydrolysate significantly decreased the SBP of SHRs by -17.9 mmHg at 200 mg/kg body weight (BW) after 2 h following oral administration (Udenigwe et al., 2012). Moreover, the blood pressure of hypertensive patients was decreased from 140.6±11.7/ 90.3±5.8 mmHg to 137.0±14.4/ 87.7±6.6 mmHg after receiving 125 mL of a milk drink supplemented with whey peptides for 12 weeks (Lee et al., 2007). From these studies, it could be suggested that corn peptides could help stabilize blood pressure *in vivo*.

2.5 Stability of bioactive peptides upon gastrointestinal (GI) digestion

One of the greatest challenges of nutraceutical and functional food development is *in vivo* efficacy. Recently, simulated GI digestion has been applied for the evaluation of the gastrointestinal stability of peptides. Previous studies

demonstrated peptides showing high content of acidic amino acids and peptides with proline and hydroxyproline residues showed high resistance to GI digestive enzymes (Segura-Campos et al., 2011; Wang et al., 2016). In addition, peptides with branched-chain aliphatic and aromatic amino acids, such as L, I, Y, and V as well as the highest L and I as N-terminal residues might be stable upon GI digestion (Ahmed et al., 2022). In general, di- or tri-peptides with P or hydroxyproline residues at C-terminus are resistant to digestive enzymes (Lafarga et al., 2015). Moreover, peptides with an average chain length of 4.5 ± 2.0 amino acid residues and molecular weight (MW) of 547.78 ± 233.17 Da might resist GI digestive enzymes, while peptides with an average chain length of 6.8 ± 2.1 amino acid residues and MW of 796.85 ± 207.70 Da would be unstable (Ahmed et al., 2022). Casein-derived peptide fractions with MW >3 kDa were more easily hydrolyzed during *in vitro* GI digestion than lower MW fractions (Chen and Li, 2012). Similarly, peptides with MW of 1107 and 843 Da gradually decreased during GI digestion, while the peptide with MW 107 Da seemed to be resisted during GI digestion (Martínez-Alvarez et al., 2016). In addition, peptides containing H might be stable during GI digestion due to unfavorable steric interactions at the active site of trypsin that cleaved basic residues with high specificity for R or L (Olsen et al., 2004). Moreover, H is unfavored by pepsin due to electrostatic incompatibility at the enzyme active site (Hamuro et al., 2008). Thus, stability of bioactive peptides towards GI digestion was affected by MW, hydrophobicity, net charge, C-and N-terminal amino acid residues, amino acid sequence, and amino acid composition. Ideally, bioactive peptides must be absorbed intact into blood stream and being transported to target organs to exert biological properties. Therefore, the resistance of bioactive peptides against digestive enzymes is one of the important properties to assure the biological properties of the peptide *in vivo*.

2.5.1 Simulated *in vitro* and *in silico* GI digestion

The assessment of peptide digestion *in vivo* is the most reliable technique, but it is not always technically, ethically, and financially possible (Li et al., 2020). Therefore, *in vitro* and *in silico* models have been used to evaluate the change in peptide structure and their bioactivity upon GI digestion. A general standard and practical static *in vitro* GI digestion based on physiologically relevant

conditions that can be applied for various endpoints have been developed by the INFOGEST (Minekus et al., 2014). This method has been widely used in many studies (Dave et al., 2016; Gallego et al., 2020; Zhang et al., 2020). Additionally, the bioinformatics tools based on the computer-aided method have been used for *in silico* GI digestion to predict the peptide fragments and cleaving site of peptides under the GI tract using pepsin, trypsin, and/or chymotrypsin. Recently, the available online tools for protein or peptide digestion have been developed, such as PeptideCutter, BIOPEP, and mMass tools. In PeptideCutter tool, only one protein or peptide and various enzymes could be put in the program to predict cleavage sites, and bioactivities against other databases (Gasteiger et al., 2005). BIOPEP and mMass tools are a combination of *in silico* digestion and bioactive peptide prediction option where BIOPEP requires one peptide or protein sequence and up to three enzymes can be entered, whereas mMass allows only one sequence and enzyme (Anekthanakul et al., 2018). Therefore, using *in vitro* and *in-silico* techniques would be helpful to evaluate peptide digestibility. However, each technique has advantages and limitations. For example, the *in vitro* model is more reliable with *in vivo* digestion than *in silico* model because it represents physiological conditions of *in vivo* (Kęska and Stadnik, 2016). However, the *in vitro* digestion generally requires chemicals and enzymes, such as pepsin, trypsin, and chymotrypsin to carry out the experiment. Peptide identification from *in vitro* digestion of protein is also time-consuming and complicated from peptides presence in the commercial enzymes (Senadheera et al., 2022). On the other hand, there is still the limitation of *in silico* digestion which is the physiological conditions such as pH, temperature, incubation time, and ionic strength in the digestion system which might affect or undermine the reliability of results (FitzGerald et al., 2020).

2.6 *In vitro* cellular system of neurodegenerative disorder studies

Cell cultures derived from humans or animals mimicking brain environment and neuronal and glial cell interaction have been used to evaluate the neuroprotective effect of compounds of interest (Slanzi et al., 2020). Any neurodegenerative diseases including AD, PD, and prion disease, have different

pathological factors. Therefore, using appropriate cell culture models would gain more understanding underlying mechanism of disease. PD is the second-most common age-related neurodegenerative disorder. The hallmark of PD is Lewy bodies that are an insoluble aggregation of the presynaptic neuronal protein α-synuclein, leading to damage to nigrostriatal dopaminergic neurons which resulted in several motor symptoms such as bradykinesia, rigidity, resting tremor, and postural instability (Dauer and Przedborski, 2003; Kalia and Lang, 2015). To understand the molecular basis of neuronal degeneration in PD and develop therapeutic strategies, several cell culture systems based on immortalized cell lines have been applied. The human embryonic kidney 293 (HEK293) cell, human neuroglioma (H4), and pheochromocytoma (PC12) cells that derived from rat adrenal medulla have been used. HEK293 has been used to study α-synuclein aggregation and mutation, but it is a non-neuronal cell type and a high passage number could lead to genetic and epigenetic alteration (Falkenburger and Schulz, 2006). Recently, the human neuroblastoma cell line, SH-SY5Y, is widely used because it consists of the dopaminergic phenotype typical of PD pathology (Xicoy et al., 2017). However, SH-SY5Y cells also have limitations such as a lack of standardized protocol to maintain cells in culture, leading to variable cell growth and inconsistent experimental results (Xicoy et al., 2017). Treatment of the POP inhibitor, KYP-2407 [4-phenylbutanoyl-*L*-prolyl-2(*S*)-cyanopyrrolidine], significantly reduced α-synuclein oligomers in SH-SY5Y cells by increasing autophagic flux (Rostami et al., 2020). In addition, KYP-2407 also reduces the production of ROS by activating protein phosphatase 2A in SH-SY5Y cells (Eteläinen et al., 2021). Previous studies demonstrated that SH-SY5Y cells could be used as a cellular model for studying the mechanism of compounds to improve neurocognitive functions.

2.7 Antihypertensive effect of ACE inhibitory peptides

Although *in vitro* experiments could perform in a more controlled setup and in a larger number than *in vivo* studies, they could not be totally relevant to living organism. *In vitro* testings of novel foods, functional foods, nutraceutical products, and pharmaceutical products are not sufficient to guarantee their health benefits.

Therefore, the *in vivo* study is needed to gain more biological information. There are many studies on the antihypertensive activity of ACE inhibitory peptides in SHRs for many decades. The SHRs are a common model for hypertensive experiments. This rat possesses multiple genes involved in the development of hypertension similar to human hypertension (Gardner et al., 2011). The development of hypertension starts at 6-7 weeks of age and reaches a stable level at 17-19 weeks of age (Reckelhoff et al., 2018). Peptide FQIN[M(O)]CILR and TGAPCR were identified from *Gracilariopsis lemaneiformis* protein hydrolysates reduced SBP by 34 mmHg and 28 mmHg, respectively, after 2 h of oral gavage dose at 10 mg/ kg BW (Deng et al., 2018). In addition, the blood pressure of SHRs significantly decreased after administering peptide RVPSL which was isolated from egg white protein for 4 weeks, and the mRNA expression levels of renin, ACE, and AT1 receptor in the kidney also decreased (Yu et al., 2014). Peptides LRVIQY and VLAGF derived from porcine skeletal myosin lowered blood pressure activity after 6 h orally fed to SHRs at a dose of 10 mg/kg BW (Muguruma et al., 2009). Furthermore, corn peptides with MW <3 kDa at a dose of 100 mg/kg BW exerted an antihypertensive effect in SHRs by reducing SBP in both acute (7 h) and long-term studies (24 days) (Huang et al., 2011). Moreover, peptide VSKRLNGDA derived from chicken blood with ACE inhibitory activity reduced SBP by -41.83 mmHg within 12 h of oral administration to SHRs at a dose of 50 mg/kg BW (Wongngam et al., 2022). In addition, this peptide downregulated the expression of renin, AT1 receptor after 4 weeks of peptide feeding (Wongngam et al., 2022). Additionally, corn peptide fraction with MW <1000 Da prepared from pilot-scale hydrolysis of alkaline proteases from *Bacillus licheniformis* and neutral proteases from *Bacillus subtilis* decreased SBP (-40 mmHg) after 6 weeks of oral administration at a dose of 450 mg/kg BW (Lin et al., 2011). Furthermore, the oral administration of rice peptides prepared by alkaline protease and trypsin hydrolysis at a dose of 100 mg/kg BW to SHRs showed a reduction of SBP by 17.1 mmHg at 6 weeks (Dong et al., 2022). Previous studies indicated that peptides derived from peptides showed antihypertensive effects, which might have potential applications as functional food or nutraceutical products for stabilizing blood pressure.

2.8 References

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CHAPTER III

PROLYL OLIGOPEPTIDASE INHIBITORY ACTIVITY AND ANTIOXIDANT PROPERTIES OF CORN GLUTEN MEAL HYDROLYSATE

3.1 Abstract

Neurocognitive disorder is a group of symptoms caused by increasing level of oxidative stress and inflammation of nerve cells in central nervous system. Prolyl oligopeptidase (POP) is one of possible causes of neurocognitive disorder. Corn gluten meal hydrolysate (CGM-H) exhibiting POP inhibitory activity was developed and its cellular antioxidant capacity was investigated. This would ultimately lead to valorization of corn gluten meal, a byproduct from corn starch processing, as nutraceutical ingredients. The CGM-H prepared by 1% pepsin and its *in vitro* digesta exhibited POP inhibitory activity, ABTS radical scavenging, ferric reducing power, and hydroxyl radical scavenging activities. Activities of the former 3 assays decreased after *in vitro* gastrointestinal (GI) digestion. In addition, CGM-H protected cells against hydrogen peroxide (H_2O_2) better than did its digested counterparts. Furthermore, peptides from CGM-H and CGM-H digesta appeared to reduce oxidative stress in SH-SY5Y cells by scavenging ROS and up-regulated the expression of *CAT* gene. Therefore, the POP inhibition, antioxidant activities, and ROS reducing capacity in SH-SY5Y cells of CGM-H suggested that CGM could be a potential raw material for nutraceutical products for brain function.

Keywords: Antioxidant peptide, Corn gluten meal, Prolyl oligopeptidase inhibitory peptide, SH-SY5Y cells

3.2 Introduction

Corn worldwide production was approximately 1,060 million tons per year and corn gluten meal (CGM) was produced approximately 5% of dry weight of wet-milling corn starch production. It contains at least 60% protein and is used for animal feed

production. Recently, corn peptides prepared from enzymatic hydrolysis have been reported to possess various bioactive properties, including antioxidant activity, antihypertensive activity, immunomodulatory of tumor, and protection of alcohol-induced liver injury (Jin, Liu, Zheng, Wang, & He, 2016; G. Li et al., 2019; J.-T. Li et al., 2013; Liu, Fang, Feng, Li, & Gu, 2020; L. Wang et al., 2016).

Dementia or neurodegenerative disorder is a group of symptoms that involve the damage of nerve cells in central nervous system and more commonly happened in the older people. Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anion, singlet oxygen, hydroxyl radicals, and other free radicals, are generated through physiological mechanisms involving in cell signaling and homeostasis (L. Wang et al., 2016). The over expression of free radicals leads to oxidative stress occurs within the cells, causing oxidative damage to cell membranes, lipids, proteins, and DNA. This would eventually lead to aging and further development of chronic diseases, including neurodegenerative disorder, cardiovascular disease, and diabetes (Jin et al., 2016; L. Wang et al., 2016). Therefore, the prevention of oxidative stress would reduce the pathological risk of chronic diseases.

Prolyl oligopeptidase (POP) also known as prolyl endopeptidase (PEP) or post-proline cleaving enzyme (EC 3.4.21.26) plays a role in the metabolism of proline-containing neuropeptides, such as arginine-vasopressin (AVP), substance P (SP), neurotensin, oxytocin, and thyrotropin-releasing hormone (TRH). These neuropeptides have been reported to improve learning ability and memory in animals (Toide, Shinoda, Fujiwara, & Iwamoto, 1997). Thus, a decrease of these neuropeptides by POP would affect memory and learning ability. Almost POP inhibitors were chemically synthesized based on the N-acyl-L-prolyl-pyrrolidine structure. Some of them could be derived from nature as phenolic compounds (Kobayashi et al., 2002; Wilson, Hayes, & Carney, 2011). Recently, POP inhibitory peptides from animal and plant sources have been reported. Peptides derived from in silico digestion of meat proteins, including serum albumin, collagen, and myosin (Pro-Pro-Leu, Ala-Pro-Pro-His, Ile-Pro-Pro, and Pro-Pro-Gly) have been reported to inhibit POP activity (Lafarga, O'Connor, & Hayes, 2015). In addition, the peptide Pro-Ile-His-Asn-Ser-Leu-Pro-Gln-Asn-ile-Pro-Pro-Leu-Thr-Gln-Thr-Val-Pro derived from β-

casein showed POP inhibitory activity with IC50 value of 29.8 µM (Hsieh, Wang, Hung, Hsieh, & Hsu, 2016). Moreover, POP inhibitory peptides from Carbernet Sauvignon wine have been identified as Val-Glu-Ile-Pro-Glu and Tyr-Pro-Ile-Pro-Phe that inhibited degradation of neuropeptides, such as substance P, vasopressin, and neurotensin more than 80% (Yanai, Suzuki, & Sato, 2003). However, the study of POP inhibitor peptides is still limited in plant proteins, especially CGM.

The greatest challenge of bioactive peptides is its stability in gastrointestinal (GI) tract. Digestive enzymes in GI tract can degrade these peptides into free amino acids, tri- or di-peptides. Changes of peptide structure would certainly affect their biological activities, which have never been systematically studied in CGM. Therefore, the objectives of this study were to determine POP inhibitory activity of CGM hydrolysate (CGM-H) and evaluate antioxidant activities based on both chemical and cellular-based assays using neuroblastoma SH-SY5Y cells. In addition, changes of bioactivity of CGM-H upon in vitro GI digestion were elucidated.

3.3 Materials and methods

3.3.1 Materials

The CGM was gifted by Friendship Corn Starch Industry (Samutprakarn, Thailand). POP from Flavobacterium sp. and a substrate Z-glycyl-L-proline-4-nitroanilide (Z-gly-Pro-pNA) were obtained from Sigma-Aldrich Trading Co. Ltd. (Shanghai, China). Pepsin from porcine stomach mucosa (400 U/mg), pancreatin from porcine pancreas (5.53 U/mg), trinitrobenzenesulfonic acid (TNBS, 5.0%, w/v, BioReagent), amino acid standard, 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′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). L-ascorbic acid was obtained from CARLO ERBA Reagents S.A.S (Rodano, Italy). 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. Other chemicals were of analytical grade.

3.3.2 Preparation of CGM-H

The CGM was hydrolyzed by pepsin at enzyme: substrate ratio of 1:100 (w/w) at 37°C (pH 2.0) for 2, 4, 6, 8 and 12 h, in a shaking water bath. Subsequently, samples were heated at 95°C for 10 min to inactivate the enzyme and cooled on ice. The mixture suspension was centrifuged at 10,000×g for 10 min and adjusted to pH 7.0. The volume of supernatants was recorded and total protein was determined using Kjeldahl method (AOAC, 1995). The protein conversion factor for cereal protein of 5.84 was used (Fujihara et al, 2008). Protein recovery was calculated using the equation (1). The hydrolysate at 12 h was lyophilized and the weight was recorded to estimate yield using the equation (2)

$$\text{Protein recovery (\%)} = \frac{\text{Protein content in CGM}-\text{H}}{\text{Protein content in CGM}} \times 100 \qquad (1)$$

$$\text{Yield (\%)} = \frac{\text{Weight of lyophilized CGM}-\text{H}}{\text{Weight of CGM used for hydrolysis}} \times 100 \qquad (2)$$

The degree of hydrolysis (DH) was determined by measuring the α-amino content based on the TNBS method described by Adler-Nissen (1979) using L-leucine as the standard. CGM-H were stored at -80°C until use.

3.3.3 Amino acid analysis

Total amino acid of CGM was analyzed according to AOAC method with some modifications. Twenty milligrams of CGM and lyophilized CGM-H were hydrolyzed with 3 ml of 6 N HCl at 110°C for 24 h. After cooling, it was adjusted to pH 7 using 10 N NaOH. Amino acid composition was determined using an Amino Acid Analyzer (Biochrom 30 plus, Biochrom Ltd., Cambridge, UK) equipped with cation exchange column (u-3183 High resolution, 200 mm of bed length and 4.6 mm of diameter, Biochrom Ltd., Cambridge, UK). Sodium system was used as a mobile phase. Amino acids were detected by post-column ninhydrin derivatization. The amino acid standard solution: AA-S 18 (Sigma Chemical Co., St. Louis, MO., USA) and 1 mg/ml of nor-leucine were used as external standards and an internal standard, respectively. Amino acid contents were calculated and expressed as mg/ g sample.

3.3.4   *In vitro* GI digestion of CGM-H

The *in vitro* GI digestion was performed according to the method of Minekus et al., (2014) with slight modifications. Lyophilized CGM-H samples (1 g) were dissolved with 5 mL of deionized water and added with 5 mL of stimulated gastric fluid (SGF) (the mixture solution of 6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.1 mM $MgCl_2(H_2O)_6$, and 0.5 mM $(NH_4)_2CO_3$, pH 3.0), followed by $CaCl_2$ to achieve 0.075 mM in the final concentration and adjusted to pH 3.0. Then, pepsin was added to achieve 2000 U/mL of the final concentration. The gastric digestion was carried out in a shaking water bath at 37°C and 150 rpm for 2 h. After 2 h. incubation, the mixture was adjusted to pH 7.0, and added 10 mL of simulated intestinal fluid (SIF) (the mixture solution of 6.8 mM KCl, 0.8 mM KH_2PO_4, 85 mM $NaHCO_3$, 38.4 mM NaCl, and 0.33 mM $MgCl_2(H_2O)_6$, pH 7.0) and followed by $CaCl_2$ to achieve 0.3 mM in the final concentration. Thereafter, pancreatin was added to achieve 100 U/mL of final concentration. The intestinal digestion was carried out in a shaking water bath at 37°C and 150 rpm for 2 h. The digestion was stopped by heating at 95°C for 10 min and immediately cooled on ice for 10 min. The digesta was centrifuged at 10,000xg at 4°C for 10 min (Minekus et al., 2014). The supernatant was collected and referred to as CGM-H digesta samples. They were measured **α**-amino content using TNBS method to determine degree of digestion. CGM-H digesta samples were stored at -80°C until use.

3.3.5   Measurement of POP inhibitory activity

POP inhibitory activity was performed in a 96-well microplate using Z-gly-Pro-pNA as a substrate according to the method of Hsieh et al., (2016) with slight modifications. CGM-H or digesta (12.5 μL) was added 25 μL of 2.5 mM substrate (in 40% 1,4-dioxane to dissolve the substrate) and 50 μL of 100 mM Tris-buffer (pH 7.0). The mixture was incubated at 30°C for 10 min. Subsequently, POP at 0.5 U/ml (12.5 μL) was added. Deionized water was used as a control. The mixture was incubated at 30°C for up to 45 min and the absorbance was measured at 410 nm using a microplate reader (Varioskan LUX, Thermo Scientific, Vantaa, Finland) (Hsieh et al., 2016). Changes in absorbance were recorded in 2-min intervals for 30 min. POP activity was estimated from the slope (**Δ** Abs/min). POP inhibitory activity of peptides was calculated as followed:

$$\text{POP inhibition (\%)} = \frac{\text{Slope of control} - \text{Slope of samples with added inhitor}}{\text{Slope of control}} \times 100$$

3.3.6 Measurement of antioxidant activities

3.3.6.1 ABTS radical scavenging activity assay

ABTS radical scavenging activity assay was performed according to the method of Re et al., (1999) with some modifications. CGM-H and digesta (5 μL) was added to 200 μL of fresh ABTS solution into a 96-well plate. The mixture was then incubated in the dark for 5 min, and measured the absorbance at 734 nm using a microplate reader (Varioskan LUX, Thermo Scientific, Vantaa, Finland) (Re et al., 1999). Trolox solution was used as a standard, and the antioxidant activity was expressed as mM Trolox equivalent (TEQ).

3.3.6.2 Ferric-reducing antioxidant power assay (FRAP assay)

FRAP assay was performed according to the method of Benzie and Strain (1996) with some modifications. Ten microliters of CGM-H and digesta was added to 100 μL of fresh FRAP reagent. The mixture was then incubated at 37°C for 15 min, and immediately measured the absorbance at 593 nm (Benzie & Strain, 1996). Trolox solution was used as a standard, and the reducing power is expressed as mM Trolox equivalent (TEQ).

3.3.6.3 Hydroxyl radical scavenging activity

Hydroxyl radicals scavenging activity was performed according to Sangsawad et al., (2016) with some modifications. CGM-H or digesta (100 μL) was mixed with 0.45 mM of 0.2 M sodium phosphate buffer (pH 7.4), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM $FeSo_4$-EDTA, 0.15 mL of 10 mM hydrogen peroxide (H_2O_2), and 0.5 mL of deionized water. The mixture was incubated at 37°C for 4 h. Subsequently, 0.75 mL of 2.8% trichloroacetic acid (TCA) and 0.75 mL of 1.0% thiobarbituric acid (TBA) were added. Deionized water was used instead of CGM-H and digesta for a control. The mixture was boiled for 10 min and cooled on ice. The absorbance was measured at 532 nm. Deionized water was used as a control (Sangsawad, Kiatsongchai, Chitsomboon, & Yongsawatdigul, 2016). Degree of scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

3.3.7 Molecular weight (MW) distribution of CGM-H and CGM-H digesta

MW distribution of CGM-H and its digesta was determined by size exclusion chromatography using a Superdex Peptide 10/300 GL column (10×300 mm, GE Healthcare, Piscataway, NJ, USA) equipped with Fast Protein Liquid Chromatography (FPLC) (AKTA PURE, GE Healthcare, Uppsala, Sweden). CGM-H and its digesta (100 μL) were loaded into a column and separation was performed using 30% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min and absorbance was monitored at 214 nm. Peptide standards included cytochrome C (12,000 Da), aprotinin (6,512 Da), AGNQVLNLQADLPK (1,713 Da), NTFLFFK (1,023 Da), DLE (411 Da), and tyrosine (181 Da).

3.3.8 Neurotoxicity effect measurement

The SH-SY5Y neuroblastoma cells were cultured according to Jaroonwitchawan et al. (2017). Cells were maintained in DMEM supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS), L-glutamine (1% v/v), penicillin/streptomycin (1% v/v), and 0.05% trypsin-EDTA. Cells were incubated at 37°C in 5% CO_2 and replaced the medium every 2 days (Jaroonwitchawan, Chaicharoenaudomrung, Namkaew, & Noisa, 2017). Cytotoxicity of CGM-H and digesta against SH-SY5Y cells was determined by cell viability using MTT assay according to Jaroonwitchawan et al., (2017) with some modifications. Briefly, cells were cultured in a 96-well plate with various content of CGM-H and its digesta (2x, 4x, 8x, 16x, 32x, and 64x dilution) for 24 h. The MTT was added to the medium to contain final concentration of 0.5 mg/ml and incubated for 3 h at 37°C in the dark. The medium was removed, and formazan crystal was solubilized in DMSO. The absorbance was measured at 570 nm, using a microplate reader (BMG Labtech, Ortenberg, Germany). Cells treated with the medium (without CGM-H or digesta) were used as a control. Absorbance of the control was taken as 100% viability (Jaroonwitchawan et al., 2017).

3.3.9 Neuroprotection and intracellular ROS reduction

Neuroprotection was determined by cell viability using MTT assay. SH-SY5Y cells were incubated with the CGM-H or its digesta at various nontoxic

concentrations (8x, 16x, 32x, and 64x dilution) for 2 h. Cells were then washed with PBS and incubated with 0.75 mM H_2O_2 at $37^{\circ}C$ for 3 h. Subsequently, cells were washed twice with PBS. The MTT solution was added at final concentration of 0.5 mg/ml and incubated for 3 h at $37^{\circ}C$ in the dark. Cells treated with only medium were used as a control. The MTT assay was followed as described above. The neuroprotection capacity of CGM-H and digesta was compared with H_2O_2-treated cells (damage cells).

Intracellular ROS scavenging activity of CGM-H and digesta was assessed by DCFH-DA oxidation according to Jaroonwitchawan et al., (2017) with some modifications. SH-SY5Y cells were incubated with various contents of the CGM-H and its digesta at various dilution of 8x, 16x, 32x, and 64x for 2 h. Subsequently, 0.5 mM H_2O_2 and 20 μM DCFH-DA were added. Cells were incubated at $37^{\circ}C$ for 1 h. Fluorescent intensity was measured using a microplate reader (Varioskan LUX, Thermo Scientific, Vantaa, Finland) at an excitation wavelength of 480 nm and an emission wavelength of 530 nm (Jaroonwitchawan et al., 2017). Ascorbic acid at concentration of 1 mM was used as a positive control. Cells treated with H_2O_2 were defined as damage cells. Results were expressed as "Cellular Antioxidant Activity Unit (CAA Unit)" following the equation:

$$CAA\ Unit = (1 - \frac{Fluorescent\ intensity\ of\ sample}{Fluorescent\ intensity\ of\ oxidative\ damage}) \times 100$$

Where oxidative damage group was referred to cells treated by H_2O_2 without CGM-H or digesta pretreatment.

3.3.10 The expression of antioxidant enzymes gene

Cells were cultured in a 6-well plate at density 4×10^5 cells/well for 24 h. The CGM-H and its digesta at various concentrations (8x, 16x, and 32x dilution) were added and incubated for 12 h. H_2O_2 (1 mM) was then added, and incubation was continued for 30 min. Total RNA was isolated by NucleoSpin RNA kit (Macherey-Nagel, Düren Germany), according to the manufacturer's protocol. One microgram of RNA was used for complementary DNA (cDNA) synthesis using 2-step RT-PCR Kit (Vivantis Technologies Sdn. Bhd., Selangor Darul Ehsan, Malaysia). PCR processing was

operated in a Biorad/C1000Touch Thermocycle (Biorad, CA, USA) with specific primers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The amplified cDNA products were separated using electrophoresis with 1.5% agarose gel and staining with ethidium bromide. Primers used in the experiment were as followed: *GAPDH*, 5'- ACC TGA GCC GTC TAG AA -3' (forward) and 5' – TCC ACC CTG TTG CTG TA – 3' (reverse); *SOD*, 5' – CTA GCG AGT TAT GGC GAC (forward) and 5' – CAT TGC CCA AGT CTC CAA C (reverse); *GPX*, 5' – CGC CAA GAA CGA AGA GAT TC (forward) and 5' – CAA CAT CGT TGC GAC ACA C (reverse); Catalase (*CAT*), 5' - TCC GGG ATC TTT TTA ACG CCA TTG (forward) and 5' – GGT GCC TAC GTT CTG ATC TGT G (reverse). The relative expression levels of mRNAs of individual genes were normalized with the expression of the housekeeping gene.

3.3.11 Statistic analysis

Data were analyzed by one-way analysis of variance (ANOVA) with 3 independent replications (n=3) using SPSS program. Mean values were compared using Dancan's multiple range test with significant differences of $p = 0.05$.

3.4 Results and discussion

3.4.1 Degree of hydrolysis (DH) and POP inhibitory activities of CGM-H

At 12 h of hydrolysis, the protein recovery was estimated to be 19.30± 1.07% with the yield of 25.67±0.57% and DH of 2.37±0.06%. This relatively low yield of protein recovery and DH was attributed to limited enzyme accessibility to the substrate. CGM exhibited low water solubility because of the presence of the hydrophobic proteins, zein and glutelin (Jin et al. 2016).
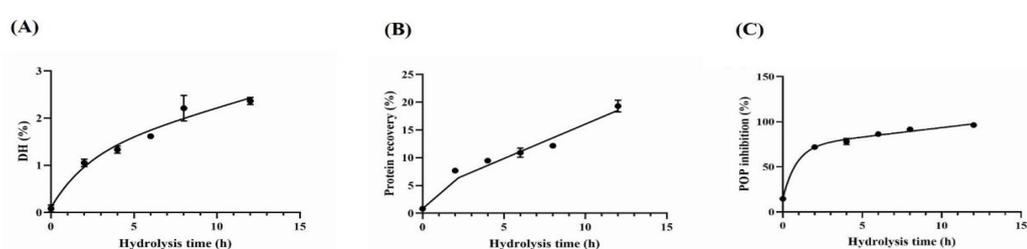
Figure 3.1 DH (A) and protein recovery (B) of CGM hydrolyzed by pepsin, and POP inhibitory activity of CGM-H after various hydrolysis times (C). Values are expressed as means±SDs (n=3).

Zein is a largely antiparallel helical structure comprising nine helical segments with nonpolar AAs in the central section that form a hydrophobic face inside a superhelix (Momany et al. 2006). CGM-H obtained using pepsin exhibited strong POP inhibitory activity with a half-maximum inhibitory concentration (IC_{50}) value of 37.55± 1.22 µg leucine/mL (0.56 mg solid/mL, Table 3.1). According to Martínez-Alvarez et al., (2016), the hydrolysate prepared from tuna heads and viscera using Alcalase exhibited POP inhibitory activity at IC_{50} values of 3.30±1.05 and 4.59±0.47 mg dry weight/mL, respectively, which were lower than that noted in this study. Hydrolysates from wheat gluten, sodium caseinate, and fish skin gelatin contained ≥ 10 mol% of Pro and exhibit adequate POP inhibition 57.9–72.4%, 77.1–86.8%, and 32.2–64.4% at 5 mg/mL, respectively (Hsieh et al. 2016). Additionally, synthetic peptides derived from γ-zein, His-Leu-Pro-Pro-Pro-Val, and His-Leu-Pro-Pro-Pro-Val-His-Leu-Pro-Pro-Pro-Val, inhibited POP with IC_{50} values of 80 µM and 30 µM, respectively, whereas collagen-derived peptide and Gly-Pro-Hyp-Gly-Pro-Ala exhibit a lower inhibitory activity (Maruyama et al. 1992). Pro-rich protein sources can be potential substrates for producing hydrolysates with POP inhibitory activity. Pro content was approximately 8% of the total AAs content (Table 3.2); therefore, it is considered a high proline food, with a strong POP inhibitory activity. To the best of our knowledge, this study is the first to report POP inhibitory activity of CGM-H.

3.4.2 Antioxidant activities of CGM-H

CGM-H exhibited various antioxidant activities that showed higher ABTS scavenging activity than reducing power with half maximum effective concentration (EC_{50}) value of 2.92±0.25 and 109.11±3.96 µg leucine/ml, respectively. Hydroxyl radical scavenging activity was so low that EC_{50} could not be determined and was reported as 35.47±0.79% at 3.5 mg leucine/ml. (Table 3.1). ABTS radical scavenging ability is based on hydrogen and electron donating ability (Zhuang, Tang, & Yuan, 2013), while reducing power capacity determined the ability of electron-donating ability of an antioxidant (X. Zhang, Noisa, & Yongsawatdigul, 2020). Hydroxyl radicals are the most reactive oxygen species and induce several damages to biomolecules, such as lipids, proteins, and DNA. One or more residues of hydrophobic amino acids such as His, Pro, Met, Cys, Tyr, Trp, Ala, and Phe can enhance antioxidant activities of peptides by donating proton (Chalamaiah, Hemalatha, & Jyothirmayi, 2012). In addition, the

presence of hydrophobic amino acids at the N-terminus, aromatic amino acids (Trp, Tyr, and Phe), and sulfur-containing amino acids (Cys and Met) contribute to effective radical scavenging property of peptides. These amino acids exhibit strong proton donating capacity (Elias, Kellerby, & Decker, 2008; Samaranayaka & Li-Chan, 2011).

Table 3.1 POP inhibitory and antioxidant activities of CGM-H by pepsin hydrolysis.

Bioactive activities	Capacity
IC_{50} value of POP inhibitory activity (µg leucine/ml)	37.55±1.22
EC_{50} value of ABTS radical scavenging activity (µg leucine/ml)	2.92±0.25
EC_{50} value of ferric reducing power (µg leucine/ml)	109.11±3.96
Hydroxyl radical scavenging activity at 3.5 mg leucine/ml (% inhibition)	35.47±0.79

The values were expressed as mean±standard deviation (SD) (n=3).

CGM-H contained considerable amount of Ala, Pro, Tyr, and Phe (Table 3.2) that could contribute to its hydrogen donating ability. Peptides Leu-Pro-Phe, Leu-Leu-Pro-Phe and Phe-Leu-Pro-Phe derived from corn protein hydrolysate have been reported to show strong ABTS radical scavenging activity with EC_{50} value of 2.70, 2.11, and 2.83 mM, respectively (Zhuang et al., 2013). Similar result was also reported by Tang et al., (2010) that zein peptides contained Leu at the N- terminus exhibited high antioxidant activity. Moreover, antioxidant activity of the soybean peptide, Leu-Leu-Pro-His-His, was largely contributed from His and Pro. (Chen, Muramoto, Yamauchi, & Nokihara, 1996). Therefore, hydrophobic amino acids would be a vital element, contributing to antioxidant activities of peptides. CGM-H contained high amount of hydrophobic amino acids, such as Leu, Pro, Phe, and Tyr that would be associated with radical scavenging activity. However, hydroxyl radical scavenging activity of CGM-H was not prominent. The peptide, Pro-Arg, isolated from salmon protamine hydrolysate showed high capacity to scavenge a hydroxyl radical (Y. Wang, Zhu, Han, & Wang, 2008). The basic peptides fraction derived from grass carp muscle protein hydrolysate had greater ability to scavenge hydroxyl radical than acidic and neutral peptides fraction (J. Ren et al., 2008). In addition, Arg- and Lys-tagged multi walled

carbon nanotubes showed highest hydroxyl radical scavenging activity when compared with His-, Cys-, and Asp-tagged (Amiri, Memarpoor-Yazdi, Shanbedi, & Eshghi, 2013). Xu et al., (2018) also reported that both Lys and Arg effectively inhibited the oxidation of lipids and proteins by scavenging hydroxyl. Moreover, mung bean hydrolysate contained high Lys and Arg that showed hydroxyl radical scavenging activity of 39.91% at 2 mg/ml (Sonklin, Laohakunjit, & Kerdchoechuen, 2018). Lys and Arg are positively charged at physiological pH (7.4) that would directly interact with hydroxyl radicals. Relatively low amounts of basic amino acids (Arg, Lys, and His, Table 3.2) in CGM-H might explain its low hydroxyl radical scavenging activity.

3.4.3 Amino acids (AAs) composition of CGM and CGM-H

The AAs composition of CGM and CGM-H was listed in Table 3.2. Major AAs in both samples were Asp/Asn, Glu/Gln, Leu, Ala, and Pro. Total hydrophobic amino acid contents of CGM and CGM-H were comparable, accounting for 44.36, and 43.13%, respectively ($p>0.05$, Table 3.2). In addition, all AAs were increased after pepsin hydrolysis. CGM contained ~18% (w/w) starch and other components, resulting in lower protein content. Thus, total AAs content of CGM-H was higher than CGM counterpart. However, total hydrophobic AAs of CGM-H was lower than CGM because pepsin which is an endo-protease that has broad specific for aromatic or carboxylic L-amino acids inside the protein structure, especially cleaves C-terminal to Phe and Leu, resulting in high hydrophobic amino acids in hydrolysate. CGM and CGM-H were also contained higher concentration of essential AAs (~35% of total AAs) that could be a good source to produce supplement nutrition. Li.X.x et al., (2008) reported that Glu/Gln and Leu were main AAs in CGM and about 41% of AAs was hydrophobic amino acids. In addition, Zhou et al., (2015) reported that CGM protein was rich in Glu/Gln, Leu, Pro and Ala. These are in agreement with our study. The major AAs composition of rice bran protein were Phe, Tyr, Val, Ile, Leu, Typ, and Ala that resulting in high antioxidant functions of rice bran hydrolysate (Phongthai & Rawdkuen, 2020), while Pro was inconspicuous component (~9 mg/g sample).

Table 3.2 Total AA composition of CGM and CGM-H.

Name	AA content (mg/g sample)	
	CGM	CGM-H
Ala	55.65±4.80	63.87±0.69
Asp / Asn	147.99±12.52	190.82±4.60
Arg	22.61±2.55	23.21±2.22
Glu / Gln	142.94±7.01	157.30±2.19
Gly	18.89±2.66	21.62±1.03
His	10.60±2.42	11.66±1.36
Ilu	29.76±1.89	35.48±1.37
Leu	92.25±6.55	99.05±0.06
Lys	6.56±2.10	9.33±1.18
Phe	40.68±5.16	50.24±2.64
Pro	62.46±3.32	71.96±0.23
Ser	33.79±4.40	42.88±2.21
Thr	22.81±2.60	26.30±1.82
Tyr	30.16±4.92	38.22±0.25
Val	32.90±2.40	36.64±1.15
Total hydrophobic AAs	332.59±26.78	378.86±6.58
Total essential AAs	235.60±23.12	268.70±9.45
Total AAs	750.04±65.30	878.58±21.92

The values were expressed as mean±SD (n=3).

Mung bean hydrolysate fraction from ultrafiltration which contains high proportion of aromatic amino acids, including Tyr, Phe, and Trp exhibited highest radical scavenging activity than that other fractions (Sonklin et al., 2018). Therefore, AAs such as Leu, Pro, Phe, His, Arg, Tyr, Glu, and Asp were believed to play an important role for antioxidant activities of peptides (Chalamaiah et al., 2012; X. x. Li, Han, & Chen, 2008; Zhuang et al., 2013), while Pro was important for POP inhibition. CGM-H contained high concentration of Leu, Pro, Tyr that might be a reason of high POP inhibitory and antioxidant activities.

3.4.4 The effect of in vitro GI digestion on MW and bioactive activities of CGM-H

MW distribution of digestive enzyme blank, CGM-H and CGM-H digesta are presented in Fig 3.2. Most peptides in CGM-H showed MW greater than 1000 Da, while digested CGM-H were mostly in the range of 200–500 Da (Table 3.3). Hydrolysis of CGM-H by digestive enzymes (pepsin + pancreatin) resulted in peptides with MW < 200 Da.
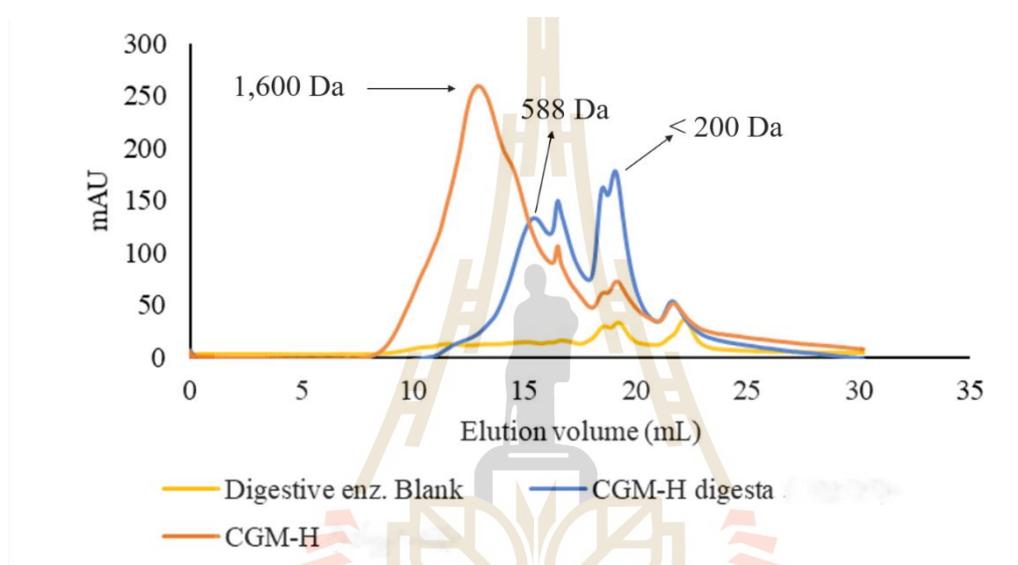
Figure 3.2 Size exclusion chromatograms of digestive enzyme blank, CGM-H, and CGM-H digesta.

Table 3.3 MW distribution of peptides in CGM-H and CGM-H digesta.

MW of peptides	Relative area of peptide (%)		
	Enzyme blank	CGM-H	CGM-H digesta
<200	43.81±0.50[c]	16.92±0.35[a]	33.30±0.81[b]
200–500	23.83±0.66[b]	13.76±0.63[a]	33.72±0.64[c]
500–1000	15.03±0.95[b]	16.48±1.04[a]	22.70±0.55[c]
>1000	17.32±0.45[b]	52.85±0.58[c]	10.28±0.42[a]

Different letters (a, b, c) indicate significantdifference ($p \le 0.05$, n=3) between sample group at the same row. The values are expressed as mean±SD.

These changes have an effect on POP inhibitory and antioxidant activities of CGM-H as shown in Table 3.4. The POP inhibitory activity was noticeably decreased upon *in vitro* GI digestion (Table 3.4). The quantitative structure–activity relationship between POP and POP inhibitory peptide derived from β-casein which identified to be competitive inhibitor have been reported by Pripp, (2006) that POP binds to adjacent residues to the prolyl group at carboxyl side in the peptide structure to form inhibitory site. In addition, ring stacking between the indole ring of Trp595 of enzyme and inhibitor Pro residue might enhance the hydrogen bond forming between Arg643 of catalytic side and inhibitor, which inactivated the enzyme (Polgar, 2002). Moreover, large hydrophobic residues at the N-terminus of peptides could increase the affinity between enzyme and peptides inhibitors (Pripp, 2006). Therefore, POP inhibitory peptides should contain Pro residues in the structure and more hydrophobic amino acid residue at the N-terminus to greatly improved their inhibition potency.

Most of large peptides degreaded to small peptides upon GI digestiont (Table 3.3), reducing bulkiness of hydrophobic amino acid residues modifying at the N-terminal end of peptides, which would result in a decrease of POP inhibitory activity (Table 4). Fülöp et al., (1998) reported that the S3 subsite in the active site of POP provides a rather nonpolar environment and is further connected with several nonpolar residues, thus this subsit prefers to interact with a hydrophobic residues of substrate or inhibitor, suggustion that POP was almost completely inactivated by bulky nonpolar molecule, whereas a small molecule showed partly inhibition. Maruyama et al., (1992) reported that peptides with MW higher than 1000 Da (Pro-Arg-Pro-Gln-Pro-His-Pro-Gln-Pro-His-Pro) corresponding to the sequence of γ-zein was more potent POP inhibitor than peptides with MW ~600 Da (His-Leu-Pro-Pro-Pro-Val). However, the peptide Leu-Pro-Pro-Pro-Val-His with MW ~600 Da showed higher POP inhibition than the peptide His-Leu-Pro-Pro-Pro-Val with same MW. Thus, there is no concensus about the size of POP inhibitor peptides. Amino acids and their position in the peptide sequence appear to be critical factors governing POP inhibitory activity. Although, CHM-H were digested by GI digestive enzyme, POP inhibitory activity was still measured (Table 3.4). This could imply that POP inhibitory peptides still exist upon GI digestion.

Table 3.4 POP inhibitory and antioxidant activities of CGM-H and CGM-H digesta.

Bioactive activities	Enzyme blank	CGM-H	CGM-H digesta
IC_{50} value of POP inhibition (mg leucine/ml)	ND	0.07 ± 0.30^a	4.73 ± 8.37^b
ABTS radical scavenging activity (mM TEQ) [A]	1.88 ± 0.01^a	17.08 ± 0.20^b	12.92 ± 1.17^c
Ferric reducing power (mM TEQ) [A]	0.04 ± 0.00^a	0.29 ± 0.00^b	0.25 ± 0.01^c
Hydroxyl radical scavenging activity (% inhibition) [A]	28.50 ± 0.53^a	46.77 ± 0.21^b	52.23 ± 1.30^c

[A] Antioxidant activities were determined at the same volume of samples. ND=not detected. The values are expressed as mean±SD (n=3). Values in the same row followed by different letters (a, b, c) indicate significant differences ($p<0.05$).

ABTS radical scavenging activity and ferric reducing power also decreased after *in vitro* GI digestion (Table 3.4). Approximately 15% of these activities was attributed from enzyme blanks. Hydroxyl radical scavenging activity appeared to increase upon GI digestion. However, about 50% of the observed value was attributed from the enzyme blanks. Therefore, digested corn peptides likely exhibited lower hydroxyl radical activity than the original CGM-H. These results suggested the instabity of bioactive peptides in CGM towards digestive enzymes. This poses technological challenge for stability enhancement. Similarly, ABTS radical scavenging activity of soy bean hydrolysate was decreased after stimulated GI digestion (Q. Zhang et al., 2018). DPPH scavenging capacity and reducing power of corn protein hydrolysate prepared from Alcalase decreased upon pepsin digestion and largly increased from original hydrolysate at the end of intestinal digestion, while hydroxyl radical scavenging ability dramatically decreased during pepsin digestion and slightly increased at the end of intestinal phase (X. Ren et al., 2018). Pepsin is an endopeptidase targetting aromatic amino acids or Leu residues in both P_1 and $P_{1'}$, while pancreatin is a mixture of endo- and exopeptidases, which possess a broad specificity (X. Zhang et al., 2020). CGM-H digesta contained high amount of peptides with MW <200 Da (33.30%, Table 3.3). Free amino acids were released by pancreatin after intestinal digestion. Amino acids have been reported to have limited antioxidant

activities when compared to peptides (Elias et al., 2008; Rival, Boeriu, & Wichers, 2001). This explained why antioxidant activities decreased after GI digestion. In addition, hydrophobic amino acids at C-terminal ends largely contribute to antioxidant activity of peptides than N-terminal region (Y.-W. Li & Li, 2013). Amino acids at C-terminal region of peptides can change during GI digestion that could also affect antioxidant activity. Our results demonstrtaed that bioactive peptides in CGM-H were unstable against digestive enzymes. However, CGM-H still contained bioactive peptides for brain function after oral administration.

3.4.5 Cytoprotective effect against SH-SY5Y cells

CGM-H and its digesta were nontoxic against SH-SY5Y cells at concentrations ranging from 0.35-2.77 mg Leucine/ml and 0.91 to 14.50 mg Leucine/mL, respectively. Thus, these concentrations were applied to investigate the neuroprotection and cellular antioxidant activities. CGM-H and its digesta showed cytoprotection against H_2O_2–induced oxidative damage at dilution of 8x to 64x, corresponding to 0.17–1.38 mg Leucine/ml and 0.91–7.25 mg Leucine/ml respectively (Figure 3 A). However, digestive enzyme blank at concentration of 0.21–0.84 mg Leucine/ml (32x to 8x) also showed neuroprotective effect, but lower than CGM-H and some dilution showed comparable results to those of CGM-H digesta.These results suggested that peptides or compounds presenting in commercial pepsin and pancratin contributed to neuroprotection of CGM-H digesta at high dilution.

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(A)

(B)
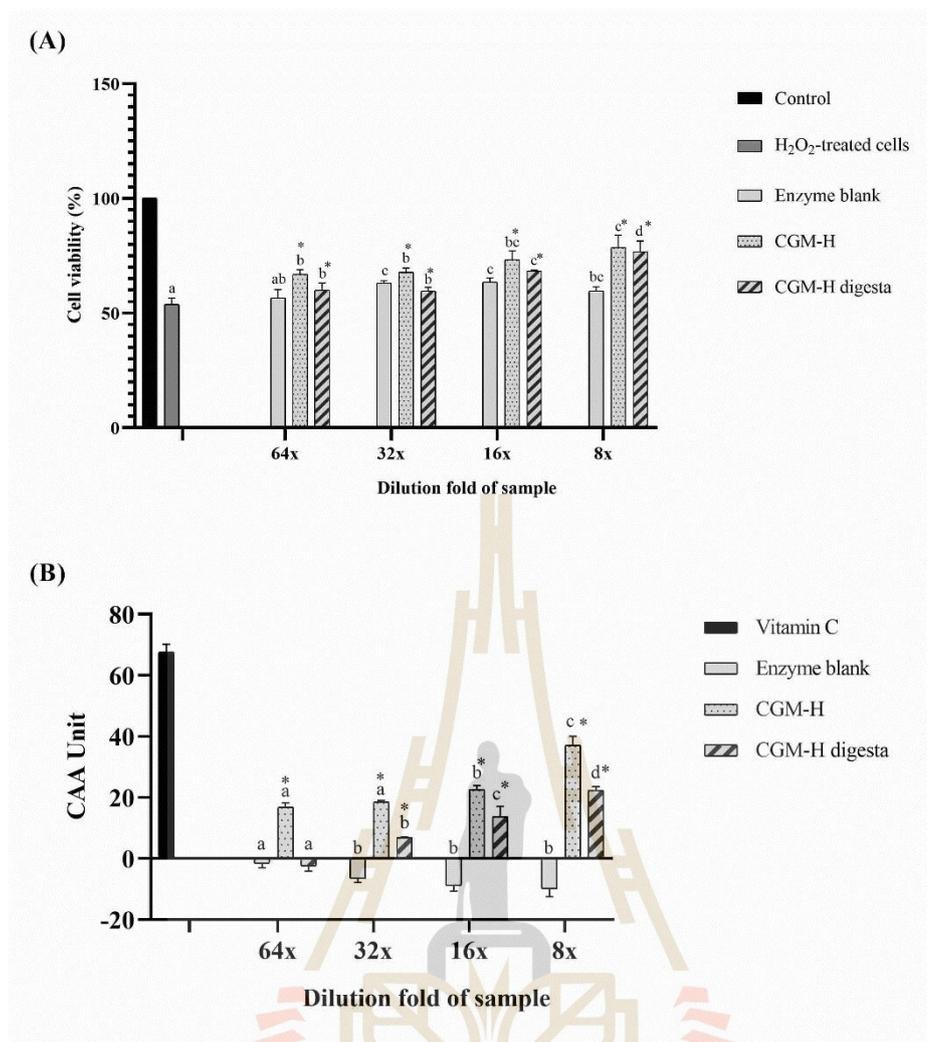
Figure 3.3 Neuroprotection (A) and intracellular ROS reduction capacity (B) against SH-SY5Y cells of digestive enzyme, CGM-H, and digesta. *indicates difference of each sample group when compared with the enzyme blank at the same dilution fold. Different lowercase letters indicated differences at various peptide content in the same sample group ($p<0.05$, n=3).

At 8x dilution of CGM-H digesta, corresponding to 7.25 mg Leucind/ml exhibited greater cytoprotection as compared to its enzyme blank (Figure 3.3A). This would imply that peptides resulted from *in vitro* digestion of CGM-H were capable to protect oxidative stress induced from OH radicals. It should be mentioned that CGM-H digesta exhibited lesser antioxidant cellular capacity than the original CGM. These results were in agreement with those of antioxidant activity assay based on synthetic radicals.

Both CGM-H and its digesta exhibited lower intracellular ROS scavenging activity when compared to 1 mM ascorbic acid (Figure 3.3B). Furthermore, CGM-H was still more effective than its digesta, while digestive enzymes did not exhibit intracellular ROS scavenging activity. Therefore, the observed CAA of digesta was mainly contributed from digested peptides.This study suggested that larger peptides showed better ability to reduce intracellular ROS in SH-SY5Y cells than smaller peptides. Similar results reported that larger peptide Ser-Glu-Glu-Tyr derived from walnut meal protein hydrolysate significantly protected oxidative damge SH-SY5Y cells to a greater extent than the smaller peptide, Glu-Ser-His (Feng et al., 2019). In contrast, Wang et al. (2016) reported that corn peptides with MW<1000 Da displayed greater intracellular ROS scavenging activity than larger peptides (MW 1000–3000 Da) based on H_2O_2-induced HepG2 cells. The authors explained that effective peptides with smaller size had more potency to penepetrate into the cells than larger peptides (L. Wang et al., 2016). Discrepancies of these studies suggested that molecular mass of peptides might not be a prime factor determining cellular antioxidant ability of peptides. Our study revealed that cellular antioxidant activity of CGM-H well correlated with ABTS scavenging activity and ferric reducing power.Thus, these 2 assays could be used as rapid screening methods to determine potential corn hydrolysates with biological antioxidant activity. Previous studies have reported different trends. The peptide Thr-Tyr derived from walnut meal protein hydrolysate showed high ABTS radical scavenging activity, but negligible protective effect againt oxidative damage on SH-SY5Y cells induced by H_2O_2 (Feng et al., 2019). Therefore, our study demonstrated that CGM-H could improve neurodegenerative disorder by reducing of oxidative stress generated in nervous cells.

3.4.6 Gene expression of antioxidant enzymes

The expression of genes encoding antioxidant enzymes of H_2O_2-induced SH-SY5Y cells pre-treated with CGM-H and its digesta are shown in Figure 3.4. Under oxidative stress condition, SOD converts superoxide anion to H_2O_2, then CAT converts H_2O_2 to water (H_2O) and oxygen (O_2). In addition, GPx eliminates H_2O_2 by converting it to H_2O (X. Zhang et al., 2020). In the presence of H_2O_2, expression of *CAT* gene was downregulated, while that of *GPx* was upregulated, and *SOD* was not affected (Figure 3.4B, 3.4D, 3.4C). Both CGM-H and its digesta samples upregulated *CAT*

under oxidative damage condition (Figure 3.4B), while they did not significantly affected *SOD* and *GPx* expression (Figure 3.4C, 3.4D). In addition, CGM-H digesta was likely upregulated *SOD* at concentration of 3.63 mg Leucine/ml (16x dilution). HepG2 cells exhibited lower SOD and CAT activity in the presence of H_2O_2, but these activities were all increased after pre-treatment with corn peptides (L. Wang et al., 2016). Wang et al., (2018) reported that *GPx3*, *GPx5*, and *SOD3* genes were also down-regulated in HepG2 cells treated with H_2O_2, but they were up-regulated by pre-treatment of CGM hydrolysate with MW less than 1000 Da (L. Wang et al., 2018).
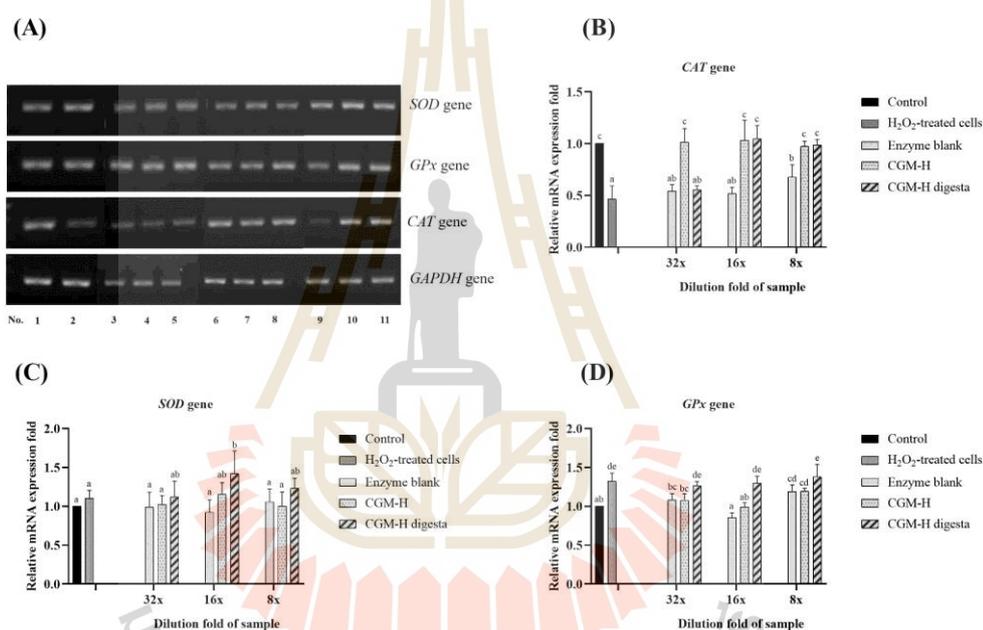
Figure 3.4 Representative 1.5% agarose gel electrophoresis of genes encoding antioxidant enzymes (A) of cell control (No.1), H_2O_2-treated cells (No.2). Pre-treated cells with digestive enzyme blank, CGM-H and CGM-H digesta at 32x, 16x, and 8x dilution fold of peptide content (No.3, 4, 5.; No. 6, 7, 8.; No.9, 10, 11, respectively). Relative mRNA expression fold of *CAT* (B), *SOD* (C), and *GPx* (D) gene of cell control, H_2O_2-treated, and peptides pre-treated H_2O_2- induced SH-SY5Y cells. Different letters (a, b, c) indicate significant difference at various concentrations of the enzyme blank, CGM-H, and CGM-H digesta ($p<0.05$, n=3).

Our present study revealed that CGM-H upregulated *CAT* to a higher extent than its digesta at low concentration, but they performed the same at high concentration (Figure 3.4B) although ROS reducing capacity of CGM-H was better than its digesta at dose-dependent manner (Figure 3.3B). Our results suggested that size of peptide might not be a main factor influencing the expression of antioxidant enzymes. Our results revealed that peptides in CGM-H and its digesta might decrease intracellular ROS by upregulating *CAT* expression and other mechanisms, such as hydrogen-donating ability. Other studies have been reported that peptides derived from soybean at 0.25 mg/ml upregulated *SOD* expression better than at higher concentrations, while upregulation of *CAT* occurred to the greatest extent at 0.5 mg/ml (Yi, Din, Zhao, & Liu, 2020). These results suggested that peptides in hydrolysate might affect expression of antioxidant enzyme genes differently.

3.5 Conclusions

CGM-H exhibited strong POP inhibitory and comparable antioxidant activities, but these activities decreased after *in vitro* GI digestion, In addtion, CGM-H and its digesta were effective to protect against H_2O_2-induced SH-SY5Y cells damage and reduced intracellular ROS. Futhermore, both CGM-H and its digesta upregulated *CAT* gene in H_2O_2-induced SH-SY5Y cells. Therefore, CGM-H showed potential as a nutraceutical ingredient.

3.6 References

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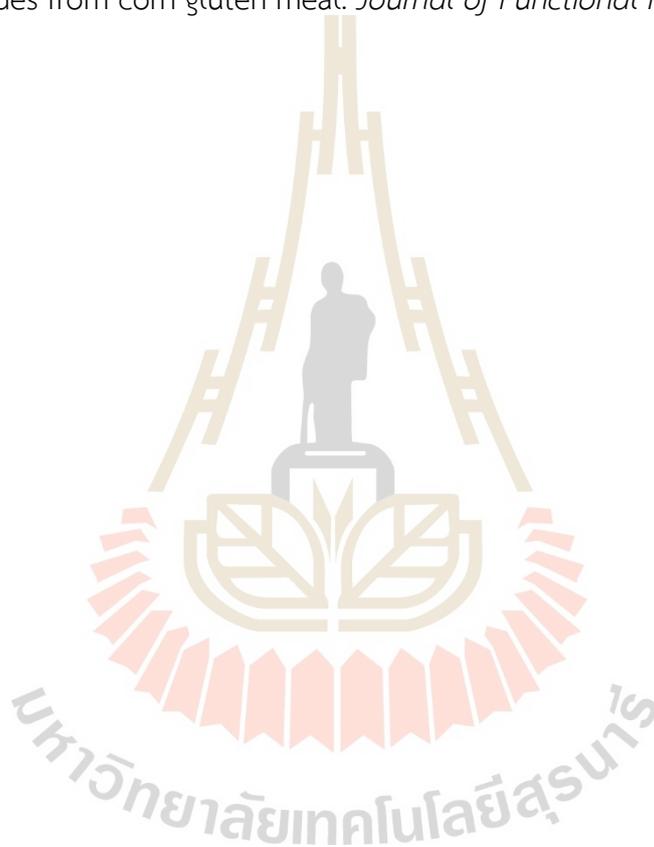
CHAPTER IV

CORN GLUTEN MEAL PEPTIDES INHIBIT PROLYL OLIGOPEPTIDASE AND MODULATE α-SYNUCLEIN AGGREGATION IN KCL-INDUCED SH-SY5Y CELLS

4.1 Abstract

Prolyl oligopeptidase (POP) is known to be related with neurocognitive disorder as it degraded neuroactive peptides. POP inhibitory peptides from CGM hydrolysate (CGM-H) were identified and characterized. After series of chromatographic separation, ALLTLSPLGPA was identified to be the most effective POP inhibitor with IC_{50} value of 0.79±0.004 mM. Its *in-silico* gastrointestinal (GI) digested peptide, SPLGPA, exhibited 3-time lower POP inhibitory activity, while higher inhibition was observed in SH-SY5Y cells when compared to its parent peptide. In addition, 25 µM ALLTLSPLGPA notably reduced α-synuclein aggregation of KCl-treated SH-SY5Y cells. ALLTLSPLGPA and SPLGPA, were characterized as a mixed-type and uncompetitive inhibitor, respectively. They bound to POP via hydrogen bonds at β-propeller domain. These results demonstrated that peptides derived from CGM could have potential for developing nutraceutical products targeting brain health.

Keyword: Maize, *in-silico* digestion, Prolyl oligopeptidase inhibitory peptide, α-synuclein aggregation

4.2 Introduction

Corn gluten meal (CGM) is a major by-product from corn starch processing, containing 67–71% protein. Zein is a major protein in CGM with hydrophobic nature (Trinidad-Calderón, Acosta-Cruz, Rivero-Masante, Díaz-Gómez, García-Lara, & López-Castillo, 2021), which limits its use in food industry.Enzymatic hydrolysis of CGM is an effective strategy to overcome the limited functionalities of CGM, which would increase its utilization. CGM hydrolysates with various bioactivities, including

antioxidant, anti-inflammatory, anti-hypertensive, hepatoprotective, anti-cancer, antimicrobial, and dipeptidyl peptidase IV-inhibitory activities, have been reported (Duvick, Rood, Rao, & Marshak, 1992; Liang, Chalamaiah, Liao, Ren, Ma, & Wu, 2020; Ma, Hou, Shi, Liu, & He, 2015; Mochida, Hira, & Hara, 2010; Trinidad-Calderón et al., 2021; Wang, Ding, Yu, Zhang, Ma, & Liu, 2016). Bioactivities related to brain function have been rarely explored.

Prolyl oligopeptidase (POP) also known as prolyl endopeptidase or post-proline cleaving enzyme (POP, EC 3.4.21.26). POP is a member of serine protease family which cleaves peptide bonds at the carboxyl side of proline residues (Wilson, Hayes, & Carney, 2011). Previous studies reported that POP could be involved in several physiological functions, such as cell division and differentiation, learning and memory, signal transduction and psychiatric disorder (García-Horsman, Männistö, & Venäläinen, 2007). Moreover, POP has been claimed to be related with neurological disorders, such as Alzheimer's disease (AD), amnesia, depression, schizophrenia (Wilson et al., 2011), and Parkinson's disease (García-Horsman et al., 2007). POP has also been involved in the aggregation of α-synuclein via direct protein-protein interaction (Savolainen, Yan, Myöhänen, & Huttunen, 2015; Svarcbahs, Julku, Norrbacka, & Myöhänen, 2018). The α-synuclein oligomerization has been shown to be a main component of Lewy bodies, leading to dopamine neuron death in the brain which is a cause of Parkinson's disease (Cui et al., 2021). Therefore, POP inhibition is considered to be an alternative means to improve neurodegenerative disorders. Previous published POP inhibitors are substrate-like inhibitors, such as pramiracetam, baicalin, JTP-4819, KYP-2047, and S-17092 that are synthesized based on N-*acyl-L-*prolyl-pyrrolidine structure (Hsieh, Wang, Hung, Hsieh, & Hsu, 2016). The peptide, IYPFVEPT, derived from human β-casein has been reported to inhibit POP with IC_{50} value of 8 μM (Asano, Nio, & Ariyoshi, 1991). Peptides isolated from a sake also inhibited POP with IC_{50} value between 11.8–42.8 μM (Saito, Ohura, Kawato, & Suginami, 1997). POP inhibitory peptides derived from CGM has not been elucidated. Therefore, the aims of this study were to produce hydrolysate with POP inhibitory activity and to identify POP inhibitory peptides. Furthermore, ability of POP inhibitory peptides to reduce aggregation of α-synuclein protein in SH-SY5Y cells was determined.

4.3 Materials and methods

4.3.1 Materials

CGM was obtained from Friendship Corn Starch Industry (Samutprakarn, Thailand). POP from *Flavobacterium* sp., Z-glycyl-L-proline-4-nitroanilide (Z-gly-Pro-pNA), 2,4,6-trinitrobenzenesulfonic acid solution (TNBS, 5% w/v), and pepsin from porcine stomach mucosa (400 U/mg) were purchased from Sigma- Aldrich Inc. (St. Louis, MO., USA). The **α**-synuclein monoclonal antibody was purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Rockford, IL, USA). 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) of HPLC grade were used. Other chemicals used were of an analytical grade.

4.3.2 CGM hydrolysate (CGM-H) preparation

CGM was hydrolyzed using pepsin at 1% of CGM (w/w) at 37°C, pH 2.0 for 12 h in a shaking water bath. To stop the reaction, the mixture was heated at 95°C for 10 min and cooled on ice immediately. The hydrolysates were centrifuged at 10,000×g for 10 min. Supernatant was adjusted to pH 7.0 by 6 N NaOH. Degree of hydrolysis (DH) was determined using α- amino content based on TNBS assay using L-leucine as a standard. CGM-H was lyophilized before stored at -80°C until use.

4.3.3 POP inhibitory activity assay

The *in vitro* POP inhibitory activity was performed using a 96-well plate according to Hsieh et al., (2016) with slight modifications. CGM-H (12.5 µl) was added to 25 µL of 2.5 mM Z-gly-Pro-pNA solubilized in 40% 1,4-dioxane and 50 µL of 100 mM Tris-buffer (pH 7.0). Reaction mixtures were then incubated at 30°C for 10 min before 12.5 µL of POP at 0.5 U/mL were added to initiate the reaction. Samples were incubated at 30°C and absorbance was measured at 410 nm for 2-min intervals for 30 min using a microplate reader (Varioskan LUX, Thermo Scientific, Vantaa, Finland). Deionized (DI) water was replaced CGM-H for the control samples. Absorbance values were plotted as a function of time and POP activity was calculated from the slope. The POP inhibition was calculated based on the following equation:

$$\text{POP inhibition (\%)} = \frac{\text{slope of control} - \text{slope of test sample}}{\text{slope of control}} \times 100$$

The half maximum inhibition concentration (IC$_{50}$) value was also calculated at various concentrations of peptide in CGM-H.

4.3.4 Purification of POP inhibitory peptides

CGM-H was firstly fractionated using centrifugal ultrafiltration (UF) membrane (Vivaspin, Sartorius, Lower Saxony, Germany) with molecular weight cutoff (MWCO) 10 kDa and centrifuged at 5,000×g for 30 min. Permeate and retentate fractions were collected and measured for POP inhibitory activity as described in 2.3. Retentates were lyophilized and stored at -20°C until use.

The lyophilized UF-retentate fraction (30 mg) was dissolved in 1 mL of DI water. One hundred µL was applied onto a Superdex Peptide 10/300 GL column (10×300 mm, GE Healthcare, Piscataway, NJ, USA). Peptide elution was performed using Fast-Protein Liquid Chromatography (FPLC, ÄKTA pure 25, GE Healthcare, Piscataway, NJ, USA) with a stepwise mode at a flow rate of 0.8 mL/min. The elution chromatogram is shown in Fig. 1a. Peptide fractions were individually collected and monitored at 214 nm. Each fraction was measured for POP inhibitory activity and the fraction exhibiting the highest POP inhibitory activity was collected and lyophilized for reverse-phase chromatographic (RPC) separation. The lyophilized sample was solubilized in DI water and applied to a SOURCE^{TM} 5RPC ST 4.6/150 column (GE Healthcare, Piscataway, NJ, USA). The peptide elution was performed using mobile phase A (DI water containing 0.1% TFA (v/v)) and mobile phase B (ACN containing 0.1% TFA(v/v)) with gradient mode at a flow rate of 0.5 mL/min. The elution was started with 7.5–50% of mobile phase B for 30 mL, followed by 50–100% of mobile phase B for 10 mL as shown in Fig. 1b. Peptide was monitored at 214 nm. The fraction possessing the highest POP inhibitory activity was collected for peptide identification.
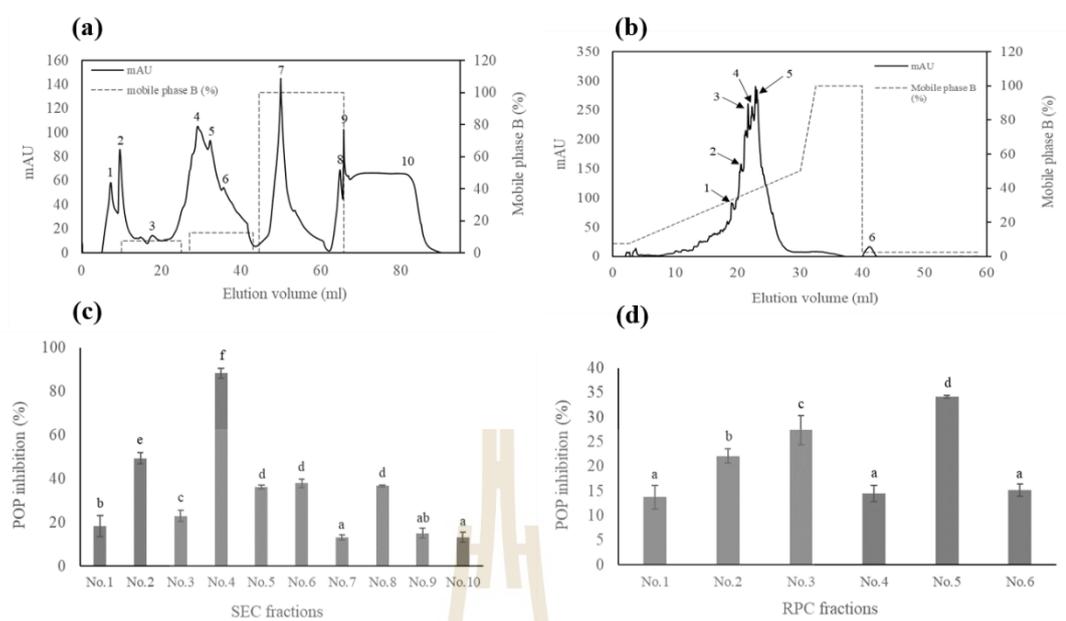
Figure 4.1 Chromatograms of CGM-H by size exclusion chromatography (SEC) (a), and revers-phase chromatography (RPC) (b). POP inhibitory activity of peptides fractions from SEC (c) and RPC (d) at the same volume. Different letters indicated significant difference at $p \leq 0.05$, n=3.

4.3.5 Peptide identification and synthesis

The *de novo* peptide sequencing was carried out using the Ultimate 3000 Nano/Capillary LC System (Dionex Ltd., Surrey, UK) coupled to a Hybrid quadrupole Q-TOF impact IITM (Bruker, Daltonics, Germany) with a nano-captive spray ion source. The peptide fraction was performed using a µ-Precolumn 300 µm i.d. × 5 mm C18 Pepmap 100, 5 µm, 100 A° (Thermo Scientific, Altrincham, UK) and separated on 175 µm i.d. × 15 cm which packed with Acclaim PepMap RSLC C18, 2 µm, 100 A°, nanoViper (Thermo Scientific, Altrincham, UK). Mobile phase A (DI water containing 0.1% formic acid) and mobile phase B (80% ACN containing 0.1% formic acid) were applied. The peptide fraction was eluted using gradient mode starting from 5 to 55% of mobile phase B at a constant flow rate of 0.3 µL/min for 30 min. The electrospray ionization was carried out at 1.6 kV using the CaptiveSpray. Mass spectra (MS) and MS/MS spectra were obtained on the positive-ion mode over the range (m/z) 150 to 1000 (Compass 1.9 software, Bruker Daltonics). The peptide

spectra were analyzed using the PEAKS Studio 10.0 software (Waterloo, ON, Canada). Peptides with at least 70% *de novo* score were selected for synthesis. The homologous proteins of identified peptides were searched by the NCBI standard protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) and/or UniProtKB (peptide search; https://www.uniprot.org/peptidesearch/) using *Zea may* (Maize; taxid: 4577) as a database. The selected peptides were synthesized using a solid-phase peptide synthesis method (GL Biochem, Shanghai, Ltd.) The synthetic peptides were purified to 98% purity.

The *in silico* gastrointestinal (GI) digestion were performed using BIOPEP tool (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008). Digestive enzymes, including pepsin (pH 1.3), trypsin, and chymotrypsin A, were used for the *in-silico* digestion. Peptide fragments from *in silico* digestion were also synthesized as described above.

4.3.6 Mode of POP inhibition

Parent peptide (ALLTLSPLGPA) showing the highest POP inhibitory activity) and its *in silico* GI digested fragment (SPLGPA) were determined for mode of inhibition. Various concentrations of the parent peptide (0, 0.94, 1.25, and 1.88 mM), and the *in-silico* fragment (0, 1.25, 1.875, and 3.125 mM) were incubated with POP in the presence of various concentrations of Z-gly-pro-pNA (0.5, 1, 1.5, and 2.0 mM). The inhibition kinetic was determined using Lineweaver-Burk by plotting 1/V versus 1/[S]. Kinetic parameters, namely V_{max}^{app} and K_m^{app} were calculated by linear regression from Lineweaver-Burk graph. Inhibitor constant (K_i) was calculated using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA).

4.3.7 Molecular docking

The structure of peptides was generated by PyMol 2.5. The crystal structures of prolyl oligopeptidase (POP) (PDB:3DDU) was selected for this study as it has good resolution of 1.56 Å. The structure of substrate (Z-gly-Pro-pNA) was obtained from the PubChem database. The prolyl oligopeptidase was prepared by removing water molecules and co-crystal ligands. Molecular docking was performed using AutoDocking Vina (Trott and Olson, 2010). The parameters of Autodock Vina tool were set as follows: grid centers were designed at dimensions x = -6.792, y =13.536 and z = 30.038: grid sizes were set as x = 15, y =15 and z = 15. Other

parameters were left as default. Since the peptide ALLTLSPLGPA was determined to be a mixed-type inhibitor, docking was performed in both free enzyme and an enzyme-substrate complex. The best docking score (lowest binding energy) was chosen. For an uncompetitive inhibitor (SPLGPA), substrate was allowed to interact with an enzyme before docking with the peptide.

4.3.8 Intracellular POP activity

Neuroblastoma SH-SY5Y cells were seeded in T-25 flasks at a density of 10^6 cell/flask and incubated for 24 h. Subsequently, cells were treated with the synthetic peptide at various concentrations for 72 h. The medium was removed and replenished with fresh medium containing peptides for every 24 h. Cells were then homogenized with lysis buffer (50 mM KH_2PO_4, 1.5 mM $MgCl_2$, 10 mM NaCl, 1 mM EDTA (pH 7.4)) and centrifuged at 16,000×g for 10 min at 4°C. Supernatants were collected and lyophilized, which were then determined for POP activity and protein content. The POP activity was carried out as described in 2.3 except for the reaction time was extended to 45 min. Protein content was determined using Bradford's method and bovine serum albumin (BSA) as a standard. The control was prepared using the medium only. Intracellular POP inhibitory activity was calculated as the shown in 2.3.

4.3.9 Immunofluorescence of α-synuclein aggregation in SH-SY5Y cells

The effect of the parent and *in silico* digested peptide on α-synuclein aggregation was evaluated using immunofluorescence following by the method of Vijiyakumaran et al., (2019) with slight modifications. Cells were cultured in a 24-well plate with coverslips at a density of 5×10^5 cell/mL and allowed to grow in the medium at 37°C and 5% CO_2 for 24 h. Peptides were added to SH-SY5Y cells at various concentrations and incubated for 48 h. Thereafter, cells were treated with 50 mM potassium chloride (KCl) to induce α-synuclein aggregation after peptide removal. Cells treated with the medium containing KCl in the absence of peptide were used as the aggregation group, while cells treated with the medium alone were used as the control group. Cell fixation was carried out using 4% paraformaldehyde solution, non-specific binding was blocked by incubation with 10% normal goat serum for α-synuclein in PBS (pH 7.4) for 30 min. Primary antibody (1:1000 Mouse IgG1 α-synuclein monoclonal antibody) was added and incubated overnight at 4°C.

After washing with 0.2% Triton-X in PBS, samples were incubated with secondary antibody (1:500 goat anti-mouse fluorescein-conjugated for **α**-synuclein) for 2 h. The coverslips were mounted on slides using Vectashield with 4',6-diamidio-2-phenylindole (DAPI) as a nuclear marker (wavelength 358 nm (ex) and 461 (em)) (Vijayakumaran, Nakamura, Henley, & Pountney, 2019). Control staining was carried out by omission of primary antibody. Immunofluorescent photomicrographs were captured by a laser scanning confocal microscope with 40✕ objective lens (Nikon A1, Nikon Crop., Tokyo, Japan).

4.3.10 Statistical analysis

All measurements were carried out in triplicate. Statistical analysis was performed using SPSS version 25.0 (SPSS Inc., Chicago, IL, USA). Differences in mean values were analyzed using analysis of variance (ANOVA) and Duncan's multiple range test at a significance level of $p<0.05$.

4.4 Results and discussion

4.4.1 Purification and identification of POP inhibitory peptides

After separation by size exclusion chromatography (SEC), the fraction .4 showed the highest POP inhibition (Figs. 1a, c.) with an increase in purity of 2.5 folds (Table 4.1). Other fractions from SEC also exhibited POP inhibition but to a lesser extent, suggesting that POP inhibitor peptides exhibited in varied sizes. After reverse-phase chromatography (RPC), the fraction 5 showed the highest POP inhibition (Figs. 1b, d.) with a 18.3-fold increase in purity (Table 4.1). However, only 1.13% yield was obtained. It could be mentioned that POP inhibitory peptides were also present in discarded fractions of SEC and RPC, which would be a reason for lower peptide yield.

Table 4.1 Purification fold of POP inhibitory peptides isolations.

Purification step	Protein content (mg)	Inhibitory activity (IC_{50}) (µg leucine/ml)	Purification fold	Yield (%)
Crude hydrolysate	181.90	32.98	1.00	100
Retentate UF fraction (MW cut off 10 kDa)	31.02	23.50	1.40	17.05
SEC	14.17	13.19	2.50	7.79
PC	2.05	1.80	18.32	1.13

After purification by RPC, the fraction 5 was collected for peptide identification. Three novel peptides were identified (Fig. 4.2) and ALLTLSPLGPA derived from zein exhibited the highest POP inhibition (Table 4.2). POP inhibitory peptides typically contain one or more proline residues, which are assumed to form hydrogen bonds with Arg643 of the catalytic site of POP (Polgar, 2002). Peptides derived from sodium caseinate, namely AVPYPQRDMPIQAFLLY, PIHNSLPQNIPPLTQTPV, and HPHPHLSFMAIPP containing at least one and up to four internal proline residues showed high POP inhibitory activity with IC_{50} values between 29.8 to 48.2 µM (Hsieh et al., 2016).
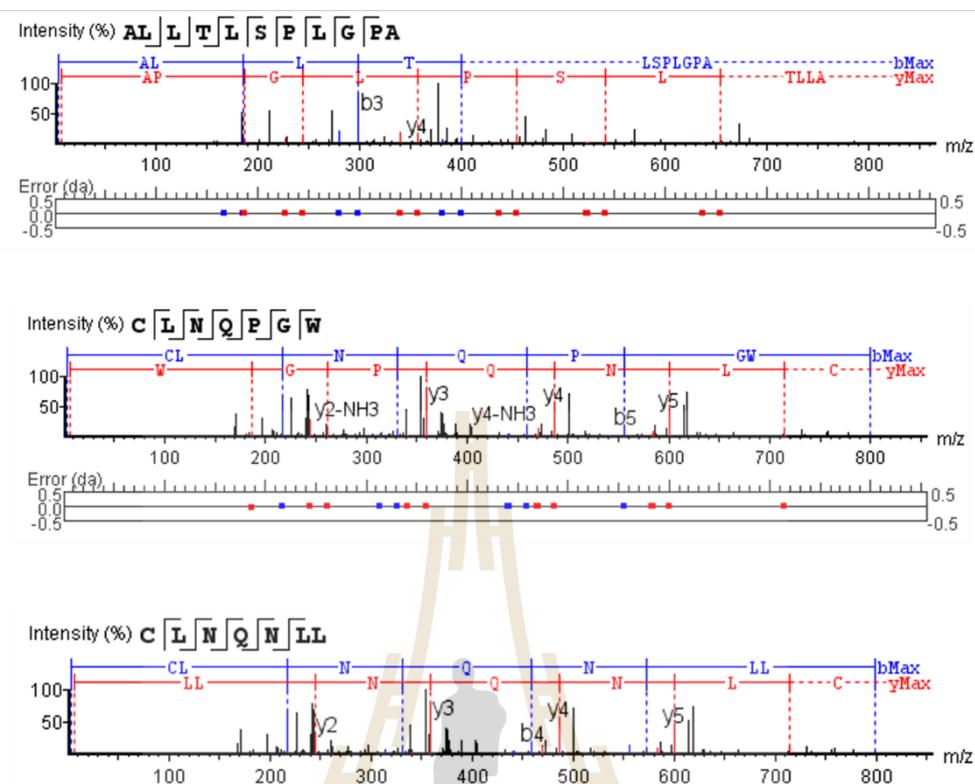
Figure 4.2 *De novo* sequencing MS/MS spectra of identified peptides derived from CGM-H.

In addition, peptides VEIPE and YPIPF from red wine containing one and two proline residues showed POP inhibitory activity with IC_{50} value of 17.0 and 87.8 μM, respectively (Yanai, Suzuki, & Sato, 2003). Based on previous studies, POP inhibitory activity of peptides may not be totally dependent on a number of Pro residues in the sequence. Hsieh et al., (2016) reported that both VPLGTQYTDAPSFSDIP and SIITSTPETPTVAVPTT derived from casein, containing 3 Pro residues in their structures showed opposite trend of POP inhibition with IC_{50} value of 650.5 and 79.8 μM, respectively. In our study, the corn peptide CLNQNLL without Pro exhibited the lowest POP inhibition (Table 4.2). Although CLNQPGW contained one Pro residue, its inhibition efficacy was also low with only about 17% inhibition at 1.0 mM (Table 4.2). The potential POP inhibitors were suggested to share the structure of X_3-X_2-X_1-X_1'-X_2'-X_3', when Pro located at X_1 is a preferred position, and hydrophobic amino acids, such as Ala, Val, Leu, or Ile, were suggested to be in the position X_2, X_3, and X_1'. In addition,

high hydrophobic amino acids at the N-terminal end could increase the POP inhibitory activity (Pripp, 2006). Peptide ALLTLSPLGPA contained more hydrophobic amino acids surrounding Pro, especially Leu and Gly or Ala at position X_3 and $X_1^{'}$, which could be one of reasons of its high inhibitory activity. In contrast, CLNQPGW contained Asn and Gly at X_3 and $X_1^{'}$, respectively. Amino acids at adjacent positions of X_3 and $X_1^{'}$ like Leu or Ala could be critical for enhancing the inhibition potency. Thus, not only the numbers of Pro but also its position and adjacent hydrophobic amino acids plays a vital role in potency of a POP inhibitor peptide.

The *in silico* GI digestion of identified peptides resulted in peptide fragments, which exhibited certain degree of POP inhibitory activity (Table 4.2). All digested peptides showed lower POP inhibition than their parent counterparts (Table 4.2). The peptide SPLGPA showed the most potent inhibitor among digested peptides, but the POP inhibitory activity was decreased approximately 3 times when compared to its parent counterpart with IC_{50} value of 2.40±0.28 mM, whereas the parent ALLTLSPLGPA exhibited POP inhibition with IC_{50} value of 0.79±0.004 mM (Table 4.2). The *in silico* digested peptides, QPGW and QNLL, showed relatively low POP inhibitory activity, while CL did not show any inhibitory activity. The peptide SPLGPA contains Leu and Ala at position X_3 and $X_1^{'}$, respectively, while QPGW only has Gly at position $X_1^{'}$. These results confirmed the important role of Pro and its adjacent amino acids in POP inhibition. In addition, when peptides are digested with digestive enzymes, smaller peptide fragments are formed with lower hydrophobic residues at the N-terminal end compared to the parent peptides, leading to decreased POP binding ability. Our results reveal that long peptide showed more potency to inhibit POP than short peptide. The S2 subsite at the active site of POP contains several nonpolar side chain residues that prefer to interact with hydrophobic amino acids of a substrate or an inhibitor (Fülöp, Böcskei, & Polgár, 1998). This explains why bulky nonpolar molecules surrounding Pro contribute to inhibition potency. Although POP inhibitory peptides derived from CGM-H are likely to be digested upon GI digestion, POP inhibition would still be detected based on their *in silico* digested peptides.

Table 4.2 Peptide sequences of peptides isolated from corn gluten meal hydrolysate and their *in-silico* GI digestion peptides and POP inhibitory activity.

Identified Peptide sequences	Mass (Da)	*de novo* score	Parent protein	Database	POP inhibition (%) at 1.0 mM	POP inhibition (IC_{50}) (mM)	*In-silico* GI digested peptide based on BIOPEP and their activity		
							Peptide fragments	POP inhibition (%) at 2.5 mM	POP inhibition (IC_{50}) (mM)
CLNQPGW	817	73	Nitrate reductase	UniProtKB id: B2C4D0.1	17.56±1.17[b]	ND	CL	ND	ND
							QPGW	11.42±2.95[a]	
CLNQNLL	817	75	30S ribosomal protein S18, chloroplastic	UniProtKB id: Q5GJ60.1	10.36±1.89[a]	ND	QNLL	13.96±3.59[a]	ND
ALLTLSPLGPA	1052	57	Zein-alpha 19D1	UniProtKB id: P06678.1	55.04±0.88[c]	0.79±0.004	SPLGPA	54.63±2.71[b]	2.40±0.28

The values are expressed as a mean±SD (n=3). Different letters (a, b, c) indicated significant difference ($p \leq .05$) in the same column table. ND indicated not detected.

4.4.2   Mode of POP inhibition and molecular docking

Lineweaver-Burk plots, K_m^{app}, V_{max}^{app}, and K_i value of POP in the absence and presence of peptides ALLTLSPLGPA and SPLGPA are shown in Fig. 4.3a, and 4.2b, respectively. The K_m^{app} value of POP-catalyzed reaction appeared to be constant in the presence of ALLTLSPLGPA at concentrations up to 0.94 mM, then slightly increased with peptide concentration, while V_{max}^{app} value decreased with increasing peptide concentrations (Fig. 4.3a). Therefore, peptide ALLTLSPLGPA showed a mixed-type inhibitor of competitive and non-competitive characteristics. The competitive inhibitor affects substrate binding at the active site, while a substrate and a non-competitive inhibitor bind independently to the enzyme at different sites. Therefore, ALLTLSPLGPA can interact with POP at the active site and other sites. The K_m^{app} and V_{max}^{app} of the reaction containing SPLGPA decreased with peptide concentrations, indicating the uncompetitive characteristic (Fig. 4.3b). Previous report indicated that peptides PIHNSLPQNIPPLTQTPV, HPHPHLSFMAIPP, and AVPYPQRDMPIQAFLLY derived from sodium caseinate showed a competitive inhibitor characteristic, but their K_i values were not reported (Hsieh et al., 2016). K_i value of ALLTLSPLGPA and SPLGPA was 0.255 and 0.510 mM, respectively (Fig. 4.3a, b), indicating that ALLTLSPLGPA appeared to bind to POP more effectively than did SPLGPA. This was well correlated with higher inhibitory activity of ALLTLSPLGPA.

(a)

Parameters	Concentrations of ALLTLSPLGPA			
	0 mM	0.94 mM	1.25 mM	1.88 mM
Vmax (ΔA 410/min)	0.2206	0.0546	0.0470	0.0320
Km (mM)	1.9128	1.8904	2.1720	2.7685
Ki (mM)	0.2550			

(b)

Parameters	Concentrations of SPLGPA			
	0 mM	1.25 mM	1.875 mM	3.125 mM
Vmax (ΔA 410/min)	0.2208	0.1277	0.0848	0.0514
Km (mM)	1.9130	1.2120	0.8532	0.6130
Ki (mM)	0.5098			
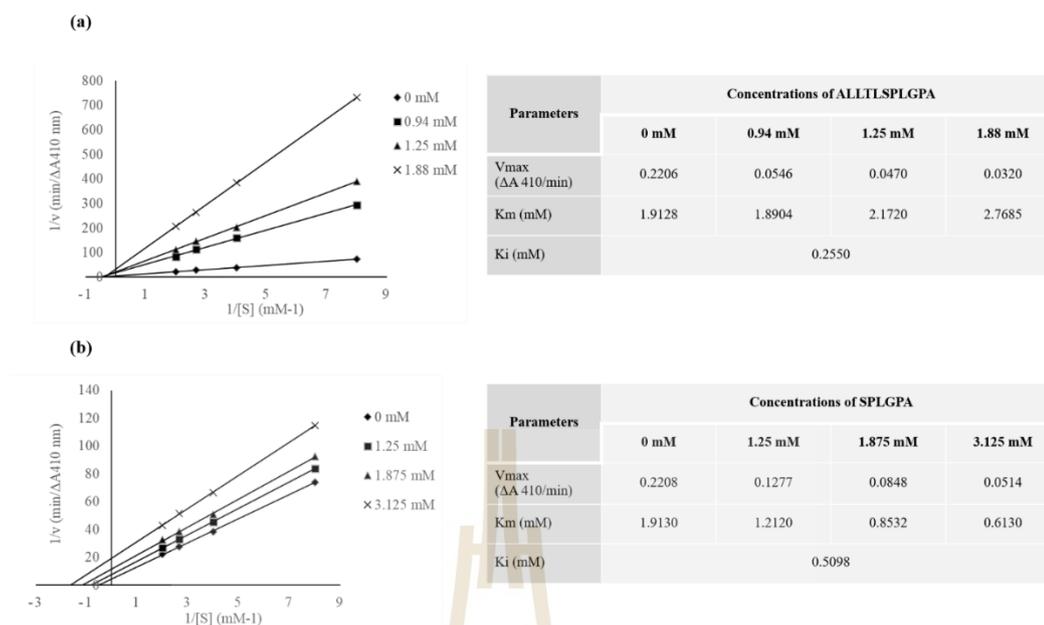
Figure 4.3 Lineweaver-Burk plot analysis and V_{max}^{app} (maximum velocity), K_m^{app} (Michaelis constants), and K_i (enzyme-inhibitor dissociation constant) in the absence and presence of ALLTLSPLGPA (a) and SPLGPA (b), respectively, at varying concentrations of substrate (Z-Gly-Pro-pNA).

Molecular docking was performed to understand how ALLTLSPLGPA and SPLGPA bind to POP (Fig 4.4). The binding energy between POP and ALLTLSPLGPA was -8.6 kcal/mol, which was lower than that of SPLGPA, -7.1 kcal/mol, (Fig. 4.4). The parent peptide ALLTLSPLGPA appeared to interact with POP to a greater extent than SPLGPA. Peptide SPLGPA bound reversibly to the enzyme-substrate complex, resulting in enzyme-substrate-inhibitor complex, leading to enzyme conformational changes and a decrease in activity. The active site of POP is located in a large cavity at the interface of two domains, catalytic and β-propeller domains, and is shaped by a catalytic triad containing Ser554, Asp641, and His680, which provide ideal position for hydrogen bond formation (Khan, Waqas, Khan, Halim, Rehman, & Al-Harrasi, 2022; Laszlo Polgar & Szeltner, 2008). The active site of POP is grouped into 3 subsites, namely S1 pocket which is composed of Phe476, Asn555, Val580, Trp595, Try599, and Val 644. This subsite forms hydrophobic interactions with a substrate or the aromatic rings of an inhibitor (Kumar et al., 2017). The second subsite, S2, contains Arg643, and the third pocket, S3, contains Phe173, Met235, Cys255, Ile591, and

Ala594, which can also form hydrophobic interactions with a substrate (Kumar et al., 2017).
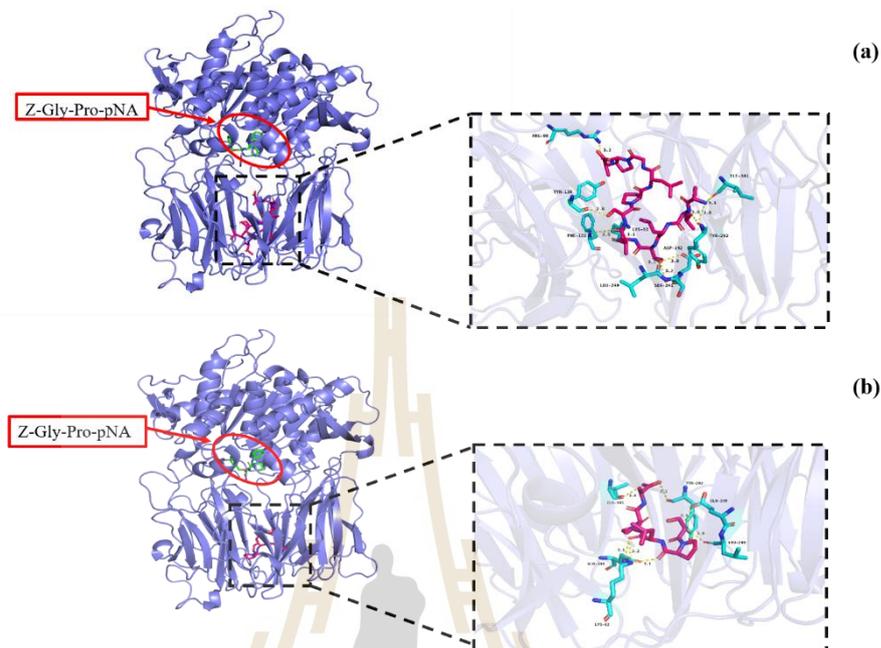
Figure 4.4 Molecular docking between POP and ALLTLSPLGPA (a) and SPLGPA (b) in the presence of substrate (Z-Gly-Pro-pNA). Interactions between peptides (red) and POP (aqua blue) are shown in the square box. The yellow dotted lines represent hydrogen bonds.

The peptide ALLTLSPLGPA formed seven hydrogen bonds with Arg98, Tyr130, Phe132, Ser241, Asp242, Tyr292, and Ile34 (Fig. 4.4a), while the peptide SPLGPA formed hydrogen bonds with Ile341, Gln388, Leu249, Glu239, and Tyr292 (Fig. 4.4b). These 2 peptides interacted with POP at β-propeller domain, which is different site from the active site. These results confirmed that ALLTLSPLGPA and SPLGPA derived from CGM-H can inhibit POP by forming hydrogen bonds at different sites from the active site, inducing conformational changes and eventually leading to POP inhibition.

4.4.3 Intracellular POP inhibition

The POP inhibitory effect of ALLTLSPLGPA and SPLGPA at the cellular level was evaluated using a neuroblastoma SH-SY5Y cells, which exerted similar morphological, neurochemical, and electrophysiological properties to neurons (Xiong et al., 2021). The POP inhibition of SH-SY5Y cells exposed to peptides increased in a dose-dependent

manner (Fig. 4.5). At 25 μM, SPLGPA was more effective to inhibit POP than its parent peptide (p<0.05, Fig. 4.5). Their inhibitory activity was comparable at concentrations of 5-10 μM. Small peptides with high hydrophobic residues could easily passthrough lipid membrane layer of cell membrane that might increase the POP inhibitory potency. This result was quite different from the *in vitro* enzyme assay, indicating that ALLTLSPLGPA showed higher inhibition than SPLGPA (Table 4.2). In the enzyme assay, a peptide inhibitor directly interacted with POP and induced conformational changes of POP. In the cellular system, a peptide needs to be transported across cell membrane before exerting their inhibitory activity. Important parameters for transmembrane diffusion of a compound are polarity and size, particularly small nonpolar molecule can cross cell membrane by passive diffusion (Yang & Hinner, 2015). Efficiency of an amphipathic arginine-rich peptide consisting of 17 amino acid residues to cross cell membrane increased with hydrophobic moment (Takechi-Haraya et al., 2022). Peptide ALLTLSPLGPA showed slightly higher hydrophobicity (72.73%) than did SPLGPA (66.67%), with higher mass of 1052 Da of the former as compared to 541 Da of the latter.
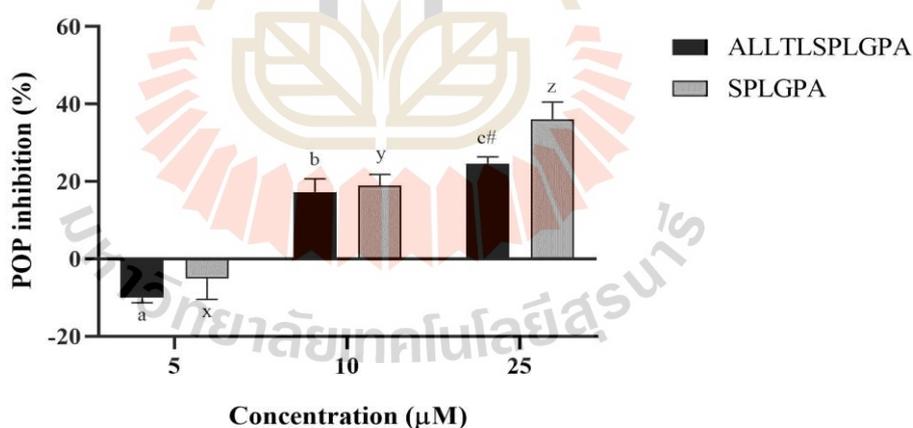
Figure 4.5 Intracellular POP inhibitory activity of synthetic parent and *in silico* digested peptides at various concentrations after incubated with neuroblastoma SH-SY5Y cells for 72 h. Data were expressed as mean ± SD. Different letters of a, b, c and x, y, z indicated significant difference among parent and *in silico* fragment peptide, respectively. # indicated significant difference between parent and digested peptide at each peptide concentration (*p*=0.05), (n=3).

A larger peptide might have limited cell membrane permeability, decreasing its cellular inhibitory capacity. Results from the enzyme assay were inconsistent with those from cellular model, which revealed that digested peptides exhibited higher POP inhibition potency in the cellular system. Derivatized peptides, Z-Pro-Pro-aldehyde-dimethylacetal and Boc-Asn-Phe-Phe-aldehyde, have been reported to inhibit POP activity in SH-SY5Y cells (Klimaviciusa et al., 2012). Intracellular POP inhibition of food peptides has never been reported. This is the first study demonstrating that corn peptides and its *in silico* GI digested fragment inhibited POP activity at the cellular level.

4.4.4 α-Synuclein aggregation in SH-SY5Y cells

Oligomerization of α-synuclein leads to a loss of brain function which is one cause of PD (Cui et al., 2021). Factors promoting aggregation of α-synuclein include oxidative stress and defect in protein synthesis (Myöhänen et al., 2012). The machanism of α-synuclein aggregation induced by POP remains unclear. It has been reported that POP bound directly to α-synuclein, enhancing dimerization under *in vitro* chemical assay (Brandt et al., 2008; Savolainen et al., 2015). Thus, it would be plausible that a POP inhibitor would help reduce the pathological conditions caused by α-synuclein aggregation. The control cells and peptide-treated cells showed α-synuclein in both cytoplasm and nucleus (Fig. 4.6a). Strong red fluorescence representing aggregation of α-synuclein in SH-SY5Y cells induced by KCl was evident in the sample without peptides (Fig. 4.6b). Aggregation of α-synuclein decreased in ALLTLSPLGPA pre-treated cells at concentrations of 5 and 10 μM as less α-synuclein aggregates were observed (Fig. 4.6c, d). At 25 μM, significant reduction of α-synuclein aggregates was notable (Fig. 4.6e). In contrast, SPLGPA did not reduce α-synuclein aggregation at studied concentrations (Figs. 4.6f, g, h). Our study demonstrated that the parent CGM peptide was likely to mediate reduction of α-synuclein aggregation. However, the digested peptide was more potent cellular POP inhibitor (Fig. 4.5). POP cellular inhibition did not well correlate with the ability to reduce α-synuclein aggregation. It can be implied that POP might not be a prime factor mediating oligomerization of α-synuclein. Cations, such as aluminum (III), copper (II), iron (III), and lead (II) as well as oxidative stress have been report to induce aggregation of α-synuclein (Fink, 2006; Myöhänen et al., 2012). Reduction of α-synuclein aggregation

by ALLTLSPLGPA could involve metal chelation and radical scavenging activities at the cellular level, which deserves further investigation. It is notable from our results that peptides from CGM-H show potency to reduce the aggregation of **α**-synuclein in cellular system. Mechanisms involved in reduction of **α**-synuclein aggregation of corn peptides should also be explored.
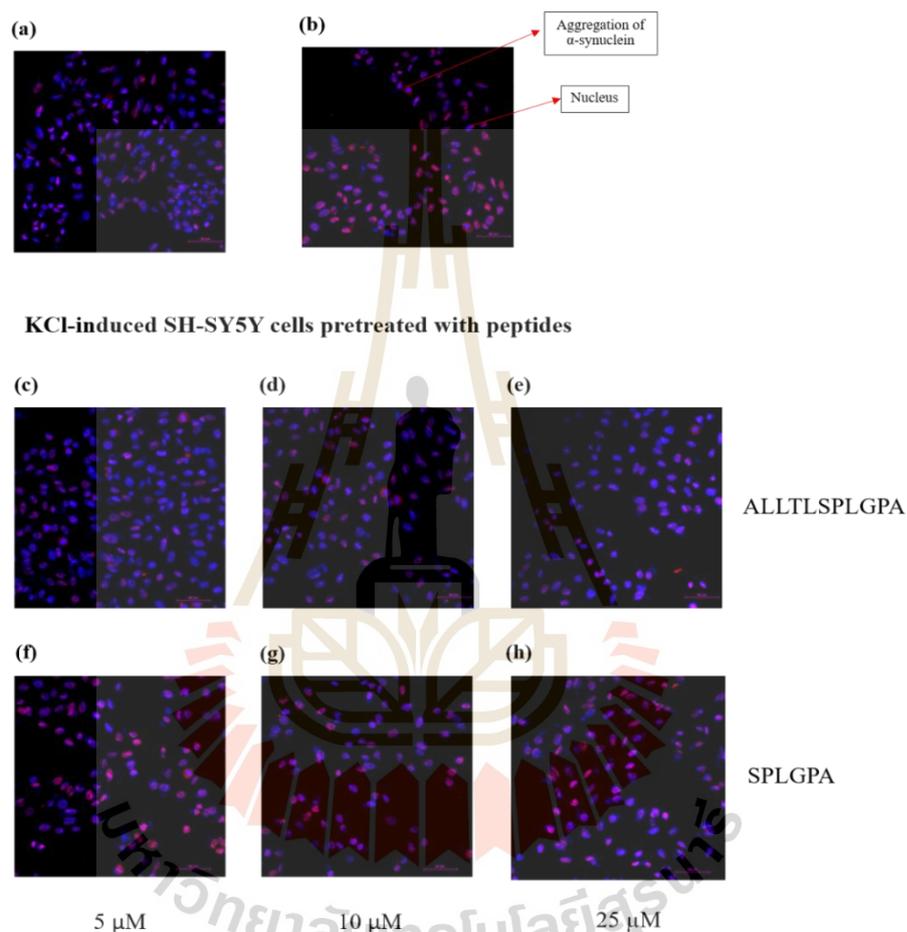
Figure 4.6 Immunofluorescence images (magnification 40✕) of KCl-induced **α**-synuclein aggregation of SH-SY5Y cells after pre-treatment with and without peptides. (a) images of the control without peptide and KCl; (b) KCl- induced cells without peptides pretreatment; (c), (d), and (e) KCl-induced cells in the presence of ALLTLSPLGPA pretreatment at 5, 10, and 25 µM, respectively; (f), (g), and (h) KCl- induced cells with SPLGPA pretreatment at 5, 10, and 25 µM, respectively. Nucleus were counterstained with DAPI as shown in blue and **α**-synuclein proteins are shown in red.

4.5 Conclusion

CGM-H hydrolyzed by pepsin exhibited POP inhibitory activity. The novel peptide ALLTLSPLGPA showed the highest POP inhibitory activity. The *in-silico* GI digested peptide, SPLGPA, exhibited less *in vitro* inhibitory activity than its parent counterpart. The peptide ALLTLSPLGPA exhibited a mixed-type inhibitor characteristic, while SPLGPA was an uncompetitive inhibitor. SPLGPA inhibited POP activity in SH-SY5Y cells to a greater extent than its parent peptide. However, 25 μM ALLTLSPLGPA profoundly decreased aggregation of α-synuclein in the KCl-treated SH-SY5Y cells. CGM-H could be used to develop nutracuticals products for brain health. Ability to cross blood brain barrier of peptides and *in vivo* studies should be further investigated.

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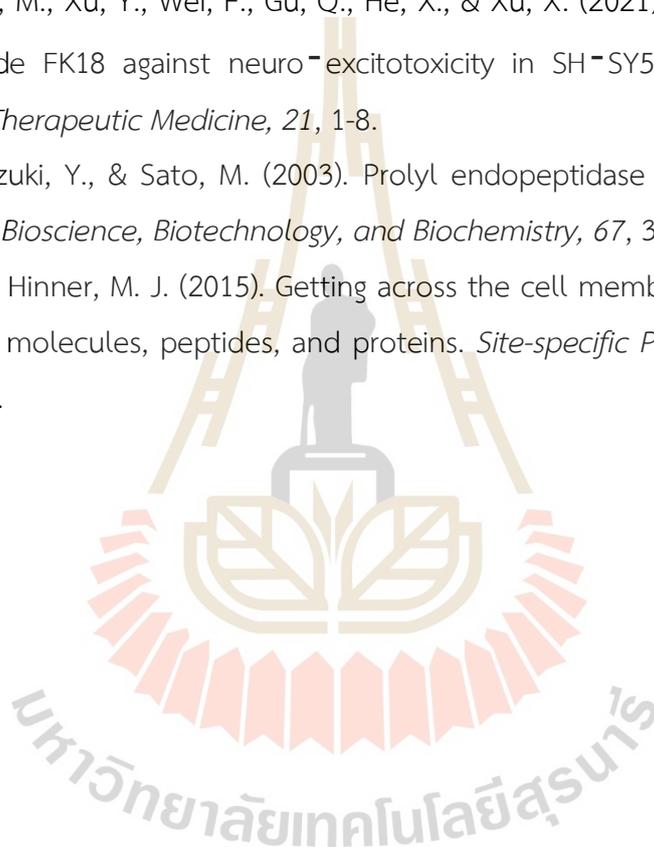
CHAPTER V

ANTIHYPERTENSIVE EFFECT OF CORN GLUTEN MEAL HYDROLYSATE, PEPTIDES, AND THEIR IN-SILICO GASTROINTESTINAL-DIGESTED FRAGMENTS

5.1 Abstract

The objectives of this study were to isolate and identify angiotensin-I-converting enzyme (ACE) inhibitory activity of corn gluten meal hydrolysate (CGM-H). In addition, ACE inhibitory activity and antihypertensive effect of the identified peptides and their gastrointestinal (GI)-digested fragments were compared along with binding energy obtained from molecular docking. CGM-H showed lower ACE and higher renin inhibitory activity when compared to ultrafiltrate fractions of < 1 kDa and 1-3 kDa. In addition, CGM-H exerted greater systolic blood pressure (SBP) reduction of -21.30 mmHg after 2 h oral administration in spontaneously hypertensive rats (SHRs). Seven peptides from CGM hydrolysate were identified after a series of chromatographic separation. Among them, KQLLGY showed the most effective inhibition with an IC_{50} value of 0.08±0.01 mg/mL. The selected peptides, namely KQLLGY, PPYPW, and PGALPGAL, were degraded upon *in silico* GI digestion, leading to changes of ACE inhibitory activity. Parent peptides KQLLGY, PPYPW, and PGALPGAL showed lower K_i value of 0.0061, 0.1733, and 0.2456 mg/mL, respectively, than their *in-silico* GI-digested fragments, namely KQL, PPY, and PGAL with K_i of 0.5749, 0.6310, and 2.0870 mg/mL, respectively. In addition, the parent peptides also showed lower binding energy than their GI-digested fragments, confirming greater ACE binding. Only KQL showed slight antihypertensive effect in SHRs at maximum reduction of -7.83 mmHg after 8 h oral administration despite of its low ACE inhibitory activity. This study revealed that ACE inhibition is not the main pathway for blood pressure lowering activity of CGM-H and peptide KQL. In addition, binding energy obtained from molecular docking and K_i value could not predict the antihypertensive efficacy of peptides.

5.2   Introduction

Hypertension is one of major public cardiovascular diseases, leading to death and disability worldwide. Angiotensin converting enzyme (ACE; EC 3.4.15.1) is a key enzyme involved with the blood pressure regulation through renin-angiotensin system (RAS). The ACE inhibitory peptides have been reported in many different protein sources (Daskaya-Dikmen et al., 2017; He et al., 2013; Sangsawad et al., 2017; Vercruysse et al., 2005). Due to the current trend of meat abstinence, plant-based protein has become an alternative for consumers. Therefore, bioactive peptides from plant proteins could gain more attention. Corn gluten meal (CGM) is a main by-product from corn starch production, which contains at least 60% protein and is typically used for animal feed production (Li et al., 2019). To increase utilization of CGM, protein hydrolysates with various bioactivities have been studied, such as antioxidant activity, antihypertensive activity, immunomodulatory of tumor, and protection of alcohol-induced liver injury (Hu, Chen, & Li, 2020; Hu et al., 2022; Kopparapu et al., 2022; Li et al., 2019; Liu et al., 2020). The greatest challenges of bioactive peptides are its stability in gastrointestinal (GI) tract because digestive enzymes can degrade these peptides into free amino acids, tri- or di-peptides. Forty-eight identified peptides isolated from green macroalga *Ulva lactuca* hydrolysate were further hydrolyzed by *in-silico* GI digestion, which their fragments exhibited ACE inhibitory activity (Garcia-Vaquero et al., 2019). The *in-silico* GI digestion has been used to evaluate the stability of bioactive peptide against GI proteases and predict their fragments and bioactivity after GI digestion.   ACE inhibitory activity of peptide TQVY derived from rice protein decreased after simulated GI digestion, but the GI-digested fragments were not identified (Li et al., 2007). In addition, peptide DLTDY showed better ACE inhibition than DY which was GI-digested fragment (Shiozaki et al., 2010). Therefore, changes of ACE inhibitory activity of peptides after simulated GI digestion might affect their antihypertensive activity. Peptides could exert their antihypertensive when they retain their intact form or degrade to more active fragments after the action of GI proteases.   Peptide YPI exhibiting IC_{50} value of >1000

µg/mL was a digested product of YAEERYPIL exhibiting IC_{50} of 5.4 µg/mL. Despite of its low ACE inhibition *in vitro*, YPI showed greater antihypertensive effect than its parent peptide at 2 mg/kg body weight (BW) (Miguel et al., 2006). In addition, FFGRCVSP (IC_{50} = 0.36 µg/mL) and ERKIKVYL (IC_{50} = 1.26 µg/mL) isolated from egg ovalbumin showed strong ACE inhibition, but they did not exert any antihypertensive activity (Iroyukifijita et al., 2000). Thus, GI digested-peptides should be elucidated to warrant its health benefits as bioactive peptides.

Previous studies reported that peptide fraction from CGM-H with molecular weight (MW) < 3 kDa showed lower IC_{50} value for ACE inhibition than peptide fractions with MW < 1 kDa and 5 kDa and could reduce systolic blood pressure (SBP) by -26.57 mmHg in spontaneously hypertensive rats (SHRs) after 2 h oral administration at 100 mg/kg BW, but the antihypertensive effect of other fractions was not reported (Huang et al., 2011). Antihypertensive effect of CGM-H compared to their ultrafiltrated fractions has not been widely elucidated. Casein hydrolysate exerted strong antihypertensive activity to SHRs at 6 h after oral administration, while isolated peptide YLVPQL showing high ACE inhibition exhibited no antihypertensive effect (Maeno et al., 1996). In contrast, lactoferrin hydrolysate with MW less than 3 kDa reduced SBP about -15 mmHg at a dose of 200 mg/kg BW, which was less effective than LIWKL isolated from lactoferrin hydrolysate with SBP reduction of -25.3±3.5 mmHg at 10 mg/kg after 1 h post- oral administration (Ruiz-Giménez et al., 2012). Based on these previous studies, isolated peptides might not always exhibit antihypertensive effect *in vivo*. Thus, antihypertensive effect of CGM-H and peptide fractions as well as identified peptides should be evaluated to assure its health benefit as a functional food. Therefore, this study aimed to purify and identify ACE inhibitory peptides from CGM-H. In addition, ACE inhibitory activity and stability under *in silico* GI digestion of parent and their GI-digested fragment peptides were evaluated. Moreover, antihypertensive activity of CGM-H and their UF fractions as well as the identified peptides and their GI-digested fragments was also determined.

5.3 Materials and methods

5.3.1 Materials and chemicals

CGM was received from Friendship Corn Starch Industry (Samutprakarn, Thailand). Angiotensin-I-Converting enzyme (ACE) from rabbit lungs, N-[3-(2-Furyl) acryloyl]-Phe-Gly-Gly (FAPGG) used as a substrate for ACE and pepsin (400 U/mL) (from porcine stomach mucosa) were purchased from Sigma Chemical Co. (St. Louis, MO., USA). Peptide sequences were synthesized using a solid phase peptide synthesis method by Genscript Inc. (Piscataway, NJ, USA). Acetonitrile (ACN), and trifluoroacetic acid (TFA) with HPLC grade were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Other chemicals were of analytical grade.

5.3.2 Preparation of CGM hydrolysate (CGM-H) and ultrafiltration fractions

CGM was hydrolyzed by pepsin with enzyme to substrate ratio of 1:100 at 37°C, pH 2.0 for 12 h in a 5-L bioreactor (New Brunswick Scientific Co., Inc., Edison, NJ, USA) with continuous stirring at 350 rpm. Then, the mixture was heated in water bath at 95°C for 15 min to stop the enzymatic reaction. Supernatants were collected after centrifugation at 10,000 ×g for 10 min and adjusted pH to 7.0 with 6 N NaOH. Protein content was determined by Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

CGM-H was separated through an Amicon stirred ultrafiltration cells (Merck KGaA, Darmstadt, Germany) using membrane with molecular weight cut-off (MWCO) 1 and 3 kDa (Merck KGaA, Darmstadt, Germany). Firstly, CGM-H was passed through a 1-kDa membrane and permeate referred to as ultrafiltration fraction (UF)< 1 kDa was collected. The retentate was mixed with deionized water at an equal volume and passed through a 3-kDa membrane, then the permeate was collected as a UF 1-3 kDa. All samples were lyophilized and their protein content was determined using Lowry method (Lowry et al., 1951).

5.3.3 Purification of ACE inhibitory peptides

Lyophilized UF fraction (30 mg) with highest ACE inhibitory activity was dissolved in 1 ml of deionized water and 100 µL of sample was loaded on a Superdex Peptide 10/300 GL column (10 × 300 mm, GE Healthcare, Piscataway, NJ, USA) using DI water as mobile phase A and 30% acetonitrile containing 0.1% TFA as mobile phase B. Peptides were separated using Fast-Protein Liquid Chromatography

(FPLC) (AKTA purifier, GE Healthcare, Piscataway, NJ, USA) in a stepwise mode at a flow rate of 0.8 mL/min. The elution was carried out from 0.5% to 100% of mobile phase as shown in Fig.1 (a). The peptide fractions were individually collected and monitored at 214 nm. ACE inhibition of each fraction was measured and fractions with the highest ACE inhibitory activity were collected and lyophilized for further purification.

The fraction No.1 obtained from size exclusion chromatography (SEC) was solubilized in DI water and applied to a SOURCE™ 5RPC ST 4.6/150 column (GE Healthcare, Piscataway, NJ, USA). The peptide elution was performed using mobile phase A (DI water containing 0.1% (v/v) TFA) and mobile phase B (ACN containing 0.1% (v/v) TFA) with gradient mode at flow rate of 0.5 mL/min. Peptides were eluted by a stepwise mode starting from 10, 20, 30, 40, and 100% of mobile phase B as shown in Fig.1 (b). Fraction with the highest ACE inhibitory activity was collected for peptide identification.

5.3.4 ACE inhibitory activity assay

In vitro ACE inhibitory activity was carried out according to the method of Martinez-Villaluenga et al., (2012) with some modifications. The CGM-H or synthetic peptides (20 μL) was added into a 96-well microtiter plate, then 10 μL of 0.25 U/mL ACE was added. The mixture was incubated at room temperature for 10 min. Thereafter, 0.5 mM FAPGG (170 μL) was added. The control was performed in the same manner as the sample, but buffer was added instead of CGM-H or synthetic peptides. The absorbance was measured at 340 nm, 37°C for 30 min (Martinez-Villaluenga et al., 2012). Absorbance values were recorded every 2 min using a microplate reader (BioTek powerwave XS2 spectrophotometer, Winooski, Vermont, USA), and plotted *versus* incubation time. ACE activity was calculated using the following equation:

$$ACE\ inhibition\ (\%) = \frac{slope\ of\ control - slope\ of\ test\ sample}{slope\ of\ control} \times 100$$

In addition, half maximum inhibition concentration (IC_{50}) value was determined.

5.3.5 Renin inhibitory activity assay

In vitro renin inhibitory activity was performed using the manufacturer's instructions by the renin inhibitory screening assay kit (Cayman Chemical, Ann Arbor, Michigan, USA) with slight modification. Before assay, 50 mM Tris-HCl buffer containing 100 mM NaCl (pH 8.0) was used to dilute concentrated renin.by 20 times. A substrate (Arg-Glu(EDANS)-Ile-His-Pro-PheHis-Leu-Val-Ile-His-Thr-Lys(Dabcyl)-Arg) was diluted with dimethylsulfoxide (DMSO) to achieve concentration of 47.5 µM before use. The reaction was performed using 96-well plate by adding 20 µL diluted substrate, 150 µL assay buffer, and 10 µL deionized water (for control well) or peptide at final concentration of 5 mg protein/mL (for inhibitor well). The mixture was pre-warmed at 37°C for 10 min in fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA). Then, 10 µL diluted renin was added into each well. The fluorescence intensity (FI) was recorded every 1 min for 15 min at excitation wavelength of 340 nm and emission wavelength of 490 nm. The renin inhibition was calculated as followed:

$$Renin\ inhibition\ (\%) = \frac{FI\ of\ control\ well - FI\ of\ inhibitor\ well}{FI\ of\ control\ well} \times 100$$

5.3.6 Peptide identification and *in silico* gastrointestinal digestion of synthetic peptides

The RPC fraction with the highest ACE inhibition was submitted for amino acid sequencing by the Ultimate 3000 Nano/Capillary LC System (Thermo Scientific, Altrincham, UK) with a Nano-captive spray ion source coupled to a Hybrid quadrupole Q-TOF impact IITM (Bruker, Daltonics, Germany). The peptide fraction was loaded into a µ-Precolumn 300 µm i.d. × 5 mm C18 Pepmap 100, 5 µm, 100 A$^{\circ}$ (Thermo Scientific, Altrincham, UK) and separated on 175 µm i.d. × 15 cm packed with Acclaim PepMap RSLC C18, 2 µm, 100 A$^{\circ}$, nanoViper (Thermo Scientific, Altrincham, UK). The peptide fractions were eluted using gradient mode with mobile phase A (DI water containing 0.1% formic acid) and mobile phase B (80% ACN containing 0.1% formic acid) which started from 5 to 55% of mobile phase B at a flow rate of 0.3 µL/min for 30 min. The electrospray ionization was performed at 1.6

kV using the CaptiveSpray. Mass spectra (MS) and MS/MS spectra were obtained on the positive-ion mode over the range (m/z) 150 to 1000 (Compass 1.9 software, Bruker Daltonics). The peptide mass spectra were analyzed using the PEAKS Studio 10.0 software (Waterloo, ON, Canada). Peptides with ≥70% *de novo* score were selected for synthesis. The homologous protein of peptide were searched by the NCBI standard protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) and/or UniProtKB (peptide search; https://www.uniprot.org/peptidesearch/) using *Zea may* (Maize; taxid: 4577) as a database. The selected peptides were synthesized by Genscript Inc. (Genscript Inc, NJ, USA) with 99% purity using solid phase peptide synthesis method. Synthetic parent peptides with the highest and lowest ACE inhibition were selected for *in silico* gastrointestinal (GI) digestion. Digestive enzymes; including pepsin (pH 1.3), trypsin, and chymotrypsin A, were used for the *in-silico* digestion by BIOPEP (Minkiewicz et al., 2008). Peptide fragments from *in silico* digestion were searched for antihypertensive activity using BIOPEP database (http://crdd.osdd.net/raghava/ahtpdb/) and fragments that have not been reported were selected for synthesis and evaluation.

5.3.7 Mode of ACE inhibition

Parent peptides (KQLLGY, PPYPW, and PGALPGAL) and their selected *in silico* fragments exhibiting the highest ACE inhibition were determined for inhibition kinetics. Peptides at various concentrations were incubated with ACE in the presence of various concentrations of FAPGG (0.0625, 0.125, 0.25, and 0.5 mM). Inhibition kinetic was determined using Lineweaver-Burk plot. Kinetic parameters, V_{max}^{app}, K_m^{app}, and inhibitor constant (K_i) were calculated using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA).

5.3.8 Molecular docking

The peptide structure was generated by PyMol 2.5 and 3-dimentional structure of ACE (PDB: 1O86) was downloaded from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (http://www.rcsb.org). The chemical structure of FAPGG was obtained from the PubChem compound database. ACE was prepared by removing water molecules and co-crystal ligands before docking analysis. Molecular docking was performed using AutoDocking Vina with free energy scoring function to determine the possible peptide-enzyme complex (Trott

and Olson, 2010). The parameters of Autodock Vina tool were set as follows: grid boxes were designed at dimensions x=50 y=70 and z=50 to cover all active pocket around the zinc (Zn (II)) prosthetic group. Other parameters were set as default. The best docking score with lowest binding energy was chosen.

5.3.9 Animal experiment

Six-week-old male SHRs were purchased from Charles River (Montreal, PQ, Canada) and housed under a 12 h day and night cycle at 20°C with regular chow feed and tap water provided *ad libitum*. All animal experiments were performed following the Canadian Council on Animal Care Ethics guidelines that approved by University of Manitoba Animal Ethics Committee. Rats were surgically implanted with telemetry sensors according to the previously protocols (O'Keeffe et al., 2017) after acclimation for 1 week. Rats were allowed to recover for another 2 weeks prior to feeding trials. Rats were grouped into 7 treatments using 4 rats/group as follows: phosphate buffered saline (PBS), captopril (50 mg/kg BW dissolved in PBS), parent synthetic peptides, including KQLLGY, PPYPW, and PGALPGAL (30 mg/kg BW dissolved in PBS), *in silico* digested fragment peptides (KQL, PPY, and PGAL) (30 mg/kg BW dissolved in PBS), CGM-H, UF fraction with molecular weight cut off (MWCO) less than 1 kDa (UF < 1 kDa), and UF fraction with MWCO 1-3 kDa (UF 1-3 kDa). Each rat was orally gavaged with 1 mL solution using a disposable plastic syringe. Systolic blood pressure (SBP) was continuously measured in a quiet room with each rat cage placed on the respective receiver (Model RPC-1, DSI Instruments, St. Paul, MN, USA). Real time experiment data were recorded continuously for 24 h in free moving rats using the Ponemah 6.1 data acquisition software (DSI instrument, St. Paul, MN, USA). The APR-1 atmospheric pressure monitor (DSI instrument, MN, USA) was attached to the system and used to normalize the transmitted pressure values to ensure that the recorded blood pressure signals are independent of atmospheric pressure changes. Data were expressed as SBP values at zero time subtracted from values obtained at 2, 4, 6, 8, and 24 h.

5.3.10 Statistical analysis

Statistical analysis was performed using SPSS version 25.0 (SPSS Inc., Chicago, IL, USA). Differences in mean values were analyzed using analysis of variance (ANOVA) and Duncan's multiple range test at a significance level of $p < 0.05$, n=3.

Blood pressure lowering activity were analyzed by t-test at p< 0.05 (n=4), which compare changes between blood pressure of SHRs fed peptides and the control group at the same period of time.

5.4   Results and Discussion

5.4.1   Purification and identification of ACE inhibitory peptides

The permeate fraction from UF showed the most potent ACE inhibition with IC_{50} value of 17.70±0.71 µg leucine/mL. This fraction was collected and applied to size exclusion chromatography (SEC). The peptide fraction 1 from SEC showed the highest ACE inhibitory activity (Fig. 5.1 a, c) and was collected for further fractionation by reverse-phase chromatography (RPC). Peptide yield decreased to 8.51% after SEC. Other SEC fractions also contained ACE inhibitory peptides, which were not selected for further purification (Fig. 5.1 c), rendering its relatively low yield. The purity of ACE inhibitory peptides increased about 8 folds after reverse-phase chromatography (RPC), whereas peptide yield decreased to 0.5 % (Table 5.1). After RPC, fraction no.11 showed the highest ACE inhibition (Fig. 5.1b, d) at 50% mobile phase B, while fractions eluted earlier at 10–20% mobile phase B exhibited lowest inhibition. These results suggested that ACE inhibitory peptides would likely be less polar. Similarly, the RP-high pressure liquid chromatography (HPLC) fraction of lemon basil seed derived peptides with higher retention time exhibited greater ACE inhibitory activity, which suggested high level of hydrophobic amino acid residues (Kheeree et al., 2020). It should be noted that fractions 2-3 from SEC and fractions no. 6–10 from RPC also exerted ACE inhibitory activity (Figs. 5.1c, 5.1d), which were discarded during purification. This could contribute to limited yield of purification.

Table 5.1 Purification table of ACE inhibitory peptides from series of chromatographic separation.

Separation methods	Peptide content (mg)	IC$_{50}$ (µg leucine/mL)	Purification folds	Yield (%)
Crude CGM-H	181.90	26.60	1.00	100.00
Permeated-UF 10 kDa	164.96	17.70	1.50	90.69
Size exclusion chromatography	15.48	6.09	4.37	8.51
Reverse-phase chromatography	0.916	3.09	8.62	0.50

Seven novel ACE inhibitory peptides from CGM were revealed (Table 5.2). It has been reported that peptides with V and I at N-terminus could bind to the active site of ACE, and peptides with P and/or aromatic residues, such as W, Y, or F and/or a branched aliphatic amino acid residues (V, I, or L) at the C-terminus strongly inhibit ACE (Liu et al., 2020; Zheng et al., 2019). Most identified peptides contained P, W, Y, or L at the C-terminus, but the ACE inhibitory activity varied. Several peptides having P or Y at C-terminal end showed a potent ACE inhibitor (Shamloo et al., 2015). In addition, peptides contained amino acids with positively charged group at the N-terminus effectively inhibited ACE (Wu et al., 2006). In the present study, peptide KQLLGY containing Y at the C-terminus and K at the N-terminus was the most effective inhibitor. The peptide NGPAGLP exhibited less inhibitory efficacy than others despite of P at the C-terminus (Table 5.2). In addition, the peptide PGALPGAL containing L at the C-terminal end showed the lowest ACE inhibition. Thus, the amino acids of P, Y, L at C-terminus are not always the essential requirement governing potency of ACE inhibition. The quantitative structural-activity relationship of peptides containing more than 4 amino acids residues indicated that four amino acid residues from C-terminal end of peptides containing Y or C at the first, H, W, or M at the second, I, L, V, or M at the third, and W at the fourth position from C terminus might directly interact with HEMGH located at the catalytic site of ACE,

resulting in enzyme inactivation (Wu, et al., 2006a). Inhibitory potency of KQLLGY was probably due to the presence of Y at the first and L at the third position from C-terminus.
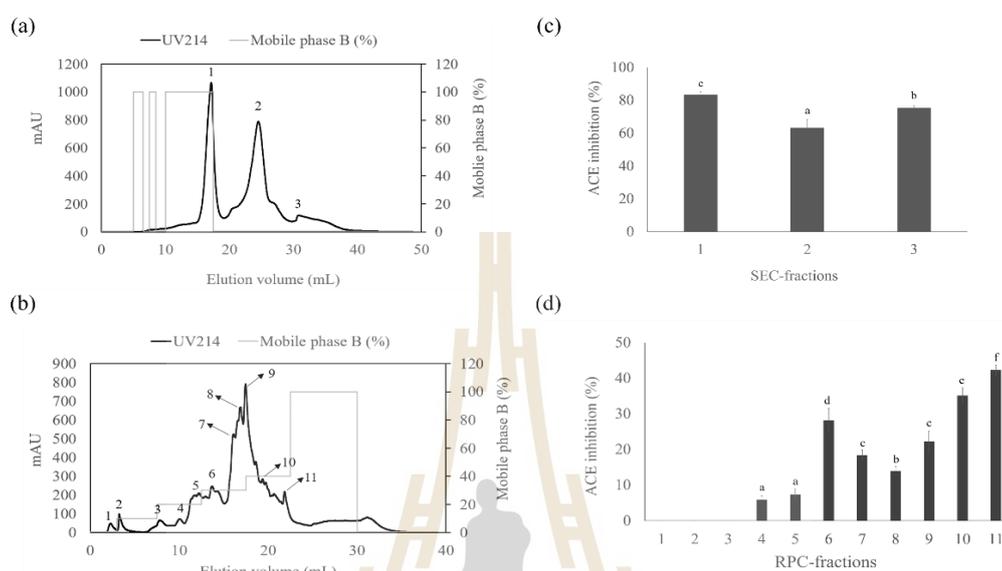
Figure 5.1 Size exclusion chromatography (SEC) (a) and reverse-phase chromatography (RPC) (b) of CGM-H. The ACE inhibition of peptide fractions from SEC (c) and RPC (d) at 10 uL. Different lower-case letters indicated significant difference at $p \leq 0.05$, n=3.

5.4.2 Effect of *in silico* digestion on ACE inhibitory activity

The most potent peptides, KGLLGY and PPYPW, and the least effective peptide inhibitor, PGALPGAL, were selected to evaluate *in silico* GI digestion. ACE inhibitory activity of *in silico* digested peptides, KQL, PPY, and PW, were decreased as compared to their parent counterparts (Table 5.3). Only tetrapeptide, PGAL, showed higher ACE inhibition than its parent (Table 5.3, 5.2). GI digestion is expected to modify peptide structure and ACE inhibition potency. Digested peptides of GY and PG were not determined for their IC_{50} as their inhibitory activity have been previously reported (Table 5.3). Previous studies reported that peptides with chain length more than 4 amino acid residues were easily digested by GI enzymes, resulting in di-, tripeptides and/or free amino acids (Sangsawad et al., 2018; Vermeirssen et al., 2004). Tripeptides containing hydrophobic residues, such as W, Y, F, and P at the C-terminal

region significantly influence binding to ACE at subsite S_1, S_1', and S_2' (Sheih et al., 2009). Peptide KQLLGY showed greater ACE inhibitory activity than its fragment, KQL, which was probably due to the presence of Y at C-terminus. However, peptide fragments PPY and PW exerted ACE inhibition lower than their parent, PPYPW, although they contain Y and W at the C-terminus, respectively. The quantitative structure-activity relationship of ACE inhibitory peptides revealed that increasing of side chain hydrophobicity of amino acid in the C-terminal position would enhance ACE inhibitory activity (Pripp et al., 2004). This might be one of reasons for higher ACE inhibitory activity of PPYPW than PPY and PW. Peptide fragment PGAL has L at C-terminus as same as its parent, PGALPGAL, but its ACE inhibitory activity was about 3 times higher (Tables 5.2, 5.3). Therefore, the amino acid residue in the ultimate position of C-terminal region might not always a determining factor of ACE inhibitory activity. In addition, previous studies pointed out that C-terminal end of tripeptide strongly influenced ACE inhibition, while no concrete relationship between N-terminus and ACE inhibitory activity has been established (Pripp et al., 2004). Moreover, P at N-terminal position was the least favorable for binding to ACE for dipeptides (Hong et al., 1980), which might be a reason for low ACE inhibitory activity of PW. PGAL showed higher ACE inhibitory activity than its parent counterpart, which was probably due to a decrease in steric hindrance. Therefore, the ACE inhibitory activity of peptides is significantly related to hydrophobicity, positive charge and molecular volume of the amino acids at C-terminus. Our study revealed that ACE inhibitory peptides derived from CGM-H were likely unstable against GI digestive enzymes, leading to modifications of peptide structure, but their ACE inhibitory activity remained after GI digestion.

Table 5.2 ACE inhibitory activity of identified corn gluten meal peptides.

Peptide sequences	Mass (Da)	*de novo* score	Parent protein	Database	ACE inhibition (IC$_{50}$, mg/mL)
DGPLPQ	625.66	84	Pyruvate, phosphate dikinase regulatory protein	UniProtKB ID: Q195N6.1	0.66±0.03[c]
NGPAGLP	624.68	80	Glutamyl-tRNA (Gln) amidotransferase subunit A	UniProtKB ID: B6U151.1	0.94±0.03[d]
MSGGAAPA	660.73	79	60S acidic ribosomal protein P2B	UniProtKB ID: O24415.1	0.56±0.01[bc]
PPYPW	658.74	78	Zealexin A1 synthase	UniProtKB ID: B4FVP3.1	0.49±0.03[b]
GYLPE	577.62	75	Cytochrome P450 88A1	UniProtKB ID: Q43246.1	1.05±0.05[e]
KQLLGY	720.85	73	ATP synthase subunit 9	UniProtKB ID: P00840.2	0.08±0.01[a]
PGALPGAL	694.81	72	Dimethylnonatriene synthase	UniProtKB ID: A0A1D6HSP4.1	1.77±0.15[f]

Data are expressed as a mean±SD (n=3). Different letters indicated the significant difference between peptide groups ($p<0.05$).

Table 5.3 The *in-silico* GI digested fragments of selected corn gluten meal peptides and their ACE inhibitory activities.

Parent peptides	*In silico* GI digested fragments	Mass (Da)	BIOPEP database IC$_{50}$ of ACE inhibition	ACE inhibitory activity (IC$_{50}$) (mg/mL)
KQLLGY	KQL	387.46	NA	0.60±0.007[c]
	GY	238.23	97 µg/mL (from: soybean (Nakahara et al., 2010)	ND
PPYPW	PPY	375.41	NA	0.54±0.010[a]
	PW	301.32	NA	0.99±0.060[d]
PGALPGAL	PGAL	356.41	NA	0.57±0.005[b]
	PG	172.17	>3.44 mg/mL (Wu et al., 2006b)	ND

NA = Not available. ND = not determined. Data are shown as a mean±SD and different letters identify significant difference among samples. (*p*<0.05, n=3).

5.4.3 Kinetic of ACE inhibitory peptides

Three parent peptides, KQLLGY, PPYPW and PGALPGAL, and their peptide fragments, KQL, PPY and PGAL, were selected for kinetic studies. Kinetic parameters, K_m^{app}, V_{max}^{app}, and K_i value, were illustrated in Figs. 5.2a-b. Parent peptides of KQLLGY, PPYPW, and PGALPGAL, showed mixed-mode, competitive, and uncompetitive characteristic, respectively. Peptide fragments, KQL, PPY, and PGAL, were identified to be a mixed-mode, competitive, and non-competitive inhibitor, respectively. These suggested that peptides KQLLGY and its fragments could bind to free enzyme at different sites from active site to form enzyme-inhibitor-substrate (EIS) complex. Peptide PPYPW and PPY could bind to ACE at the active site of enzyme. The PGALPGAL bound only to the enzyme-substrate complex as it was

characterized as an un-competitive inhibitor and PGAL was identified to be a non-competitive inhibitor, thus it could react with either free enzyme to generate enzyme-inhibitor complex or with enzyme-substrate complex to form enzyme-substrate-inhibitor complex. The K_i value described the binding affinity between inhibitor and enzyme. A smaller value indicates tighter binding in most case (Burlingham and Widlanski, 2003). The K_i values of parent peptides, KQLLGY and PPYPW, were lower than their fragments, KQL and PPY (Figs. 5.2a, b), implying that parent peptides bound to ACE more effectively than their digested counterparts. This was correlated with higher ACE inhibitory activity of parent peptides. However, the K_i value of PGALPGAL and PGAL did not correspond to their inhibitory activity. Digested peptide, PGAL, with higher K_i value of 2.087 mg/mL exerted higher ACE inhibition than its parent peptide PGALPGAL, which showed approximately 10-fold stronger affinity with K_i value of 0.246 mg/mL. Mode of inhibition of ACE inhibitory peptides from other plant proteins have been reported. Peptide VVSLSIPR derived from pigeon pea was identified to be a competitive inhibitor for ACE (Nawaz, et al., 2017). In addition, peptides VNP and VWP from rice protein were both characterized to be a competitive ACE inhibitor (Chen, et al., 2013). Peptide AY derived from Alcalase-hydrolyzed CGM showed strong ACE inhibitory activity with IC_{50} value of 14.2 μM, but the K_i value and type of inhibitor were not reported (Yang et al., 2007). Therefore, this study clearly revealed that peptides derived from CGM-H could react with ACE by different mode of action.
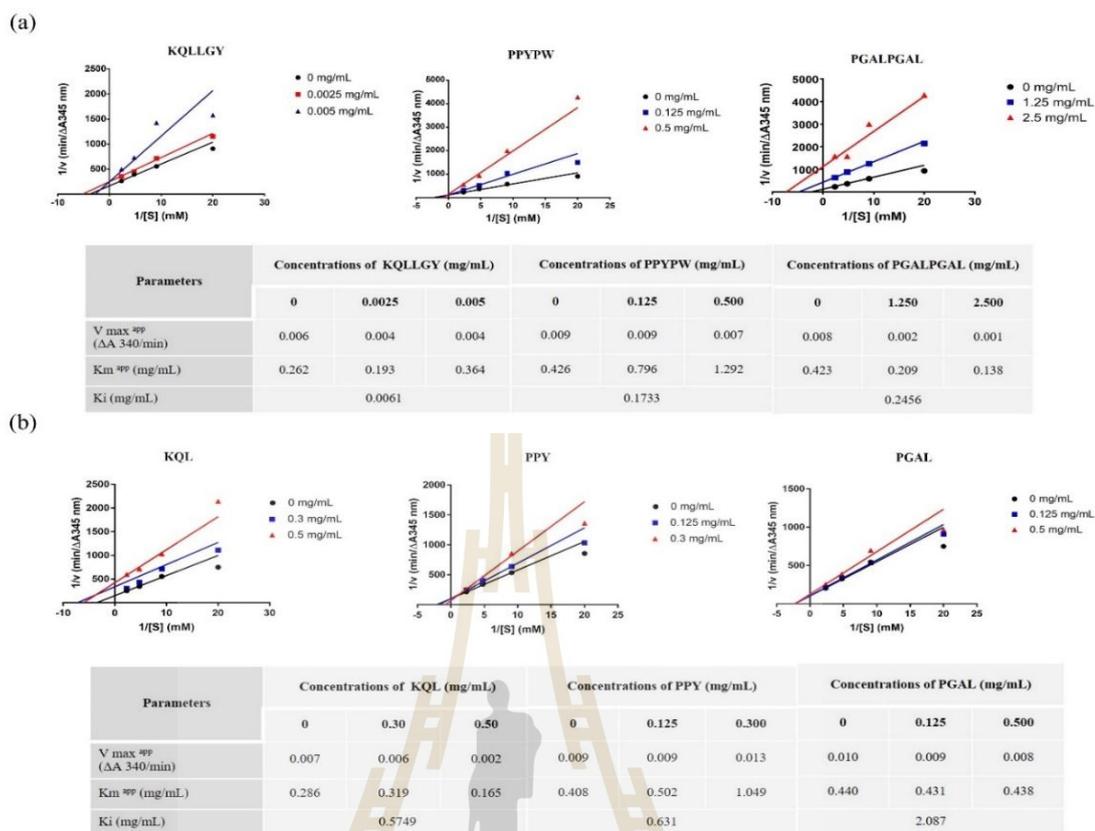
Figure 5.2 Lineweaver-Burk plot analysis, V_{max}^{app} (maximum velocity), K_m^{app} (Michaelis constants), and K_i (enzyme-inhibitor dissociation constant) in the absence and presence of parent peptides; KQLLGY, PPYPW, and PGALPGAL (a) and *in silico* digested fragments; KQL, PPY, and PGAL, (b), at varying concentrations of substrate.

5.4.4 Molecular docking

Interactions between ACE and peptides were observed in Fig. 5.3. The docking simulation of peptide KQLLGY was performed based on competitive and un-competitive characteristics because it was identified to be a mixed-mode inhibitor. Peptide KQLLGY showed lower binding energy than KQL, suggesting higher binding affinity of KQLLGY. This also correlated with their ACE inhibitory activity in which peptide KQLLGY showed more potent inhibition. ACE consists of three active site pockets, namely S$_1$, S$_2$, and S$_1^{'}$, that contains Ala354, Glu384, and Tyr523 residues in S$_1$, while S$_2$ contains Gln281, His353, Lys511, His513, and Tyr520 residues, and S$_1^{'}$ contains Glu162 (Rohit et al., 2012; Wu et al., 2016). Peptide KQLLGY formed 11

hydrogen (H) bonds with Ala356, Tyr523, His353, Ala354, His513, Tyr520, Lys 511, Gln281, Thr282, and Lys454, while KQL formed 8 H-bonds with Glu378, Glu162, His353, Gln281, His513, Ala354, and Glu384 in ACE structure (Fig. 5.4). Both KQLLGY and KQL mainly interacted with S_1 and S_2 pocket sites of ACE, where KQLLGY was likely to bind to ACE stronger than KQL. In addition, the interaction between Zn(II) and L in KQLLGY was observed in this simulation. Lisinopril, an antihypertensive drug, binds to ACE at S_1 and S_2 pocket and directly interacts with Zn(II) in the ACE structure, while peptide TLS showed no interaction with Zn(II), resulting in lower ACE inhibitory activity than lisinopril (Jimsheena & Gowda, 2010; Pan et al, 2012; Wu et al., 2016). It could be suggested that interaction with Zn(II) in ACE would enhance the inhibitory activity, which would be one of reasons of higher ACE inhibitory activity of KQLLGY. Peptide PPYPW formed 4 H-bonds with Ala354, Cys370, Gln281, and Tyr520 at S_1 and S_2 pocket site of ACE, while PPY formed 3 H-bonds with Asp415, His353, and His513 at S_2 pocket sits of ACE. PPYPW formed more H-bonds than PPY, which might be one of reasons of lower binding energy and higher ACE inhibition of PPYPW. In addition, many hydrophobic interactions were observed. It might be suggested that H-bond and hydrophobic interaction were major driving force, resulting in enzyme conformational changes and deactivation. Peptide PGALPGAL and its fragment bound to ACE at different sites. PGALPGAL established 4 H-bonds with Arg124, Asn66, Asn85, and Tyr62 residues of ACE, while PGAL only formed 1 H-bond with Gly404 residue. These results might explain why PGAL exhibited less binding energy, corresponding to higher K_i value (Fig. 5.2, Table 5.5). However, PGAL showed better ACE inhibitory activity than its parent peptide (Table 5.4). Similarly, peptides PFPGPIPN derived from casein showed lower ACE inhibitory activity (IC$_{50}$ value of 12.79 μM) than LYIPIQ (IC$_{50}$ value of 7.28 μM), with higher binding energy (Lin et al 2017). In addition, the molecular docking revealed that the binding energy of captopril with ACE was higher than that of peptides, while the IC$_{50}$ value was much lower (Luna-Vital et al., 2015). It should be noted that both binding energy and K_i did not directly imply inhibitory efficacy.
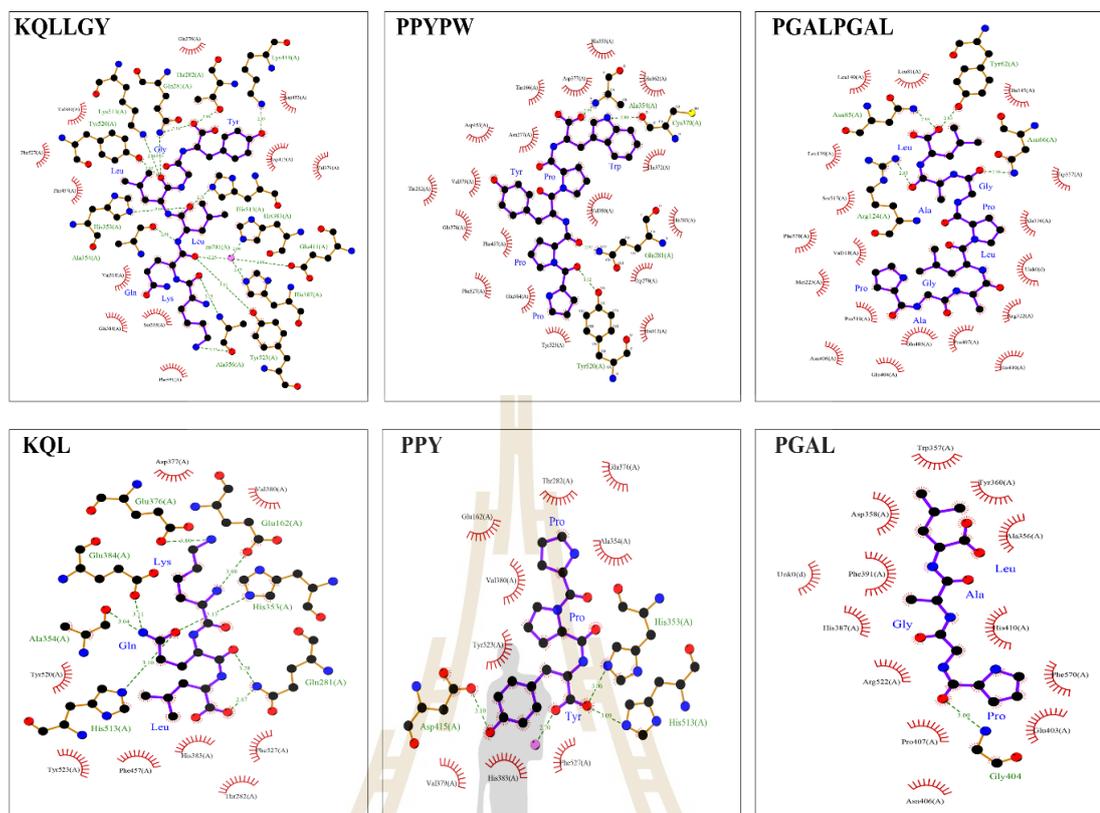
Figure 5.3   The 2D illustration of expected interactions between synthetic ACE inhibitory peptides; KQLLGY, PPYPW, PGALPGAL, KQL, PPY, PGAL and ACE with binding energy of -9.77, -11.10, -9.14, -7.13, -8.78, and -7.38 kcal/mol, respectively. Purple line with blue letters indicated amino acids in the peptide molecule. Amino acids denoted in green letter belong to ACE. Green dots represent hydrogen bonds between peptide and ACE. Red eye symbols indicate hydrophobic interactions among a peptide inhibitor and ACE.

5.4.5 Antihypertensive effect in SHRs

UF Fractionation resulted in an increase in ACE inhibitory activity (Table 5.4). The peptide fraction with MWCO 1-3 kDa showed the highest ACE inibitory activity. Similar findings were observed in gelatin hydrolysate with MW < 3 kDa, which exerted higher ACE inhibition than crude hydrolysate (Mahmoodani et al., 2014). In addition, UF fractions from pea protein and whey protein hydrolysate with

low molecular weight showed higher ACE inhibitory activity (Li and Aluko, 2010; Pan et al., 2012). Moreover, peptide fraction from mungbean hydrolysate with MW < 1 kDa showed the highest ACE inhibition with IC_{50} value of 500 μg protein/mL when compared to the crude mungbean hydrolysate with IC_{50} value of 690 μg protein/mL (Sonklin et al., 2020). In contrast, crude hydrolysate from hemp seed protein showed more potent ACE inhibition (IC_{50} value of 670 μg protein/mL) than the fraction with MW < 1 kDa and 1-3 kDa (IC_{50} value of 1005 and 1170 μg protein/mL, respectively) (Girgih et al., 2011). Therefore, the effect of UF on ACE inhibitory activity appeared to vary with sources of protein hydrolysate.

Table 5.4 ACE and renin inhibitory activity of CGM-H and UF-fractions.

Samples	IC_{50} value of ACE inhibition (μg protein/mL)	Renin inhibition (%)*
CGM-H	140.97±8.27[c]	63.40±4.47[c]
UF < 1 kDa	129.75±0.64[b]	50.59±4.13[b]
UF 1-3 kDa	117.55±1.06[a]	42.68±3.69[a]

Data are expressed as a mean±SD (n=3). Different letters indicated the significantly difference between sample group. *Renin inhibition was performed using CGM-H and peptide fractions with final concentration of 5 mg protein/mL.

In addtion, CGM-H also showed higher renin inhibition (63.40±4.47%) than UF fractions at concentration of 5 mg protein/mL but the activity was decreased after fractionation (Table 5.4). Renin inhibitory activity of hemp seed protein hydrolysate also decreased after UF fractionation (Girgih et al., 2011). In contrast, UF fraction from trypsin-hydrolyzed canola protein showed higher renin inhibition than crude hydrolysate (Alashi et al., 2014). CGM-H significantly reduced systolic blood pressure (SBP) by-21.30 mmHg after 2 h oral administration at dose of 100 mg/kg BW and maximum reduction effect of -24.21 mmHg was achieved at 24 h when compared with saline group (Fig. 5.4c). Meanwhile, UF fractions did not exert the antihypertension. It is obvious from the present study that antihypertensive effect was not correlated with *in vitro* ACE inhibition as shown in Table 5.4. However, CGM-

H exhibiting higher renin inhibition (Table 5.4) that might contribute to high antihypertensive activity. Peptide fraction from cod muscle protein hydrolysate obtained from reverse-phase high performance liquid chromatography (RP-HPLC) showed higher renin inhibitory activity and SBP reduction in SHRs than its crude hydrolysate at dose of 30 mg/kg BW (Girgih et al., 2015). In addition, crude moringa protein hydrolysate showed higher *in vitro* renin inhibitory activity and stronger blood pressure lowering ability than peptide fractions obtained from the UF fraction (<1 kDa, 1-3 kDa, 3-5 kDa, 5-10 kDa, and >10 kDa) after 8 h oral administration at dose of 200 mg/kg BW, although UF fractions showed higher ACE inhibition in the enzyme assay (Aderinola et al., 2019). Furthermore, peptide fraction prepared from ultrafiltration of CGM using enzymatic membrane reactor showed long-term antihypertensive effect (-29.06 mmHg) to SHRs within 2 weeks of oral administration at dose of 1000 mg/kg BW by decreasing of renin and angiotensin II and increasing of nitric oxide (NO) level, which is vasodilator factor, in serum (Gue et al., 2020). Therefore, antihypertensive effect of CGM-H could partly be attributed from its ability to inhibit renin.

Peptide KQL reduced SBP by -7.83 mmHg ($p < 0.05$) at 8-h post-oral adinimistration when compared to the saline group (Fig. 5.4b), while other peptides did not exert antihypertensive effect (Figs. 5.4 a-b). This study suggested that peptide KQL might be absorbed into blood stream as intact form to exert their antihypertensive activity. Among GI-digested peptides, PPY and PGAL showed higher ACE inhibitory activity (Table 5.5) than KQL, but they did not exert antihypertensive activity. Peptide KQL tighty bound to ACE than PPY and PGAL as evidenced by lower K_i value (Fig. 5.2). This would be one of reasons of better antihypertensive activity of KQL. It is well known that peptides with ACE inhibitory activity do not necessarily show antihypertensive effect. Previous study demonstarted that FKGRYYP derived from chicken meat, FFGRCVSP and ERKIKVYL derived from ovalbumin showed strong ACE inhibition with IC$_{50}$ value of 0.51, 0.36, and 1.26 µg/mL, respectively, but they did not exert antihypertensive activity (Iroyukifijita et al., 2000). It was suggested that these peptides could be digested by GI tract and transformed to new inactive peptides. In contrast, YPI showed weak ACE inhibition with IC$_{50}$ value of >1000 µg/mL, but it significantly decreased SBP by-31.6 mmHg at 2 mg/kg BW after 2-4 h of

oral administration to SHRs (Miguel et al, 2006). Furthermore, peptide YP derived from casein had a lower ACE inhibitory activity (IC_{50} value=200 μg/mL) than tripeptides IPP and VPP (IC_{50} value=1.63 and 2.80 μg/mL, respectively), however, the former showed the same antihypertensive effect with IPP and VPP (Yamamoto et al, 1999). These results could mention that these peptides might exert more other bioactive functions *in vivo* to stabilized blood pressure rather than ACE inhibition.

Table 5.5 IC_{50} value, K_i , binding energy, and antihypertensive effect of CGM-H derived peptides and their *in-silico* GI-digested fragments.

Peptides	IC_{50} value (mg/mL)	K_i value (mg/mL)	Binding energy (kcal/mol)	Antihypertensive effect*
KQLLGY	0.08 ± 0.01[a]	0.0061	-9.72	No effect
PPYPW	0.49 ± 0.03[b]	0.1733	-11.10	No effect
PGALPGAL	1.77 ± 0.15[f]	0.2456	-9.14	No effect
KQL	0.60 ± 0.01[e]	0.5749	-7.13	-7.83 mmHg
PPY	0.54 ± 0.01[c]	0.6310	-8.78	No effect
PGAL	0.57 ± 0.01[d]	2.0870	-7.38	No effect

Data are expressed as a mean±SD (n=3). *Antihypertensive effect in SHRs at 8 h after oral administration (p<0.05, n=4).

There are several mechanisms which may improve hypertension. The lactokinin (ALPMHIR), which is an ACE inhibitory peptide released from milk protein by tryptic digestion, inhibited the release of ET-1, which is an endothelial peptide induced contractions in smooth muscle cells (Maes et al., 2004). In addition, YADLVE isolated from mungbean hydrolysate exhibited high renin inhibition of 97.06% at 1 mg protein/mL and antihypertensive effect in SHRs (-27 mmHg) after 24 h, although it showed limited *in vitro* ACE inhibition (Sonklin et al., 2020). The authors suggested that renin inhibition might modulate blood pressure to a greater extent than ACE inhibition (Sonklin et al., 2020). Furthermore, peptide VGINYW derived from α-lactalbumin decreased SBP of SHRs by -21 mmHg after 2 h oral administration by inhibiting ACE activity, upregulating the expression of angiotensin-converting enzyme II and angiotensin

type 2 receptor, and downregulating the expression of angiotensin type 1 receptor in SHRs (Xie et al., 2022). On the other hand, tripeptide LRW showed emphatically inhibit ACE with IC_{50} value of 0.21 µg/mL, but it did not show any hypertensive activity due to its low transepithelial permeability (Fan et al., 2022). Therefore, further studies on how KQL contributes to a slight blood pressure reduction should be elucidated.
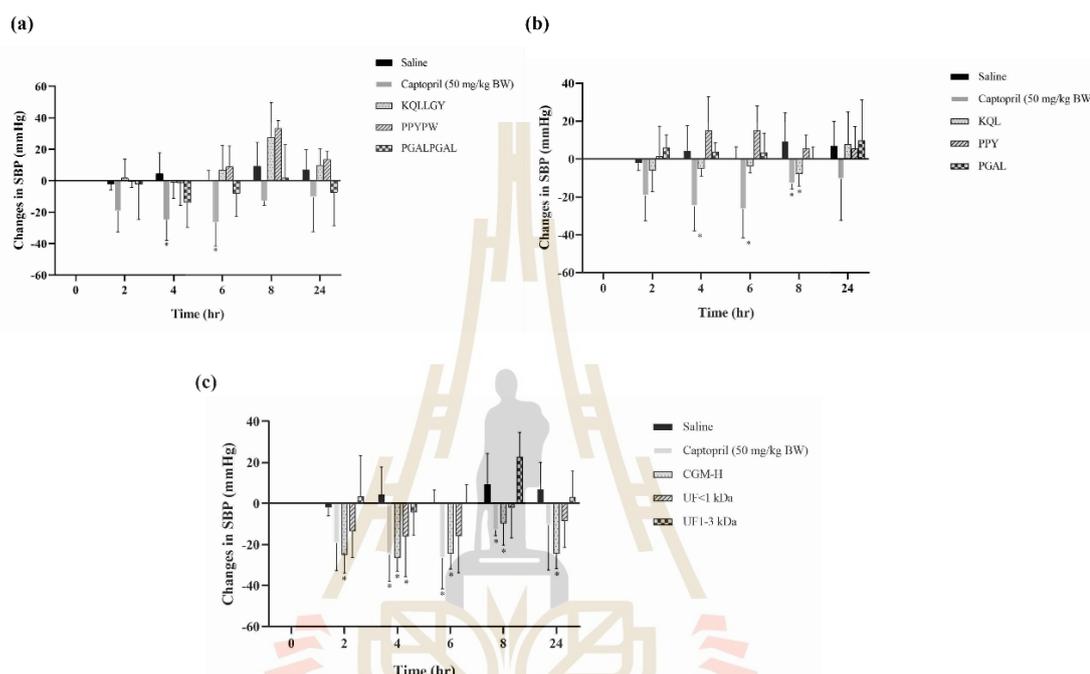

Figure 5.4 Changes in systolic blood pressure after oral administration of (a) parents and (b) GI-digested fragments of ACE inhibitory peptides at dose of 30 mg/kg BW, and (c) CGM-H and peptide fractions at dose of 100 mg/kg BW in SHRs. Data are expressed as mean±SD (n=4). *indicated significant difference between peptide and saline group at the same period time (*p*<0.05, n=4).

5.5 Conclusion

CGM-H could be a potential source of ACE inhibitory peptides. However, peptides isolated from CGM could not resist to GI digestive enzymes and degraded into di-, tri-, and tretapeptides with changes of ACE inhibition. Binding energy obtained from molecular docking and K_i did not correlate with inhibitory activity. CGM-H showed higher renin inhibitory activity than UF fractions and significantly

reduced blood pressure in SHRs during 2 - 24 h after oral administration at dose of 100 mg/kg BW. In addition, peptide KQL which is a GI-digested fragment of KQLLGY exerted a slight antihypertensive activity in SHRs at dose of 30 mg/kg BW, although it showed lower ACE inhibition than its parent counterpart. CGM-H could be used to develop food ingredients and/or functional foods with antihypertensive property. In addition, *in vitro* ACE inhibitory activity, K_i, and bindingenergy obtained from docking cannot be used to predict *in vivo* antihypertensive effect.

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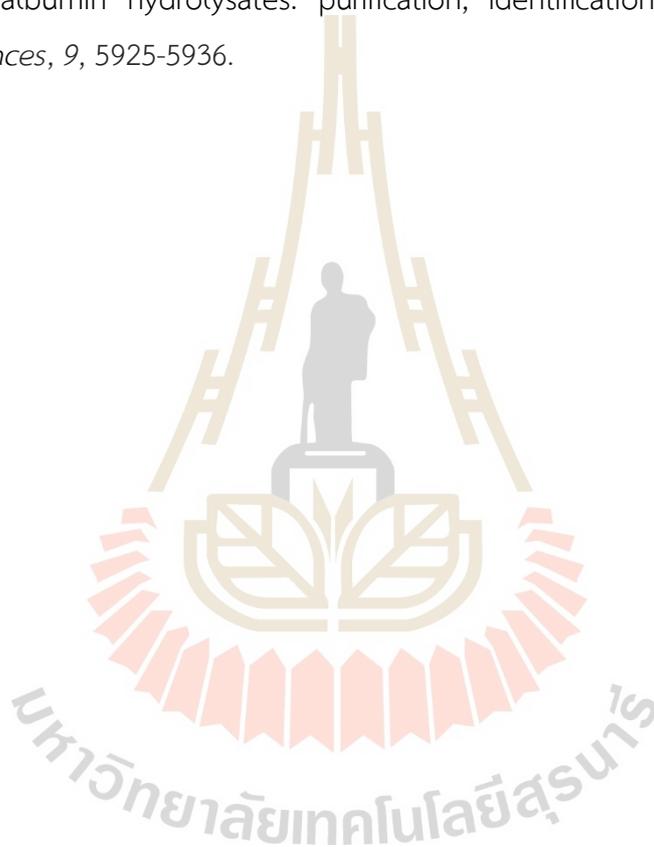
CHAPTER VI

SUMMARY

Prolyl oligopeptidase (POP) inhibitory and antioxidant activities of corn gluten meal hydrolysate (CGM-H) prepared using pepsin changed upon *in vitro* GI digestion. Both CGM-H and its digesta protected neuroblastoma SH-SY5Y cell damages by reducing intracellular reactive oxygen species (ROS) as well as upregulating the expression of catalase (*CAT*) gene. Three novel POP inhibitory peptides were identified from CGM-H. The most effective POP inhibitory peptide, namely ALLTLSPLGPA, degraded by *in silico* gastrointestinal (GI) digestion into SPLGPA, which showed 3-times lower POP inhibition than its parent peptide. In contrast, the GI-digested fragment exhibited higher POP inhibition in SH-SY5Y cells than its parent. Moreover, the peptide ALLTLSPLGPA potentially reduced α-synuclein aggregation of KCl-treated SH-SY5Y cells. POP inhibitory activity of peptide was not correlated with reduction of α-synuclein aggregation. Pathway and role of CGM peptides on α-synuclein aggregation should be further elucidated. These results demonstrated that CGM-H could be considered as a source for peptides with brain functions.

Additionally, seven novel angiotensin-I-converting enzyme (ACE) inhibitory peptides were identified from CGM-H, but ACE inhibitory activity of peptides degraded upon *in silico* GI digestion, leading to a decrease in inhibitory activity. The identified parent and GI-digested peptides interacted with ACE by hydrogen bond (H)-bonds and hydrophobic interactions at S_1 and S_2 pocket site of active sites. Moreover, the binding energy value of parent peptides was lower than that of their GI-digested counterparts, correlating with higher ACE inhibition and lower K_i value. Furthermore, CGM-H at dose of 100 mg/kg BW and peptide KQL at dose of 30 mg/kg BW which was derived from KQLLGY exerted antihypertensive effect in spontaneously hypertensive rats (SHRs) in short term study of 2 and 8 h, respectively. These results reveal that the *in vitro* ACE inhibitory of peptides was not always related with antihypertensive effect. Thus, CGM-H and ACE inhibitory peptides should be further characterized for long term study *in vivo* to understand antihypertensive mechanisms.

BIOGRAPHY

Phiromya Chanajon was born in September 21, 1992, at Uttaradit, Thailand. She studied for her high school diploma at Nampadchanupathum School, Uttaradit, Thailand (2005- 2011). In 2015, she received Bachelor's degree of Science (Food Technology) with the first-class honor from Suranaree University of Technology (SUT). After graduation, she received the Royal Golden Jubilee Ph.D. program Scholarships to pursue Ph.D. program in Food Technology at SUT. During her Ph.D. study, she received the second runner up in oral graduate student competition from The Food Innovation Asia Conference in 2021 (Bangkok, Thailand) under the title of " Isolation and identification of prolyl oligopeptidase and angiotensin-I-converting enzyme inhibitory peptides derived from corn gluten meal hydrolysate". She has also published her research work under the title of " Prolyl oligopeptidase inhibition and cellular antioxidant activities of corn gluten meal hydrolysate" in Cereal Chemistry (volume 99, issue 6, page 1183–1195) in 2022. Her research works has prepared for publication under the title of "Corn gluten meal peptides inhibit prolyl oligopeptidase and modulate α-synuclein aggregation in KCl-treated SH-SY5Y cells" and "Identification and hypotensive effect of Angiotensin-I-converting enzyme (ACE) inhibitory peptides from corn gluten meal hydrolysate and their in-silico GI-digested fragments"