ANTIDIABETIC PROPERTIES OF COMPOSITION OF WHEY PROTEIN CONJUGATED WITH β-GLUCAN AND ITS APPLICATION

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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 2023

สมบัติการต้านโรคเบาหวานของส่วนประกอบของเวย์โปรตีนควบคู่กับเบต้า-กลูแคนและการประยุกต์ใช้

นางกุ้งนาง บุญเสริม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
มหาวิทยาลัยเทคโนโลยีสุรนารี
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กุ้งนาง บุญเสริม : สมบัติการต้านโรคเบาหวานของส่วนประกอบของเวย์โปรตีนควบคู่กับเบต้า-กลูแคนและการประยุกต์ใช้ (ANTIDIABETIC PROPERTIES OF COMPOSITION OF WHEY PROTEIN CONJUGATED WITH β-GLUCAN AND ITS APPLICATION) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.ศิวัฒ ไทยอุดม, 93 หน้า.

คำสำคัญ : การต้านโรคเบาหวาน/เวย์โปรตีน/เบต้า-กลูแคน/พอลิแซ็กคาไรด์-โปรตีน/การควบคู่/เยลลี

เวย์โปรตีนเป็นแหล่งของสารยับยั้ง dipeptidyl peptidase-IV (DPP-IV) โดย DPP-IV คือเอนไซม์ที่สามารถย่อยสลาย glucagon-like peptide-1 (GLP-1) ซึ่งเป็นฮอร์โมนที่นำไปสู่การควบคุมระดับน้ำตาลในเลือดในผู้ป่วยโรคเบาหวานชนิดที่ 2 (T2D) การศึกษานี้จึงวิเคราะห์คุณสมบัติต้านเบาหวานหลังจากการย่อยสารละลายเวย์โปรตีนไอโซเลต (WPI) ในหลอดทดลอง เปรียบเทียบกับสารละลายเวย์โปรตีนแต่ละชนิด (สารละลาย β-lactoglobulin (β-LG) และ α-lactalbumin (α-LA)) ที่ให้ความร้อนด้วยอุณหภูมิต่างกัน (65, 75 และ 85°C) เป็นเวลา 30 นาที พบว่า α-LA hydrolysate มีค่า degree of protein hydrolysis (DH) น้อยที่สุด ($p < 0.05$) โดยระดับของการหลั่ง GLP-1 และฤทธิ์ยับยั้ง DPP-IV ของ α-LA hydrolysate ดีกว่า β-LG hydrolysate ($p < 0.05$) ซึ่ง WPI hydrolysate ลดการทำงานของ DPP-IV ได้น้อยกว่า α-LA hydrolysate แต่การหลั่ง GLP-1 ที่เกิดจาก WPI และ α-LA hydrolysate ไม่แตกต่างกันอย่างมีนัยสำคัญ ($p \geq 0.05$) ส่วนผลของอุณหภูมิแสดงให้เห็นว่า protein hydrolysate ทั้งหมดที่ให้ความร้อน 75°C สามารถยับยั้งการทำงานของ DPP-IV ได้ดีกว่าเมื่อเทียบกับ protein hydrolysate อื่น ๆ ที่ให้ความร้อน 65 และ 85°C ($p < 0.05$) นอกจากนี้ การหลั่ง GLP-1 ที่เพิ่มขึ้นสูงสุดยังสังเกตได้จากการให้ความร้อนแก่สารละลายโปรตีนที่ 75°C hydrolysate จากโปรตีนนมควบคู่กับคาร์โบไฮเดรตที่เกิดจากปฏิกิริยาเมลลาร์ดสามารถป้องกันโรค T2D ได้ นอกจากนี้ ผลของสารควบคู่ระหว่าง α-LA และ β-glucan ด้วยความร้อนที่ 75°C เป็นเวลา 30 นาที ต่อสมบัติต้านเบาหวานหลังจากการย่อยในหลอดทดลองได้รับการทดสอบ โดยเปรียบเทียบอัตราส่วนที่แตกต่างกันของ α-LA ต่อ β-glucan (5:0, 5:1, 5:3, 5:5 และ 0:10 (ร้อยละน้ำหนักต่อปริมาตร)) ที่ pH ต่างกัน (pH 3, 5 และ 7) ทั้งนี้พบว่า hydrolysate จากสารควบคู่ที่อัตราส่วน α-LA ต่อ β-glucan ที่ 5:1 และ 5:3 (ร้อยละน้ำหนักต่อปริมาตร) ที่ pH 3 ให้ค่า DH สูงสุดเมื่อเทียบกับสารควบคู่ที่อัตราส่วน 5:5 (ร้อยละน้ำหนักต่อปริมาตร) ที่ pH 3, 5 และ 7 ($p < 0.05$) อย่างไรก็ตาม ค่า DH ของ hydrolysate ทั้งหมดจากสารควบคู่ระหว่าง α-LA และ β-glucan นั้นน้อยกว่าค่า DH ของ α-LA hydrolysate ที่ pH เดียวกัน ($p < 0.05$) ซึ่งที่ pH 3 การหลั่ง GLP-1 และการยับยั้ง DPP-IV ของ hydrolysate จากสารควบคู่ที่อัตราส่วน 5:1 และ 5:3 (ร้อยละน้ำหนักต่อปริมาตร) ดีกว่าที่อัตราส่วน 5:0, 5:5 และ 0:10 (ร้อยละน้ำหนักต่อปริมาตร) อย่างไรก็ตาม สารควบคู่ระหว่าง α-LA และ β-glucan ที่ 5:3 (ร้อยละน้ำหนักต่อปริมาตร) มีลักษณะปรากฏ

เป็นเนื้อเดียวกันมากกว่าที่ 5:1 (ร้อยละน้ำหนักต่อปริมาตร) จึงได้รับการคัดเลือกเป็นสภาวะในการผลิต jelly-paste ที่มีสารควบคู่ดังกล่าวสำหรับผู้บริโภคที่มีความเสี่ยงต่อโรคเบาหวานและผู้ป่วย T2D โดยใช้ซูคราโลสทดแทนน้ำตาลที่ให้พลังงานใน jelly-paste ดังกล่าว เมื่อศึกษาสมบัติต้านเบาหวานและประสาทสัมผัสของ jelly-paste นี้ พบว่า jelly-paste ช่วยเหนี่ยวนำให้เกิดการหลั่ง GLP-1 สูงกว่าตัวอย่างควบคุมทางการค้า นอกจากนี้ เมื่อทดสอบการยอมรับโดยรวมของผลิตภัณฑ์จากการประเมินผลทางประสาทสัมผัสโดยผู้บริโภคที่มีความเสี่ยงต่อโรคเบาหวานและผู้ป่วย T2D พบว่า อยู่ในช่วงชอบเล็กน้อยถึงชอบปานกลาง อย่างไรก็ตาม ความน่าจะเป็นในการซื้อ jelly-paste นี้อยู่ในช่วงที่อาจจะซื้อถึงซื้อแน่นอนคิดเป็นร้อยละ 60 ของผู้บริโภคดังกล่าว นอกจากนี้ หลังจากที่ผู้บริโภคเหล่านั้นได้รับการอธิบายเกี่ยวกับประโยชน์ของ jelly-paste ส่งผลให้ความน่าจะเป็นในการซื้อเพิ่มขึ้นจากร้อยละ 60 เป็นร้อยละ 73 ข้อมูลที่ได้ยืนยันว่า jelly-paste ในการศึกษาครั้งนี้มีศักยภาพที่จะได้รับการยอมรับและอาจเป็นอาหารเสริมทางเลือกสำหรับผู้บริโภคที่มีความเสี่ยงต่อโรคเบาหวาน

สาขาวิชาเทคโนโลยีอาหาร
ปีการศึกษา 2566

ลายมือชื่อนักศึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

KUNGNANG BUNSROEM : ANTIDIABETIC PROPERTIES OF COMPOSITION OF WHEY PROTEIN CONJUGATED WITH β-GLUCAN AND ITS APPLICATION. THESIS ADVISOR : ASSOC. PROF. SIWATT THAIUDOM, Ph.D., 93 PP.

Keyword: ANTIDIABETIC/WHEYPROTEIN/β-GLUCAN/POLYSACCHARIDE-PROTEIN/ CONJUGATION/JELLY

Whey protein is a source of dipeptidyl peptidase-IV (DPP-IV) inhibitor. DPP-IV is an enzyme which can degrade glucagon-like peptide-1 (GLP-1), a hormone leading to regulate blood glucose levels in type 2 diabetes (T2D) patients. This study explored the antidiabetic properties after *in vitro* digestion of whey protein isolate (WPI) solution compared to individual whey protein solutions (β-lactoglobulin (β-LG), and α-lactalbumin (α-LA) solutions) with different heating temperatures (65, 75, and 85°C) for 30 min. The lowest degree of protein hydrolysis (DH) was observed in α-LA hydrolysate ($p < 0.05$). The level of GLP-1 release and DPP-IV inhibitory activity of α-LA hydrolysate were also had better results than β-LG hydrolysate ($p < 0.05$). WPI hydrolysate decreased DPP-IV activity less than α-LA hydrolysate while the release of GLP-1 induced by WPI and α-LA hydrolysate was not significantly different ($p \geq 0.05$). The result of the heating temperatures showed that all protein hydrolysates heated at 75°C provided greater inhibition of the activity of DPP-IV compared to the other protein hydrolysates at 65 and 85°C ($p < 0.05$). Also, the highest increase in GLP-1 release was observed when heated at 75°C. The hydrolysates from milk protein/ carbohydrate conjugates formed by Maillard reaction could inhibit T2D. The effect of conjugates between α-LA and β-glucan heated at 75°C for 30 min on their antidiabetic properties after *in vitro* digestion was investigated. Such conjugates which varied ratios of α-LA to β-glucan (5:0, 5:1, 5:3, 5:5, and 0:10% *w/v*) at different pH (pH 3, 5, and 7) were compared. The hydrolysates from conjugates at the ratios of α-LA to β-glucan of 5:1 and 5:3% (*w/v*) at pH 3 provided the highest DH compared to that of the conjugates at the ratio of 5:5% (*w/v*) at pH 3, 5, and 7 ($p < 0.05$). However, DH of all hydrolysates from the conjugates of α-LA and β-glucan were lower than that of α-LA hydrolysate at the same pH ($p < 0.05$). At pH 3, GLP-1 release and DPP-IV inhibition of the hydrolyzed conjugates at the ratios of 5:1 and

5:3% (*w/v*) were better than those at the ratios of 5:0, 5:5, and 0:10% (*w/v*). However, the conjugate of α-LA and β-glucan at 5:3% (*w/v*) appeared more homogeneous than that of 5:1% (*w/v*) that was selected to produce jelly-paste containing such conjugate for the targeted consumers who are at risk for diabetes and T2D patients. Calorie sugars in such jelly-paste were replaced with sucralose. The antidiabetic and sensory properties of this jelly-paste were determined. The overall acceptance of this jelly-paste with high GLP-1 release, evaluated by consumers who are at risk for diabetes and T2D patients, was in the range of liking slightly to liking moderately. However, the probability of purchasing this jelly-paste was in the range of probably will purchase to definitely will purchase by 60% of targeted consumers who are at risk for diabetes and T2D patients. Moreover, the benefit of this jelly-paste was explained to those consumers, the probability of purchasing increased from 60 to 73%. This confirms that the jelly-paste in this study possesses the potential to be accepted and could be the alternative supplement food for the diabetes risk consumers.

School of Food Technology
Academic Year 2023

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Assoc. Prof. Dr. Siwatt Thaiudom for his continued support of my studies as well as his patience, motivation, and advice. I deeply appreciate his accepting me as his student and giving me this opportunity to fulfill my ambition to become a food scientist. Special thanks go to my co-advisor Prof. Dr. Witoon Prinyawiwatkul, his guidance helped me in all aspects of my research. Without their tremendous assistance, this dissertation could not have been successfully conducted.

In addition to my advisors, my sincere thanks go also to Assoc. Prof. Dr. Parinya Noisa, who welcomed me to have access to his laboratory and research facilities for cell culture part at the laboratory of Cell-Based Assays and Innovations.

I am grateful to my thesis examining committee members, Assoc. Prof. Dr. Jirarat Anuntagool, Assoc. Prof. Dr. Ratchadaporn Oonsivilai, and Assoc. Prof. Dr. Parinya Noisa for their comments and advice on my thesis.

Additionally, I would like to thank all member colleagues in laboratory of Assoc. Prof. Dr. Siwatt Thaiudom, and Assoc. Prof. Dr. Parinya Noisa for their help and support. I also thank Miss Atchara Jaiboonma and Mr. Palakorn Kaokaen for dividing the starter cells. Also, I offer my heartfelt gratitude to Miss Chattip Sunthrarak who shared her laugh and mind support with me through 6 years of study. I extend my acknowledgement to Dr. Nipha Chaicharoenaudomrung for her invaluable guiding, teaching, and numerous suggestions.

I am sincerely grateful for the scholarships which was provided to me by the National Science and Technology Development Agency of Thailand, grant number NSTDA.RMUTI 3/2560. Rajamangala University of Technology Isan (Nakhon Ratchasima, Thailand) was also acknowledged for providing full supports.

Finally, I would like express my deepest gratitude to my parents (Mrs. Noi Tamaruay and Mr. Boonwan Tamaruay) for their patience and all their loving support throughout the course of my study, and for their faith in me and in allowing me to follow my ambitious goal. Also, I would like to thank my husband and my son (Mr.

Anan Bunsroem and Ms. Anukool Bunsroem) for their heartwarming love and support me through the completion of this work.

Kungnang Bunsroem

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LIST OF ABBREVIATIONS

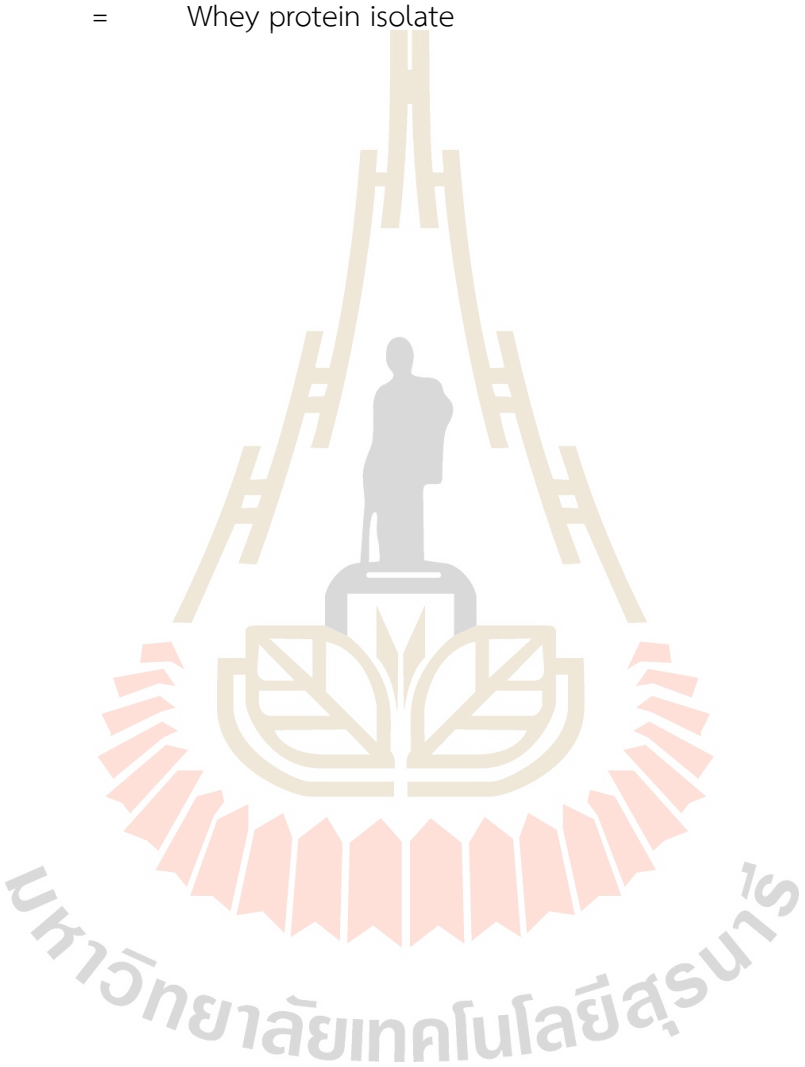
BSA	=	Bovine serum albumin
BI	=	Browning index
CO_2	=	Carbon dioxide
CFU	=	Colony Forming Unit
CLSM	=	Confocal laser scanning microscope
ca.	=	Culture area
Da	=	Dalton
℃	=	Degree Celsius
DH	=	Degree of protein hydrolysis
COO^-	=	Deprotonated carboxylic acid
DM	=	Diabetes mellitus
MTT	=	3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide
DPP-IV	=	Dipeptidyl peptidase IV
DMEM	=	Dulbecco's modified eagle medium
ELISA	=	Enzyme-linked immunosorbent assay
E: S	=	Enzyme: substrate
et al.	=	et alibi (and others)
etc.	=	et cetera (and other things)
EDTA	=	Ethylene diamine tetra-acetic acid
e.g.	=	Exempli gratia (for example)
GI	=	Gastrointestinal
GLP-1	=	Glucagon-like peptide-1
g	=	Gram
IC_{50}	=	Half-maximal inhibitory concentration
h	=	Hour
HSG	=	Human salivary gland
HCl	=	Hydrochloric acid

LIST OF ABBREVIATIONS (Continued)

kDa	=	Kilodalton
pI	=	Isoelectric point
α-LA	=	α-lactalbumin
β-LG	=	β-lactoglobulin
λ	=	Lambda
L	=	Liter
MRPs	=	Maillard reaction products
µg	=	Micrograms
µL	=	Microliter
µm	=	Micrometer
mg	=	Milligram
mL	=	Milliliter
mM	=	Millimolar
min	=	Minute
M	=	Molar
nm	=	Nanometer
n	=	Number
% *w/v*	=	Percent by weight to volume
% *w/w*	=	Percent by weight to weight
pg	=	Picogram
pM	=	Picomolar
PBS	=	Phosphate buffered saline
PTFE	=	Polytetrafluoroethylene
pH	=	Potential of hydrogen ion
PC	=	Principal component
PCA	=	Principal component analysis
NaOH	=	Sodium hydroxide
cm^2	=	Square centimeter
× *g*	=	Times gravity

LIST OF ABBREVIATIONS (Continued)

TNBS	=	2, 4, 6-trinitrobenzenesulfonic acid
T2D	=	Type 2 diabetes
UV	=	Ultraviolet
WPI	=	Whey protein isolate



CHAPTER I

INTRODUCTION

1.1 Rationale of the study

Type 2 diabetes (T2D) is a metabolic disorder in human that is of the leading public health concerns, which are increasing at an explosive rate (Song, Wang, Du, Ji, & Mao, 2017). This disease is characterized by several pathophysiological impairments which consist of insulin resistance, excessive glucose production, and pancreatic β-cell dysfunction (Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013). Insulin is a hormone playing a key role in lowering blood sugar (The_global_diabetes_community, 2019). Insulin secretion is stimulated by glucagon-like peptide-1 (GLP-1), which is secreted in the distal small intestine and also produced in the central nervous system, mainly in the brainstem (Mousa & Ayoub, 2019). It was found that dipeptidyl peptidase IV (DPP-IV) could degrade GLP-1 reaching to 95%. This makes the DPP-IV inhibition has been played attention to support of enhance the GLP-1 activity in order to use as a medicine for T2D patients (Thoma et al., 2003). Beside the DPP-IV inhibitors used as that medicine, insulin, GLP-1 analogs, α-glucosidase inhibitors, sulfonylureas, and metformin also have been used for T2D treatment (Zhang, Zhou, & Li, 2009). However, a price of those medicines seems to be very expensive and their safeness is still in a doubt (Wang, Zhao, Yang, Wang, & Kuang, 2016). The functional foods such as milk proteins are the alternative which not only are benefit to those patients, but also are much cheaper than those medicines.

Recently, whey protein and its compositions have been recognized as a functional ingredient and could be used in many foods due to its low cost and voluminous production as a by-product of many dairy manufactures such as cheese and casein manufacturing. Whey protein is also a source of protein containing many bioactive peptides (Nongonierma & FitzGerald, 2016a). Proteins initiating from major whey protein (β-lactoglobulin: β-LG and α-lactalbumin: α-LA) have several health

benefits, that includes antidiabetic (Tulipano, Faggi, Nardone, Cocchi, & Caroli, 2015). However, commercial whey products contain a total protein content in a different way; whey protein concentrate contains 34% of protein while whey protein isolate (WPI) contains 95% of protein. Therefore, the choice of source for these proteins is also an important factor in obtaining different amounts of bioactive protein, which in turn affects the antidiabetic properties. WPI in post-simulated gastrointestinal digestion could inhibit the activity of DPP-IV (Corrochano et al., 2018). However, the different amino acids sequence in WPI also plays an important role for the bioactive peptides and its potential (Nongonierma & FitzGerald, 2016b). α-LA displayed the highest content of short amino acid sequences with high potential DPP-IV inhibitory activity, which was studied in silico (Nongonierma & FitzGerald, 2014). Tulipano et al. (2015) found that the most potential DPP-IV inhibitors come from β-LG hydrolysates, according to their half maximal inhibitory concentration (IC_{50}) values. Besides, the heat treatment of milk protein isolate after hydrolysis affected the amount of peptides to inhibit DPP-IV activity in post-simulated gastrointestinal digestion (Nongonierma, Paolella, Mudgil, Maqsood, & FitzGerald, 2017).

Additionally, interaction of whey proteins with carbohydrates via Maillard reaction (glycation) has been extensively studied in milk products and in model systems. Glycation of individual whey proteins could increase their antioxidant activity (Abd El-Salam & El-Shibiny, 2018). Maheshwari, Sowrirajan, and Joseph (2019) found that the dietary fiber of β-glucan contributed to a decrease of the blood sugar and T2D. This was relevant to a study of Andrade et al. (2014) which showed that the ingestion of oat β-glucan was efficient in decreasing glucose levels of diabetic patients. Moreover, the fat binding capacity of β-glucan-dipeptiven conjugate was enhanced compared with that of using only oat β-glucan (Sun et al., 2019). However, there still lacks of report about the synergistic effect of the peptides or protein from milk conjugated with β-glucan on antidiabetic properties. Shen, Liu, Dong, Si, and Li (2015) studied the mixture system of oat β-glucan and soy protein isolate and found that the interaction between two macromolecules affected the forming of gels, which was a viscoelastic semisolid, generally found as dessert gels, jams, and jellies (Damodaran, Parkin, & Fennema, 1996). However, the appropriate conditions, such as heat treatment, pH value, and the ratio of ingredients are the important factors in

conjugation process, which in turn affects the physical and antidiabetic properties of the conjugated product.

Therefore, this study aims to: 1) investigate the antidiabetic properties of commercial WPI compared with individual whey proteins (α-LA and β-LG); 2) to determine the best condition for conjugation process between the whey protein which gives the highest antidiabetic properties (the selected whey protein) and β-glucan; and 3) to study the acceptability on sensory profile of antidiabetic jelly-paste from conjugation of the selected whey protein and β-glucan.

1.2 Research objectives

The objectives of this study were:

1.2.1 To compare the antidiabetic properties among WPI, β-LG, and α-LA solutions with different temperature of heat treatment process.

1.2.2 To determine the best condition of conjugation process between the selected whey protein and β-glucan and to qualify/quantify that conjugation.

1.2.3 To produce the jelly-paste and determine its properties (the physical, chemical, antidiabetic, microbial safety, and sensorial).

1.2.4 To carry out the acceptability of jelly-paste made from conjugated of the selected whey protein and β-glucan by consumer test.

1.3 Research hypotheses

WPI, β-LG and α-LA have antidiabetic properties, which can be conjugated with β-glucan. It may increase the efficiency of antidiabetic properties, such as incremental GLP-1 and DPP-IV inhibition. In addition, jelly-paste produced by conjugated compounds, may possess the sensorial and antidiabetic properties which may be suitable for diabetic consumers and benefit to general consumers.

1.4 Scope of the study

The study was divided into three parts which were: 1) the study of the antidiabetic properties of WPI solution, compared with β-LG and α-LA solutions at different temperature of heat treatment process; 2) the effect of pH and ratio of the

selected whey protein conjugated with β-glucan on physical, chemical, and antidiabetic properties; and 3) the production of jelly-paste from the selected whey protein conjugated with β-glucan, which was determined for the physical, chemical, antidiabetic, microbiological, and sensorial properties.

In part 1, the comparison of the antidiabetic properties (GLP-1 quantification as well as DPP-IV inhibitory activity and DPP-IV IC_{50} value *in vitro*) among WPI, β-LG, and α-LA solutions (the samples are commercial food grade) was proceeded at the same conditions. The heat treatment process used in this study was varied in three level (65, 75 and 85°C) for 30 min. The temperature control was measured by a portable thermometer.

In part 2, the conjugation of the selected whey protein and β-glucan, which varied three concentration of β-glucan (1, 3, and 5% *w/v*) and three different pH (pH 3, 5, and 7) was determined for the physical, chemical, and antidiabetic properties.

Finally, the jelly-paste was produced by the selected whey protein conjugated with β-glucan. The qualities of samples were analyzed for their physical, chemical, antidiabetic, microbiological, and sensorial properties.

1.5 Expected results

1.5.1 Receive the comparison results of antidiabetic properties of WPI, β-LG, and α-LA solutions.

1.5.2 Obtain the best condition of heat treatment which may provide the highest antidiabetic properties of WPI, β-LG, and α-LA solutions.

1.5.3 Obtain the best conjugated condition (ratio and pH) of the selected whey protein and β-glucan mixtures which may provide the highest antidiabetic properties and suitable physicochemical properties.

1.5.4 Receive the qualitative and quantitative information of the selected whey protein conjugated with β-glucan.

1.5.5 Accomplish the value-added antidiabetic jelly-paste which might be benefit for T2D patients as consumers.

1.5.6 Receive the information of consumers' acceptance between responses before and after learning the associated health benefits of jelly-paste.

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CHAPTER II

COMPARISON OF THE ANTIDIABETIC PROPERTIES AMONG WPI, β-LG, AND α-LA WITH DIFFERENT TEMPERATURES OF HEAT TREATMENT PROCESS

2.1 Abstract

The inhibition of dipeptidyl peptidase-IV (DPP-IV) and the release of glucagon-like peptide-1 (GLP-1) could normalize blood glucose levels in diabetic patients. This study evaluated the susceptibility of whey proteins to enzyme hydrolysis and the antidiabetic properties of protein hydrolysates from β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) solutions compared with whey protein isolate (WPI) solution treated at different heating temperatures (65, 75, and 85°C). α-LA hydrolysate provided the lowest degree of hydrolysis (DH). Those heating temperatures did not significantly affect the DH of all protein hydrolysates. α-LA hydrolysate significantly increased GLP-1 levels and DPP-IV inhibitory activity more than β-LG hydrolysate. WPI hydrolysate inhibited DPP-IV activity less than an α-LA hydrolysate, but they were no significant differences for GLP-1 release activity. Heat treatment could affect the antidiabetic properties of all protein hydrolysates. Heating at 75°C resulted in greater inhibition of the activity of DPP-IV than at 65 and 85°C. The highest increase in GLP-1 release was also observed by heating at 75°C. The recently obtained information is useful for the utilization of α-LA, heated at 75°C for 30 min, in the preparation of antidiabetic food supplements.

Keywords: antidiabetic, whey protein, β-lactoglobulin, α-lactalbumin, heat treatment.

2.2 Introduction

Diabetes mellitus (DM) is a metabolic disease that is of worldwide concern. The disease is characterized by hyperglycemia resulting from defects in insulin

secretion, insulin action, or both. It severely impairs peoples' quality of life, attributing to several life-threatening complications, including atherosclerosis, nephropathy, and retinopathy (Olesen, Cleal, & Willaing, 2020; Wang, Zhao, Yang, Wang, & Kuang, 2016). The current therapies for DM mainly include oral antidiabetic drugs and insulin. Some widely used drugs to treat DM patients are, for example, dipeptidyl peptidase-IV (DPP-IV) inhibitors, glucagon-like peptide-1 (GLP-1) analogs, metformin, sulfonylureas, and α-glucosidase inhibitors (Omar & Ahrén, 2014; B. B. Zhang, Zhou, & Li, 2009). However, continuous use of these drugs causes insulin resistance and side effects (Wang et al., 2016). Thus, the demand for effective, nontoxic, and affordable drugs for DM patients has gained more attention, and natural alternative foods, for instance, milk proteins, have been used for such purposes.

Whey proteins from milk contain many bioactive peptides. However, commercial whey products are different in their protein content. Whey protein concentrate and whey protein isolate (WPI) contain 65–80% and above 90% of protein on a dry basis, respectively (Andrade et al., 2019). The major components of WPI are β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) (Nongonierma & FitzGerald, 2016a). It was suggested that these bioactive peptides have several health benefits, including antidiabetic (Flaim, Kob, Di Pierro, Herrmann, & Lucchin, 2017; Tulipano, Faggi, Nardone, Cocchi, & Caroli, 2015). WPI in post-simulated gastrointestinal (GI) digestion could inhibit the activity of DPP-IV (Alberto R. Corrochano, Arranz, et al., 2018), which is a serine protease expressed in many tissues, such as kidney, liver, lung, and endothelial cells (Thoma et al., 2003). WPI hydrolysate has been recognized to inhibit DPP-IV activity, which presumably results in an increase in the GLP-1 level, leading to an antihyperglycemic effect (White, 2008). GLP-1 is a hormone that stimulates insulin secretion, which plays a key role in the regulation of blood glucose levels. Insulin deficiency can lead to the development of diabetes symptoms (Müller et al., 2019). A different amino acid sequence in WPI has been known as an index of its bioactive capability and potential (Nongonierma & FitzGerald, 2016b). α-LA possessed the highest content of short amino acid sequences, which provided the most potential DPP-IV inhibitory activity compared to β-LG, bovine serum albumin (BSA), and lactoferrin, studied in silico. In addition, α-LA

in post-simulated GI digestion recognized as α-LA hydrolysate showed a lower level of degree of hydrolysis (DH) than hydrolysates from other whey protein fractions (A. R. Corrochano et al., 2019; Nongonierma & FitzGerald, 2014). On the other hand, the β-LG hydrolysate was a better source than α-LA hydrolysate as the DPP-IV inhibitor in terms of its half-maximal inhibitory concentration (IC_{50}) values (Tulipano et al., 2015). In both previously mentioned methods (Nongonierma & FitzGerald, 2014; Tulipano et al., 2015), purified β-LG and α-LA were used without consideration of the protein solution after exposure to heat during food processing. Heat treatment is one of the most important processes in food preparation (Relkin & Mulvihill, 1996). It could modify the structure of peptides in milk proteins' powder after hydrolysis, resulting in the inhibition of DPP-IV activity of milk protein hydrolysate in post-simulated GI digestion (Nongonierma, Lalmahomed, Paolella, & FitzGerald, 2017; Nongonierma, Paolella, Mudgil, Maqsood, & FitzGerald, 2017). The heated α-LA showed higher antioxidant activity than the native α-LA during stomach digestion. Nevertheless, the antioxidant activity of β-LG significantly decreased at a temperature above 80°C (L. Zhang, Zhou, Zhang, & Zhou, 2021). Preheating BSA at the temperature range between 65 and 75°C could improve the DH values of BSA. However, increasing the temperature above the transition point did not increase the extent of hydrolysis significantly (Arrutia, Puente, Riera, Menéndez, & González, 2016). Importantly, the direct effects of heat treatment of whey protein types on GLP-1 secretion and DPP-IV inhibition after *in vitro* digestion of these proteins are still unclear and need further investigation. Therefore, this study was aimed at exploring the influence of different types of whey proteins with different heat treatment temperatures on their susceptibility to *in vitro* enzyme digestion and determining the antidiabetic properties of these protein hydrolysates.

2.3 Materials and methods

2.3.1 Materials

WPI was purchased from Mullins Whey, Inc. (Mosinee, WI, USA). Both β-LG and α-LA were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The protein content of WPI and α-LA was determined by the Macro–Kjeldahl method (AOAC, 2012) using a nitrogen conversion factor of 6.38. WPI and α-LA contained

96.52 ± 0.43 and 96.91 ± 0.87% of protein (dry basis), respectively. The protein content in β-LG was 95%, as determined by Polyacrylamide Electrophoresis (per the manufacturer's data). Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Carlo Erba Reagents (Val de Reuil, Normandie, France). Dulbecco's modified eagle medium (DMEM) was bought from GE Healthcare Life Sciences (South Logan, UT, USA). Fetal bovine serum was bought from GE Healthcare Bio-Sciences Austria GmbH (Kremplstrasse, Pasching, Austria). Penicillin/streptomycin solution was bought from Capricorn Scientific GmbH (Auf der Lette, Ebsdorfergrund, Germany). Dimethyl sulfoxide was purchased from Ameresco Inc. (Framingham, MA, USA). GLP-1 Total ELISA kit was obtained from Millipore Corporation (Saint Louis, MO, USA). Other chemicals and enzymes used in this study were of analytical grade and purchased from Sigma-Aldrich (Saint Louis, MO, USA).

2.3.2 Preparation of WPI, β-LG, and α-LA solution

The powder of WPI, β-LG, and α-LA was dissolved with distilled water to obtain a 50 mg/mL solution. It was heated at 65, 75, and 85°C for 30 min with constant stirring and then rapidly cooled to 4°C. The protein solution was stored for no more than 5 days at 4°C prior to *in vitro* digestion. The experimental design for this study was a Randomized Complete Block Design, with temperatures acting as the treatments and the different types of whey protein solutions assigned as the blocks.

2.3.3 *In vitro* digestion of whey proteins

The protein solution in part 2.3.2 was *in vitro* digested in a GI simulated system. This was carried out as described by Nongonierma, Lalmahomed, et al. (2017). Briefly, the protein solution was heated at 37°C for 30 min. The pH of the solution was adjusted to 2.0 using 1 M HCl. The protein solution was hydrolyzed with pepsin (enzyme: substrate (E: S), 2.5% *w/w*) for 90 min at 37°C. Pepsin activity was inactivated by heating the protein solution for 20 min at 90°C. An aliquot of the peptic hydrolysate was adjusted to pH 7.5 using 1 M NaOH and was subsequently hydrolyzed with pancreatin (E: S, 1% *w/w*) for 150 min at 37°C. The reaction was terminated by thermal treatment (90°C, 20 min). The protein hydrolysate was stored at 4°C until further analysis.

2.3.4 Determination of degree of hydrolysis (DH)

The protein hydrolysate in part 2.3.3 was tested for DH values by 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) colorimetry for α-amino nitrogen as described by Yi, Lin, and Johns (2021) and Gruppi, Dermiki, Spigno, and FitzGerald (2022) with a slight modification. Briefly, samples (hydrolyzed and unhydrolyzed control samples after heat inactivation of enzymes) were diluted in 1% (*w/v*) sodium dodecyl sulfate to a final protein concentration/protein equivalent of 10 mg/mL and incubated at 50°C for 60 min. Then, 10 μL of both samples and 10 μL of leucine standards at 0–5 mg/mL were loaded onto a 96-well plate with 80 μL of 0.2 M sodium phosphate buffer, pH 8, followed by 80 μL of 0.025% (*w/v*) TNBS. The sample plate was incubated at 45°C for 30 min in a microplate reader (Thermo Fisher Scientific, Vantaa, Southern Finland, Finland), and the absorbance at 420 nm was monitored every 2 min. The DH values were calculated using the equation as follows:

$$\text{DH}\ (\%) = (A - B/T) \times 100.$$

where A is the reactive α-amino nitrogen concentration determined by TNBS colorimetry, B is the reactive α-amino nitrogen concentration of the intact protein substrate, and T is the total reactive α-amino nitrogen concentration of the intact protein substrate. B/T values of WPI, β-LG, and α-LA were 0.39/8.29, 0.88/9.24, and 0.92/9.38, respectively (Yi et al., 2021).

2.3.5 Evaluation of antidiabetic properties

2.3.5.1 Glucagon-like peptide-1 (GLP-1) quantification assay

1) Cell culture

Cell lines of the human salivary gland (HSG) were obtained from the laboratory of Dr. Parinya Noisa (School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand). Cells were grown at 37°C, with 95% air and 5% CO_2 in a humidified atmosphere incubator (Thermo Fisher Scientific, Waltham, MA, USA) (Shao, Furusawa, Aoki, Matsumoto, & Ando, 2002). Cells were cultured in DMEM containing 4.0 mM L-glutamine and 4.5 g/L glucose supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Jeffers, Madden, & Webster-Cyriaque, 2009). Cells were

routinely grown in 75 cm^2 tissue culture plastic flasks. The DMEM was changed once every 1–2 days (Qiu, Qiu, Cui, & Wei, 2016). Cells were trypsinized with 0.1% trypsin-EDTA and reseeded. HSG cells were frozen at −80°C in a freezer (Thermo Fisher Scientific, Marietta, OH, USA) and thawed at 37°C before a test of cell viability and GLP-1 secretion.

2) Cell viability test

The MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) colorimetric assay adapted from Arteaga-Cardona et al. (2016) was used to determine the cell viability and cytotoxicity. Briefly, the protein hydrolysate was sterilized by filtration through a 0.2 μm PTFE membrane filter (Sigma-Aldrich, Saint Louis, MO, USA). HSG cells were maintained in 96 well culture plates (well growth area of ca. 0.32 cm^2) at a density of 10^4 cells/well and incubated in cell culture conditions for 24 h. Then, the DMEM was replaced with 100 μL of fresh DMEM containing different concentrations of the protein hydrolysate (0, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, and 10 mg/mL). Cells were further incubated for 24 h. Then, the DMEM with protein hydrolysate was removed, and 10 μL MTT solution (5 mg/mL in PBS pH 7.4) was added to each well. After further incubation in the dark for 3 h at 37°C, the MTT solution was removed, and 100 μL of dimethyl sulfoxide was added to each well. The absorbance was monitored in a microplate reader at a wavelength of 550 nm. The untreated cells were used as the control, and the cell viability was calculated using the following equation:

Cell viability (%) = (Absorbance of sample well/ absorbance of control well) × 100.

3) GLP-1 secretion test

In order to examine the GLP-1 quantification, HSG cells were maintained at 6 × 10^4 cells/well in 24 well culture plates (well growth area of ca. 1.86 cm^2) and incubated in cell culture conditions for 24 h. Then, the DMEM was replaced with 600 μL of fresh DMEM containing the protein hydrolysate at concentration levels which provided the maximum cell viability. Untreated cells were used as the control. After further incubation for 24 h, the supernatants were centrifuged at 664× *g* for 3 min at 25°C to remove the remaining cells. Total GLP-1

release levels were determined using a GLP-1 total ELISA kit according to the manufacturer's instruction (Komatsu et al., 2019).

2.3.5.2 Dipeptidyl peptidase-IV (DPP-IV) inhibition assay

1) Measurement of DPP-IV inhibitory activity

The existence of GLP-1, which was mainly metabolized by DPP-IV, was indirectly determined as the DPP-IV inhibition value (Mousa & Ayoub, 2019). The protein hydrolysate was used at the concentration providing the highest cell viability, as previously mentioned. The protein hydrolysate (25 µL) was mixed with 50 µL of 1 mM reaction substrate (Gly-Pro *p*-nitroanilide hydrochloride) in a 96-well clear microplate. Then, 25 µL of 0.2 units/mL DPP-IV was subsequently added. The microplate was incubated at 37°C for 30 min, and the absorbance of the *p*-nitroanilide, which was released at 405 nm, was monitored every 5 min in a microplate reader (Nongonierma, Lalmahomed, et al., 2017). The results were compared with that of the control (no protein hydrolysate added) (Chakrabarti et al., 2011).

2) Measurement of DPP-IV half-maximal inhibitory concentration (IC_{50})

The IC_{50} value of DPP-IV was measured in order to confirm the DPP-IV inhibitory activity of protein hydrolysate. The protein hydrolysate was varied in the range of 5 to 50 mg/mL. Diprotin A (Ile-Pro-Ile) was used as a positive control at the concentration range between 1.56 and 62.50 µg/mL. The DPP-IV IC_{50} value was determined as previously mentioned in the DPP-IV inhibitory activity measurement. The IC_{50} values were determined by plotting the percentage of inhibition and the concentration of protein hydrolysate (Nongonierma, Lalmahomed, et al., 2017).

2.3.6 Statistical analysis

All experiments and measurements were performed at least in triplicate. All results were expressed as the mean ± standard deviation. The analysis of covariance with a significance at $p < 0.05$ was used to test the lack of significant differences between the mean of cell viability values at various concentrations of different types of whey protein hydrolysates. A one-way analysis of variance was used to determine the difference between mean values with a significance at $p < 0.05$ for DH percentage, total GLP-1 release, DPP-IV inhibition percentage, and DPP-IV IC_{50}

values. A one-way analysis of variance was followed by Duncan's Multiple Range Test for multiple means comparison. Principal component analysis (PCA) was performed to identify patterns of the experimental data, investigate their similarity and difference, and determine the variation in antidiabetic properties among the different protein hydrolysates. All analyses were determined using the statistical package for the social sciences program (version 17.0, SPSS Inc., Chicago, IL, USA).

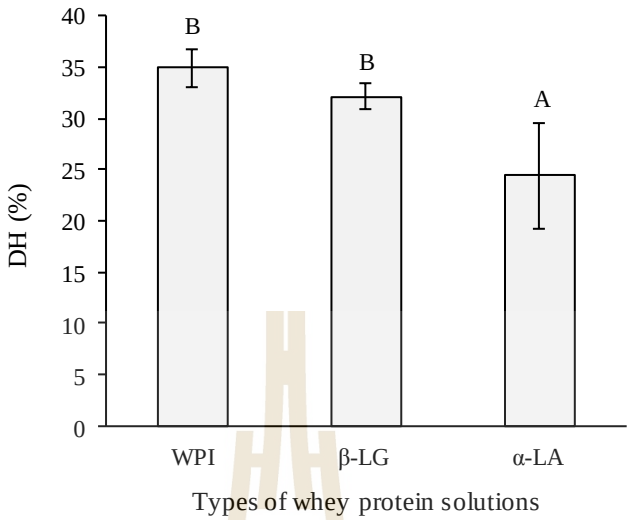
2.4 Results and discussion

2.4.1 Degree of hydrolysis of protein hydrolysate

The DH values of protein hydrolysates from WPI, β-LG, and α-LA solutions heated at different temperatures (65, 75, and 85°C for 30 min) were compared. The results are shown in Figure 2.1. α-LA hydrolysate showed the lowest DH (%) compared to WPI (1.4 times higher) and β-LG hydrolysate (1.3 times higher) ($p < 0.05$). This result was consistent with the findings of A. R. Corrochano et al. (2019) and Lagace (2012). The higher the DH value, the higher the susceptibility of the protein hydrolysate to digestive enzymes. This might be due to the effect of different protein structures. A β-sheet structure, found in β-LG, which possesses more simple structure and more accessible sites for the digestive enzyme than an α-helix form of α-LA, could be easier hydrolyzed by the enzymes (Creamer, Parry, & Malcolm, 1983; Permyakov, 2013). Moreover, β-LG is the main component found more than α-LA in WPI. Thus, the DH value of WPI hydrolysate was not significantly different from that of β-LG hydrolysate (Figure 2.1a).

In addition, the heating temperatures did not significantly affect the DH (%, Figure 2.1b). This might be because when those proteins were hydrolyzed with the same type of enzymes and hydrolysis time, finally, the particle size of protein hydrolysates should provide the same particle size distribution, resulting in the non-significantly different DH values. However, Arrutia et al. (2016) revealed that the higher DH values of BSA hydrolysate were attributed to the preheated temperature between 65 and 75°C but not at 85 and 95°C.

[a]



[b]

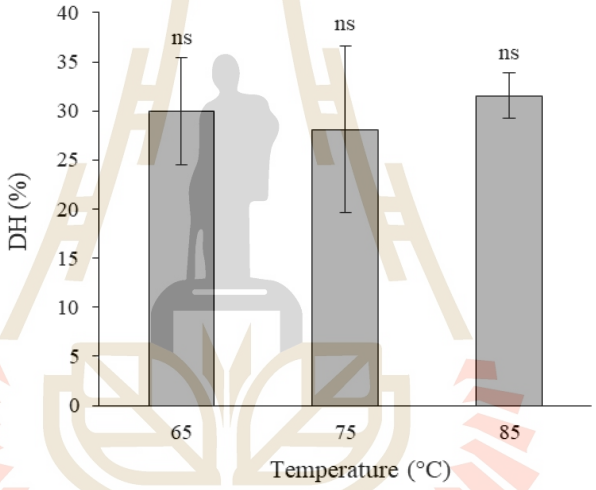


Figure 2.1 Degree of hydrolysis (DH, %) of protein hydrolysates: [a] Comparisons between different types of protein solutions (WPI, β-LG, and α-LA), and [b] comparisons between different heat treatment temperatures (65, 75, and 85°C) for 30 min for all protein solutions. Data are presented as mean ± standard deviation (n = 3). The different letters ^{A,B} indicate a significant difference at $p < 0.05$. The letter ^{ns} indicates no significant difference at $p \geq 0.05$. WPI, β-LG, and α-LA denote whey protein isolate, β-lactoglobulin, and α-lactalbumin, respectively.

2.4.2 Glucagon-like peptide-1 release activity

HSG cells were used as an *in vitro* model of GLP-1 release. This cell line has been widely used to elucidate the mechanism of GLP-1 release (Baum et al., 2010; Rowzee, Cawley, Chiorini, & Di Pasquale, 2011; Samuni & Baum, 2011). However, food digestion is known to be cytotoxic to cells, which depends on the type, concentration, processing, quantity of food, etc. (Chevalier, Chobert, Genot, & Haertlé, 2001; Diao et al., 2021). The optimum concentration of the protein hydrolysates from WPI, β-LG, and α-LA solutions at different heat temperatures (65, 75, and 85°C for 30 min) for GLP-1 quantification was determined using the MTT colorimetric assay. Figure 2.2 shows that there are two noticeably different zones of cell viability with the protein hydrolysate concentrations beyond 2.5 mg/mL and below. In the present study, significant decreases in cell viability were observed at the high concentrations of all protein hydrolysates (5–10 mg/mL). This was relevant to the generally used-optimum concentration range of the tested compounds, which was between 1.0×10^{-4} and 0.5 mg/mL (Arteaga-Cardona et al., 2016; Chevalier et al., 2001; Lagace, 2012). All protein hydrolysates at the concentration of 0.313 mg/mL were not harmful to HSG cells. Evidently, at this concentration, the cell viability (%) was the highest (92.00–156.64%, Figure 2.2).

The comparison result demonstrated that WPI and α-LA hydrolysates could induce and maintain the cell viability more than a β-LG hydrolysate at the low concentrations (0.078–1.25 mg/mL). Alberto R. Corrochano, Buckin, Kelly, and Giblin (2018) explained that direct exposure of cell lines to whey protein hydrolysate increased intracellular antioxidants such as glutathione, which was recognized as a diet for the well-being of cells. This is in line with the previous findings that α-LA did not display the cell membrane-damaging activity in an aqueous solution at a neutral pH. However, there was no evidence of the effect of α-LA hydrolysate on cell viability (Shi et al., 2020).

Moreover, at the concentrations of protein hydrolysates below or equal to 2.5 mg/mL, WPI and the α-LA hydrolysate heated at 65°C for 30 min, known as a low-temperature long time pasteurization (Fagnani, Mexia, Puppio, & Battaglini, 2016), provided the highest cell viability (86.87–156.64%), compared to those at 75

and 85°C (75.29–125.71%, Figure 2.2). However, when heating was at 75 and 85°C, each protein hydrolysate did not clearly induce cell viability.

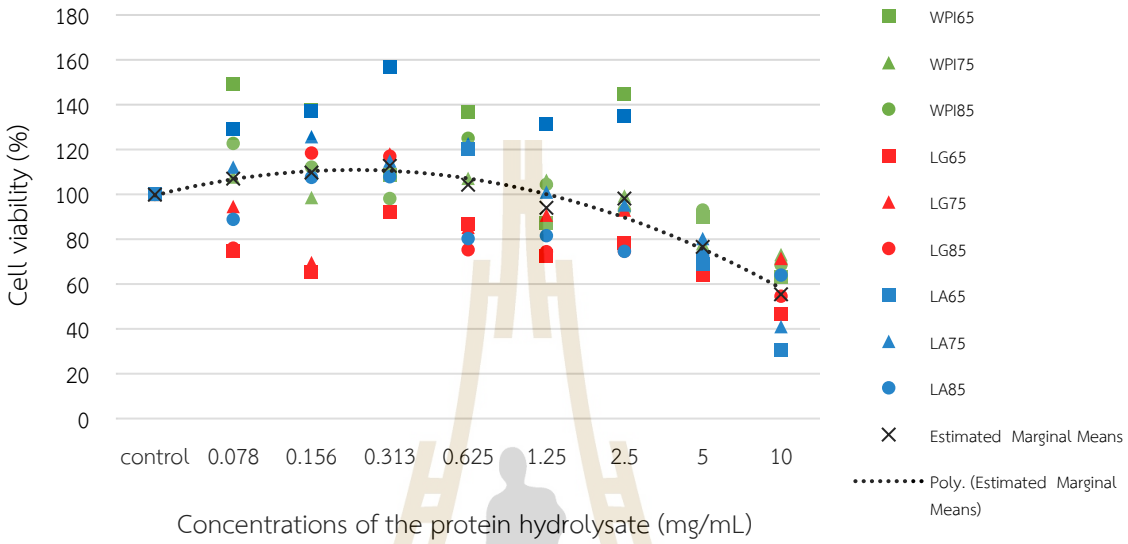


Figure 2.2 Cell viability (%) of the human salivary gland (HSG) cells after 24 h incubation with DMEM (control) and the protein hydrolysate at concentrations between 0.078 and 10 mg/mL (n = 4). WPI, LG, and LA denote whey protein isolate, β-lactoglobulin, and α-lactalbumin solutions, respectively. The number after the abbreviated protein solutions indicates the temperature (°C) of the heat treatment process.

Each protein hydrolysate at three different temperatures providing the highest cell viability was used to determine the GLP-1 release. The results are shown in Table 2.1. WPI, β-LG, and α-LA hydrolysates significantly increased the release of GLP-1 compared to the control. At all heating temperatures, WPI and α-LA hydrolysates could induce the release of GLP-1 better than β-LG hydrolysate (p < 0.05).

Furthermore, heat treatment could enhance the release of GLP-1 compared to the control. The release of GLP-1 induced by heating at 75 and 85°C

was not significantly different. However, heat treatment at 65°C seemed to provide fewer releases of GLP-1 than at 75 and 85°C ($p < 0.05$).

Table 2.1 Glucagon-like peptide-1 (GLP-1) release values (pM) of protein hydrolysates from whey protein isolate (WPI), β-lactoglobulin (β-LG), and α-lactalbumin (α-LA) solutions with different heat treatment temperatures. Untreated cells were used as the control.

Temperature (°C)	Types of whey protein hydrolysates			
	WPI	β-LG	α-LA	Control
65	12.37 ± 0.06 ^{Ba}	11.98 ± 0.08 ^{Bb}	12.34 ± 0.02 ^{Ba}	
75	12.43 ± 0.08 ^{Aa}	12.20 ± 0.07 ^{Ab}	12.41 ± 0.05 ^{Aa}	
85	12.51 ± 0.06 ^{Aa}	12.08 ± 0.02 ^{Ab}	12.37 ± 0.07 ^{Aa}	
Control				11.83 ± 0.06 ^{Cc}

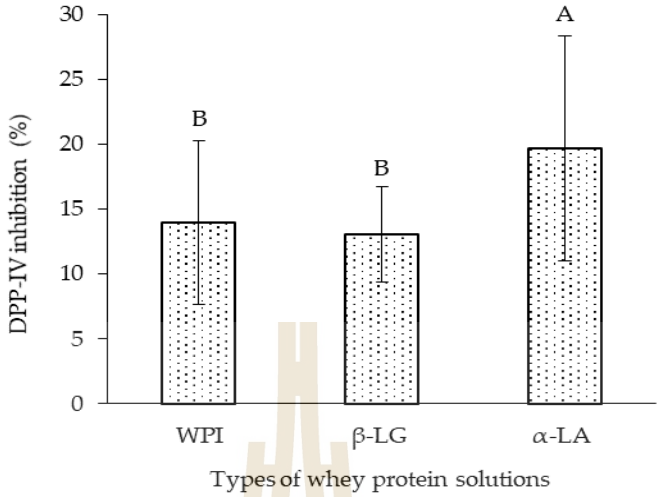
Note: Data are presented as mean ± standard deviation ($n = 6$). The different letters ^{A,B,C} in the same column indicate a significant difference at $p < 0.05$. The different letters ^{a,b,c} in the same row indicate a significant difference at $p < 0.05$.

2.4.3 Dipeptidyl peptidase-IV inhibitory activity

The DPP-IV inhibitory activity of the protein hydrolysates from WPI, β-LG, and α-LA solutions at different temperatures of the heat treatment process was determined. The concentration of protein hydrolysate at 0.313 mg/mL, providing the highest cell viability, was used (Figure 2.2). As shown in Figure 2.3a, α-LA hydrolysate showed the highest DPP-IV inhibition (%) compared to WPI (1.4 times lower) and β-LG hydrolysate (1.5 times lower) ($p < 0.05$). The percentages of DPP-IV inhibitory activity of WPI and β-LG hydrolysates were not significantly different.

Among the three heating temperatures, all protein hydrolysates heat-treated at 75°C provided the highest DPP-IV inhibitory activity (nearly two times higher) compared to the rest, while there were no significant differences in DPP-IV inhibitory activities at 65 and 85°C (Figure 2.3b).

[a]



[b]

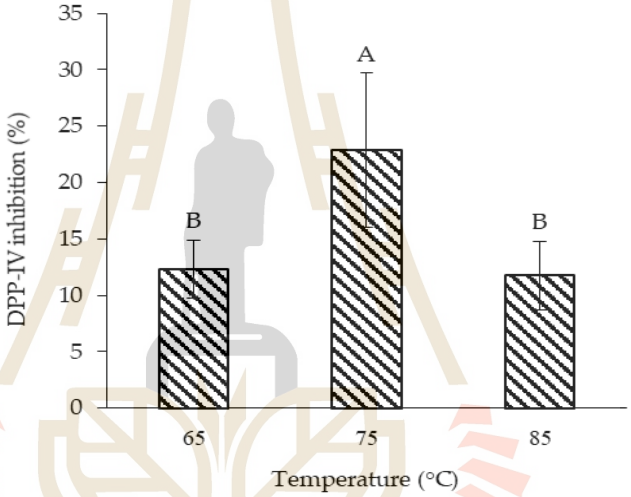


Figure 2.3 Dipeptidyl peptidase-IV (DPP-IV) inhibition values (%) of protein hydrolysates at a concentration of 0.313 mg/mL: [a] Comparisons between different types of protein hydrolysates from WPI, β-LG, and α-LA solutions; [b] comparisons between different heat treatment temperatures (65, 75, and 85°C) for 30 min for all protein hydrolysates. Data are presented as mean ± standard deviation (n = 3). The different letters ^{A,B} indicate a significant difference at $p < 0.05$. WPI, β-LG, and α-LA denote whey protein isolate, β-lactoglobulin, and α-lactalbumin, respectively.

In order to confirm the DPP-IV inhibitory activity of each protein hydrolysate, their DPP-IV IC_{50} values (mg protein/mL) were determined, and the results are shown in Table 2.2. Diprotin A was used as a reference for DPP-IV inhibitory activity, and its DPP-IV IC_{50} value was 0.0185 ± 0.0010 mg/mL in this current study. The DPP-IV IC_{50} value of the α-LA hydrolysate was in the range of 7.16 to 13.80 mg/mL, which was varied with the temperature of the heat treatment process. Regardless of the heating temperature, α-LA hydrolysate could reduce the DPP-IV IC_{50} value better (about two times higher or more) than β-LG and WPI hydrolysate ($p < 0.05$). In addition to the insulinotropic effect of whey protein hydrolysates was believed to delay the inactivation of GLP-1 release induced by DPP-IV (Tulipano et al., 2015). However, the types of whey proteins greatly influenced their antidiabetic properties. The peptide profiles undoubtedly inserted a role for these bioactivities after the simulated GI digestion (Alberto R. Corrochano, Arranz, et al., 2018). The result showed that α-LA hydrolysate significantly increased GLP-1 release level more than β-LG hydrolysate (Table 2.1). This was because α-LA hydrolysate exhibited a higher potential on the *in vitro* DPP-IV inhibitory activity than β-LG hydrolysate. The shorter amino acid residues of whey peptides contributed to their best DPP-IV inhibitory activity. The best DPP-IV inhibitor was peptides with a length of 3–6 amino acids (Nongonierma et al., 2019; Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013). However, practically, tri-peptides IPI (diprotin A) and VPL (diprotin B) have always been used as the precursor and standard of the DPP-IV inhibition (Umezawa et al., 1984). Regarding the DH values of these protein hydrolysates, the DH of α-LA hydrolysate was the lowest compared to the others. This implies that α-LA hydrolysate was the most resistant to the action of these studied digestive enzymes. The lower DH value seemed to correlate with a lower amino acid content but not with a higher amount of short-chain peptides, which could inhibit DPP-IV activity (Farup et al., 2016). Thus, α-LA hydrolysate showed less DH but high DPP-IV inhibitory activity. Even though WPI hydrolysate could inhibit DPP-IV activity less than α-LA hydrolysate, both were not significantly different in the induced GLP-1 release (Table 2.1). This might be due to the modified structure of GLP-1 by glycine in WPI hydrolysate, which may be resistant to enzyme degradation by DPP-IV (Burcelin, Dolci, & Thorens, 1999; Voutetakis et al., 2010).

For each protein hydrolysate, there were no significant differences in DPP-IV IC_{50} values at 65 and 85°C ($p \geq 0.05$), but these values were significantly ($p < 0.05$) higher than those at 75°C. The structural changes in whey protein were recognized as a consequence of heat treatment (Jiang et al., 2018). Hydrophilic interactions of proteins (hydrogen bonds, Van der Waals interactions, electrostatic interactions between charged groups, and specific binding) were weakened, while their hydrophobic interactions were strengthened by heat treatment in the temperature range of 60 to 80°C (Damodaran, Parkin, & Fennema, 1996). At natural pH (4.5–6.0) values, β-LG was denatured by temperature between 75 and 80°C (Relkin & Mulvihill, 1996). The thermal denaturation temperature of α-LA was, on average, at 63.7°C (McGuffey, Epting, Kelly, & Foegeding, 2005). The denaturation ratio of whey proteins increased approximately by 20, 45, and 98% at the heating temperatures of 65, 75, and 85°C for 30 min, respectively (Qian et al., 2017). However, three heating temperatures did not significantly affect the susceptibility of protein hydrolysates to digestive enzymes. In contrast, heating at 65°C gave less GLP-1 release than heating at 75 and 85°C, which was not significantly different in GLP-1 levels (Table 2.1). In addition, the effect of the heat treatment process on the DPP-IV inhibitory activity of the protein hydrolysates was clearly evidenced in this study (Table 2.2 and Figure 2.3b). Protein hydrolysates from heating the protein solution at 75°C resulted in a significantly higher DPP-IV inhibition percentage and lower IC_{50} value than at 65 and 85°C.

Table 2.2 The Dipeptidyl peptidase-IV half-maximal inhibitory concentration (DPP-IV IC_{50}) values (mg/mL) of protein hydrolysates from whey protein isolate (WPI), β-lactoglobulin (β-LG), and α-lactalbumin (α-LA) solutions with different heat treatment temperatures.

Temperature (°C)	Types of whey protein solutions		
	WPI	β-LG	α-LA
65	24.19 ± 0.85^{Bb}	30.81 ± 0.89^{Bb}	12.31 ± 0.52^{Ba}
75	20.41 ± 1.64^{Ab}	25.35 ± 1.10^{Ab}	7.16 ± 0.82^{Aa}
85	37.40 ± 0.36^{Bb}	26.57 ± 0.70^{Bb}	13.80 ± 0.87^{Ba}

Note: Data are presented as mean ± standard deviation (n = 3). The different letters ^{A,B} in the same column indicate a significant difference at $p < 0.05$. The different letters ^{a,b} in the same row indicate a significant difference at $p < 0.05$.

2.4.4 Principal component analysis

In order to investigate the relationship between the protein hydrolysates from different types of protein solutions with different heat treatment temperatures and their antidiabetic properties, PCA was performed. In Figure 2.4, the PCA biplot of the first and second principal components (PC1 and PC2) for the antidiabetic properties of the nine protein hydrolysates is presented. The sum of PC1 and PC2 explained 0.71 (71%) of the total variance, which was higher than the acceptable variance explained (0.60) in factor analysis for a construct (Hair, Black, Babin, & Anderson, 2019). As shown in the PC1, a group of the α-LA hydrolysates with different heat treatment temperatures was attributed to an influential type of protein solution with its best DPP-IV inhibition. In addition, the effect of 75°C heating temperature on high GLP-1 release was grouped by the PC2. This PCA plot suggests that protein hydrolysates from three protein solutions (WPI solution with the heating temperature at 65°C as well as β-LG solutions with the heating temperatures at 75 and 85°C) are correlated by the DPP-IV IC_{50} values. This is supported by the data in Table 2.2, which shows that the DPP-IV IC_{50} value of protein hydrolysates from WPI solution with the heating temperature at 65°C, and β-LG solutions with heating

temperatures at 75 and 85°C were 24.19 ± 0.85, 25.35 ± 1.10, and 26.57 ± 0.70 mg/mL, respectively.

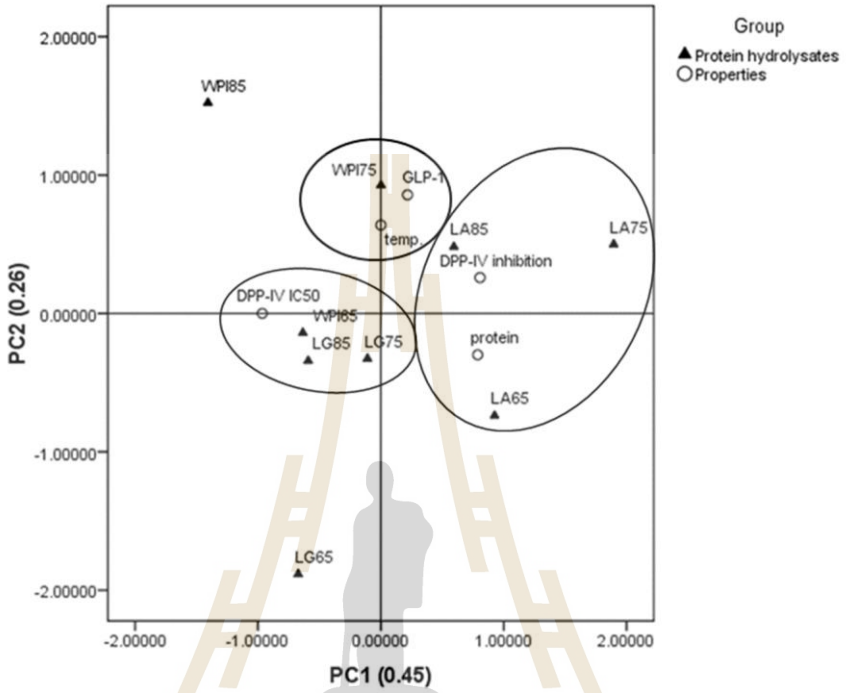


Figure 2.4 Bi-plot for the protein hydrolysates and their properties from principal component analysis (PCA). WPI, LG, and LA denote whey protein isolate, β-lactoglobulin, and α-lactalbumin solutions, respectively. The number after the abbreviated protein solutions indicate the temperature (°C) of the heat treatment process.

2.5 Conclusions

This study revealed that α-LA provided the least DH of hydrolysate compared to β-LG and WPI. The α-LA hydrolysate exhibited antidiabetic properties better than WPI and β-LG hydrolysates. However, all protein hydrolysates were harmless to HSG cells at a specific concentration, that is, 0.313 mg/mL. Evidently, α-LA hydrolysate possesses a high potential for being the enhancer for GLP-1 release and DPP-IV inhibitory activity when a thermal process is applied. Heating at 75°C for 30 min gave the best results of GLP-1 release and DPP-IV inhibition while heating at

65 and 85°C for 30 min provided both lower activities of GLP-1 release and DPP-IV inhibition. From the PCA analysis, process temperature could affect GLP-1 release while the types of whey protein hydrolysate influenced DPP-IV inhibitory activity. Conclusively, **α**-LA solution heated at 75°C for 30 min can potentially be used as a potential antidiabetic substance in the form of a food supplement for diabetic patients in the future. However, the optimal concentration of **α**-LA solution heated at 75°C for 30 min should be further investigated for such purpose. In addition, the effect of preheating temperature and enzyme inactivation temperature on antidiabetic activity of protein hydrolysate as well as the physicochemical properties, e.g., size distribution, structure, and bioactive peptides and amino acid sequences of such protein hydrolysates should be further investigated.

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CHAPTER III

EFFECT OF pH AND RATIO OF α-LACTALBUMIN CONJUGATED WITH β-GLUCAN ON CHEMICAL, PHYSICAL, AND ANTIDIABETIC PROPERTIES

3.1 Abstract

The release of glucagon-like peptide-1 (GLP-1) and the inhibition of dipeptidyl peptidase-IV (DPP-IV), the main GLP-1 degrading enzyme, has been proposed for the treatment of type 2 diabetes (T2D). The hydrolysates from conjugates between milk protein and carbohydrate via Maillard reaction can inhibit T2D. This study evaluated the physical and chemical properties of conjugate between α-lactalbumin (α-LA) and β-glucan and their antidiabetic properties of hydrolysates from the conjugates also were determined. The ratios of α-LA to β-glucan (5:0, 5:1, 5:3, 5:5, and 0:10% *w/v*) at different pH (pH 3, 5, and 7) were examined as the variable factorial treatments. The microstructures of conjugates were observed by a confocal laser scanning microscope. The results showed that some β-glucan particles could absorb onto the structure of α-LA. The browning index, showing the presence of Maillard reaction, of the conjugates at pH 3 was lower than that of conjugates at pH 5 and 7 in all variable ratios of α-LA to β-glucan, especially at the ratios of 5:1 and 5:3% *w/v*. Consequently, the degree of protein hydrolysis of those conjugates was higher than that of the conjugates at the ratio of 5:5% *w/v*, resulting in lower half-maximal inhibitory concentration of DPP-IV but higher GLP-1 of hydrolysates from the conjugates of ratios of 5:1 and 5:3% (*w/v*) of α-LA to β-glucan at pH 3. However, the appearance of conjugate of 5:3% (*w/v*) exhibited more homogenous than that of 5:1% *w/v*. Thus, the conjugate from α-LA and β-glucan at a ratio of 5:3% (*w/v*) using pH value at pH 3 is the best conjugated condition. This summarized that α-LA/β-glucan conjugate could use as a potential antidiabetic substance in the ingredient of a food supplement for T2D patients in the future.

Keywords: antidiabetic, α-lactalbumin, β-glucan, pH, ratio.

3.2 Introduction

Diabetes is a disease which has been found in many parts of the world and seems to increase more and more from past to present. Type 2 diabetes (T2D) is the most prevalent form of diabetes, which is about 90% of all cases (Nongonierma et al., 2019). The disease is a metabolic disorder characterized by malfunctioned insulin secretion of β-cells and insulin resistance in tissues, a condition that is associated with the development of several complications, including hypertension and cardiovascular disease (Brandelli, Daroit, & Corrêa, 2015; Olesen, Cleal, & Willaing, 2020). The therapies which are available for T2D could be insulin injection and taking antidiabetic medicines such as metformin, sulfonylureas, α-glucosidase inhibitors, dipeptidyl peptidase-IV (DPP-IV) inhibitors, and glucagon-like peptide-1 (GLP-1) analogs (Zhang, Zhou, & Li, 2009). However, the existing synthetic medicines have several limitations, for instance, expensive cost, insulin resistance, and side effects such as hypoglycemia, edema, and gastrointestinal intolerance (Wang, Zhao, Yang, Wang, & Kuang, 2016; Zhang et al., 2009). Therefore, the usage of natural extracts as a substitute for synthetic medicines is probably a safer option for people at risk of diabetes and T2D patients (Arumugam, Manjula, & Paari, 2013; Ogori et al., 2022). Natural extracts such as polysaccharides from *Enteromorpha prolifera* could stimulate glucose metabolism in diabetic rats (Wang et al., 2016). In addition, camel milk protein, constituting an interesting source of naturally bioactive peptides, had the potential to inhibit the activity of the DPP-IV enzyme (Mudgil, Kamal, Yuen, & Maqsood, 2018). While milk proteins, such as whey which is hydrolyzed with digestive enzymes, could generate peptides possessing the ability to antidiabetic (Bunsroem, Prinyawiwatkul, & Thaiudom, 2022; Lacroix & Li-Chan, 2012).

α-lactalbumin (α-LA) is a composition of milk proteins found in whey and contains many bioactive peptides (Nongonierma & FitzGerald, 2016). α-LA consists of 123 amino acid residues, forming a compact globular structure, stabilized by four disulfide bonds. α-LA is calcium metalloprotein, possessing an isoelectric point (pI) at approximately pH 4.4 - 4.8 and a molecular mass of 14,200 Da without free thiol groups (Ding, Yang, Zhao, Li, & Wang, 2011; Mao et al., 2017; Nicoleta & Rapeanu, 2010). Bioactive peptides derived from α-LA can play a role as antioxidant, antimicrobial, antihypertensive, and antidiabetic activities (Brandelli et al., 2015; Flaim,

Kob, Di Pierro, Herrmann, & Lucchin, 2017). α-LA hydrolysate exhibited antidiabetic properties better than whey protein isolate (WPI) and β-lactoglobulin hydrolysates. Regarding the degree of protein hydrolysis (DH) values of these protein hydrolysates, the DH of α-LA hydrolysate was the lowest compared to the other whey proteins (Bunsroem et al., 2022). However, dairy processing may change the α-LA structure and their functional properties.

Maillard reaction via glycation is one of the dairy processing used to modify the protein with various carbohydrates, such as sugars, polysaccharides, starch, and fiber. The formation of compounds between a carbonyl group of reducing carbohydrates and an amine group of free amino acids in proteins is the non-enzymatic browning reaction (Ajandouz, Tchiakpe, Ore, Benajiba, & Puigserver, 2001; Lertittikul, Benjakul, & Tanaka, 2007). The complexes between WPI and chitosan conjugated via a Maillard reaction, provided the lowest hydrolyzed peptide bonds (%) than the complexes without conjugation under an *in vitro* digestion (Zelikina et al., 2024). The conjugated product from such reaction between α-LA and 2'-fucosyllactose could significantly increase the antioxidant activity more than using only α-LA (Tu, Xu, Ren, & Zhang, 2020). Meanwhile, the Maillard conjugation between α-LA and galacto-oligosaccharides significantly increased the surface hydrophobicity and antioxidant capacity of conjugated product better than using only α-LA (Joubran, Moscovici, Portmann, & Lesmes, 2017). However, studies on changes in the antidiabetic properties of α-LA after conjugation with carbohydrates still needs further study.

β-glucan has useful effects for preventing and healing diabetes. It is an important compound contributing to glycemic control (Andrade et al., 2014; Bozbulut & Sanlier, 2019; Maheshwari, Sowrirajan, & Joseph, 2019). Moreover, the research of Sun et al. (2019) showed that the β-glucan/dipeptiven conjugate has the better fat binding capacity compared to the native β-glucan. The short linear glucan conjugated with lysine was a significant improvement for antioxidant activity (Lin et al., 2019). However, the characteristics and antidiabetic activity of Maillard reaction products (MRPs), formed by α-LA and β-glucan, remain unrevealed.

In addition, the appropriate conditions, such as the ratio between α-LA and β-glucan, pH value, and heat treatment, are the important factors in conjugation process. Heating at 75 °C for 30 min gave the best antidiabetic properties of the α-LA, compared

to that at 65 and 85 °C (Bunsroem et al., 2022). Regarding the pH values of α-LA, heating from 70 °C for 30 min resulted in the unfolding of α-LA at pH 6.7 but not at pH 5.8, suggesting that the denatured structure of α-LA increased with the decrease of pH from 6.7 to 5.8 due to the closeness of the dispersion pH to pI of the α-LA (Picone, Takeuchi, & Cunha, 2011). On the other hand, the effect of pH on MRPs from amino acid conjugated with fructose or glucose has been studied at approximately pH 4-12 (Ajandouz et al., 2001). The WPI/dextran conjugate had better heat stability than WPI when heated at 80 °C for 30 min and was stable over the pH range from 3.2 to 7.5 (Zhu, Damodaran, & Lucey, 2010). Rich and Foegeding (2000) reported that the concentrations of lactose and ribose in a range of 13.5 - 15% (*w/v*) could stabilize WPI solutions by conjugation at pH 6 to 9. However, adding sucrose at low concentration (0 to 10% *w/w*) to WPI solutions (10% *w/w*, pH 7, heated at 75 °C for 15 min) decreased the rate of whey protein aggregation and gelation (Wijayanti, Bansal, & Deeth, 2014). Importantly, the direct effects of pH and ratio of α-LA conjugated with β-glucan on their antidiabetic properties after the *in vitro* digestion are still unclear and need further investigation.

DPP-IV inhibitory activity and GLP-1 secretion values always have been used to be the index of antidiabetic properties for such product (Borah, Ahmed, & Borah, 2022; Komatsu et al., 2019; Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013). Salivary glands have been used to study the release of GLP-1 which is a hormone that stimulates insulin secretion (Ono, Watari, Kubono-Mizumachi, & Ono, 2015; Rowzee, Cawley, Chiorini, & Di Pasquale, 2011; Voutetakis et al., 2010). Therefore, the objective of this study was to determine the pH and ratio of α-LA to β-glucan in the occurrence of conjugation that provided the best antidiabetic properties, determined from GLP-1 secretion of human salivary gland (HSG) cells and DPP-IV inhibitory activity values. In addition, the physical and chemical properties of conjugated product were determined.

3.3 Materials and methods

3.3.1 Materials

The chemicals used in this study are divided into two groups: food and analytical grade.

3.3.1.1 The food-grade chemicals

α-LA was purchased from Sigma-Aldrich (Saint Louis, MO, USA). The protein content in α-LA was 96.91 ± 0.87% (dry basis), as determined by the Macro–Kjeldahl method (AOAC, 2012) using a nitrogen conversion factor of 6.38. Oat β-glucan was bought from L'eternel World (Aurora, OH, USA). The β-glucan content of material was determined using a β-glucan assay kit (Megazyme, Bray, Co. Wicklow, Ireland) according to the manufacturer's instruction. Oat β-glucan contained 72.90 ± 0.64 % of β-glucan. Sodium bicarbonate was purchased from R&B Food Supply Public Company Limited (Wangnoi, Ayutthaya, Thailand). Citric acid was supplied by Thai Citric Acid (Bangkhunthian, Bangkok, Thailand).

3.3.1.2 The analytical grade chemicals

Hydrochloric acid, sodium hydroxide, sodium sulfite, sodium dodecyl sulfate, and sodium phosphate buffer were bought from Carlo Erba Reagents (Val de Reuil, Normandie, France). L-Leucine, diprotin A, Gly-Pro *p*-nitroanilide hydrochloride, and calcofluor were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Rhodamine B was supplied by Acros Organics (Geel, Antwerpen, Belgium). 2, 4, 6-trinitrobenzenesufonic acid was purchased from G-Biosciences (Saint Louis, MO, USA). Dulbecco's modified eagle medium (DMEM) was bought from GE Healthcare Life Sciences (South Logan, UT, USA). Fetal bovine serum was purchased from GE Healthcare Bio-Sciences Austria GmbH (Krempl strasse, Pasching, Austria). Penicillin/ streptomycin solution was supplied by Capricorn Scientific GmbH (Auf der Lette, Ebsdorfergrund, Germany). Dimethyl sulfoxide was bought from Ameresco Inc. (Framingham, MA, USA). The enzymes used in this study were of analytical grade and obtained from Sigma-Aldrich (Saint Louis, MO, USA). Cell lines of human salivary gland (HSG) were provided by the laboratory of Cell-Based Assays and Innovations (Suranaree University of Technology, Nakhon Ratchasima, Thailand).

3.3.2 Solution preparation of α-lactalbumin conjugated with β-glucan

The solution was prepared by mixing powdered α-LA with powdered β-glucan. The concentrations of β-glucan were varied to 1, 3, and 5% (*w/v*) while the concentration of α-LA was fixed at 5% (*w/v*) of mixture. The negative controls for this part of study were the mix ratios of α-LA to β-glucan at 5:0 and 0:10% *w/v*. The ratios of α-LA to β-glucan to be studied are 5:1, 5:3, and 5:5% *w/v*, with the highest total

percentage being 10 %, so 0:10% *w/v* was used as a negative control while at the ratio of 5:0 % *w/v* was the negative control without conjugation. Each mixture was dissolved in distilled water. After that, pH values of mixture were adjusted to different pH at 3, 5 and 7 with citric acid or sodium bicarbonate. Then, the mixture was heated at 75°C for 30 min with constant stirring and rapidly cooled to 4°C. After that, the solution was stored for no more than 5 days at 4°C prior to determination of the chemical, physical, and antidiabetic properties. The experimental design for this study was a 5 × 3 full factorials in Completely Randomized Design.

3.3.3 Evaluation of α-lactalbumin conjugated with β-glucan

3.3.3.1 Microstructure study

A confocal laser scanning microscope (CLSM; Nikon A1R, Nikon, Minata-ku, Tokyo, Japan) was used to observe the microstructure of the solution. Calcofluor at 0.01% (*w/v*) was used for β-glucan staining, and the stained β-glucan was photographed by CLSM with a UV stimulus filter λ at 343 and an emission filter λ at 420 nm. Rhodamine B (0.02% *w/v*) was used to stain α-LA with the excitation filter λ at 543 nm and emission filter λ at 580 nm (Sharafbafi, Tosh, Alexander, & Corredig, 2014). To achieve optimal dying, the samples were placed on a slide glass and carefully distributed with a spatula. Then, the cover glass was used to cover the slide and the samples were visualized under the CLSM, with a 40× objective lens. At least ten photographs were taken of each sample, using various sections.

3.3.3.2 Determination of chemical properties

1) Browning index (BI)

The BI was considered as a major indicator to evaluate the extent of Maillard reaction (Zhong et al., 2019a). The BI was determined according to Ajandouz et al. (2001) and Lertittikul et al. (2007) with the slight modifications. Before the measurement, the sample was diluted to 3-fold concentration with distilled water and filtered with a Whatman filter paper No. 1 (GE Healthcare, Amersham, Buckinghamshire, UK). The BI of the sample was measured at 420 nm using a spectrophotometer (Genesys 10 UV, Thermo Electron, Madison, WI, USA).

2) Degree of protein hydrolysis (DH)

The sample was digested in an *in vitro* gastrointestinal system. This was carried out as described by Nongonierma, Lalmahomed, Paolella, and

FitzGerald (2017). The hydrolysate was tested for DH values by 2, 4, 6-trinitrobenzenesufonic acid (TNBS) colorimetry for α-amino nitrogen as described by Yi, Lin, and Johns (2021) and Gruppi, Dermiki, Spigno, and FitzGerald (2022) with a slight modification. Briefly, samples (hydrolyzed and unhydrolyzed control samples) were diluted in 1% (*w/v*) sodium dodecyl sulfate to a final protein concentration as protein equivalent of 10 mg/mL and incubated at 50°C for 60 min in an incubator (JP Selecta, Abrera, Barcelona, Spain). Then, 10 µL of both samples and 10 µL of leucine standards at concentrations which ranged from 0 - 5 mg/mL were loaded into a 96-well plate with 80 µL of sodium phosphate buffer (0.2 M, pH 8), followed by 80 µL of 0.025% (*w/v*) TNBS. The sample plate was incubated at 45°C for 30 min in a microplate reader (Thermo Fisher Scientific, Vantaa, Southern Finland, Finland), and the absorbance at 420 nm was monitored every 2 min until 30 min. The DH values were calculated using the equation as follows:

$$DH\ (\%) = (A - B/T) \times 100.$$

where *A* is the reactive α-amino nitrogen concentration determined by TNBS colorimetry, *B* is the reactive α-amino nitrogen concentration of the intact protein substrate, and *T* is the total reactive α-amino nitrogen concentration of the intact protein substrate. *B/T* value of α-LA was 0.92/9.38 (Yi et al., 2021).

3.3.3.3 Determination of antidiabetic properties

1) Dipeptidyl Peptidase-IV (DPP-IV) inhibition

The existence of GLP-1, which was mainly metabolized by DPP-IV, was indirectly determined as the DPP-IV inhibition value (Mousa & Ayoub, 2019). The half-maximal inhibitory concentration (IC_{50}) value of DPP-IV was used to measure the DPP-IV inhibitory activity of hydrolysate. The hydrolysate was varied in the range of 0.025 to 50 mg/mL. Diprotin A (Ile-Pro-Ile) was used as a positive control at the concentration range between 1.56 and 62.50 µg/mL. The hydrolysate (25 µL) was mixed with 50 µL of 1 mM reaction substrate (Gly-Pro *p*-nitroanilide hydrochloride) in a 96-well clear microplate. Then, 25 µL of 0.2 units/mL DPP-IV was subsequently added. The microplate was incubated at 37°C, and the absorbance of the *p*-nitroanilide, releasing at 405 nm, was monitored every 5 min for 30 min in a microplate

reader (Nongonierma et al., 2017). The results were compared with that of the control (no protein hydrolysate added) (Chakrabarti et al., 2011). The IC_{50} values were determined by plotting the percentage of inhibition and the concentration of protein hydrolysate (Nongonierma et al., 2017).

2) Glucagon-like Peptide-1 (GLP-1) secretion

In order to examine the GLP-1 quantification, cell lines of HSG were used. Cells were grown at 37°C, with 95% air and 5% CO_2 in a humidified atmosphere incubator (Thermo Fisher Scientific, Waltham, MA, USA) and were cultured in DMEM containing 4.0 mM L-glutamine and glucose 4.5 g/L, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Jeffers, Madden, & Webster-Cyriaque, 2009; Shao, Furusawa, Aoki, Matsumoto, & Ando, 2002). HSG cells were maintained at 6×10^4 cells/well in 24 well culture plates (well growth area of ca. 1.86 cm^2) and incubated at the same condition as mentioned above for 24 h. Then, the DMEM was replaced with 600 µL of fresh DMEM containing the hydrolysate at concentration levels, providing the maximum cell viability (Arteaga-Cardona et al., 2016). Untreated cells were used as a control. After further incubation for 24 h in a humidified atmosphere incubator, the supernatants were centrifuged at 664× g for 3 min at 25°C to remove the remaining cells by refrigerated centrifuge (Hermle Labortechnik GmbH, Wehingen, Germany). GLP-1 release levels were determined using a human GLP-1(7-36) simple step ELISA kit (Abcam, Discovery Drive, Cambridge, UK), following the manufacturer's instruction (Komatsu et al., 2019). The standard curve of Human GLP-1 was constructed by plotting the average of the absorption values for each human GLP-1 standard on the vertical axis (Y) versus the concentration of the human GLP-1 standard on the horizontal axis (X) and drawing the optimal curve through the points on the graph. The standard curve obtained from this current study are shown in Figure 3.1. GLP-1 release value (pg/mL) of the hydrolysate was calculated using the second order polynomial equation.

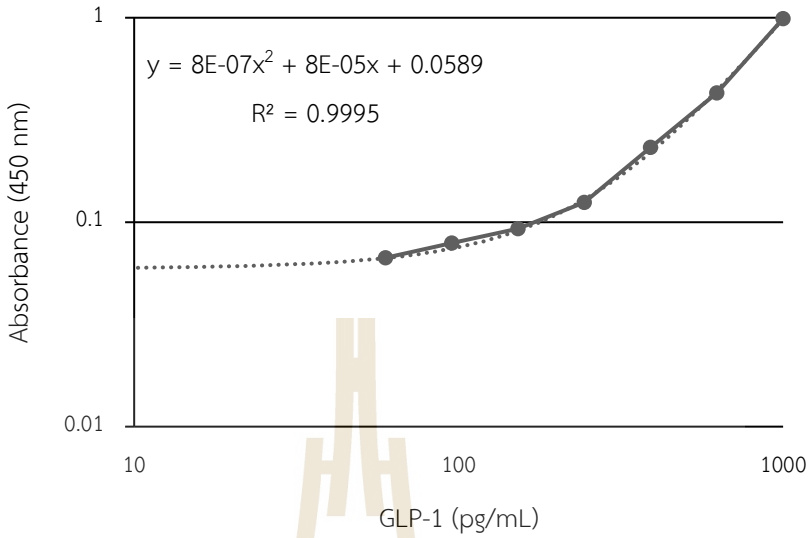

Figure 3.1 Human Glucagon-like peptide-1 (GLP-1) ELISA standard curve.

3.3.4 Statistical analysis

All experiments and measurements were performed at least in triplicate. All results were expressed as the mean ± standard deviation. A two-way analysis of variance was used to determine the difference between mean values with a significance at $p < 0.05$, which was followed by Duncan's Multiple Range Test for multiple means comparison. All statistical analyses were determined using the statistical package for the social sciences program (version 17.0, SPSS Inc., Chicago, IL, USA).

3.4 Results and discussion

3.4.1 Microstructure

Representative CLSM images showing the microstructure of the solution from α-LA conjugated with β-glucan at different pH (pH 3, 5, and 7) and ratios of α-LA to β-glucan (5:0, 5:1, 5:3, 5:5, and 0:10% *w/v*) are shown in Figure 3.2. The structure of α-LA was in a red color and exhibited a classic molten globule (Figure 3.2A.1, B.1, and C.1). However, the structures of α-LA were aggregated (Figure 3.2B) at pH 5 close to its isoelectric point (pI ~ 4.4 to 4.8) due to the denaturation of protein (Ding et al., 2011; Mao et al., 2017). β-glucan was in a blue color with homogenous distribution of its particles (Figure 3.2A.5, B.5, and C.5). However, in this study, the amorphous agglomeration of β-glucan was formed when the pH was decreased. de

Souza et al. (2015) also found that an increase in acetic anhydride concentration from 4 to 6% in acetylation of β-glucan resulted in much smoother surface and denser structure of β-glucan. When α-LA was mixed with β-glucan, some β-glucan particles could absorb onto the structure of α-LA which was observed by a purple color created from the mix of red and blue colors (Figure 3.2B.2). The overlapping of red and blue color was presented in Figure 3.2A.2-4, B.2-4, and C.2-4, confirming the interaction between α-LA and β-glucan (Thaiudom & Pracham, 2018). In fact, β-glucan contains functional aldehyde groups, which allows for conjugation through Maillard reaction to protein molecules (Kaur, Sharma, Ji, Xu, & Agyei, 2020). Therefore, the amount of overlapping of red and blue color was increased when the concentration of β-glucan increased. However, at pH 3, the structure of α-LA conjugated with β-glucan was smaller and finer than that at pH 5 and 7. This result was consistent with the findings of Zhong et al. (2019b) who found that when pH was below 5, oat protein isolate/*Pleurotus ostreatus* β-glucan conjugate stabilized the solution by an increased magnitude of electric charge from determination of the ζ-potential of emulsion droplets. This was because the deprotonated carboxylic acid (COO^-) groups on *Pleurotus ostreatus* β-glucan could counteract the amount of hydrogen ion when pH was decreased.

3.4.2 Chemical properties

3.4.2.1 Browning index (BI)

Browning intensity at the final stages in the non-enzymatic browning reaction of α-LA conjugated with β-glucan at different pH (pH 3, 5, and 7) and ratios of α-LA to β-glucan (5:0, 5:1, 5:3, 5:5, and 0:10% *w/v*) after heating were studied by measuring the absorbance at 420 nm. The most of UV-absorbance curves suggested that the brown color was developed at increasing β-glucan and pH values are shown in Figure 3.3. At pH 3, no decrease in the absorbance at 420 nm of the solution was observed when the concentration in terms of percentage of β-glucan was increased. Generally, an increase in pH of the solution influences in an increase rate of the Maillard reaction (Ajandouz et al., 2001; Lertittikul et al., 2007; Zhong et al., 2019a). The lower BI of conjugates seemed to correlate with a lower pH (pH 3), which could give a finer and smaller structure of α-LA conjugated with β-glucan (Figure 3.2A.2-4). However, the increased ratio of α-LA to β-glucan from 5:3 to 5:5% (*w/v*) at

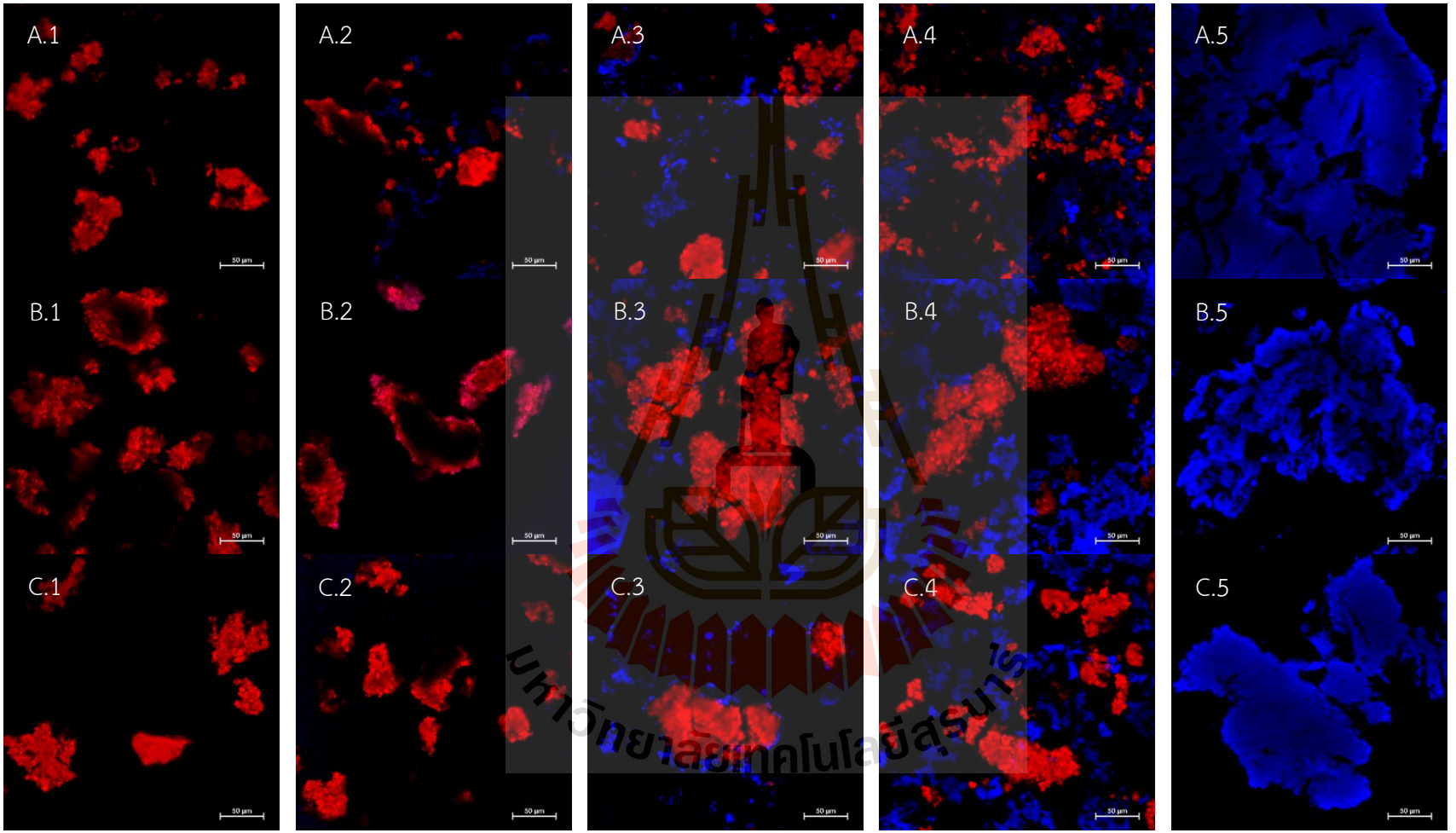

Figure 3.2 Confocal images of **α**-LA conjugated with **β**-glucan. A, B, and C denote micrographs of mixtures at pH 3, 5, and 7, respectively. The number indicates the ratios of **α**-LA to **β**-glucan (1 = 5:0; 2 = 5:1; 3 = 5:3; 4 = 5:5 and 5 = 0:10% *w/v*). Scale bar is 50 μm.

pH 5 and 7 could not cause an improved production of such conjugates because the steric hindrance of β-glucan limiting the extent of Maillard reaction (Oliver, Melton, & Stanley, 2006).

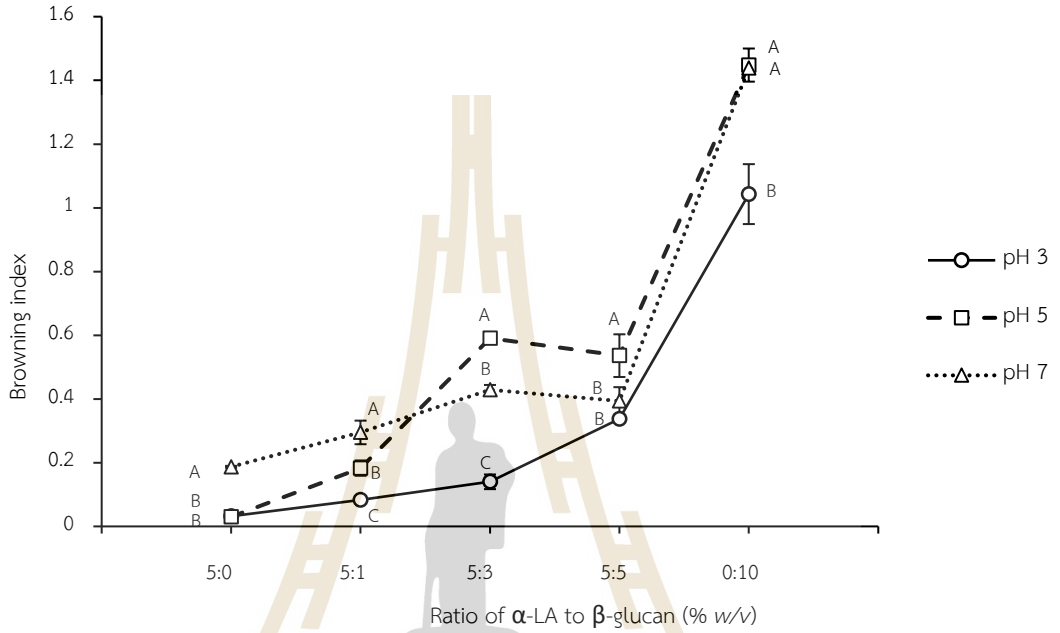

Figure 3.3 Browning index of solutions from α-lactalbumin (α-LA) conjugated with β-glucan at different pH and ratios of α-LA to β-glucan after heating at 75°C for 30 min. Bars indicate the standard deviation (n = 4). The different letters ^{A,B,C} in the same ratio indicate a significant difference at $p < 0.05$.

3.4.2.2 Degree of protein hydrolysis (DH)

The DH values of hydrolysates from α-LA conjugated with β-glucan at different pH (pH 3, 5, and 7) and ratios of α-LA to β-glucan (5:0, 5:1, 5:3, 5:5, and 0:10% *w/v*) were compared. The results are shown in Table 3.1. At all pH values, α-LA hydrolysate provided the highest DH (%) compared to all hydrolysates of α-LA/β-glucan conjugates at each pH ($p < 0.05$). This might be because the native α-LA structure possesses more accessible sites for the digestive enzyme than the α-LA/β-glucan conjugates, could be easier hydrolyzed by enzymes than the conjugates. Zhong et al. (2019a) explained that the oat protein isolate/*Pleurotus ostreatus* β-

glucan conjugate had higher random coil and lower α-helix than oat protein isolate. Moreover, the addition of a carbohydrate to a α-LA solution increased the density of the conjugate solution (Velusamy & Palaniappan, 2016). However, the percentages of DH of hydrolysates from α-LA/β-glucan conjugates at the ratios of 5:1 and 5:3% (*w/v*) were not significantly different ($p > 0.05$). The hydrolysate of conjugate was formed at the ratio of 5:5% *w/v* (α-LA to β-glucan) showed the lowest DH (%), compared to that at the ratios of 5:0, 5:1, and 5:3% *w/v* ($p < 0.05$). Even though the ratio of α-LA to β-glucan from 5:3 to 5:5% (*w/v*) at pH 5 and 7 were not significantly different in the BI of conjugates (Figure 3.3). This might be due to the steric hindrance of β-glucan which limited the susceptibility to digestive enzymes (Oliver et al., 2006).

Among the three pH values, the hydrolysate from α-LA solution at pH 5 seemed to provide the least DH value, compared to that at pH 3 and 7 ($p < 0.05$). This may be due to the effect of pI value of α-LA which is at approximately pH 4.4 - 4.8 (Ding et al., 2011; Mao et al., 2017). The hydrolysis of α-LA was difficult to perform, as the compact globular structure was relatively resistant toward enzymatic proteolysis (Nicoleta & Rapeanu, 2010). Therefore, the conjugates at pH 5 also resulted in the lowest DH (%) of their hydrolysates compared to the rest ($p < 0.05$). While conjugation at pH 3 for all hydrolysates could provide higher percentages of DH than those at pH 7 ($p < 0.05$). This might be due to an increased magnitude of electric charge at pH 3 representing increase in the α-LA surface by unfolding of α-LA structure in solution (Litwińczuk et al., 2014), resulting in easier hydrolysis of α-LA. In addition, the lower BI of conjugates was observed at pH 3, compared to those at pH 5 and 7 (Figure 3.3), which was relevant to the lower Maillard reaction of α-LA and β-glucan at pH 3, compared to the rest of pH.

Table 3.1 Degree of protein hydrolysis (DH, %) of hydrolysates from **α**-lactalbumin (**α**-LA) conjugated with **β**-glucan at different pH and ratios of **α**-LA to **β**-glucan after heating at 75°C for 30 min.

pH	Ratio of **α**-LA to **β**-glucan				
	5:0	**5:1**	**5:3**	**5:5**	**0:10**
3	20.17 ± 1.15 ^{Ba}	14.81 ± 1.40 ^{Ab}	13.87 ± 2.35 ^{Ab}	8.92 ± 0.48 ^{Ac}	ND
5	13.95 ± 0.59 ^{Ca}	10.28 ± 1.84 ^{Cb}	10.22 ± 1.37 ^{Cb}	7.81 ± 2.15 ^{Cc}	ND
7	24.49 ± 2.38 ^{Aa}	10.82 ± 0.55 ^{Bb}	11.20 ± 0.92 ^{Bb}	8.21 ± 2.51 ^{Bc}	ND

Note: Data are presented as mean ± standard deviation (n = 4). The different letters ^{A,B,C} in the same column indicate a significant difference at $p < 0.05$. The different letters ^{a,b,c} in the same row indicate a significant difference at $p < 0.05$. ND denote the DH values were not determined as the hydrolysates had no or low DH values.

3.4.3 Antidiabetic properties

3.4.3.1 Dipeptidyl Peptidase-IV (DPP-IV) inhibition

The DPP-IV IC_{50} value of hydrolysates from α-LA conjugated with β-glucan at different pH (pH 3, 5, and 7) and ratios of α-LA to β-glucan (5:0, 5:1, 5:3, 5:5, and 0:10% *w/v*) was determined, and the results are shown in Table 3.2. Diprotin A was used as a reference for DPP-IV inhibitory activity, and its DPP-IV IC50 value was 0.0168 ± 0.0017 mg/mL in this current study. The highest DPP-IV inhibitory activity was observed in the hydrolysates from conjugated products of ratios between α-LA to β-glucan at 5:1 and 5:3% (*w/v*), compared to those at the ratios of 5:0, 5:5, and 0:10% *w/v* ($p < 0.05$). This might be due to the moderate DH of the hydrolysates from α-LA/β-glucan conjugates at the ratios of 5:1 and 5:3% *w/v* (α-LA to β-glucan), compared to those at the other ratios (Table 3.1). The moderate DH value seemed to correlate with a moderate amino acid content but not with a higher amount of short-chain peptides, compared to the higher DH value (Farup et al., 2016). While the lower DH value was consistent with a lower amino acid content and a higher number of long-chain peptides. This was relevant to the α-LA/β-glucan conjugates at the ratios of 5:1 and 5:3% (*w/v*) at pH 3 attributed to the moderate Maillard reaction between α-LA and β-glucan.

The conjugated condition at pH 3 and 7 resulted in greater inhibition of the activity of DPP-IV than at pH 5 ($p < 0.05$). This was because the conjugates at pH 3 and 7 gave higher DH (%) of their hydrolysates compared to those of the conjugates at pH 5 ($p < 0.05$). The shorter amino acid residues of peptides contributed to their best DPP-IV inhibitory activity. Silveira et al. (2013) and Nongonierma et al. (2019) revealed that the best DPP-IV inhibitor was peptides with a length of 3-6 amino acids. Moreover, tri-peptides IPI (diprotin A) and VPL (diprotin B) have always been used as the precursor and standard of the DPP-IV inhibition (Umezawa et al., 1984).

3.4.3.2 Glucagon-like Peptide-1 (GLP-1) secretion

HSG cells were used as an in vitro model of GLP-1 release. This cell line has been widely used to elucidate the mechanism of GLP-1 release (Baum et al., 2010; Rowzee et al., 2011; Samuni & Baum, 2011). However, food digestion is known

Table 3.2 The Dipeptidyl peptidase-IV half-maximal inhibitory concentration (DPP-IV IC_{50}) values (mg/mL) of hydrolysates from **α**-lactalbumin (**α**-LA) conjugated with **β**-glucan at different pH and ratios of **α**-LA to **β**-glucan after heating at 75°C for 30 min.

pH	Ratio of α-LA to β-glucan				
	5:0	**5:1**	**5:3**	**5:5**	**0:10**
3	22.69 ± 3.20 ^{Ac}	3.21± 0.47 ^{Aa}	3.83 ± 1.12 ^{Aa}	9.55 ± 0.24 ^{Ab}	ND
5	26.01 ± 3.76 ^{Bc}	5.36 ± 0.29 ^{Ba}	7.48 ± 1.07 ^{Ba}	16.11 ± 3.32 ^{Bb}	ND
7	25.03 ± 2.54 ^{Ac}	0.72 ± 0.21 ^{Aa}	2.26 ± 0.09 ^{Aa}	7.63 ± 0.25 ^{Ab}	ND

Note: Data are presented as mean ± standard deviation (n = 3). The different letters ^{A,B} in the same column indicate a significant difference at $p < 0.05$. The different letters ^{a,b,c} in the same row indicate a significant difference at $p < 0.05$. ND denote IC_{50} values were not determined as the hydrolysates had no or low inhibitory activity.

to be cytotoxic to cells, which depends on the type, concentration, processing, and quantity of food, etc. (Chevalier, Chobert, Genot, & Haertlé, 2001; Diao et al., 2021). In the present study, the best concentration of each hydrolysate at different pH and ratios of α-LA to β-glucan providing the highest cell viability (Table 1A in Appendix A.) was used to determine the GLP-1 release. The results are shown in Table 3.3. All hydrolysates significantly increased the release of GLP-1 compared to the control, which was 16.92 ± 6.60 pg/mL in this current study. Table 3.3 shows that the hydrolysate at different pH and ratios of α-LA to β-glucan significantly affected the release of GLP-1. The ratios of α-LA to β-glucan from 5:1 and 5:3% (*w/v*) at pH 3 and from 5:5% (*w/v*) at pH 5 provided the highest GLP-1 release. The effect of protein hydrolysates was believed to delay the inactivation of GLP-1 release induced by DPP-IV. An increase in GLP-1 released from HSG cells was found in the hydrolysates from α-LA/β-glucan conjugates at the ratios of 5:1 and 5:3% (*w/v*) at pH 3 (Table 3.3). This might be because the hydrolysates from that α-LA/β-glucan conjugates at those conditions could inhibit the activity of DPP-IV. Regarding the DH values of these hydrolysates, the hydrolysates from α-LA/β-glucan conjugates at the ratios of 5:1 and 5:3% (*w/v*) at pH 3 showed higher DH than that of the other conjugates (Table 3.1). However, the DH of α-LA without the conjugation with β-glucan showed higher DH at pH 3 and 7.

Even though the hydrolysate from α-LA/β-glucan conjugate at the ratio of 5:5% (*w/v*) at pH 5 provided the DH value less than that from the other ratios and pHs (Table 3.1), this hydrolysate could induce HSG cells to release the highest GLP-1. This result might be due to the increase in percentage of β-glucan. Wongkrasant et al. (2020) explained that the higher GLP-1 levels in supernatant of the intestinal secretin tumor cell line were induced by high glucose media, compared to that induced by low glucose media. Moreover, the hydrolysate from α-LA solution influenced the increase in GLP-1 release as well. Therefore, the hydrolysate from α-LA/β-glucan conjugate at a ratio of 5:5% (*w/v*) significantly increased GLP-1 release level more than the hydrolysate from β-glucan solution at concentration 10% (*w/v*) when adjusting pH at 5.

The result of the effect of the pH values showed that the hydrolysate from α-LA solution at pH 5 could induce GLP-1 release less than that of

hydrolysate at pH 3 and 7. This was because the hydrolysate from α-LA solution at pH 5 provided the least DH value, compared to that at pH 3 and 7 ($p < 0.05$). When α-LA conjugated with β-glucan at the ratio of 5:5% *w/v* (α-LA to β-glucan) and pH 5, the release of GLP-1 induced by its hydrolysate was the highest compared to that of the conjugate at pH 3 and 7 at the same ratio of α-LA to β-glucan. This believed that the HSG cells could readily hydrolyze nutrients at pH 5, known as the lysosomal pH (pH 4.5 - 5) which is the optimal pH for maintaining cellular homeostasis of lysosomes. Lysosome is an organelle in animal cell containing numerous hydrolytic enzymes, degrading biological polymers such as proteins and polysaccharides (Zeng, Shirihai, & Grinstaff, 2020). Therefore, the hydrolysis at pH 5 presumably resulted in an increase in the GLP-1 level of its hydrolysate. This was in line with the previous findings that hadrazone/β-glucan conjugates were readily hydrolyzed in the environment of the lysosomes at pH 5 (Tuse, Mohagheghpour, Dawson, Hobbs, & Winant, 1996).

3.4.4 Appearance property

In order to select the best conjugated condition from the ratios of α-LA to β-glucan between 5:1 and 5:3% (*w/v*), the appearance of those conjugated products was observed. As shown in Figure 3.4, the conjugate ratio of α-LA to β-glucan at 5:1% (*w/v*) showed phase separation while the ratio of α-LA to β-glucan at 5:3% (*w/v*) exhibited homogeneous appearance. This may be because more β-glucan added into the conjugated samples could help overall viscosity of those samples increase, consequently in more homogeneity in those samples.

Table 3.3 Glucagon-like peptide-1 (GLP-1) release values (pg/mL) of hydrolysates from α-lactalbumin (α-LA) conjugated with β-glucan at different pH and ratios of α-LA to β-glucan after heating at 75°C for 30 min.

pH	Ratio of α-LA to β-glucan				
	5: 0	**5: 1**	**5: 3**	**5: 5**	**0: 10**
3	69.71 ± 8.46 ^{NSab}	71.10± 7.97 ^{Aab}	77.71 ± 9.80 ^{Aa}	16.92 ± 6.60 ^{Cc}	50.49 ± 6.22 ^{Bb}
5	67.67 ± 7.51 ^{NSb}	70.18 ± 8.77 ^{Ab}	31.44 ± 7.13 ^{Cc}	87.31 ± 6.44 ^{Aa}	62.22 ± 7.88 ^{ABb}
7	72.70 ± 10.20 ^{NSa}	46.99 ± 3.67 ^{Bc}	58.56 ± 3.30 ^{Bb}	67.67 ± 7.51 ^{Ba}	72.91 ± 5.09 ^{Aa}

Note: Data are presented as mean ± standard deviation (n = 4). Untreated cells were used as the control, and GLP-1 release value of the control was 16.92 ± 6.60 pg/mL. The letter ^{NS} in the same column indicates no significant difference at $p \geq 0.05$. The different letters ^{A,B,C} in the same column indicate a significant difference at $p < 0.05$. The different letters ^{a,b,c} in the same row indicate a significant difference at $p < 0.05$

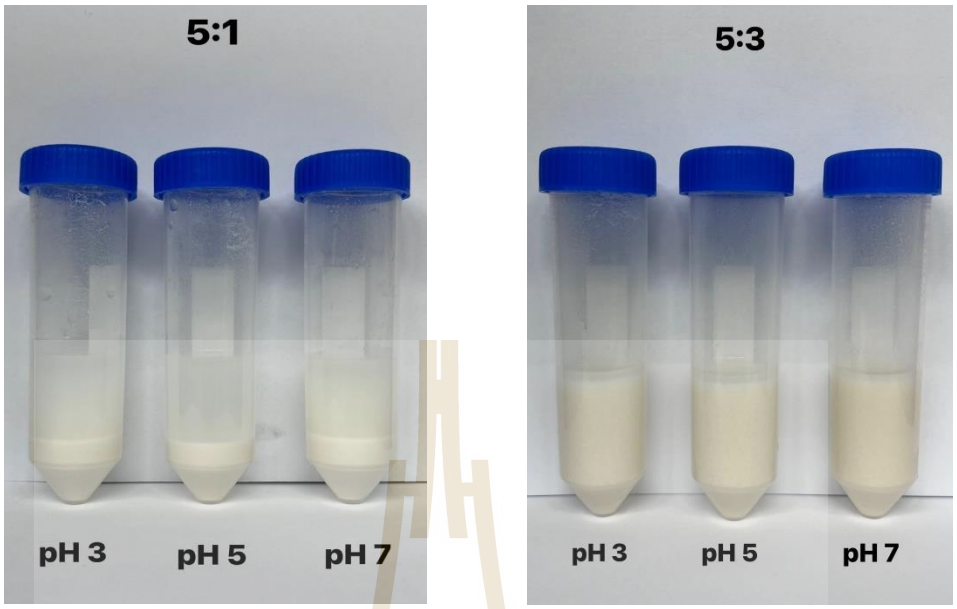

Figure 3.4 The appearance of solutions from α-lactalbumin (α-LA) conjugated with β-glucan at different pH after heating at 75°C for 30 min: Comparisons conjugated products of ratios between α-LA to β-glucan at 5:1 and 5:3% (*w/v*).

3.5 Conclusions

This study revealed that the microstructure of α-LA/β-glucan conjugate at pH 3 was smaller than that at pH 5 and 7. The result was consistent with the BI value. The conjugates at pH 3 provided the least BI value than the conjugates at pH 5 and 7. Also, the conjugates at pH 3 exhibited the highest DH (%) compared to that of the conjugates at pH 5 and 7. In addition, the hydrolysates from conjugated product at ratios of α-LA to β-glucan of 5:1, and 5:3% (*w/v*) at pH 3 provided GLP-1 release and DPP-IV inhibition better than those at the ratios of 5:0, 5:5, and 0:10% (*w/v*) at the same pH. The appearance of the conjugate between α-LA and β-glucan at a ratio of 5:3% (*w/v*) showed greater homogenous than that of 5:1% (*w/v*). This obtained information can be used as a guideline for producing a potential antidiabetic food supplement such as, sol, jelly or paste in the future. However, the jelly or paste containing α-LA/β-glucan conjugate at the ratio of 5:3% (*w/v*) at pH 3 should be further experimented for a possibility in terms of its production, physicochemical properties, and acceptance from the target consumers.

3.6 References

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CHAPTER IV
THE PRODUCTION OF THE JELLY-PASTE FROM α-LACTALBUMIN/β-GLUCAN CONJUGATE

4.1 Abstract

Jelly-paste is a novel food product combining jelly and paste characteristics that contents high amount of calorie sweeteners. To reduce the risk of being diabetes by consuming such product, sucralose can be used to replace calorie sweeteners. Regarding diabetes, a prior study discovered that the hydrolysate from the compound of α-lactalbumin (α-LA) and β-glucan exhibited antidiabetic properties. However, the physicochemical characteristics and sensorial acceptance of this jelly-paste might impact by such ingredients. Thus, this study evaluated the antidiabetic and physicochemical properties of jelly-paste containing the α-LA/β-glucan conjugate at the ratio of 5:3% (*w/w*) at pH 3 and using sucralose as sweetener. The sensorial evaluation of such jelly-paste was also determined by the targeted consumers who are risk to the diabetes and diabetic patients (n = 81). The results of this study showed that the degree of protein hydrolysis of hydrolysate from the jelly-paste containing α-LA/β-glucan conjugate was lower than that of the commercial jelly-paste ($p < 0.05$), resulting in higher GLP-1 release than that of the commercial jelly-paste. The overall acceptance of this jelly-paste, evaluated by those consumers was ranged from liking slightly to liking moderately (6.02 ± 1.71 to 6.88 ± 1.48 out of 9). The probability of purchasing this jelly-paste was increased from 60 to 73 % after the explanation of health benefits that was provided to the consumers. This obtained information confirms that the jelly-paste containing the α-LA/β-glucan conjugate has the potential acceptance and could be the supplement food for diabetic patients in the future. However, the jelly-paste syneresis cannot be prevented by adding only α-LA/β-glucan conjugate. The effect of types and concentrations of stabilizers on physicochemical properties, e.g., syneresis, texture, and rheology of such jelly-paste as well as antidiabetic activity of its hydrolysate should be further investigated.

Keywords: antidiabetic; α-lactalbumin; β-glucan; jelly; sensory

4.2 Introduction

Jelly is one of the oldest foods preservation processes. Fruits, sugar, pectin, and edible acids are the compositions of jelly. These ingredients are of paramount importance for the texture, structure and overall quality of jelly (Kamal, Song, Zhang, Zhu, & Tan, 2018) whereas paste, composing of starch and fat as general ingredients, is always viscous. Sweeteners and oil can be added to the paste for improving texture and mouthfeel (Luo, 2018). This study was focus on combining jelly and paste characteristics to produce novel food product called jelly-paste. The example of commercial jelly-paste such as GU Energy Gel (GU Energy Labs, Berkeley, CA, USA) composes of carbohydrates (maltodextrin and fructose) and amino acids (L-leucine, L-valine, and L-isoleucine) with natural flavor, citric acid, sodium citrate, potassium citrate, calcium carbonate, sea salt, green tea, malic acid, gellan gum, sunflower oil, sodium benzoate, and potassium sorbate (GU, 2020). GU Energy Gel is designed for the athletes requiring the endurance which can be used anytime for boosting energy (VitalThai, 2020). However, carbohydrates in GU might not be suitable for the consumers who are risk to the diabetes disease. Thus, replacing those carbohydrates with reduced calorie sweeteners such as aspartame, acesulfame K, saccharin, and sucralose (McClements, 2005) might reduce the risk of being diabetes. The continuous consume of sucralose could reduce acute insulin response and enhance glucagon-like peptide-1 (GLP-1) release in healthy consumers (Lertrit et al., 2018). However, replacement of calorie sugars by non-calorie sweeteners might interfere in flavor and texture of the jelly-paste. Sugar-free jellies had more pronounced syneresis over time, whereas jellies with calorie sugar such as sucrose presented a significant hardening during storage (Di Monaco, Miele, Cabisidan, & Cavella, 2018). However, the production of jelly-paste containing Maillard reaction products could solve such problems (Feng et al., 2023). The apparent viscosity of oat β-glucan increased largely after Maillard reaction (Sun et al., 2019), a heat-induced browning reaction between the amino groups and the reducing sugars (Lin et al., 2019). The highest apparent viscosity was

also observed in dipeptiven/β-glucan conjugate, compared to that of glutathione/β-glucan conjugate and L-glutamine/β-glucan conjugate (Sun et al., 2019).

Moreover, the conjugate of α-lactalbumin (α-LA) and β-glucan resulted in the highest inhibition of the activity of dipeptidyl peptidase-IV (DPP-IV), enzyme mainly metabolized hormone GLP-1, compared to that of α-LA without conjugation after an *in vitro* digestion. According to previous study, the conjugate from α-LA and β-glucan at a ratio of 5:3% (*w/v*) at pH 3 showed better antidiabetic properties and homogenous appearance than the conjugates at the ratios of 5:0, 5:1, 5:5, and 0:10% (*w/v*) at pH 3, 5 and 7. Thus, the jelly-paste containing that conjugate should be further experimented for a possibility in terms of its production. Sucralose was used as a sweetener in production as a replacement of calorie sugars for the consumers who are risk to the diabetes disease. However, such ingredients of the jelly-paste might affect the physicochemical properties and sensorial acceptance of consumers.

Sensory processing refers to the mechanism by which the central nervous system receives the inputs from the senses and integrates those inputs to generate an appropriate behavioral response (Jones, Hanley, & Riby, 2020). Sensory evaluation is a scientific method to measure, analyze, and interpret responses to products through sight, smell, touch, taste, and hearing. In an ever competitive marketplace, sensory evaluation methods are increasingly being used to help make vital decisions about food products such as product improvement, quality control, and new product development (Leatherhead food research, 2020).

Appearance of product is the first impression for a consumer. Appearance is one of the major attributes of quality involving size, shape, texture, gloss, color, and others (Chantrapornchai, 2018). The appearance, texture, and temperature of food affected the perception of the flavor and taste of food products in turn impacting food acceptability (Jiang, King, & Prinyawiwatkul, 2014). However, it remains questionable whether the concept of overall acceptability or hedonic liking is a sufficient benchmark for sale prediction or product success. Consideration of food with health claims could lead to rational thinking that may lead to the judgment for consumers' purchase (Jiang et al., 2014). O'Brien et al. (2017) concluded that the kefir beverage scored significantly higher for overall liking after the health benefits were explained (6.5 ± 1.8 and 7.0 ± 1.7 out of 9 before and after the explanation of health benefits, respectively).

Participants showed a high intent to purchase after they learned about the health benefits of the kefir beverage, compared to before they learned about that (75% of participants indicated an intent to purchase, and 89% after they learned about the health benefits). Therefore, the objective of this study was to determine the physicochemical, microbiological, and antidiabetic properties of jelly-paste containing the conjugate from α-LA and β-glucan at the ratio of 5:3% (*w/w*) at pH 3, adding sucralose as sweetener. In addition, the sensorial evaluation through the consumer test of such jelly-paste was determined.

4.3 Materials and methods

4.3.1 Materials

The chemicals used in this study are divided into two groups: food and analytical grade.

4.3.1.1 The food-grade chemicals

α-LA was purchased from Sigma-Aldrich (Saint Louis, MO, USA). The protein content in α-LA was 96.91 ± 0.87% (dry basis), as determined by the Macro–Kjeldahl method (AOAC, 2012) using a nitrogen conversion factor of 6.38. β-glucan was received from L'eternel World (Aurora, OH, USA). The β-glucan content of material was 70%, according to the manufacturer's information. Mandarin orange (*Citrus reticulata*) was bought from Ex-Chai Distribution System Company Limited (Muang Nakhon Ratchasima, Nakhon Ratchasima, Thailand). Sucralose was supplied by Krungthepchemi (Khan Na Yao, Bangkok, Thailand). Sunflower oil was purchased from Thai Vegetable Oil Public Company Limited (Nakhonchaisi, Nakhonpathom, Thailand). Orange flavor was bought from Sensient Technologies (Patumwan, Bangkok, Thailand). Citric acid was supplied by Thai Citric Acid (Bangkhunthian, Bangkok, Thailand).

4.3.1.2 The analytical grade chemicals

Hydrochloric acid, sodium hydroxide, sodium sulfite, sodium dodecyl sulfate, and sodium phosphate buffer were purchased from Carlo Erba Reagents (Val de Reuil, Normandie, France). L-Leucine, and calcofluor were bought from Sigma-Aldrich (Saint Louis, MO, USA). Rhodamine B was supplied by Acros Organics (Geel, Antwerpen, Belgium). 2, 4, 6-trinitrobenzenesufonic acid was purchased

from G-Biosciences (Saint Louis, MO, USA). Dulbecco's modified eagle medium (DMEM) was supplied by GE Healthcare Life Sciences (South Logan, UT, USA). Fetal bovine serum was bought from GE Healthcare Bio-Sciences Austria GmbH (Krempl strasse, Pasching, Austria). Penicillin/streptomycin solution was purchased from Capricorn Scientific GmbH (Auf der Lette, Ebsdorfergrund, Germany). Dimethyl sulfoxide was bought from Ameresco Inc. (Framingham, MA, USA). The enzymes used in this study were of analytical grade and received from Sigma-Aldrich (Saint Louis, MO, USA). Cell lines of human salivary gland (HSG) were provided by the laboratory of Cell-Based Assays and Innovations (Suranaree University of Technology, Nakhon Ratchasima, Thailand).

4.3.2 Preparation of jelly-paste from α-lactalbumin/β-glucan conjugate

The jelly-paste was prepared by mixing 5% (*w/w*) powdered **α**-LA with 3% (*w/w*) powdered **β**-glucan, 0.1% (*w/w*) sunflower oil, and 0.04% (*w/w*) sucralose. The mixture was dissolved in Mandarin orange juice. The flavor of mixture was adjusted with synthetic orange flavor. pH value of the mixture was adjusted to pH 3 with 25% (*w/v*) citric acid. After that, the mixture was homogenized using a second-stage APV Gaulin homogenizer (1500/500 bar; Model: 1515MR-8TA, SPX FLOW, Inc., NC, USA) and then heated at 75°C for 30 min with constant stirring. The mixture was aseptic-filled into previously sterilized package and rapidly cooled to 4°C. The prepared jelly-paste sample was then stored in hermitically sealed packaging at 4°C not longer than 5 days for analysis. The physical, chemical, microbiological, and sensorial properties of the sample and of commercial jelly-paste (GU Energy Gel that Mandarin orange flavored; GU Energy Labs, Berkeley, CA, USA), as a comparable sample, were determined.

4.3.3 Evaluation of the jelly-paste

4.3.3.1 Determination of physical properties

1) Microstructure study

A confocal laser scanning microscope (CLSM; Nikon A1R, Nikon, Minata-ku, Tokyo, Japan) was used to observe the microstructure of the samples. Calcofluor at 0.01% (*w/v*) was used for **β**-glucan staining, and the stained **β**-glucan was photographed by CLSM with a UV stimulus filter **λ** at 343 and an emission filter **λ** at 420 nm. Rhodamine B (0.02% *w/v*) was used to stain **α**-LA with the excitation filter **λ** at 543 nm and emission filter **λ** at 580 nm (Sharafbafi, Tosh, Alexander, & Corredig,

2014). To achieve optimal dying, the samples were placed on a slide glass and carefully distributed with a spatula. Then, the cover glass was used to cover the slide and the samples were visualized under the CLSM, with a 40× objective lens. At least ten photographs were taken of each sample with its various sections.

2) Syneresis

The sample was weighed and then placed in a Büchner funnel with a Whatman filter paper No. 1 (GE Healthcare, Amersham, Buckinghamshire, UK) at the base. The funnel with the weighed sample was covered with a plastic film to avoid dehydration. Then, the funnel was put in an Erlenmeyer (previously weighed) to collect the liquid lost from the sample by gravity. After 24 h at 4°C, the funnel with the drained sample and the Erlenmeyer with the liquid were weighed. Syneresis (water loss) was calculated as:

$$Syneresis = \frac{m_i - m_f}{m_i}$$

where m_i and m_f are the initial and final weights of the samples. Results express as g (water)/ 100 g (product) (Figueroa & Genovese, 2019).

4.3.3.2 Determination of chemical properties

1) Degree of protein hydrolysis (DH)

The sample was digested in an *in vitro* gastrointestinal system. This was carried out as described by Nongonierma, Lalmahomed, Paolella, and FitzGerald (2017). The hydrolysate was tested for DH values by 2, 4, 6-trinitrobenzenesufonic acid (TNBS) colorimetry for α-amino nitrogen as described by Yi, Lin, and Johns (2021) and Gruppi, Dermiki, Spigno, and FitzGerald (2022) with a slight modification. Briefly, samples (hydrolyzed and unhydrolyzed control samples) were diluted in 1% (*w/v*) sodium dodecyl sulfate to a final protein concentration as protein equivalent of 10 mg/mL and incubated at 50°C for 60 min in an incubator (JP Selecta, Abrera, Barcelona, Spain). Then, 10 µL of both samples and 10 µL of leucine standards at concentrations which ranged from 0 - 5 mg/mL were loaded into a 96-well plate with 80 µL of sodium phosphate buffer (0.2 M, pH 8), followed by 80 µL of 0.025% (*w/v*) TNBS. The sample plate was incubated at 45°C for 30 min in a microplate reader (Thermo Fisher Scientific, Vantaa, Southern Finland, Finland), and the absorbance at

420 nm was monitored every 2 min until 30 min. The DH values were calculated using the equation as follows:

$$DH\ (\%) = (A - B/T) \times 100.$$

where *A* is the reactive α-amino nitrogen concentration determined by TNBS colorimetry, *B* is the reactive α-amino nitrogen concentration of the intact protein substrate, and *T* is the total reactive α-amino nitrogen concentration of the intact protein substrate. *B/T* value of α-LA was 0.92/9.38 (Yi et al., 2021).

2) Glucagon-like Peptide-1 (GLP-1) secretion

In order to examine the GLP-1 quantification, cell lines of HSG were used. Cells were grown at 37°C, with 95% air and 5% CO_2 in a humidified atmosphere incubator (Thermo Fisher Scientific, Waltham, MA, USA) and were cultured in DMEM containing 4.0 mM L-glutamine and glucose 4.5 g/L, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Jeffers, Madden, & Webster-Cyriaque, 2009; Shao, Furusawa, Aoki, Matsumoto, & Ando, 2002). HSG cells were maintained at 6×10^4 cells/well in 24 well culture plates (well growth area of ca. 1.86 cm^2) and incubated at the same condition as mentioned above for 24 h. Then, the DMEM was replaced with 600 µL of fresh DMEM containing the hydrolysate at a selected concentration level, providing the maximum cell viability (Arteaga-Cardona et al., 2016). Untreated cells were used as a control. After further incubation for 24 h in a humidified atmosphere incubator, the supernatants were centrifuged at 664× g for 3 min at 25°C to remove the remaining cells by refrigerated centrifuge (Hermle Labortechnik GmbH, Wehingen, Germany). GLP-1 release levels were determined using a human GLP-1(7-36) simple step ELISA kit (Abcam, Discovery Drive, Cambridge, UK), following the manufacturer's instruction (Komatsu et al., 2019). The standard curve of Human GLP-1 was constructed by plotting the average of the absorption values for each human GLP-1 standard on the vertical axis (Y) versus the concentration of the human GLP-1 standard on the horizontal axis (X) and drawing the optimal curve through the points on the graph. The standard curve obtained from this current study are shown in Figure 4.1. GLP-1 release value (pg/mL) of the hydrolysate was calculated using the second order polynomial equation.

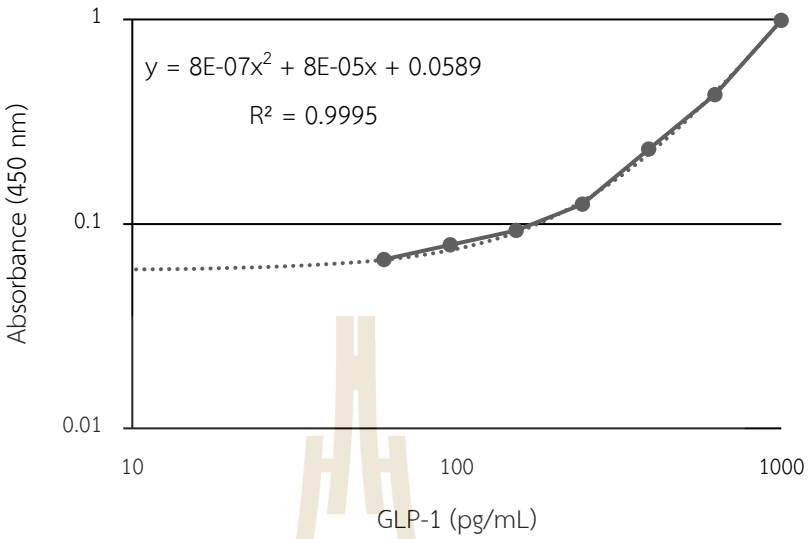

Figure 4.1 Human Glucagon-like peptide-1 (GLP-1) ELISA standard curve.

4.3.3.3 Microbiological study

In order to confirm the microbial safety of the sample and the commercial jelly-paste prior to the sensory evaluations, their microorganisms were determined. A 10 g of sample and 10 g of commercial jelly-paste were diluted with 90 mL of peptone water 0.1% (*w/v*) and homogenized with a paddle blender for 2 min. Ten-fold serial dilutions of each sample were prepared in peptone water. Then, a 1 mL aliquot of each dilution was plated onto duplicate of 3M Petrifilm Aerobic Count Plates, 3M Petrifilm Yeast and Mold Count Plates, and 3M Petrifilm Rapid E. coli/ Coliform Count Plates (3M Health Care, Saint Paul, MN, USA) for each dilution. Each plate was incubated in microorganism culture condition and enumerated all microorganisms, following the manufacturer's instruction. Plates containing greater than 150 colonies were recorded as too numerous to count. Typical colonies in the countable range (15-150) were enumerated using a colony counter (Model: 570, Suntex Instruments, Lingya, Kaohsiung, Taiwan). Final results were determined by multiplying the counts by the dilution factor for that plate and were recorded as Colony Forming Unit (CFU)/g (Bird et al., 2020). When plates from both dilutions yield fewer than 15 colonies, reported the count as less than 15 × 1/*d* where *d* is the dilution factor for the dilution from which the first counts were obtained. When plates

from all dilutions had no colonies, the results were reported as “less than 1 times of the corresponding lowest dilution used” (Bacteriological Analytical Manual, 2001).

4.3.3.4 Determination of sensorial properties

For the consumer test, participants were consumers who are risk to the diabetes and diabetic patients (*n* = 81; female *n* = 55, and male *n* = 26) and were aged in the range of 27 to 85 years. The overall profile rate for those participants who selected to take the questionnaire of consumer behavior and satisfaction on the jelly-paste containing α-lactalbumin/β-glucan conjugate is displayed in Table 2A and 3A (Appendix A).

1) Acceptance test

Thirty-two grams of the sample was contained in spout pouch package prior to the sensory session. The sensory evaluations of jelly-paste were determined by serving sample to group of the consumer at risk of diabetes and diabetic patients. The jelly-paste sample were evaluated for 8 sensorial attributes: appearance, aroma, flavor, syneresis, viscosity, smoothness, overall texture, and overall acceptance using a 9-point hedonic scale (1 = dislike extremely, 5 = neither dislike nor like, 9 = like extremely) (Hannum, Forzley, Popper, & Simons, 2021). The scores of sample’s attributes were averaged. The consumers could write additional comments on the bottom of the sensory evaluation form. Evaluation of acceptance test was performed before and after learning that the sample contained α-LA/β-glucan conjugate and the associated health benefits of jelly-paste.

2) Attributes satisfaction

Eighty-one of the same consumer group determined their attributes satisfaction for jelly-paste sample (Ramírez-Rivera et al., 2018). Participants were asked to rate the intensity of 5 sensorial attributes: orange aroma, sourness, sweetness, viscosity, and smoothness using the Just-About-Right (JAR) scale, which were consisted of 3 categories (1 = not enough, 2 = just about right, 3 = too much) (O'Brien et al., 2017).

3) Purchase intent

The consumer purchasing decisions were determined before and after receiving information about product benefits which was its antidiabetic property. The test was obtained from the same consumer group (81 consumers). The

consumer responses were based on ranking scale (1 = definitely will not buy; 5 = definitely will buy) (Suwonsichon, 2018).

All procedures were approved by the Human Research Ethics Committee (Suranaree University of Technology, Nakhon Ratchasima, Thailand), and the project code is EC-66-44 (Certificate of Exemption in Appendix B).

4.3.4 Statistical analysis

All experiments and measurements were performed at least in triplicate. All results were expressed as the mean ± standard deviation. A 2-related sample t-test was used to determine the difference between mean values with a significance at $p < 0.05$. All statistical analyses were determined using the statistical package for the social sciences program (version 23.0, SPSS Inc., Chicago, IL, USA).

4.4 Results and discussion

4.4.1 Physical properties

4.4.1.1 Microstructure

Representative CLSM images exhibiting the microstructure of the jelly-paste containing α-LA/β-glucan conjugate and the commercial jelly-paste are shown in Figure 4.2. The structures of α-LA (the ingredient in the jelly-paste sample) and amino acids (L-leucine, L-valine, and L-isoleucine from the ingredient in the commercial jelly-paste) were in a red color (Figure 4.2a and 4.2b, respectively). However, the structures of α-LA were exhibited a classic molten globule (Figure 4.2a) while the structures of amino acids were ungranulated residues (Figure 4.2b). This images were consistent with α-LA as a monomer globulin consisting of 123 amino acid residues, and its relative molecular weight is approximately 14.2 kDa (Mao et al., 2017) while the molecular weight of amino acids that are between 75 and 204 Da, the smallest protein unit, did not exhibit the same spherical structure as α-LA (Barea et al., 2023). β-glucan and carbohydrates (maltodextrin and fructose from the ingredient in the commercial jelly-paste) were in a blue color with homogenous distribution of its particles (Figure 4.2a and 4.2b, respectively). When α-LA was mixed with β-glucan in the jelly-paste, some β-glucan particles could absorb onto the structure of α-LA which was observed by a purple color created from the mix of red and blue colors

(Figure 4.2a). A purple color in CLSM image of the commercial jelly-paste was also observed, due to some carbohydrate particles could absorb onto the structure of amino acids (Figure 4.2b). The overlapping of red and blue color confirmed the interaction between protein and carbohydrate of both jelly-pastes. In fact, β-glucan or other carbohydrates contain functional aldehyde groups, which allows for conjugation through Maillard reaction to protein or amino acid molecules (Kaur, Sharma, Ji, Xu, & Agyei, 2020; Lertittikul, Benjakul, & Tanaka, 2007).

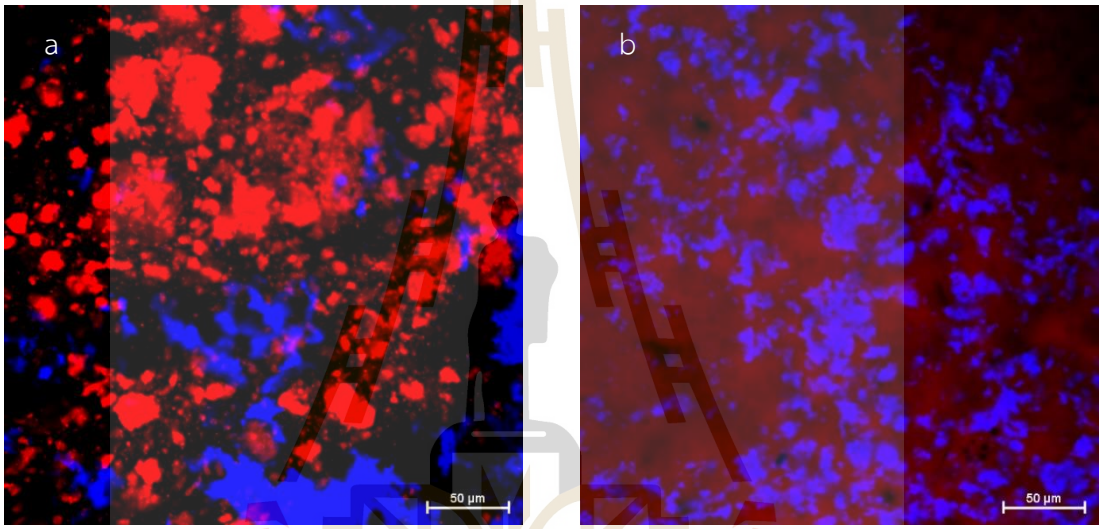

Figure 4.2 Confocal images of (a) the jelly-paste containing α-lactalbumin/β-glucan conjugate and (b) the commercial jelly-paste. Magnification 40× and scale bar 50 µm.

4.4.1.2 Syneresis

The syneresis (water loss) of the jelly-paste containing α-LA/β-glucan conjugate and the commercial jelly-paste was determined, and the results are shown in Table 4.1. It was found that jelly paste containing α-LA/β-glucan conjugates showed significant syneresis compared to commercial jelly paste. Even though the molecular weight of β-glucan was increased after Maillard reaction resulting in the increase in apparent viscosity (Sun et al., 2019), the addition of only α-LA/β-glucan conjugate could not reduce syneresis of jelly-paste. The less syneresis in the case of commercial jelly-paste could be associated with stabilizing and gelling properties of

gellan gum found as the ingredient in that jelly-paste (GU, 2020) but it was not found in jelly-paste containing α-LA/β-glucan conjugate. Moreover, the structure of α-LA in the jelly-paste containing α-LA/β-glucan conjugate was larger than of amino acids in the commercial jelly-paste (Figure 4.2), resulting in higher syneresis. This might be because higher molecular weight of α-LA, compared to amino acids found in commercial jelly-paste could easily induce the precipitation, leading to easier separation of layers or easier syneresis (Barea et al., 2023; Mao et al., 2017).

4.4.2 Chemical properties

4.4.2.1 Degree of protein hydrolysis (DH)

The DH values of the hydrolysates from the jelly-paste containing α-LA/β-glucan conjugate and the commercial jelly-paste were compared. The results are shown in Table 4.1. The jelly-paste containing α-LA/β-glucan conjugate possessed less DH (%) of its hydrolysate compared to that of hydrolysate from the commercial jelly-paste ($p < 0.05$). The result was correlated with the CLSM images of both jelly-pastes (Figure 4.2). α-LA has a globular structure, resulting in resistance to proteolysis enzymes (Nicoleta & Rapeanu, 2010) while amino acids could be easier hydrolyzed by those enzymes than α-LA when both were conjugated with carbohydrates in jelly-pastes.

4.4.2.2 Glucagon-like Peptide-1 (GLP-1) secretion

HSG cells were used as an *in vitro* model of GLP-1 release. This cell line has been widely used to elucidate the mechanism of GLP-1 release (Baum et al., 2010; Rowzee, Cawley, Chiorini, & Di Pasquale, 2011; Samuni & Baum, 2011). However, food digestion is known to be cytotoxic to cells, which depends on the type, concentration, processing, and quantity of food, etc. (Chevalier, Chobert, Genot, & Haertlé, 2001; Diao et al., 2021). In the present study, the optimum concentration of each hydrolysate from the jelly-paste containing α-lactalbumin/β-glucan conjugate and the commercial jelly-paste, providing the highest cell viability was used to determine the GLP-1 release. The results are shown in Table 4.1. The hydrolysate from the jelly-paste containing α-lactalbumin/β-glucan conjugate significantly increased the release of GLP-1 compared to the hydrolysate from the commercial jelly-paste and the control. However, there was no significant difference in the GLP-1 release by the hydrolysate from the commercial jelly-paste and the control. Untreated cells were

used as the control, and its GLP-1 release was 16.919 ± 6.604 pg/mL in this present study. Regarding the DH values of hydrolysates from both jelly-pastes, the lower DH value seemed to correlate with the higher GLP-1 release (Table 4.1). Lower DH values implied that a large number of short-chain dipeptides were formed, which could inhibit DPP-IV activity, believed to delay the inactivation of GLP-1 release (Farup et al., 2016; Tulipano, Faggi, Nardone, Cocchi, & Caroli, 2015).

Table 4.1 The syneresis, degree of protein hydrolysis (DH), and glucagon-like peptide-1 (GLP-1) release values of the jelly-paste containing α-lactalbumin (α-LA)/β-glucan conjugate and the commercial jelly-paste.

Properties	Samples	
	Jelly-paste containing α-LA/β-glucan conjugate	Commercial jelly-paste
Syneresis (%)	15.163 ± 2.739 [b]	0.010 ± 0.002 [a]
DH (%)	20.500 ± 2.164 [a]	43.127 ± 3.425 [b]
GLP-1 release (pg/mL)	67.712 ± 5.312 [a]	16.919 ± 6.604 [b]

Note: Data are presented as mean ± standard deviation (n = 4). Untreated cells were used as the control for GLP-1 release value and its value was 16.919 ± 6.604 pg/mL. The different letters [a,b] in the same row indicate a significant difference at $p < 0.05$.

4.4.3 Microbiological property

In order to confirm the microbial safety of sample and commercial jelly-pastes prior to the sensory evaluations, their microorganisms were determined. The results are shown in Table 4.2. The numerous amount of total plate count and E. coli/ coliform in the jelly-paste containing α-LA/β-glucan conjugate (7 and 14 days of storage) and that of the commercial jelly-paste were lower than the amount announced by a Notification of the Ministry of Public Health No. 355 B.E. 2556 (2013), Thailand (Food and Agriculture Organization of the United Nations, 2017). However, the amount of yeast and mold in the jelly-paste, containing α-LA/β-glucan conjugate and kept for 14 days, was higher than that of the hygienic criteria established by that notification. While the amount of yeast and mold of the commercial jelly-paste was

lower than 100 CFU/g. This means that the shelf life of jelly-paste sample in this study was not more than 7 days in hermetically sealed packaging at 4°C.

Table 4.2 The amount of the microorganisms (CFU/g) in the jelly-paste containing α-lactalbumin/β-glucan conjugate (jelly paste LA&G) at different storage and the commercial jelly-paste.

Samples	Total plate count (CFU/g)	Yeast & Mold (CFU/g)	Coliform & *E. coli* (CFU/g)
commercial jelly plate	<150	<100	<10
jelly paste LA&G 7 day	<100	<100	<10
jelly paste LA&G 14 day	<100	<150	<10

Note: Data are presented as mean (n = 4).

4.4.4 Sensorial properties

The jelly-paste attributes were measured after tasting before the explanation of health benefits. As shown in Figure 4.3, average scores of sensory liking ranged from 6.02 ± 1.71 (appearance) to 6.88 ± 1.48 (overall texture). These scores were related to subjects "liking slightly to liking moderately".

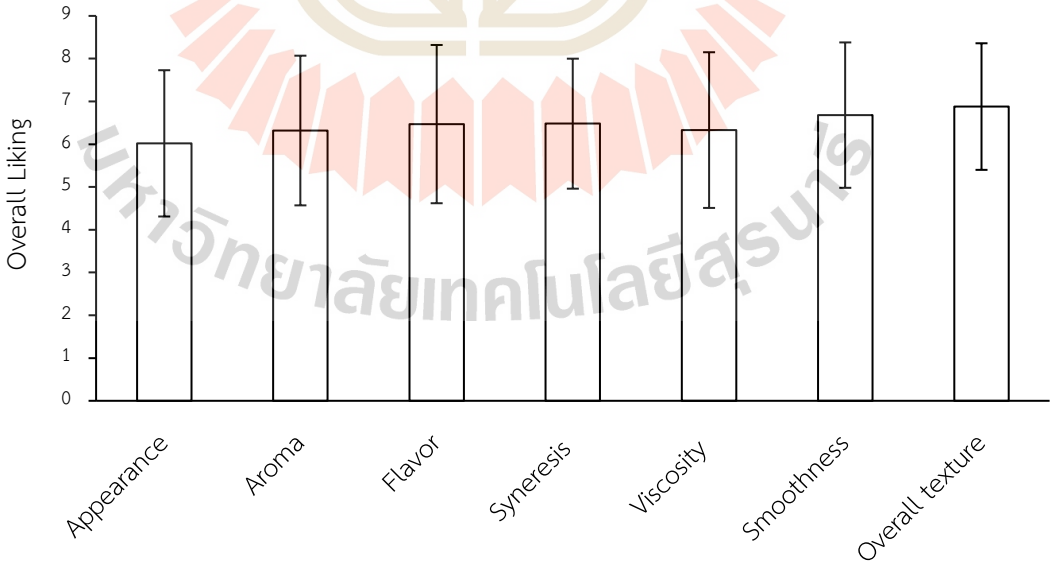

Figure 4.3 Sensory liking scores for appearance, aroma, flavor, syneresis, viscosity, smoothness, and overall texture of the jelly-paste based on a 9-point

hedonic scale before learning that it contained α-lactalbumin/β-glucan conjugate and the health benefits associated with α-lactalbumin/β-glucan conjugate. Data are presented as mean ± standard deviation (*n* = 81).

Overall liking of the jelly-paste containing α-lactalbumin/β-glucan conjugate scored 6.91 ± 1.70 and 6.95 ± 1.37 out of 9 before (sample 1) and after (sample 2) the explanation of health benefits, respectively (Figure 4.4). There were no significant differences in overall liking scores between samples 1 and 2. However, the overall liking score of sample 2 had a higher frequency of individual scores in the range of 5 to 8 than sample 1.

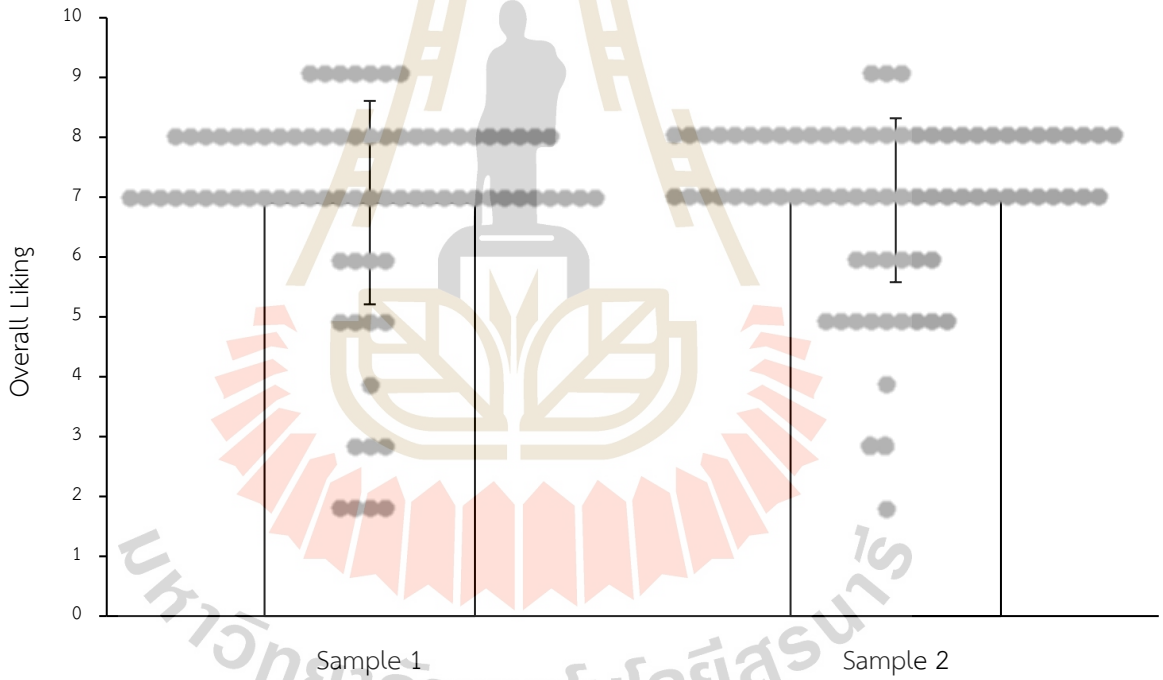

Figure 4.4 Overall liking scores of the jelly-paste before (sample 1) and after (sample 2) learning that it contained α-lactalbumin/β-glucan conjugate and the health benefits associated with α-lactalbumin/β-glucan conjugate. Data based on a 9-point hedonic scale are presented as individual data points (gray circles) and mean ± standard deviation (*n* = 81).

The average JAR score of orange aroma, sourness, sweetness, viscosity, and smoothness for the jelly-paste containing α-LA/β-glucan conjugate was 1.89 ± 0.35, 2.01 ± 0.34, 1.98 ± 0.45, 2.14 ± 0.41, and 1.81 ± 0.50, respectively, suggesting a 'just about right' or close to ideal quality (Figure 4.5). Data based on JAR score was consisted of 3 categories (1 = not enough, 2 = just about right, 3 = too much) (O'Brien et al., 2017).

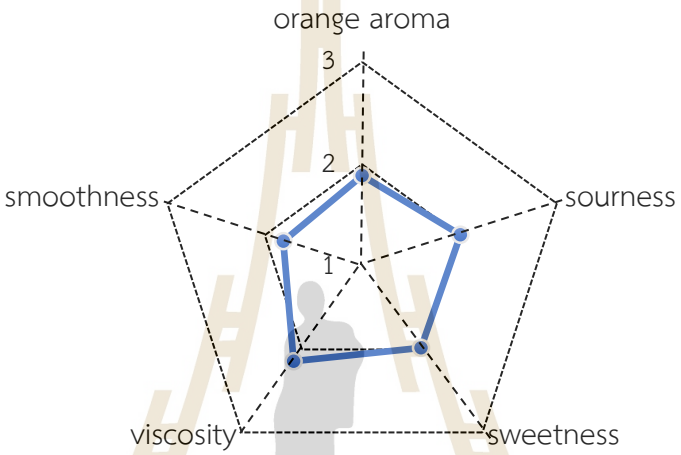

Figure 4.5 Just-About-Right scores of the jelly-paste containing α-lactalbumin/β-glucan conjugate (Blue dot). Data are presented as mean (*n* = 81) which based on the 3 categories (1 = not enough, 2 = just about right, 3 = too much).

A high intent to purchase before participants about the health benefits: 49 out of 81 (60%) indicated a desire to purchase after sample 1, and this number increased to 59 out of 81 (73%) after sample 2 (Table 4.2). A possible health halo associated with jelly-paste may have been associated with initial high overall scores. Although participants were not initially informed that they were eat jelly-paste containing α-LA/β-glucan conjugate, they knew that they were participated in a 'antidiabetic food product' study.

Table 4.3 The possibility of purchasing of the jelly-paste before (sample 1) and after (sample 2) learning that it contained α-lactalbumin/β-glucan conjugate and the health benefits associated with α-lactalbumin/β-glucan conjugate.

	Study Participants (%)	
	Sample 1	*Sample 2*
Definitely will not purchase	14	5
Probably will not purchase	6	7
Might or might not purchase	20	15
Probably will purchase	26	32
Definitely will purchase	34	41

4.5 Conclusions

This study shows that consumers accepted jelly-paste containing α-LA/β-glucan conjugate which indicated that this jelly-paste had the potential to be produced for commercial sale. In addition, the consumption of jelly-paste containing α-LA/β-glucan conjugate might increase GLP-1 secretion. However, the syneresis of the jelly-paste containing α-LA/β-glucan conjugate was higher than that of the commercial jelly-paste. Therefore, the application of stabilizers, such as gellan gum, locust bean gum, or carrageenan in jelly-paste containing α-LA/β-glucan conjugate should be further studied to reduce the syneresis of such jelly-paste. A larger consumer study of such jelly-paste should be further also investigated to warrant the results of its sensorial acceptance.

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CHAPTER V

SUMMARY

This study exposed that, with heat treatment, the hydrolysate from α-lactalbumin (α-LA) provided the least degree of protein hydrolysis (DH) compared to that of β-lactoglobulin (β-LG) and whey protein isolate (WPI). The α-LA hydrolysate exhibited antidiabetic properties better than WPI and β-LG hydrolysates. Evidently, α-LA hydrolysate possesses a high potential for being the enhancer for glucagon-like peptide-1 (GLP-1) release and dipeptidyl peptidase-IV (DPP-IV) inhibitory activity. Heating at 75°C for 30 min gave the best results of GLP-1 release and DPP-IV inhibition while heating at 65 and 85 C for 30 min provided both lower activities of GLP-1 release and DPP-IV inhibition. The effect of conjugates between α-LA and β-glucan heated at 75°C for 30 min on their antidiabetic properties after *in vitro* digestion was found in this study as a function of pH and ratio of α-LA to β-glucan. The conjugates at pH 3 exhibited the highest DH (%) compared to that of the conjugates at pH 5 and 7. In addition, the hydrolysates from conjugated product at ratios of α-LA to β-glucan of 5:1, and 5:3% (*w/v*) at pH 3 provided GLP-1 release and DPP-IV inhibition better than those at the ratios of 5:0, 5:5, and 0:10% (*w/v*) at the same pH. The appearance of the conjugate between α-LA and β-glucan at a ratio of 5:3% (*w/v*) showed greater homogenous than that of 5:1% (*w/v*). The jelly-paste containing α-LA/β-glucan conjugate at the ratio of 5:3% (*w/v*) at pH 3 was produced for the targeted consumers who are at risk for diabetes and type 2 diabetes (T2D). With the consumer test, this study showed that jelly-paste containing α-LA/β-glucan conjugate had the commercially potential to be produced. In addition, the consumption of jelly-paste containing α-LA/β-glucan conjugate might increase GLP-1 secretion in human body. However, the jelly-paste syneresis cannot be prevented by adding only α-LA/β-glucan conjugate. The effect of types and concentrations of stabilizers on physicochemical properties, e.g., syneresis, texture, and rheology of such jelly-paste as well as antidiabetic activity of its hydrolysate should be further

investigated. In addition, the potential antidiabetic activity of jelly-paste containing **α**-LA/**β**-glucan conjugate should be confirmed by animal study in further experiment.

APPENDICES

APPENDIX A

Table 1A Cell viability (%) of the human salivary gland (HSG) cells after 24 h incubation with DMEM (control) and the **α**-LA/**β**-glucan conjugates at concentrations between 0.020 and 2.500 mg/ml.

Conc. (mg/ml)	Ratio of α-LA to β-glucan														
	pH 3					pH 5					pH 7				
	5:0	5:1	5:3	5:5	0:10	5:0	5:1	5:3	5:5	0:10	5:0	5:1	5:3	5:5	0:10
control	100.00±16.79	100.00±10.47	100.00±11.78	100.00±9.17	100.00±4.75	100.00±7.87	100.00±6.99	100.00±0.51	100.00±11.79	100.00±7.42	100.00±9.72	100.00±6.62	100.00±2.98	100.00±2.42	100.00±5.34
0.020	108.75±8.74	101.59±2.59	110.49±9.61	99.68±8.16	102.50±9.85	101.17±7.02	139.02±4.21	113.41±3.99	110.28±6.76	114.33±6.18	118.58±7.05	96.81±5.24	103.48±7.47	86.08±5.00	100.12±5.31
0.039	122.36±14.39	107.13±8.14	101.75±6.31	94.20±2.37	86.49±7.55	101.80±11.03	111.04±6.51	115.64±4.63	100.28±9.04	103.15±13.73	108.41±6.96	100.39±10.64	118.27±5.00	78.55±5.85	101.93±11.62
0.078	102.09±4.57	134.55±16.92	103.99±3.90	99.41±2.18	96.26±6.03	104.53±9.81	134.33±3.06	119.14±8.87	121.30±9.20	103.97±2.23	135.20±0.16	108.62±16.65	120.03±7.00	93.25±4.99	114.22±6.65
0.156	118.58±9.43	125.11±9.42	106.83±10.26	99.35±1.98	95.42±6.11	111.40±16.69	160.55±4.33	119.75±4.79	105.49±2.35	114.56±10.01	118.09±6.12	103.40±5.02	145.60±2.69	99.14±5.51	105.86±3.78
0.313	123.88±12.64	102.84±2.66	104.54±7.25	84.35±2.57	92.43±0.69	104.13±6.41	100.20±1.34	117.33±4.98	98.23±6.11	105.59±9.91	103.81±10.94	93.47±5.83	117.80±10.31	92.15±1.21	107.68±4.98
0.625	92.94±1.56	97.33±0.92	94.37±6.57	102.22±3.46	91.82±8.08	116.89±14.85	102.10±0.38	111.29±4.04	116.38±8.31	110.31±9.11	98.09±8.84	88.51±4.78	111.02±1.71	81.50±6.24	97.66±4.99
1.250	71.08±11.47	74.48±3.11	84.84±7.24	87.03±9.30	84.22±9.45	61.47±9.75	83.75±2.18	98.85±4.63	86.06±3.65	102.82±6.75	84.92±4.84	64.18±11.78	87.86±0.61	75.65±6.76	85.61±4.89
2.500	54.17±3.30	61.59±3.92	61.93±3.46	61.97±7.77	72.44±2.55	55.13±6.25	55.04±2.59	74.50±4.92	70.30±2.81	74.19±11.73	64.07±2.14	54.84±4.46	69.40±3.65	47.36±2.58	69.70±2.39

Note: Data are presented as mean ± standard deviation (n = 4).

Table 2A Demographics for of those participants completing the questionnaire of consumer behavior and satisfaction on the jelly-paste containing α-lactalbumin/β-glucan conjugate.

	Study Participants (%)
	Sex
Female	68
Male	32
	Age
20-29 years old	4
30-39 years old	4
40-49 years old	7
50-59 years old	31
60-69 years old	31
70-79 years old	17
80-89 years old	6
	Job
Government officer/ State enterprise	6
Private employee	10
Personal occupation	25
Farmer	9
Homemaker	27
Others	6
Stay at home	17

Table 3A General consumer behavior of those participants completing the questionnaire of consumer behavior and satisfaction on the jelly-paste containing α-lactalbumin/β-glucan conjugate.

	Study Participants (%)
	How often do you eat snacks or desserts?
Do not eat snacks or desserts	7
Less than once a day	36
Once a day	28
2 times a day	20
More than 2 times a day	9
	What are reasons for buying snacks or desserts? (More than one answer possible)
I like to eat	29
People in the family like to eat	27
Ingredients with health benefits	11
Cheapness	12
Convenient and easy to buy	18
Long shelf-life	1
Others	2
	Where do you usually buy snacks or desserts? (More than one answer possible)
Markets	31
Convenience stores	25
	Where do you usually buy snacks or desserts? (More than one answer possible)
Retail stores	25
Department stores	11
Wholesale shops	2

Table 3A (Continue).

	Study Participants (%)
Food truck	6
	Have you ever heard / known about the orange jelly paste?
Ever	17
Never	83
	Have you ever consumed the orange jelly paste?
Ever	9
Never	91

APPENDIX B

COE no. 42/2566

Human Research Ethics Committee, Suranaree University of Technology

Certificate of Exemption

Human Research Ethics Committee, Suranaree University of Technology, Nakhon Ratchasima, Thailand, has exempted the following study which is to be carried out in compliance with the international guidelines for human research protection as Declaration of Helsinki, The Belmont Report, CIOMS Guideline, International Conference on Harmonization in Good Clinical Practice (ICH-GCP) and 45CFR 46.101(b)

Title of Project : Antidiabetic properties of composition of whey protein conjugated with β-glucan and its application

Project Code : EC-66-44

Principal Investigator : Mrs. Kungnang Bunsroem

Department : Institute of Agricultural

Co-Investigator : –

Document Reviewed : Protocol, Information sheet, Informed consent, Questionnaire (Version 1.0, 21 March 2023)

Criteria of exemption : Research on flavor, quality of food and consumer satisfaction in general

Signature.....Chairman
(Asst. Prof. Dr. Benjamart Chitsomboon)
Human Research Ethics Committee, Suranaree University of Technology

Date of Exemption: 26 April 2023

Note: 1. No progress review required.
2. Submit protocol amendments, if there are changes to the initial protocol (AF/01-11/02.0)
3. Submit the final report when finished. (AF/01-13/02.0)

ผ่านการพิจารณาจาก
คณะกรรมการจริยธรรมการวิจัยในมนุษย์
มหาวิทยาลัยเทคโนโลยีสุรนารี แล้ว


Thai MOOC
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แพลตฟอร์มเพื่อการเรียนรู้ออนไลน์ตลอดชีวิต | กระทรวงการอุดมศึกษา วิทยาศาสตร์ วิจัยและนวัตกรรม
ผ่านการพิจารณาจาก
คณะกรรมการจริยธรรมการวิจัยในมนุษย์
มหาวิทยาลัยเทคโนโลยีสุรนารี แล้ว
กุ้งนาง บุญเสริม
ได้ผ่านการเรียนออนไลน์ตามเกณฑ์การวัดผลในรายวิชา
จริยธรรมการวิจัยในมนุษย์เบื้องต้น (5 ชั่วโมงการเรียนรู้)
พัฒนารายวิชาโดย มหาวิทยาลัยนเรศวร
รศ.ดร.พัชราภรณ์ สุดชาฎา
เลขานุการและกรรมการจริยธรรมการวิจัยในมนุษย์ Panel 1 & 3
มหาวิทยาลัยนเรศวร
นายแพทย์สมบูรณ์ ตันสุภสวัสดิกุล
ประธานคณะกรรมการจริยธรรมการวิจัยในมนุษย์ Panel 1 & 3
มหาวิทยาลัยนเรศวร
ผู้ช่วยศาสตราจารย์ ดร.วนาวัลย์ ดาดี้
ประธานคณะกรรมการจริยธรรมการวิจัยในมนุษย์ Panel 2
มหาวิทยาลัยนเรศวร
(กุ้งนาง บุญเสริม)
21 มีนาคม 2566
หมายเลขใบรับรอง:
20 ตุลาคม 2022

View article

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The influence of whey protein heating parameters on their susceptibility to digestive enzymes and the antidiabetic activity of hydrolysates

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Authors	Kungnang Bunsroem, Witoon Prinyawiwatkul, Siwatt Thaiudom
Publication date	2022/3/14
Journal	Foods
Volume	11
Issue	6
Pages	829
Publisher	MDPI

Description: The inhibition of dipeptidyl peptidase-IV (DPP-IV) and the release of glucagon-like peptide-1 (GLP-1) could normalize blood glucose levels in diabetic patients. This study evaluated the susceptibility of whey proteins to enzyme hydrolysis and the antidiabetic properties of protein hydrolysates from β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) solutions compared with whey protein isolate (WPI) solution treated at different heating temperatures (65, 75, and 85 °C). α-LA hydrolysate provided the lowest degree of hydrolysis (DH). Those heating temperatures did not significantly affect the DH of all protein hydrolysates. α-LA hydrolysate significantly increased GLP-1 levels and DPP-IV inhibitory activity more than β-LG hydrolysate. WPI hydrolysate inhibited DPP-IV activity less than an α-LA hydrolysate, but they were no significant differences for GLP-1 release activity. Heat treatment could affect the antidiabetic properties of all protein hydrolysates. Heating at 75 °C resulted in greater inhibition of the activity of DPP-IV than at 65 and 85 °C. The highest increase in GLP-1 release was also observed by heating at 75 °C. The recently obtained information is useful for the utilization of α-LA, heated at 75 °C for 30 min, in the preparation of antidiabetic food supplements.

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BIOGRAPHY

Kungnang Bunsroem was born on January 12th, 1984 in Saraburi, Thailand. She attended Phu Khae Witthaya School (1996-2002), and in 2006 she received the Bachelor degree of Science in Food Science and Technology from Maejo University, Thailand. In 2009 she received the degree of Master of Science (Food Science and Technology) from Chaingmai University, Thailand. She worked as lecturer at Rajamangala University of Technology Isan Sakonnakon Campus, Sakonnakon (2009 – 2014) and Rajamangala University of Technology Isan, Nakhon Ratchasima (2014 - present). In 2017 – 2023 she received a scholarship from the National Science and Technology Development Agency Scholarship of Thailand (grant number NSTDA.RMUTI 3/2560) and studied the Doctoral degree of Philosophy in Food Technology at Suranaree University of Technology. During her graduate study, she published this research project in 2022.