ENCAPSULATION OF ADZUKI BEAN *Vigna angularis* HY DROLYSATE BY ALGINATE-BASED BEADS

Miss Thasanporn Sangsukiam

A Dissertation Submitted in Partial Fulfillment of the Requirements
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เอนแคปซูเลชันของไฮโดรไลเสตจากถั่วอะซูกิ *Vigna angularis* ด้วยเม็ดบีดฐานแอลจิเนต

น.ส.ฐสรรพร แสงสุขเอี่ยม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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By	Miss Thasanporn Sangsukiam
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Thesis Advisor	Assistant Professor KIATTISAK DUANGMAL, Ph.D.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

.... Dean of the FACULTY OF SCIENCE
(Professor POLKIT SANGVANICH, Ph.D.)

DISSERTATION COMMITTEE

.... Chairman
(Associate Professor Kanitha Tananuwong, Ph.D.)

.... Thesis Advisor
(Assistant Professor KIATTISAK DUANGMAL, Ph.D.)

.... Examiner
(Associate Professor CHALEEDA BOROMPICHAICHARTKUL, Ph.D.)

.... Examiner
(Sarisa Suriyarak, Ph.D.)

.... External Examiner
(Assistant Professor Wanwimol Klaypradit, Ph.D.)

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CHULALONGKORN UNIVERSITY

ฐูสรรพร แสงสุขเอี่ยม : เอนแคปซูเลชันของไฮโดรไลเสตจากถั่วอะซูกิ *Vigna angularis* ด้วยเม็ดบีดฐานแอลจิเนต. (ENCAPSULATION OF ADZUKI BEAN *Vigna angularis* HYDROLYSATE BY ALGINATE-BASED BEADS) อ.ที่ปรึกษาหลัก : ผศ. ดร.เกียรติศักดิ์ ดวงมาลย์

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาอิทธิพลของวิธีการปรุงสุกที่แตกต่างกันต่อสารออกฤทธิ์ทางชีวภาพและการออกฤทธิ์ทางชีวภาพของถั่วปรุงสุกและน้ำต้มถั่ว เทคนิคการห่อหุ้มที่ต่างกันต่อกักเก็บไฮโดรไลเสตจากถั่วอะซูกิ ลักษณะการปลดปล่อยสารของอนุภาคแอลจิเนตที่ผ่านการทำแห้งแบบแช่เยือกแข็งภายหลังการจำลองการย่อยในระบบทางเดินอาหารในหลอดทดลอง ผลการศึกษาแสดงให้เห็นว่าภาวะการปรุงสุก (การต้ม 60 นาที การใช้ความร้อนร่วมกับความดัน 10 นาที และการใช้ความร้อนร่วมกับความดัน 60 นาที) ทำให้ปริมาณของโปรตีนหยาบ เพปไทด์ที่ละลายได้ในสารละลายกรดไตรคลอโรแอซิติก หมู่แอมิโนอิสระ สารประกอบฟีนอลทั้งหมด และฟลาโวนอยด์ในเนื้อถั่วปรุงสุกลดลงอย่างมีนัยสำคัญ ($p \leq 0.05$) ในขณะที่การปรุงสุกส่งผลต่อการเพิ่มขึ้นอย่างมีนัยสำคัญของปริมาณเพปไทด์ที่ละลายได้ในสารละลายกรดไตรคลอโรอะเซติก หมู่อะมิโนอิสระ สารประกอบฟีนอลทั้งหมด และฟลาโวนอยด์ในน้ำต้มถั่ว วิธีการปรุงสุกที่แตกต่างกันส่งผลต่อชนิดและปริมาณของกรดอะมิโนและสารประกอบฟีนอล การใช้ความร้อนร่วมกับความดัน 10 นาทีเป็นภาวะที่เหมาะสมสำหรับปรุงสุกถั่วอะซูกิเนื่องจากสามารถรักษาปริมาณกรดแอมิโนและปลดปล่อยสารประกอบฟีนอลได้มากขึ้น โดยเนื้อถั่วปรุงสุกและน้ำต้มถั่วที่ได้จากภาวะนี้มีปริมาณสารออกฤทธิ์ทางชีวภาพและแสดงการออกฤทธิ์ทางชีวภาพสูงเมื่อเปรียบเทียบกับภาวะปรุงสุกอื่น ดังนั้นจึงนำเนื้อถั่วสุกและน้ำต้มถั่วจากภาวะที่ใช้ความร้อนร่วมกับความดัน 10 นาทีผสมกันเพื่อใช้เป็นสารตั้งต้นสำหรับการย่อยโปรตีนด้วยเอนไซม์ Flavouryzme ความเข้มข้นร้อยละ 7 ที่อุณหภูมิ 50 องศาเซลเซียส เป็นเวลา 6 ชั่วโมง จากนั้นนำไฮโดรไลเสตจากถั่วอะซูกิที่เตรียมได้ไปกักเก็บโดยใช้แอลจิเนตเป็นสารห่อหุ้มด้วยวิธีการห่อหุ้มที่แตกต่างกันสองวิธี (บีดของแคลเซียมแอลจิเนตและพาร์ทิเคิลของโซเดียมแอลจิเนต) ศึกษาผลเทียบกับไฮโดรไลเสตจากถั่วอะซูกิที่ผ่านการทำแห้งแบบแช่เยือกแข็งเป็นตัวอย่างควบคุม พาร์ทิเคิลของโซเดียมแอลจิเนตมีความสามารถในการกักเก็บสารประกอบฟีนอลและเพปไทด์ที่ละลายได้ในสารละลายกรดไตรคลอโรแอซิติกสูงเมื่อเปรียบเทียบกับบีดของแคลเซียมแอลจิเนต การใช้ฟูเรียร์ทรานส์ฟอร์มอินฟราเรดยืนยันการมีอันตรกิริยาระหว่างไฮโดรไลเสตจากถั่วอะซูกิและโครงสร้างของแอลจิเนต ค่า FRAP ferrous chelating, ORAC และ ACE-I inhibitory activity ของพาร์ทิเคิลภายหลังการจำลองการย่อยในระบบทางเดินอาหารเมื่อเทียบกับค่าดังกล่าวของบีดเพิ่มขึ้นอย่างชัดเจน ในขณะที่ค่า ABTS scavenging activity ของบีดสูงกว่าพาร์ทิเคิล ค่า DPP-IV inhibitory activity ของพาร์ทิเคิลและบีดไม่แตกต่างอย่างมีนัยสำคัญ ทั้งนี้เนื่องจากเทคนิคการห่อหุ้มส่งผลอย่างมีนัยสำคัญต่อการกักเก็บสารออกฤทธิ์ทางชีวภาพและลักษณะสมบัติการควบคุมและปลดปล่อยสาร

สาขาวิชา	เทคโนโลยีทางอาหาร	ลายมือชื่อนิสิต ....
ปีการศึกษา	2562	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

5772812123 : MAJOR FOOD TECHNOLOGY
KEYWORD: Cooking condition Encapsulation technique Bioactive compound Antioxidant activity ACE-I Converting Enzyme (ACE-I) inhibitory activity Dipeptidyl peptidase-4 (DPP-4) inhibitory activity

Thasanporn Sangsukiam : ENCAPSULATION OF ADZUKI BEAN *Vigna angularis* HYDROLYSATE BY ALGINATE-BASED BEADS. Advisor: Asst. Prof. KIATTISAK DUANGMAL, Ph.D.

The aims of this research were to study the effect of different cooking conditions on bioactive compounds and biological activities of cooked adzuki beans and cooking water. The different encapsulation methods to entrap adzuki beans hydrolysate were investigated. The release characteristics of different freeze-dried alginate particles were investigated after *in vitro* simulated gastrointestinal digestion. The results showed that all cooking conditions (60-min boiling, 10-min autoclaving and 60-min autoclaving) caused a significant reduction in crude protein, TCA-soluble peptides, free amino groups, total phenolic content (TPC) and total flavonoid content (TFC) in cooked adzuki beans ($p \leq 0.05$). Meanwhile, cooking resulted in a significant increase in TCA-soluble peptides, free amino group content, TPC and TFC in cooking water. The different cooking conditions affected amino acid composition and phenolic compounds. The 10-min autoclaving was the appropriate condition as it could retain more amino acids and release more bound phenolics. The obtained cooked beans and its cooking water contained higher bioactive compounds and showing higher biological activity compared to other conditions. Thus, mixed cooked beans and its cooking water from 10-min autoclaving, substrate for protein hydrolysis, were hydrolyzed with 7% Flavouryzme at 50°C, pH 6.0 for 6 hours. The obtained beans hydrolysate was entrapped in alginate-based via different encapsulation techniques (calcium alginate beads and sodium alginate particles). Freeze-dried adzuki beans hydrolysate was prepared as the control. The sodium alginate particles had a higher encapsulation efficiency of TPC and TCA-soluble peptides compared to calcium alginate beads. Fourier-transform infrared result confirmed the interaction of functional groups between adzuki beans hydrolysate and alginate structure. A remarkable increase in FRAP, ferrous chelating, ORAC value and ACE-I inhibitory after *in vitro* GI tract digestion were observed in the particles. Whilst beads exhibited higher ABTS scavenging activity after *in vitro* GI tract digestion compared to particles. DPP-IV inhibitory activity of particles and beads were not significantly different. This might be because encapsulation technique had a significant effect on the entrapped bioactive compounds and controlled-release characteristic of sample.

Field of Study:	Food Technology	Student's Signature ....
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CHAPTER I
INTRODUCTION

Climate change has become a major concern to agricultural crops. Legumes are considered to be drought-resistant crops. Legume seeds are the important diet especially source of protein in many countries. They also contain carbohydrate, dietary fiber, vitamins, minerals and phenolic compounds. Recently, the bioactive compounds from adzuki beans (Vigna angularis) become more interesting. This is because adzuki beans are excellent sources of high-quality protein and phenolic compounds related to health-promoting benefits, such as cardiovascular disease and diabetes, and antioxidant activities. Although, adzuki beans have a high protein quality and nutritional-rich value, their utilization is limited due to the presence of antinutrients. The presence of antinutrients reduces the nutrient absorption in small intestine; therefore, legumes are usually cooked before consumption. Cooking could improve digestibility and nutrient absorption of pulses via destroying antinutrients and inducing protein denaturation.

Enzymatic hydrolysis is one of the most effective ways to produce bioactive peptides from legume proteins. May researches showed that bioactive peptides obtained from legumes exhibit anticarcinogenic, hypocholesterolemic, blood pressure-lowering (angiotensin converting enzyme (ACE)-inhibitory), antioxidant activity and antimicrobial activity. During hydrolysis, phenolic compounds in protein-bound polyphenols transform into free polyphenols. Both bioactive peptides and phenolic compounds have been reported to show health-promoting benefits and antioxidant activities. The application of encapsulation in food industry includes the entrapment of bioactive compounds within a matrix in particulate form in order to achieve one or more desirable effects such as protection sensitive ingredients, extending shelf life and controlling the release of active compounds. The alginate-based encapsulation is a useful technique to entrap bioactive compounds within gel networks. This system can controlled-release bioactive compounds in adzuki bean hydrolysate to the specific target site.

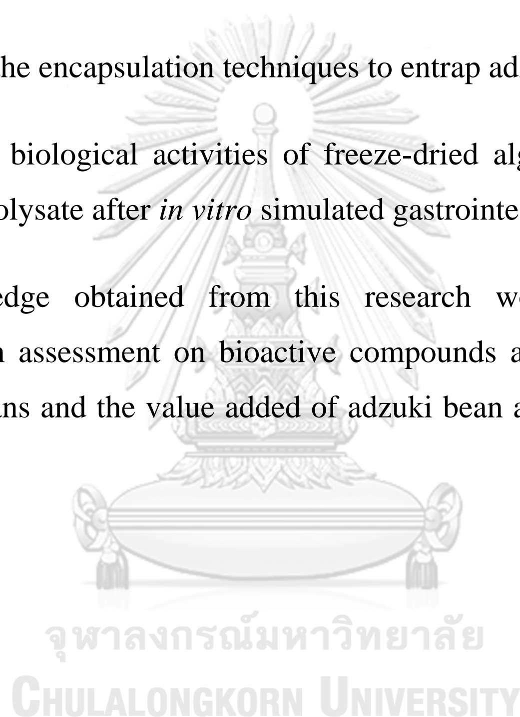
The cooked adzuki bean and adzuki bean hydrolysate have been reported to have potent health-promoting benefits and antioxidant in foods but very little published information was obtained. In addition, information on adzuki bean hydrolysate with the different encapsulation techniques under stimulated gastrointestinal condition is scarce. Therefore, the objectives of this thesis were as follows:

1) To study the effect of cooking conditions on bioactive compounds and the biological activities of cooked adzuki bean and its cooking water

2) To study the encapsulation techniques to entrap adzuki beans hydrolysate

3) To study biological activities of freeze-dried alginate particles containing adzuki beans hydrolysate after *in vitro* simulated gastrointestinal digestion

The knowledge obtained from this research would provide meaningful information for an assessment on bioactive compounds and biological activities of cooked adzuki beans and the value added of adzuki bean as functional ingredients in food industry.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CHAPTER II
LITERATURE REVIEW

2.1 Grain legume

Legumes, belonging to the leguminosae family (Fabaceae), consist of oilseeds and pulses. According to Food and Agriculture Organization (FAO), the dry seeds harvested from 1 of 12 leguminous crops are pulses which are a subgroup of legumes. The oil crop legumes and leaf vegetable legumes are excluded. Dry seeds are also referred to grain legumes or pulse grains. Common bean, pea, chickpea, faba bean, cowpea, lentil, pigeon pea, peanut, mung bean, black bean, rice bean, azuki bean, grass pea and horsegram, are dominant grain legumes in diet for human (Bisht & Singh, 2013).

Grain legumes have a high proportion of carbohydrate, around 50 to 60% in their seeds. The fraction of grain carbohydrate consists of starch, monosaccharide, disaccharide, low molecular weight oligosaccharide and a group of cell wall polysaccharide (Hedley, 2001). Generally, grain legumes are particularly high in resistance starch (RS) around 1.7–4.2 g/100 g cooked legumes. The ratio of amylose to amylopectin influences the starch digestibility and causes either slowly starch digestion rate and/or resistant to digestion (Clemente & Olias, 2017). Moreover, grain legume also contains both insoluble and soluble fiber, which are non-digestible carbohydrates, as well as α-galactosides, which are considered prebiotics (Guillon & Champ, 2002). Insoluble fiber has water-holding capacity yielding the fecal bulking. Whist gut bacteria is fermented soluble fiber and produced physiologically active by-products and gases in the colon (McCrory, Hamaker, Lovejoy, & Eichelsdoerfer, 2010). The resistance to digestion of these complex carbohydrates makes grain legume a low GI index food (Clemente & Olias, 2017). In addition, the combination of grain legumes classified as low glycemic index and high glycemic index food (i.e. white rice) could produce intermediate-glycemic index foods (Tan & Azrina, 2017)

The storage protein accumulates during the development of grain legume. The major abundant storage protein is the globulins, representing approximately 70% of total protein (López-Barrios, Gutiérrez-Uribe, & Serna-Saldívar, 2014). The globulins

are generally classified as vicilin (7S) and legumin (11S). The 7S globulins have trimeric structures (oligomeric protein). Vicilin are often glycosylated, but lack disulfide bridges between the subunits because vicilin protein is lack of cysteine. The 11S globulins have hexameric quaternary structures. Each subunit of legumin is composed of two polypeptide chains. The larger α chain is hydrophilic and the smaller β chain is more hydrophobic (Duranti, 2006).

Grain legumes are a good source of phenolic compounds. Phenolic compounds are defined as natural metabolites arising biogenetically from either the Shikimate pathway or phenylpropanoid pathway (Saltveit, 2010). In grain legumes, phenolic acids and flavonoids are the dominant phenolic compounds. Most of the phenolic compounds are concentrated in the seed coat more than the cotyledon. The type of individual phenolic compounds depends on variety and agronomic conditions of grain legume growth (Singh, Singh, Kaur, & Singh, 2017).

2.2 Vigna bean species

Vigna species belong to Leguminosae family. More than twenty Vigna species are found in tropical regions and closely related to Phaseolus species. Some previously placed in Phaseolus are renamed Vigna. In many developing countries, vigna bean species such as mung beans (*Vigna radiata* (L.) Wilczek), black beans (*Vigna mungo* (L.) Hepper), adzuki beans (*Vigna angularis* (Willd.) Ohwi & Ohashi), rice beans (*Vigna umbellata* (Thunb. Ohwi & Ohashi)), cowpeas (*Vigna unguiculata* (L.) Walp.), bambara groundnuts (*Vigna subterra*nea (L.) Verdn.), and mat beans (*Vigna aconitifolia* (Jacq.) Marechal) are importance in financial system as dietary staples.

2.2.1 Vigna bean and its composition

Vigna bean have a one of the most plant-based diets for humans because it is a rich source of carbohydrate (41-62 %), protein (15-29 %), fat (1-7 %) and other compounds such as vitamin, mineral and phenolic compounds. The compositions of some Vigna beans are shown in Table 2.1. The varieties of Vigna bean influence on composition in the seed as described in Table 2.1.

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Table 2. 1 Carbohydrate, protein and fat of some Vigna beans

	Carbohydrate %	**Protein %**	**Fat %**	**Reference**
Mung bean (*Vigna radiata*)	54.9 - 58.9	20.8-23.7	5.63-7.24	(Shi, Yao, Zhu, & Ren, 2016)
	40.6% - 48.9	20.00–24.27	1.9-2.2	(Abbas & Shah, 2007)
Black bean (*Vigna mungo*)	54.81-58.13	25.07-28.60	5.13-6.22	(Zia-Ul-Haq et al., 2014)
Adzuki bean (*Vigna angularis*)	50.18	21.68	-	(Katoch, 2013)
Rice bean (*Vigna umbellata*)	52.23–55.65	23.17–25.57	1.92–3.42	(Sritongtae, Sangsukiam, Morgan, & Duangmal, 2017)
Cowpea (*Vigna unguiculata*)	53.3	25.9	2	(Frota, Lopes, Silva, & Arêas, 2017)
Bambara groundnut (*Vigna subterranea*)	51.6 - 61.9	15.2 - 22.2	2.47 - 6.99	(Olaleye, Adeyeye, & Adesina, 2013)

2.2.2 Cooking process on Vigna beans

Food processing plays an important role in modifying the compact proteolysis-resistant structures of grain legumes (Verma, Kumar, Das, & Dwivedi, 2012). The nutritional quality and health benefits of Vigna beans are influenced by the preparation and cooking process. Normally, legumes are usually cooked before consumption. After preparation and cooking process, bean products have lower amount of antinutrients and higher nutritional value (Khandelwal, Udipi, & Ghugre, 2010).

Mubarak (2005) reported that the amount of raffinose and stachyose after food processing (germination, boiling, autoclaving or microwave cooking) was significantly lower than those in raw seeds. Moreover, all processes can improve *in vitro* protein digestibility. Protein digestibility of mung bean after *in vitro* digestion was significantly higher after autoclaving compared to raw seeds. The increasing in protein digestibility may be due to protein denaturation by thermal process. Rehman and Shah (2005) investigated the effect of autoclaving conditions on protein and

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starch digestibility in red kidney beans, black grams, chickpeas, lentils, white kidney beans, and red kidney beans. They found that autoclave condition at 121 °C for 10 minutes provided higher protein and starch digestibility compared to raw seed and boiling. Torres, Rutherfurd, Muñoz, Peters, and Montoya (2016) reported that protein digestion of *Canavalia brasiliensis*, *Lablab purpureus* and *Vigna unguiculate* increased around 31 % to 78 % after soaking and autoclaving at 121 °C for 5 min.

Raw legumes are reported to have low protein digestibility, and presence of antinutrients. In order to decrease antinutrients and increase protein digestibility, legumes are usually cooked before consumption. Heating can destruct or inactivate heat-labile antinutrients (Mubarak, 2005). In addition, soaking, dehulling or germination can also reduce antinutrients such as trypsin inhibitors or polyphenols (Torres et al., 2016).

Processing, such as heating, soaking, dehulling or germination, can reduce the presence of, or inactivate, some anti-nutritional factors such as trypsin inhibitors or polyphenols and are therefore commonly used to improve the nutritional value and palatability of legumes (Torres et al., 2016). Many researchers documented protein digestibility of different species and varieties of legumes in *in vitro* and *in vivo* studies after treatment process. All results indicated that treatment process such as boiling, autoclaving, microwave cooking and germination can be improved protein digestibility of all legume varieties.

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Cooking is not only modifying the compact proteolysis-resistant structures of legume seed but also releasing the bound phenolics from different matrix, resulting in increasing phenolic content. Heating also leads to changes in phenolic structure and its biological and antioxidant activity to a certain degree. After compact proteolysis-resistant structures of bean are modified by heat, proteins are easier to be digested by proteolytic enzymes. Endopeptidase cleaves the peptide linkage in the primary sequence of a protein into small peptides of various size and free amino acids. Several studies show that peptides and some free amino acids possess an antioxidant activity. However, the antioxidant activity of hydrolysate depends on composition and sequence of amino acids in peptides (Luna-Vital, Mojica, de Mejía, Mendoza, & Loarca-Piña, 2015; Sarmadi & Ismail, 2010)

2.2.3 Vigna bean and its bioactive properties

The bioactive peptides obtained from Vigna beans also exhibit bioactive properties such as angiotensin I-converting enzyme (ACE) inhibitors, antioxidant activities and anticancer. Mamilla and Mishra (2017) reported that ACE inhibitory activity increased from 25.33% (IC_{50}= 0.14 mg/mL) in non-germinated mung bean extract to 82.34% (IC_{50}=0.03 mg/mL) after 5 day of germination.

Wongsiri, Ohshima, and Duangmal (2015) studied the effect of a short germination period on antioxidant activity of germinated mung bean. They reported that the germinated mung beans exhibited higher DPPH radical scavenging activity and FRAP than non-germinated mung beans. The increasing antioxidant activity may be due to an increase in phenolics content. Luo, Cai, Wu, and Xu (2016) reported that the seed coat of mung beans and adzuki beans had the highest amount of total phenolic content followed by the whole bean and cotyledon. It have been reported that phenolic compounds in grain legume exhibit several biologically activities such as protection against oxidative stresses and degenerative diseases. Mamilla and Mishra (2017) investigated phytochemicals and their antioxidant activity of germinated mung beans. They reported that germination of mung bean caused an increase in phenolic compounds such as vanillic acid, caffeic acid, *p*-coumaric acid and ferulic. Type and amount of phenolic compounds in beans related to DPPH radical scavenging activity. Sritongtae et al. (2017) studied effect of germination period and acid pretreatment on antioxidant activity of rice beans. They reported that both germination period and citric acid pretreatment affected an increase in total phenolic content, DPPH radical scavenging activity and FRAP of rice beans. Catechin and rutin were found as major phenolic compounds in rice bean sprouts.

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2.2.4 Vigna bean hydrolysate

In order to improve the functional properties or produce biologically-active peptides, dietary proteins are usually hydrolysed by proteolytic enzymes. In addition, enzymatic hydrolysis affects the releasing of phenolic compounds, improving the overall antioxidant activity. This is because the protein-bound polyphenols are released into free form during hydrolysis (Sangsukiam & Duangmal, 2017). Thus,

specific peptides and increasing phenolic content can be obtained via enzymatic modification of cooked beans under the controlled hydrolysis condition. The enzymatic hydrolysate has been reported to improve the antioxidant activity based on degree of hydrolysis (DH) which varies depending on enzyme specificity, hydrolysis conditions and extent of proteolysis (Panyam & Kilara, 1996). Therefore, hydrolysates have been used to improve the digestion and absorption of poorly digestible protein sources, but more recently have also been cited as a potent method to produce bioactive peptides exhibiting antihypertensive and antioxidative activity.

Nowadays, short-chain peptides with specified molecular size are highly desired for specific applications as it possesses biological activities. Recently, research information on bioactive peptides obtained from legumes possess anticarcinogenic, hypocholesterolemic, angiotensin converting enzyme (ACE)-inhibitory activity, antioxidant activity and antimicrobial activity has been reported. Some researchers have shown the biological activity of predominant phenolics present in beans. G. H. Li, Wan, Le, and Shi (2006) hydrolysed defatted mung bean protein isolate with 4% alcalase at 55 ^{o}C, pH 8, for 2 hours. The obtained protein hydrolysate was passed through a 6 kDa cut off ultrafiltration membrane to yield purified nine individual fractions. The fraction containing the highest ACE inhibitory activity was subjected to RP-HPLC with a peptide C18 column. The purified ACE inhibitory peptide sequences were analysed by MALDI-TOF MS/MS. The intense fragment ions were observed at m/z 439.33 of peptide Val-Thr-Pro-Ala-Leu-Arg, at m/z 605.44 of peptide Lys-Lue-Pro-Ala-Gly-Thr-Leu-Phe and at m/z 694.35 of peptide Lys-Asp-Tyr-Arg-Leu with the IC_{50} value of 82.4 μM, 26.5 μM and 13.4 μM. Rui, Boye, Simpson, and Prasher (2013) investigated ACE-I inhibitory activity of small red bean protein hydrolysate under *in vitro* gastrointestinal digestion. Small red beans protein isolate was hydrolysed by alcalase (50 ^{o}C at pH 7 for 90 minutes) and papain (60 ^{o}C at pH 6.5 for 90 minutes). The obtained protein hydrolysate was subjected to *in vitro* gastrointestinal digestion model. The permeate fraction, smaller than 5 kDa, exhibited a significantly higher ACE-I inhibitory activity than the retentate fraction. The highest ACE-I inhibitory activity was further characterized through Mass spectrometric. The peptide was identified as Pro-Val-Asn-Asn-Pro-Glu-Ile-His which demonstrated an IC_{50} value of 206.7 μM.

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2.3 Adzuki bean

Adzuki bean (*Vigna angularis*) is one of popular traditional dessert recipes in Asia. Adzuki beans are a decent source of protein and phenolic compounds. Globulins, vicilin and legumin, are the major storage protein in adzuki bean. The total globulin fractions include 80% of globulin and about 10% legumin (Durak, Baraniak, Jakubczyk, & Świeca, 2013). Most of polyphenols are found in adzuki bean seed coat. Catechin glycosides, quercetin glycosides, myricetin 3-rhamnoside, anthocyanin, and procyanidin dimers are dominantly polyphenols found in adzuki beans seed coat (Lin & Lai, 2006). Moreover, some phenolic compounds identified in adzuki bean included caffeic acid, chlorogenic acid, catechin, epicatechin glucosides, *p*-coumaric acid, ferulic acid and sinapic acid (Amarowicz, Estrella, Hernandez, & TROSZYŃSKA, 2008; Yao, Cheng, Wang, Wang, & Ren, 2011).

Recently, adzuki bean has been acclaimed as being good plant-based diet for functional components and health benefits. This consideration can be deduced from the reports on adzuki bean extract-dependent suppression of obesity, blood pressure, cholesterol and hyperglycaemia. The presence of bioactive peptide, bioactive oligosaccharide and phenolic compounds in adzuki bean are appeared to contribute to the health beneficial effects.

2.3.1 Potential health benefits and antioxidant activity

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The extruded adzuki bean protein was developed as functional food product and investigated for the α-glucosidase inhibitory activity under *in vivo* mice model for 42 days . The consumption of extruded adzuki bean protein resulted in a decrease in blood glucose concentration compared to control group. Further studies showed that the extracted phenolic compounds obtaining from adzuki beans possessed antioxidant and immune-regulatory activities (Yang Yao, Xue, Zhu, Gao, & Ren, 2015).

Durak et al. (2013) isolated four protein fractions (albumin, globulin, prolamin and glutelin) from adzuki bean and hydrolysed each protein fraction using pepsin. They reported that released biologically active peptides showed antiradical activity, ACE inhibitory activity and chelation of iron and copper ions. Phenolic

compound from adzuki bean extract powder was determined antioxidant activities. The results indicated that adzuki bean extract powder contained proanthocyanidins and anthocyanins. These compounds exhibited radical-scavenging and antioxidant activity (Han et al., 2015). Sangsukiam and Duangmal (2017) studied the impact of germination time on antioxidant activity of adzuki bean sprouts hydrolysate. 27-germinated beans and non-germinated beans were hydrolysed with 7% Flavourzyme at 50 ^{o}C for 6 hours pH6. The hydrolysate was subjected to free amino groups, total phenolic content, and antioxidant activity determination. They reported that gallic acid, *p*-coumaric acid, vitexin, catechin, and rutin were found in both adzuki bean hydrolysate. Adzuki bean hydrolysate provided higher DPPH radical scavenging activity and FRAP value than non-germinated adzuki bean hydrolysate. The result indicated that bean hydrolysate containing free amino groups, antioxidant amino acids, and phenolic compounds exhibited both hydrogen-donating and hydrogen-reducing activities.

Nowadays, beans hydrolysate is mainly used in an extensive variety of food application to improve their functional properties in liquid foods (Clemente et al., 1999) and meat product (Cumby, Zhong, Naczk, & Shahidi, 2008). In addition, they have been decided as functional foods that show health-promoting antioxidant activities (Betancur-Ancona, Sosa-Espinoza, Ruiz-Ruiz, Segura-Campos, & Chel-Guerrero, 2014). Health-promoting biological activity and antioxidant activity of beans could be verified after consumption. The study on *in vitro* simulation of gastrointestinal food digestion could prove this hypothesis. Moreover, *in vitro* gastrointestinal of the hydrolysate in model system could explain more for the application of bean hydrolysate in foods.

The above review shows production of hydrolysate containing both phenolics and bioactive peptides may result in a better antioxidant activity and health-promoting biological activity due to the synergistic effects.

2.4 Alginate-based particles

The benefit gained from the process of confining bioactive compounds depends on the potential to conserve the stability/activity of these compounds. Drying is a simple technique for encapsulation of bioactive compounds. Spray-drying and freeze-drying techniques are common approach, which can increase product stability during storage. Nevertheless, these techniques can cause degradation of sensitive compounds, including bioactive peptides, phenolic compounds, essential oils, vitamins and minerals due to high temperature or long processing time (Balanč et al., 2016). One of effective approaches to preserve biological activity of bioactive compounds is to incorporate them into a polymer matrix for extending shelf life and controlling the release of bioactive compounds. The materials used in the encapsulation should be food grade, low toxicity, low hygroscopicity, friendly environment as well as capability of forming a barrier between the internal and the external phase. The potential benefits of biopolymer beads are to protect and to transport bioactive compounds to target sites and to maintain the stability of bioactive compounds during processing and storage.

Biopolymer hydrogels are polymeric networks with 3-dimensional configuration capable of imbibing high amount of water or fluids. These networks can be physically or chemically cross-linked in order to produce hydrogel (Kopeček & Yang, 2012). Its unique properties offer great potential for the utilization of hydrogels in applications for cosmetics and pharmaceuticals (Caló & Khutoryanskiy, 2015). Various methods have been employed in order to prepare spherical hydrogel beads with encapsulated bioactive compounds.

Alginates are extensively used in the food, cosmetic and pharmaceutical industries and have been apply as a wall material for the entrapment of bioactive compounds. Alginate has several unique properties that have enabled it to be used as a matrix for entrapment and delivery bioactive compounds. Alginates are a naturally appearing an anionic polymerization from brown seaweed or bacterial fermentation. Alginates are comprised of two different monosaccharaides as guluronic acid and mannuronic acid. These two monosaccharaides are linearly linked together with glycosidic bonds. Alginate polymer segments have at three different forms as

polyguluronic acid, polymannuronic acid or combination. Alginate has several unique properties such as biocompatibility, non-toxicity, low cost and mild gel formation. These properties are appropriate wall matrix for entrapment and control-released bioactive compounds. The alginate gel-forming occurs under mild condition encapsulation process. The divalent cations form bonding between G-blocks with yield a three-dimensional network. An egg box model has been proposed to explain the relationship between polyguluronic acid as the box and the cation as the egg. Calcium ion is well known for its ability to from junction zone in alginate solution. The formation of these zones requires at least two guluronic acid residues to be sequential in the chain. The formation of cross-linked alginate gel matrices consists of two steps are external gelation and internal gelation. The bioactive compounds were added into alginate solution and it is delivered to the cross-linking solution as extruded or atomized droplets as called external step. For internal step, alginate solution was loaded and dropped from a droplet generator into a gelling collection as well as $CaCl_2$ solution to form capsules/beads (Ching, Bansal, & Bhandari, 2017; Tsai, Chiang, Kitamura, Kokawa, & Islam, 2017; Wong, 2011). The high diffusion rates and biodegradation of obtained capsules/beads under normal physiological as a result of high gel porosity on alginate gel. The encapsulation conditions caused bioactive compounds release from alginate capsules/beads. Thus, encapsulation conditions, including alginate ratio, divalent cations concentration, bioactive compound content as well as other factors were subsequently studied (Lee & Mooney, 2012).

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Chan, Yim, Phan, Mansa, and Ravindra (2010) studied the effect of alginate M/G ratio (high-guluronic acid and high-mannuronic acid), alginate concentration (2, 3, 4 and 5 %, w/v) and extract concentration (0.25, 0.50, 0.75 and 1 w/w) on encapsulation efficiency and chemical stability of Ca-alginate beads. The higher encapsulation efficiency was found in high-mannuronic acid beads than other conditions. This was because alginate gel containing high-mannuronic acid could form well-constructed network through calcium ions coordination referred to egg-box model. The results also indicated that alginate concentration, extract concentration and beads size did not showed clear effect on overall encapsulation efficiency. Cho, Chun, Kim, and Park

(2014) investigated alginate concentration (0.5 and 1%, w/v) and $CaCl_2$ concentration (0.5 and 1 M) on encapsulation efficiency and chemical stability of Ca-alginate beads. The increasing concentration of alginate and $CaCl_2$ led to smaller microspheres and increasing encapsulation efficiency from 96.33 to 98.49 % and 95.18 to 97.29 % at 0.5 and 1% alginate concentration, respectively. New FTIR spectra peaks were observed at 2157 and 3218 cm^{-1} with increasing concentration of alginate and $CaCl_2$ indicating the crosslinking reaction between alginate and $CaCl_2$. Pasukamonset, Kwon, and Adisakwattana (2016) revealed that the concentration of calcium chloride and blue pea extract had a strong impact on Ca-alginate matrix formation as they could fulfill cavities in Ca-alginate matrix resulting in smooth surface morphology. Polyphenol degradation could be delayed and biological activity was increased after gastrointestinal digestion.

Particles, another form of hydrogel, are also formed and used for the entrapment of bioactive compounds. With this technique, the solution of bioactive compounds is mixed with the solution of alginate, inulin or other soluble polysaccharides. The solution is then dried and bioactive compounds are entrapped in the matrix (Ozkan, Franco, De Marco, Xiao, & Capanoglu, 2019).

2.5 *In vitro* simulating gastrointestinal (GI) digestion model

In vitro simulating GI digestion model is widely applied for studying gastrointestinal behaviour of foods. Although human nutritional studies are still "the best standard" for addressing diets related to health-promoting biological activity, *In vitro* method offers the advantages of being more rapid with less expensive and do not involve ethical restrictions.

2.5.1 Passage through the gastrointestinal tract

The gastrointestinal tract consists of digestion, absorption and excretion. *In vitro* gastrointestinal digestion model is reconstructed digestion system, starting from oral to anus, and nutrient absorption. Each part of gastrointestinal tract has specific functionality. The function of mouth is for mastication and mixing with saliva. Then, food is subjected though the oesophagus to stomach. In the stomach, masticated food

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is hydrolysed by protease and lipase at low pH. The chime is transported to the small intestine and neutralised by bicarbonate. The digestive juice in stomach includes trypsin, chymotrypsin and bile acid. The final of dietary carbohydrates and proteins digestion appear right on the small intestinal enterocytes by brush boarder enzyme, including maltase, sucrose-isomaltase, lactase and peptidase. The final stage, nutrients are predominantly absorbed by the enterocytes of the jejunum (Cheng et al., 2010). The simulating GI digestion model typically include the oral, gastric and small intestine phase. These methods try to mimic physiological condition of digestive system in human via using similar digestive enzymes under the specified conditions of pH, digestion time and salt concentrations.

The method of Minekus et al. (2014) was developed by members of the EU Cost Action INFOGEST, an international network joined by over 200 scientists from over 30 different countries. This method was set of conditions that are close to the physiological situation. The method included three different phases: oral, gastric and intestinal digestion. Simulated fluid in each phase was combined of electrolytes, enzymes, $CaCl_2$ and water based on human *in vivo* data. Liquid food can be subjected to the oral phase (optional) or directly to gastric phase. In oral phase, the solid samples were minced and mixed with Simulated Salivary Fluid (SSF) at the ratio of 50:50 (w/v). In the final mixture of oral phase, human salivary alpha-amylase was added to achieve 75 U/mL, followed by $CaCl_2$ concentration achieved 0.75 mM and incubated at 37 ^{o}C for 2 minutes. To start the gastric phase, porcine pepsin (EC 3.4.23.1) was adjusted to achieve a 2000 U/mL activity in the final mixture, followed by $CaCl_2$ to achieve 0.075 mM and HCl 1 N to adjust the pH to 3. The samples were then incubated at 37 °C for 2 h. Finally, the gastric phase was mixed with the simulated intestinal fluid at the ratio of 50:50 (v/v), which consisted of an electrolyte solution (eSIF), pancreatin (100 U/mL trypsin activity in the final mixture) and NaOH 1 N to adjust the pH to 7. The samples were then incubated at 37 °C for 2 h. At the end of the intestinal digestion, the digested samples were centrifuged and the supernatant was collected for further measurements.

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CHAPTER III
MATERIALS AND METHODS

3.1 Materials and Equipment

3.1.1 Raw materials

Adzuki bean seeds (*Vigna angularis*), bought in the same lot, were purchased from Royal Project Foundation (Bangkok, Thailand) in the harvest year of 2018.

3.1.2 Enzymes

α-Amylase from *Bacillus amyloliquefaciens* (EC 3.2.1.1), CAS No.: 9000-85-5, (Sigma, Müllheim, Germany)

α-Amylase from human saliva (EC 3.2.1.1), CAS No.: 9000-90-2, (Sigma, Müllheim, Germany)

Angiotensin Converting Enzyme from rabbit lung (EC 3.4.15.1), CAS No.: 9015-82-1, (Sigma, Müllheim, Germany)

Chymotrypsin (EC 3.4.21.1), CAS No.: 9004-07-3, (Sigma, Müllheim, Germany)

Pancreatic lipase (EC 3.1.1.3), CAS No.: 9001-62-1, (Sigma, Müllheim, Germany)

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Pepsin from porcine gastric mucosa (Porcine pepsin) (EC 3.4.23.1), CAS No.: 9001-75-6, (Sigma, Müllheim, Germany)

Protease from *Aspergillus oryzae* or Flavourzyme 500L (EC 3.4.11.1), Product no.: P6110 (Sigma, Müllheim, Germany)

Trypsin from porcine pancreas (EC 3.4.21.4), CAS No.: 9002-07-7, (Sigma, Müllheim, Germany)

3.1.3 Chemicals

Chemical	Grade	Company	Location
AccQ.fluor™ reagent kit	HPLC grade	Waters	MA, USA
Acetonitrile	HPLC grade	Waters	MA, USA
Amino acid standard hydrolysate	HPLC grade	Thermo scientific	MA, USA
Aluminum chloride	A.R. grade	Sigma	Mülheim, Germany
Ammonium molybdate	A.R. grade	Univar	NSW, Australia
2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH)	A.R. grade	Acros Organics	NJ, USA
Boric acid	A.R. grade	Univar	NSW, Australia
Bromocresol green	A.R. grade	Carlo Erba	Val de Reuil, France
Copper (II) sulfate pentahydrate	A.R. grade	Univar	NSW, Australia
Fluorescein sodium salt	A.R. grade	Sigma	Müllheim, Germany
Folin-Ciocalteu phenol reagent	A.R. grade	Sigma	Müllheim, Germany
N-Hippuryl-His-Leu hydrate	A.R. grade	Sigma	Müllheim, Germany
Hydrochloric acid	A.R. grade	J.T. Baker	PA, USA
L-leucine	A.R. grade	Merck	Darmstadt, Germany
β-mercaptoethanol	A.R. grade	Merck	Darmstadt, Germany
Methyl red	A.R. grade	Merck	Darmstadt, Germany
Polyvinylpyrrolidone (PVPP)	A.R. grade	Acros Organics	NJ, USA
2,4,6-tripyridyl-s-triazine (TPTZ)	A.R. grade	Sigma	Mülheim, Germany
Selenium reagent mixture	A.R. grade	Merck	Darmstadt, Germany
Sodium carbonate	A.R. grade	Univar	NSW, Australia
Sodium dihydrogen orthophosphate	A.R. grade	Univar	NSW, Australia
Sodium hydroxide	A.R. grade	Univar	NSW, Australia
di-sodium hydrogen orthophosphate	A.R. grade	Univar	NSW, Australia

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Sulphuric acid 98%	A.R. grade	J.T. Baker	PA, USA
L-tyrosine	A.R. grade	Univar	NSW, Australia
Trichloroacetic acid	A.R. grade	Carlo Erba	Val de Reuil, France
2,4,6-trinitrobenzenesulfonic acid	A.R. grade	Sigma	Müllheim, Germany

3.1.4 Equipment

Electronic balance (2 digit) (Sartorius model BP 310s, Göttingen, Germany)

Electronic balance (4 digit) (Sartorius model BSA 2245, Göttingen, Germany)

High Performance Liquid Chromatography (HPLC) system for amino acid analysis (Waters, MA, USA)

- Auto sampler (Waters model 717, MA, USA)
- Waters AccQ.Tag amino acid analysis column 5 µm, 3.9 mm × 150 mm (Waters, MA, USA)
- UV/VIS detector (Waters model 2487, MA, USA)
- Pump (Waters 515 HPLC pump, MA, USA)

Microplate reader (Synergy HT multi-detection microplate reader, Bio Tek Instruments, Inc., Winooski, VT).

Protein and nitrogen analyser

- Digestion unit (BUCHI model K-424, Flawil, Switzerland)
- Scrubber unit (BUCHI model B-414, Flawil, Switzerland)
- Distillation unit (BUCHI model B-324, Flawil, Switzerland)

Refrigerated centrifuge (Universal 32 centrifuge, DJB Labcare Ltd., Buckinghamshire, UK)

Spectrophotometer (Genesys 20 spectrophotometer 4001/4, Thermo Fisher Scientific, MA, USA)

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Part A: Effect of different cooking conditions on properties of cooked adzuki beans and its cooking water

3.2 Effect of cooking conditions on amino acids and protein in cooked adzuki bean and its cooking water

3.2.1 Sample preparation

Adzuki bean seeds were soaked in distilled water at the ratio of 1:10 at 30 ± 2 °C for 6 hours. After being drained, soaked adzuki beans were separately subjected to three different cooking methods: boiling for 60 minutes and autoclaving at 121 °C for 10 and 60 minutes, respectively. Cooking conditions were selected based on preliminary results. Cooked beans, starch granule with no birefringence, obtaining from 60-min boiling and 10-min autoclaving condition were similar in texture. 60-min autoclaving condition was selected as the extreme cooking condition.

Drained cooked adzuki beans were ground using a Waring blender at high speed for 2 minutes before further analysis. Its cooking water was also analysed. Raw adzuki seeds and 6-hour soaked adzuki beans was set as the reference and the control, respectively. All cooking conditions are summarized in Table 3.1.

Table 3. 1 The cooking conditions in this experiment

	Condition
Reference	Raw adzuki bean seeds
Control	Soaking for 6 hours (ratio of raw seeds: water = 1:10 w/w)
60-min boiling	Boiling at atmospheric pressure for 60 minutes (ratio of soaked beans: water = 1:5 w/w)
10-min autoclaving	Autoclaving for 10 minutes (ratio of soaked beans: water = 1:5 w/w)
60-min autoclaving	Autoclaving for 60 minutes (ratio of soaked beans: water = 1:5 w/w)

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3.2.2 Chemical analysis

Cooked adzuki beans and its cooking water from three different cooking conditions were analysed as follows:

3.2.2.1 Moisture content

Moisture content of the sample was determined by drying the sample in an oven at 105 °C to constant weight (AOAC, 2019).

3.2.2.2 Crude protein content

Nitrogen content of cooked adzuki beans and its cooking water were analysed using Kjeldahl method (AOAC, 2019). Crude protein was calculated by multiplying the percent Kjeldahl nitrogen by the factor 6.25 and expressed as mg tyrosine per 30 grams raw seeds (wb.) and mg tyrosine per 100 gram cooked portion (wb.).

3.2.2.3 TCA-soluble peptide analysis

The samples were prepared according to the method of Sritongtae et al. (2017). Three grams of ground cooked adzuki beans or cooking water was mixed with twenty-seven melilites of 5% (w/v) trichloroacetic acid. The suspension was continuously stirred for 2 minutes and allowed to stand in ice bath for 1 hour. After centrifugation at 10,000 *g* for 10 min, the obtained supernatant was filtered through filter paper (Whatman no.1). The obtained supernatant was determined according to the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) stated in Appendix A.1. TCA-soluble peptides content was expressed as mg tyrosine per 30 grams raw seeds (wb.) and mg tyrosine per 100 gram cooked portion (wb.).

3.2.2.4 Free amino group analysis

Cooked adzuki beans were prepared as described above in TCA-soluble peptide analysis. Free amino group content of the sample was determined using the reaction with trinitrobenzenesulphonic acid (TNBS) (Adler-Nissen, 1979) with slightly modified by Sangsukiam and Duangmal (2017). The results were

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expressed as mg leucine per 30 grams raw seeds (wb.) and mg leucine per 100 gram cooked portion (wb.), as stated in Appendix A.2.

3.2.3 Amino acid profile of cooked adzuki beans and their chemical scores

3.2.3.1 Amino acid profile determination

Cooked beans obtaining three different cooking conditions were each hydrolysed with 6 M HCl in a hydrolysis tube at 110 ºC for 24 hours under a nitrogen atmosphere. The obtained aliquot was derivatized with AccQ. Tag solution at 55 ºC. Amino acid profile of cooked adzuki bean was performed according the operation of Wongsiri et al. (2015). The high performance liquid chromatography (HPLC) system (Waters) consisted of a Waters AccQ Tag amino acid analysis column (3.9 mm i.d. × 150 mm) set at 37 ºC, preceded by a UV detector (Waters Dual 2487 Absorbance Detector) set at 254 nm. The external amino acids standard ranging from 10 to 200 *p*mol (Amino Acid Standard H, MA, USA) and the amino acid profile of cooked adzuki bean were identified and integrated by Empower software (Waters, MA, USA). Each amino acid was expressed as mg amino acid per 30 grams raw seeds (wb.).

Alkali hydrolysis condition: to determine the amount of tryptophan, cooked adzuki beans were hydrolysed with 4.5 M NaOH at 110 ºC for 19 hours under a nitrogen atmosphere. The derivatized amino acids, using *o*-phthalaldehyde reagent, were injected to Shim-pack ISC-07/S 1504 Na column equipped with a fluorescence detector (RF-20A/RF- 20A, Shimadzu) set at 55 ºC. The operation was performed using an Amino Acid Analyzer (LC-6A, Shimadzu, Kyoto, Japan). Each amino acid was expressed as mg amino acid per 30 grams raw seeds (wb.).

3.2.3.2 Chemical scores calculation

The amount of each essential amino acid in cooked beans from different cooking conditions was calculated in term of chemical score. Essential amino acids of whole egg, cow's milk and soybeans were used as the reference protein for calculation. The results were obtained using the Eq (1):

$$\text{Chemical score} = \frac{\text{mg of essential amino acid in sample}}{\text{mg same essential amino acid in refferent protein}} \times 100 \qquad \text{Eq (1)}$$

3. 3 Cooked adzuki beans and its cooking water: biological activity and compositions

3.3.1 Borate buffer-soluble fraction

Ground cooked beans and its cooking water from different cooking conditions were prepared as detailed in Section 3.2.1. To prepare borate buffer-soluble fraction, cooked beans or cooking water was blended with 20 mM borate buffer, pH 9, containing 2 mM β-mercaptoethanol at cooked beans or cooking water to borate buffer at ratio of 1:10 (w/v). Each sample mixture was incubated at 30 ^{o}C in a water bath with continuous shaking for 2 hours. The sample mixture was then centrifuged at 10,000 × g for 30 minutes. The borate buffer-soluble fraction, supernatant, was determined as follows:

3.3.1.1 Determination of antioxidant, ORAC and ACE-I inhibitory activity of borate buffer-soluble fraction

3.3.1.1.1 Antioxidant activity

Determination of ABTS radical scavenging activity was made according to the procedure of Re et al. (1999) modified by Almeida et al. (2011) as stated in Appendix A.3. ABTS radical scavenging activity was expressed as mmol trolox equivalent per 30 grams raw seeds (wb.).

Ferric reducing antioxidant power (FRAP) assay was performed using Sritongtae et al. (2017) as stated in Appendix A.4. The results were expressed as mmol trolox equivalent per 30 grams raw seeds (wb.).

Metal chelating activity on ferrous (Fe^{2+}) was measured using the method of Jamdar et al. (2010) as stated in Appendix A.5. Ferrous chelating activity was expressed as mmol EDTA equivalent per 30 grams raw seeds (wb.).

3.3.1.1.2 Oxygen radical absorbance capacity (ORAC)

ORAC assay was performed using Alashi et al. (2014) as stated in Appendix A.6. The results were expressed as mmol trolox equivalent per 30 grams raw seeds (wb.).

3.3.1.1.3 ACE-I inhibitory activity

The ACE-I inhibitory activity of borate buffer (pH 9) soluble fraction was analysed as illustrated in Hayakari, Kondo, and Izumi (1978) with a slight modification as stated in Appendix A.7. ACE-I inhibitory activity was expressed as percentage of ACE inhibitory activity. Captopril, synthetic medicine, was set as the positive control.

3.3.1.2 Determination of TCA-soluble peptides, free amino group, total phenolic compounds, total flavonoid and reducing sugar of borate buffer-soluble fraction

TCA-soluble peptides content of borate buffer-soluble fraction was determined using Lowry method as stated in Section 3.2.2.3. Data were shown as mg tyrosine equivalent per 30 grams raw seeds (wb.).

Free amino group content of borate buffer-soluble fraction was determined using the reaction with TNBS as stated in Section 3.2.2.4. The results were expressed as mg leucine equivalent per 30 grams raw seeds (wb.).

Total phenolic content (TPC) of borate buffer-soluble fraction using Folin–Ciocalteu method (Waterhouse, 2005) as stated in Appendix A.8. TPC were expressed as mg gallic acid equivalent per 30 grams raw seeds (wb.).

Total flavonoid content (TFC) of borate buffer-soluble fraction was determined based on an aluminum chloride complex (Zhishen, Mengcheng, & Jianming, 1999) as stated in Appendix A.9. TFC were expressed as mg catechin equivalent per 30 grams raw seeds (wb.).

Reducing sugar content of borate buffer-soluble fraction using Somogyi-Nelson method according to Sangsukiam and Duangmal (2017) as stated in

Appendix A.10. Reducing sugar content were expressed as mg glucose equivalent per 30 grams raw seeds (wb.).

3.3.2 Borate buffer-soluble fraction with phenolic removal of cooked adzuki beans and its cooking water

The borate buffer-soluble fraction obtaining from the supernatant in Section 3.3.1 was mixed with 5% PVPP (w/v), in order to remove phenolic compounds (Karppinen, Taulavuori, & Hohtola, 2010), in water bath shaking with continuous at ambient temperature for 30 minutes. The mixture was centrifuged at 10,000 × *g* for 10 minutes, then supernatant was filtered through filter paper (Whatman no.1). PVPP at 5% (w/v) was then mixed to the obtained supernatant. After shaking for 30 minutes, the suspension was then centrifuged. Supernatants were combined and subjected to analysed as follows:

3.3.2.1 Determination of antioxidant, ORAC and ACE-I inhibitory activity of borate buffer-soluble fraction with phenolic removal

3.3.2.1.1 Antioxidant activity

ABTS radical scavenging activity and FRAP of borate buffer-soluble fraction with phenolic removal was stated in Section 3.3.1.1.1 The results were presented as mmol trolox equivalent per 30 grams raw seeds (wb.).

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Ferrous chelating activity of borate buffer-soluble fraction with phenolic removal was stated in Section 3.3.1.1.1 The results were expressed as mmol EDTA equivalent per 30 grams raw seeds (wb.).

3.3.2.1.2 ORAC

ORAC of borate buffer-soluble fraction with phenolic removal was stated in Section 3.3.1.1.2. The results were expressed as mg trolox equivalent per 30 grams raw seeds (wb.).

3.2.2.1.3 ACE-I inhibitory activity

ACE-I inhibitory activity of borate buffer-soluble fraction with phenolic removal was stated in Section 3.3.1.1.3. The results were expressed as percentage of ACE-I inhibitory activity.

3.3.2.2 Determination of TCA-soluble peptides, free amino group, total phenolic compounds, total flavonoid and reducing sugar of borate buffer-soluble fraction with phenolic removal

TCA-soluble peptides content, free amino group content, TPC, TFC, and reducing sugar in borate buffer-soluble fraction with phenolic removal were measured as stated in Section 3.2.2.3. The data were calculated based on 30 grams raw seeds (wb.).

3.3.3 Phenolic fraction of cooked adzuki beans and its cooking water

The pellets obtaining from PVPP precipitation process were extracted with 80% methanol in a shaking water bath with continuous at ambient temperature for 2 hours. All suspensions were centrifuged at 10,000 × *g* for 10 minutes. The pellet from cooking water was extracted in the same manner. The phenolic fraction was analysed as follows:

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3.3.3.1 Determination of antioxidant, ORAC and ACE-I inhibitory activity of phenolic fraction

3.3.3.1.1 Antioxidant activity

ABTS radical scavenging activity and FRAP of phenolic fraction were stated in Section 3.3.1.1.1 Data were presented as mmol trolox equivalent per 30 grams raw seeds (wb.).

Ferrous chelating activity of phenolic fraction was stated in Section 3.3.1.1.1 The results were expressed as mmol EDTA equivalent per 30 grams raw seeds (wb.).

3.3.3.1.2 ORAC

ORAC of phenolic fraction was stated in Section 3.3.1.1.2. The results were expressed as mmol trolox equivalent per 30 grams raw seeds (wb.).

3.3.3.1.3 ACE-I inhibitory activity

ACE-I inhibitory activity of phenolic fraction was stated in Section 3.3.1.1.3. The data were presented as the percentage of ACE-I inhibitory activity.

3.3.3.2 Determination of TCA-soluble peptides, free amino group, total phenolic compounds, total flavonoid and reducing sugar of phenolic fraction

TCA-soluble peptides, free amino group, TPC, TFC and reducing sugar content in phenolic fraction were measured as stated in Section 3.2.2. The results were calculated based on 30 grams raw seeds (wb.).

The flow diagram for sample extract fractions obtaining from cooked adzuki beans and its cooking water and their biological activity and compositions determination shown in Figure 3.1.

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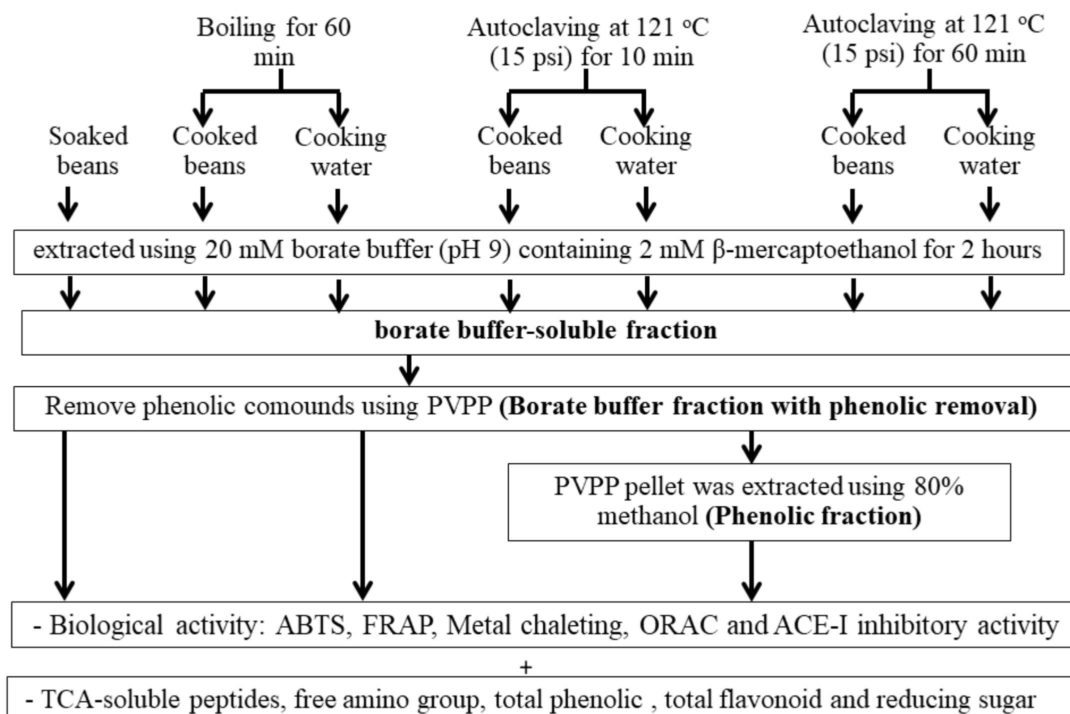

Figure 3. 1 The flow diagram for sample extract fractions obtaining from cooked adzuki beans and its cooking water and theirs biological activity and compositions determination

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3.4 Effect of cooking conditions on phenolics in cooked adzuki beans and its cooking water

Ground cooked beans and its cooking water from different cooking conditions were prepared as described above in 3.2.1. Ten grams of ground cooked adzuki bean or cooking water were combined with 95% methanol at a ratio of 1:10 (w/v) and incubated at 30 ºC in a shaking water bath for 2 hours. The obtained samples were centrifuged at 10,000 × *g* for 10 minutes and hours and filtered thought filter paper (Whatman no. 1). The methanolic extracts were collected and analysed as follows:

3.4.1 Determination of total phenolic, total flavonoid and reducing sugar content in cooked adzuki beans and its cooking water

TPC of methanolic extracts using Folin–Ciocalteu method was stated in 3.3.1.2. TPC were calculated as mg gallic acid equivalent per 30 grams raw seeds (wb.) and mg gallic acid per 100 gram cooked portion (wb.).

TFC of methanolic extracts was measured based on aluminum chloride colorimetric assay as stated in 3.3.1.2. TFC were expressed as mg catechin equivalent per 30 grams raw seeds (wb.) and mg catechin equivalents per 100 gram cooked portion (wb.).

Reducing sugar content of methanolic extracts using Somogyi-Nelson as stated in 3.3.1.2. Reducing sugar content were expressed as mg glucose equivalents per 30 grams raw seeds (wb.) and mg glucose equivalent per 100 gram cooked portion (wb.).

3.4.2 Phenolic composition and antioxidant activity of the methanolic extract from cooked adzuki beans and its cooking water

The phenolic composition of all methanolic extracts was identified using HPLC with UV detector (Waters, Dual 2487 Absorbance Detector) at 265 and 280 nm. Methanolic extracts were loaded on Luna C18 column (4.6 × 250 mm, 5 μm) and eluted with gradient elution of 0.1% formic acid (mobile phase A) and 70% methanol (mobile phase B). The gradient profile was set as follows: the initial condition started with 0% to 40% of mobile phase B over 15 minutes, then 42% of mobile phase B at 35 minutes, 55% of mobile phase B at 50 minutes, 70% of mobile phase B at 60 minutes and 100% of mobile phase B at 70 minutes. The gradient was then decreased to 0% of mobile phase B at 85 minutes. Identification of phenolic composition in the sample was done on the comparison with standard phenolic compounds (rutin hydrate, catechin, epicatechin, trans-cinnamic acid, naringenin, gallic acid, genistein, quercetin, ferulic acid, vitexin, vanillic acid, *p*-coumaric acid, sinapic acid and hydroxybenzoic acid).

Antioxidant activity (ABTS radical scavenging, FRAP and ferrous chelating activity) and ORAC of all methanolic extracts was stated in Section 3.3.1.1.1.

3.4.3 Phenolic composition in borate buffer-soluble fraction of cooked adzuki beans and its cooking water

The individual phenolic composition of borate buffer-soluble fraction, obtaining from Section 3.3.1, was descripted as stated above in Section 3.4.2. Individual phenolic content was calculated as mg equivalent per 30 grams raw seeds (wb.).

3.4.4 Phenolic composition in phenolic fraction (phenolic extract) of cooked adzuki beans and its cooking water

The individual phenolic composition of phenolic fraction or phenolic extract, obtaining from Scetion 3.3.3, was descripted as stated above in Section 3.4.2. The identified phenolic content was calculated as mg equivalent per 30 grams raw seeds (wb.).

All experiments were done in triplicates. The results were evaluated using ANOVA and mean multiple comparison was performed by Tukey’s test at the 95%confidence interval. The statistical analyses were performed using SPSS package (SPSS 17.0 for windows; SPSS Inc., Chicago, IL, USA).

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3.5. Microstructure

Microstructure of cooked beans from each cooking condition was investigated under a scanning electron microscope (JEOL, JSM-5410LV, Tokyo, Japan). The sample was covered with gold in a sputter coater (Balzers, model SCD 040, Vaduz, Liechtenstein) then observed under a scanning electron microscope at a voltage of 12 kV with 200× of magnification.

Part B: alginate-based encapsulation

3.6 Optimization of encapsulation conditions for producing Ca-alginate beads containing adzuki beans hydrolysate with high encapsulation efficiency

3.6.1 Adzuki beans hydrolysate: preparation and its properties

The cooking condition retaining high amino acids and releasing bound phenolics content obtained from Part A was used as the substrate for protein hydrolysis. Adzuki beans hydrolysate was prepared as stated in procedure of Sangsukiam and Duangmal (2017). Ground cooked adzuki beans and cooking water from the selected cooking condition were blended with 0.1 M phosphate buffer (pH 6.0) containing 0.5 g/L of $CaCl_2$ at a ratio of 1:2.5 (w/v). The addition of 1% α-amylase (BAN® 240L) into the mixture solution before heating was due to pasting of the slurry during heating. The suspension was incubated at 70 °C in a shaking water bath for 1 hour. The enzymatic hydrolysis was inactivated by heating at 85 °C for 20 minutes. Then, 7% Flavourzyme (by dry weight of substrate) was added into suspension and initiated hydrolysis by incubation at 50 ºC for 6 hours. The hydrolysis reaction was terminated by heating at 85 °C for 20 minutes. In order to remove precipitate, the suspension was centrifuged at 7,000 × g for 10 min and filtered through Whatman No.1 filter paper. The obtained hydrolysate was collected and analysed as follows:

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Free amino group content was determined using the reaction with TNBS as stated in Section 3.2.2.4. The free amino group was calculated as shown in Eq (2).

$$\text{Free amino group} = \frac{\text{Absorbance 420 nm} \times \text{mL of total reactant solution}}{\varepsilon \times 1\text{cm} \times \text{g of protein in sample}} \times 10^6 \qquad \text{Eq (2)}$$

Where ε = molar extinction coefficient (20300 M^{-1} cm^{-1})

Degree of hydrolysis (DH) of adzuki beans hydrolysate was determined based on the measurement of free amino group obtained from hydrolysis. The percentage of DH was calculated as shown in Eq (3).

$$DH = \frac{(L_t - L_0)}{(L_{max} - L_0)} \times 100 \qquad \text{Eq (3)}$$

Where L_0 = initial amount of free amino group in the solution

L_t = amount of free amino group in the solution at time t

L_{max} = amount of free amino group in the solution after acid hydrolysis

TCA-soluble peptides content of adzuki beans hydrolysate was determined using Lowry method as stated in Section 3.2.2.3. The results were expressed as mg tyrosine equivalent per 100 mL of adzuki beans hydrolysate.

Total phenolic content (TPC) of adzuki beans hydrolysate using Folin–Ciocalteu method was stated in 3.3.1.2. TPC was calculated as mg gallic acid equivalent per 100 mL of adzuki beans hydrolysate.

3.6.2 Optimization of Ca-alginate encapsulation condition containing adzuki bean hydrolysate using response surface methodology (RSM)

3.6.2.1 Experimental design

The optimization of Ca-alginate encapsulation condition containing adzuki bean hydrolysate by RSM was performed by an experimental plan based on a three factor/five level design, -α, -1, 0, 1, α (α = 1.68), referred to as a rotatable central composite design. Independent variables were alginate concentration (1 - 3 %, w/v), total phenolic content (15 - 45 mg gallic acid) and $CaCl_2$ concentration (0 - 2 %, w/v). Five levels of values of the independent variables, obtaining from Minitab software version 17.1.0 (Minitab, Inc., State Collage, PA), were expressed in their coded and uncoded forms (Table 3.1). The design matrix was comprised of 20 experimental runs including 15 factorial points with five replicates at the center point (Table 3.2). The dependent variables were encapsulation efficiency of phenolic (TPCEE) and encapsulation efficiency of TCA-soluble peptides (TCA-soluble peptidesEE). In this experiment section, wet Ca-alginate beads were studied.

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Table 3. 2 Independent variables and their coded and actual values used for optimization

	-α	-1	0	1	α
Alginate (%, w/v)	0.32	1	2	3	3.68
TPC (mg gallic acid)	4.77	15	30	45	55.23
$CaCl_2$	0.66	1	1.5	2	2.34

Table 3. 3 The design matrix for producing Ca-alginate beads containing adzuki beans hydrolysate

	CODE			UNCODE		
Run	X_1 (sodium alginate)	X_2 ($CaCl_2$)	X_3 (TPC)	X_1	X_2	X_3
1	-1	-1	-1	1	1	15
2	1	-1	-1	3	1	15
3	-1	1	-1	1	2	15
4	1	1	-1	3	2	15
5	-1	-1	1	1	1	45
6	1	-1	1	3	1	45
7	-1	1	1	1	2	45
8	1	1	1	3	2	45
9	-1.68	0	0	0.32	1.5	30
10	1.68	0	0	3.68	1.5	30
11	0	-1.68	0	2	0.66	30
12	0	1.68	0	2	2.34	30
13	0	0	-1.68	2	1.5	4.77
14	0	0	1.68	2	1.5	55.23
15	0	0	0	2	1.5	30
16	0	0	0	2	1.5	30
17	0	0	0	2	1.5	30
18	0	0	0	2	1.5	30
19	0	0	0	2	1.5	30
20	0	0	0	2	1.5	30

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3.6.2.2 Determination of physico-chemical properties of response variables

The design matrix (20 experimental conditions) was used for producing Ca-alginate beads containing adzuki beans hydrolysate. The Ca-alginate encapsulation preparation was followed as stated in procedure of Pasukamonset et al. (2016) with minor modifications. Sodium alginate was dispersed in the freshly prepared adzuki beans hydrolysate. Total volume of the sodium alginate solution was fixed at 200 mL. The sodium alginate solution was stirred with magnetic stirring until full hydration (homogeneous mixture) and left to stand for about 2 h to remove any air bubbles. A syringe pump (model Nexus 3000 Syringe Pump-Ultra-high precision; Chemyx, Stafford, United States) was used to deliver sodium alginate solution with the flow rate of 1.2 mL/min to a $CaCl_2$ solution as a gelling collection. The appropriate distance between syringe and a gelling collection was set at 9.5 cm. To obtained harden beads, the obtained beads were maintained in calcium chloride solution for further 20 minutes with magnetic stirring. Then, wet Ca-alginate beads were washed using distilled water and wiped by filter paper (Whatman no.1). The obtained wet Ca-alginate beads were analysed as follows:

3.6.2.2.1 Determination of TPC, TCA-soluble peptides content and encapsulation efficiency

In order to disintegrate the wet Ca-alginate beads containing adzuki beans hydrolysate, one gram of beads was added into 10 mL of 5% sodium citrate solution. Then, sample was sonicated for 30 minutes and centrifuged at 10,000 × *g* for 10 minutes. Determination of TPC and TCA-soluble peptides in wet Ca-alginate beads was stated in Section 3.2.2.3.

The percentage of encapsulation efficiency was presented as gallic acid or tyrosine equivalent. The encapsulation efficiency was calculated as shown in Eq (4).

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$$EE\ (\%) = \frac{\text{bioactive content encapsulated in beads}}{\text{initial bioactive content solution used for encapsulation}} \times 100 \qquad \text{Eq (4)}$$

3.6.2.3 Modeling and statistical analysis

Models were assessed based on statistically significant coefficients and R^2 values using analysis of variance (ANOVA) technique using *Design-Expert*® software, version 12 (Stat-Ease, Inc., Minneapolis, MN, USA). The experimental data were fitted to a second-order polynomial model, Eq.5, and the regression coefficients were obtained by a multiple linear regression.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i-1}^{2} \sum_{j=2}^{3} \beta_{ij} X_i X_j \qquad \text{Eq (5)}$$

Where Y = the measured response of the dependent variables

X_1, X_2, and X_3 = the independent variables

β_0 = the intercept

β_i = the linear coefficients

βii (i=1, 2, 3) = the quadratic coefficients

βij (i=1, 2, 3; j = 2, 3) = the interaction coefficients

The three-dimensional surface plots were established via fitted polynomial equation, in order to depict the dependence between the experimental variables and responses. The encapsulation condition yielding the maximum response (TPCEE and TCA-soluble peptidesEE) was used to make the Ca-alginate beads to verify the validity of the model.

3.6.2.4 Validation

In order to validate the proposed model, the maximum response TPCEE and TCA-soluble peptidesEE was carried out using the optimized

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formulation. Experimental responses of checkpoints were compared to the predicted results from the fitted model to evaluate the precision of the polynomial equations.

3.6.3 Optimization of encapsulation condition of Ca-alginate beads containing adzuki bean hydrolysate using factorial design

3.6.3.1 Experimental design

An experimental factorial design was employed to investigate the influence of $CaCl_2$ and sodium alginate concentration on TPCEE and TCA-soluble peptidesEE of wet Ca-alginate beads. Three level of sodium alginate concentrations (1, 2 and 3% w/v) and three different $CaCl_2$ concentrations (1, 1.5 and 2% w/v) were designed. All conditions contained 120 – 125 mL beans hydrolysate (equivalent to 45 mg gallic acid and 8.6 mg tyrosine) and total volume was adjusted to 200 mL with phosphate buffer (pH6). Thus, the experiment design was a 3 x 3 factorial in a completely randomized design (CRD) with 3 replications. The TPCEE and TCA-soluble peptidesEE were considered as dependent factors (response). The experimental design is shown in Table 3.4.

Table 3. 4 The experimental design for producing Ca-alginate beads containing adzuki beans hydrolysate

Run	alginate concentration (%, w/v)	$CaCl_2$ concentration (%, w/v)	Adzuki beans hydrolysate (mL)
1	1	1	
2	1	1.5	
3	1	2	
4	2	1	
5	2	1.5	120 - 125
6	2	2	
7	3	1	
8	3	1.5	
9	3	2	

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3. 6. 3. 2 Determination of physico-chemical properties of response variables

The preparation for Ca-alginate beads containing adzuki beans hydrolysate were prepared as stated above (Section 3.6.2.2). The obtained wet Ca-alginate beads were analysed as follows:

TPC and TCA-soluble peptides analysis were determined as stated in Section 3.2.2. The TPCEE and TCA-soluble peptidesEE were calculated as stated in Section 3.6.2.2.1.

3.6.3.3 Physical properties

The mechanical property of Ca-alginate beads was measured using a Texture Analyzer (Stable Micro System, model TA-XT2, Godalming, UK). Cylinder probe (P/6) was connected to a texture analyzer via probe adapter and operated with compression mode (a pre-test speed of 0.5 mm/s, a test-speed of 0.01 mm/s, a post-test speed of 2 mm/s and trigger force of 2 g). A compression of the sample was set as 60%. The mechanical property of wet Ca-alginate beads obtaining from different encapsulation conditions was presented in grams-force.

Morphological observations and size analysis of the beads were realized using a Nikon stereomicroscope (Nikon Corporation, Japan). The diameters were measured using NIS-Elements Microscope Imaging Software (Nikon Corporation, Japan). The average size and standard deviation of the beads were computed using at least 30 beads, randomly selected, from each batch.

3.6.3.4 Release characteristic

Ten grams of wet Ca-alginate beads was incubated at 37 ^{o}C in buffer solution (pH 1.2 and pH 7.4) with continuous shaking. Release characteristic of wet Ca-alginate beads obtaining from different encapsulation conditions was showed expressed as the gallic acid or tyrosine equivalent and calculated divided by the encapsulated amount of bioactive compounds and released bioactive compounds as following the Eq (6).

$$\text{Release (\%)} = \frac{\text{bioactive content released at pH 1.2 and 7.4}}{\text{encapsulated bioactive content in beads}} \times 100 \qquad \text{Eq (6)}$$

3.6.3.3 Statistical analysis

The experiment was performed in triplicate. ANOVA of the experimental data was performed. Tukey rank test was used for evaluate testing the difference between means at the 95% confidence interval.

3.7 Effect of different encapsulation techniques in alginate-based encapsulation: Physicochemical properties and release characteristics under simulated gastrointestinal digestion

3.7.1 Experimental design

Two different encapsulation techniques were investigated: calcium alginate beads formed via dipping techniques and sodium alginate entrapment formed via ionic gelation process. Ca-alginate beads and sodium alginate particles were subjected to freeze drying step. Physico-chemical properties of freeze-dried calcium alginate beads and sodium alginate particles were investigated. The release characteristics were also investigated continuously under *in vitro* gastrointestinal digestions. Freeze-dried adzuki beans hydrolysate was used as the control.

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3.7.2 Determination of physico-chemical properties of different encapsulation techniques

3.7.2.1 Physical properties of different encapsulation techniques

Freshly prepared adzuki beans hydrolysate, 2% sodium alginate and mixed hydrolysate and 2% sodium alginate suspension were subjected to zeta potential analysis using Zetasizer Nano (Malvern Co., Ltd., Worcestershire, England).

Fourier transform infrared spectrophotometer (JASCO FT-IR 6300, Japan) was used to analyze the chemical functional groups of the freeze-dried samples through a spectrum recorded in KBr pellets in the range of 400 to 4000 cm^{-1}. The spectrum was adjusted at a resolution of 4 cm^{-1}.

Microstructure of freeze-dried particles from two encapsulation methods was investigated under a scanning electron microscope (JEOL, JSM-5410LV, Tokyo, Japan). The sample was covered with gold in a sputter coater (Balzers, model SCD 040, Vaduz, Liechtenstein) then observed under a scanning electron microscope at a voltage of 12 kV with 200× of magnification.

3.7.2.2 The encapsulation efficiency of different encapsulation methods

Freeze-dried samples were prepared as state in the process of Gonzalez et al. (2019) with slightly modification. To determine the surface bioactive compounds, 5 grams of sample were dispersed in 50 mL Milli-Q water and stirred for 1 minute at room temperature. After centrifugation at 8,000 × *g* for 3 minutes, the supernatant was subjected to TPC and TCA-soluble peptides determination as stated in Section 3.2.2.3.

To determine the total bioactive compounds, 5 grams of freeze-dried particle were dispersed in 50 mL Milli-Q water and stirred until homogeneous at room temperature (approx. 2 hours). The sample was centrifuged at 10,000 × *g* for 10 min before subjected to TPC and TCA-soluble peptides determination as stated in Section 3.2.2.3. Freeze dried beads was dispersed in 5% sodium citrate instead of Milli-Q water to disintegrate the Ca-alginate structure. Then, TPC and TCA-soluble peptides were also determined as stated in Section 3.2.2.3.

The encapsulation efficiency of TPC and TCA-soluble peptides was calculated according to Eq (7) and the obtained values represented the bioactive compounds in the freeze-dried sample.

$$\text{EE (\%)} = \frac{\text{Total bioactive content} - \text{Surface bioactive content}}{\text{Total bioactive content}} \times 100 \qquad \text{Eq (7)}$$

3.7.3 Determination of release characteristics under simulated gastrointestinal (GI) digestion of different encapsulation techniques

Freeze-dried adzuki beans hydrolysate, particle and bead were subjected to *in vitro* simulated GI digestion according to the method of Minekus et al. (2014) and

as developed by members of the EU Cost Action INFOGEST, an international network joined by over 200 scientists from over 30 different countries. In oral phase, the samples were mixed with Simulated Salivary Fluid (SSF) at the ratio of 50:50 (w/v). In the final mixture of oral phase, human salivary α-amylase was added to achieve 75 U/mL, followed by $CaCl_2$ concentration achieved 0.75 mM and incubated at 37 °C for 2 minutes. To start the gastric phase, porcine pepsin (EC 3.4.23.1) was adjusted to achieve a 2000 U/mL activity in the final mixture, followed by $CaCl_2$ to achieve 0.075 mM and 1 N HCl to adjust the pH to 3. The samples were then incubated at 37 °C for 2 hours. Finally, the gastric phase was mixed with the simulated intestinal fluid at the ratio of 50:50 (v/v), which consisted of an electrolyte solution (eSIF), pancreatin (100 U/mL trypsin activity in the final mixture) and 1 N NaOH to adjust the pH to 7. The samples were then incubated at 37 °C for 2 hours. At the end of the intestinal digestion, the digested samples were centrifuged at 10,000 × *g* for 10 min and the supernatant was collected for further measurements as follows:

3.7.3.1 Antioxidant activity (ABTS, FRAP and ferrous chelating)

ABTS radical scavenging activity and FRAP of the digested samples were analysed as stated in Section 3.3.1.1.1 The results were expressed as mmol trolox equivalent per gram sample.

Ferrous chelating activity of the digested samples were analysed as stated in Section 3.3.1.1.1 The results were expressed as mmol EDTA equivalent per gram sample

3.7.3.2 ORAC

ORAC of digested samples was analysed as stated in Section 3.3.1.1.2. The results were expressed as mmol trolox equivalent per grams sample.

3.7.3.3 Health promoting activity (ACE-I inhibitory and DPP-IV inhibitory activity)

ACE-I inhibitory activity of digested samples was determined as stated in Section 3.3.1.1.3. The results were expressed as percentage of ACE-I inhibitory activity.

The DPP-IV inhibition assay was carried out as described by Nongonierma and FitzGerald (2013). 50 µL of digested sample was pipetted onto a 96 well microplate containing Gly-Pro p-nitroanilide hydrochloride (Gly-Pro-pNA) at a final concentration of 0.2 mM, a substrate for dipeptidyl peptidase-IV (DPP-IV) enzyme assay. The reaction was initiated by the addition of DPP-IV (final concentration 0.0025 units/mL). The microplate was incubated at 37 ^{o}C for 60 min in a microplate reader. The absorbance value of resulting solution was measured at 405 nm. Diprotin A was used as a positive control. DPP-IV inhibitory activity was expressed as percentage of DPP-IV inhibitory activity.3.7.3.4 TCA-soluble peptides, free amino group, total phenolic compounds, total flavonoid and reducing sugar

TCA-soluble peptides content, free amino group content, TPC, TFC, and reducing sugar in digested sample were determined as stated in Section 3.2.2.3. The results were calculated based on the equivalent weight (1 g) of freeze-dried sample.

3.7.4 Statistical analysis

The experiment was performed in triplicate. ANOVA of the experimental data was performed. Tukey rank test was used for evaluate testing the difference between means at the 95% confidence interval.

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CHAPTER IV
RESULTS AND DISCUSSION

Part A: Effect of different cooking conditions on properties of cooked adzuki beans and its cooking water

4.1 Effect of cooking conditions on amino acids and protein in cooked adzuki beans and its cooking water

4.1.1 Effect of cooking conditions on crude protein, TCA-soluble peptides, total amino acid and free amino group content in cooked adzuki beans and its cooking water

Soaking is a common process to increase water imbibition of legumes before cooking. In this experiment, the effect of cooking condition on adzuki beans was studied while 6-hour soaked adzuki beans were set as the control. Raw adzuki beans were set as reference.

Weight and moisture content of samples obtained from different cooking conditions might affect the calculated data of beans in each condition in this experiment due to the difference in moisture of samples obtaining from the different treatments. Thus, data were presented in 2 forms.

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1) data were calculated based on 30 grams of raw seeds in order to compare the effect of cooking conditions on the equivalent raw seeds weight basis.

2) data were calculated based on 100 grams of cooked portion in order to present the value based on the equivalent portion size of cooked sample.

The sample weight, moisture, crude protein, TCA-soluble peptides, total amino acids and free amino group content of cooked adzuki beans and its cooking water obtaining from different cooking conditions are presented in Table 4.1.

The weight and moisture content of soaked beans significantly increased during soaking period ($p \leq 0.05$) compared to raw seeds. This may be due to water absorption of seeds. Cooking condition resulted in a significant increase in weight of

cooked beans compared to soaked beans. Samples obtained from different cooking conditions resulted in difference in weight and moisture content of cooked beans. This might be because raising water temperature during cooking affected the increase in water absorption of beans during cooking. Boiling condition (60-min boiling) resulted in a higher cooking water weight compared to other conditions due to the leaching of soluble solids during cooking, resulting in increasing the amount of soluble solids in cooking water.

Based on the equivalent weight (30 g) of raw seeds, crude protein of cooked beans significantly decreased ($p \leq 0.05$) compared to raw seeds and soaked beans, respectively. The reduction of crude protein in cooked beans was due to the leaching of protein component from beans to cooking water (Table 4.1). Among all cooking conditions, cooked beans obtained from the cooking condition "autoclaving 10 minutes" (10-min autoclaving) showed the highest crude protein content. While, the extending autoclaving time from 10 to 60 minutes caused further cell wall breaking down, resulting in an increase in crude protein of its cooking water. It is possible that the saturated steam under pressure from autoclaving rapidly conducts the heat into bean structure resulting in seed components diffusion into the cooking water. The extreme condition (60-min autoclaving) caused more breakdown of cell wall resulting in more leaching of crude protein into the cooking water.

Based on the equivalent cooked portion (100 g), 60-min boiling was an appropriate condition to obtain cooked beans with the highest amount of crude protein. Sum of crude protein content of cooked beans and that of its cooking water (at the same portion size) also showed that boiling condition yielded significantly higher crude protein content compared to other conditions. This may be due to the difference in the starting weight of each sample, resulting from the release of some soluble solids from beans into cooking water. That is the reason why the comparison should be based on the equivalent weight (30 g) of raw seeds. Wang, Hatcher, Tyler, Toews, and Gawalko (2010) also reported that crude protein in beans and chickpeas significant ($p \leq 0.05$) increased after cooking by automated Mattson Cooker due to the loss of soluble solids during cooking, resulting in increasing the concentration of protein in cooked seeds.

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Table 4. 1 Effect of different cooking conditions on weight, free amino group and total moisture, crude protein, TCA-soluble peptides, free amino group and total amino acids content of cooked adzuki beans and its cooking water

Sample	Weight (g)	Moisture (g/100g cooked portion)	Crude protein (g)		TCA-soluble peptides (mg Tyr)		Free amino group (mg Leu)		Total amino acids (mg)	
			(/30g raw seeds wb.)	(g/100g cooked portion)	(/30g raw seeds wb.)	(g/100g cooked portion)	(/30g raw seeds wb.)	(g/100g cooked portion)	(/30g raw seeds wb.)	(g/100g cooked portion)
Raw seeds (Reference)	$30.78^{a} \pm 0.60$	$10.89^{a} \pm 0.08$	$6.74^{f} \pm 0.18$	$21.80^{h} \pm 0.30$	$7.02^{a} \pm 0.27$	23.40^{a} 0.91	$139.22^{a} \pm 9.04$	$464.07^{a} \pm 30.14$	NA	NA
Control (soaked beans)	$40.40^{b} \pm 1.08$	$66.02^{e} \pm 3.73$	$4.87^{d} \pm 0.22$	$12.39^{g} \pm 0.57$	$8.79^{b} \pm 0.40$	$22.74^{a} \pm 1.04$	$328.71^{bc} \pm 31.34$	$835.85^{b} \pm 90.41$	$511.57^{c} \pm 42.25$	$912.18^{c} \pm 79.46$
60-min boiling										
Cooked beans	$62.79^{c} \pm 0.48$	$32.28^{d} \pm 2.37$	$4.79^{cd} \pm 0.36$	$7.83^{f} \pm 0.59$	$31.24^{c} \pm 1.07$	$47.63^{b} \pm 10.46$	$287.49^{b} \pm 47.41$	$469.42^{a} \pm 74.95$	$430.56^{b} \pm 53.65$	$703.54^{b} \pm 87.66$
Cooking water	$95.43^{g} \pm 1.19$	$98.10^{g} \pm 0.18$	$0.39^{a} \pm 0.04$	$0.42^{a} \pm 0.04$	$44.95^{f} \pm 1.49$	$48.07^{b} \pm 1.60$	$655.22^{e} \pm 172.41$	$705.96^{b} \pm 190.06$	NA	NA
10-min autoclaving										
Cooked beans	$84.66^{f} \pm 0.90$	$26.97^{c} \pm 1.66$	$5.16^{e} \pm 0.33$	$6.25^{d} \pm 0.40$	$39.28^{d} \pm 2.27$	$47.77^{b} \pm 2.32$	$347.10^{bcd} \pm 51.95$	$420.40^{a} \pm 60.58$	$454.62^{bc} \pm 39.16$	$619.97^{ab} \pm 51.20$
Cooking water	$81.23^{e} \pm 0.28$	$98.28^{g} \pm 0.17$	$0.26^{a} \pm 0.02$	$0.33^{a} \pm 0.03$	$46.87^{g} \pm 1.09$	$59.28^{d} \pm 1.29$	$410.43^{cd} \pm 115.75$	518.40^{a} 146.26	NA	NA
60-min autoclaving										
Cooked bean	$85.16^{f} \pm 0.84$	$20.09^{b} \pm 0.84$	$4.62^{c} \pm 0.37$	$5.63^{c} \pm 0.39$	$44.02^{f} \pm 1.44$	$53.43^{c} \pm 1.51$	$446.57^{d} \pm 35.66$	$537.95^{a} \pm 41.01$	$359.18^{a} \pm 31.29$	$547.72^{a} \pm 47.8$
Cooking water	$74.69^{d} \pm 1.11$	$91.75^{f} \pm 0.58$	$1.09^{b} \pm 0.11$	$1.37^{b} \pm 0.13$	$41.75^{e} \pm 1.37$	$57.68^{d} \pm 1.77$	$861.39^{f} \pm 192.43$	$1186.49^{c} \pm 281.91$	NA	NA

wb., wet basis; NA, not analysis. Mean values ± SD of triplicate determinations. Mean in the same column with different letters are significantly ($p \leq 0.05$).

Thermal processing results in the cleavage of di-sulphide bounds, unfolding, aggregation and formation of dimers and larger oligomers of polypeptides (Dutson & Orcutt, 1984). This process causes a release of soluble storage compounds into cooking water. The mixture of cooked beans and its cooking water might contain various sizes of soluble peptides and free amino acids. Trichloroacetic acid (TCA) was applied to precipitate proteins in cooked beans and its cooking water. The results in Table 4.1 shows that TCA-soluble peptides content, based on the equivalent weight (30 g) of raw seeds, in soaked beans (the control) was slightly increased compared to that in raw seeds.

The different cooking conditions of adzuki beans (Table 4.1) resulted in a significant increase in the TCA-soluble peptides content compared to the control. At the same starting weight (30 g) of raw seeds, the highest TCA-soluble peptides content of cooked beans was observed in 60-min autoclaving with an increase of 47.56% , followed by 10-min autoclaving with an increase of 28.27% compared to cooked beans from 60-min boiling. Different cooking conditions resulted in releasing different amount of small peptides and amino acids into cooking water. Therefore, TCA-soluble peptides content in cooking water was determined. Among all cooking conditions, the significantly higher TCA-soluble peptides content, based on the equivalent weight (30 g) of raw seeds, in cooking water was obtained from 10-min autoclaving. This result could be explained by the fact that a higher extent of cell wall breakdown causes a higher release of storage protein which is then leaching into cooking water. In addition, thermal denaturation of protein occurs when hydrogen and other non-covalent bonds, such as ionic and van der Waals bonds, within the protein are disrupted by heat (Li-Chan & Lacroix, 2018). Heat denaturation also breaks covalent di-sulphide bonds in larger oligomers of proteins, causing an increase in TCA-soluble peptides after cooking. In this experiment, 60-min autoclaving resulted in higher amount of TCA-soluble peptides content in both cooked beans and its cooking water at the same cooked portion (100 g) compared to other conditions. This was because extreme cooking condition caused further cell wall breaking down.

Free amino group content in cooked beans and its cooking water was observed dependent on cooking condition as shown in Table 4.1. Based on the

equivalent starting weight (30 g) of raw seeds, 60-min autoclaving was an appropriate condition to obtain cooked beans and cooking water with the highest amount of free amino group content. It was noted that the extreme condition could cleave more disulfide bond in the storage protein of adzuki beans compared to other conditions. The leaching effect caused an increasing free amino group in cooking water. At the same cooked portion (100 g), 60-min autoclaving also resulted in higher amount of free amino group content in both cooked beans and its cooking water at the same cooked portion (100 g) compared to other conditions.

Based on the equivalent weight of raw seeds (30 g), total amino acids in cooked beans significantly decreased ($p \leq 0.05$) compared to soaked beans as shown in Table 4.1. Cooking caused a reduction in total amino acids content due to heat destruction. The result showed that 60-min autoclaving had the highest degree of total amino acid destruction (around 31%) while degree of amino acid destruction under 10-min autoclaving was minimal (around 8 %). In this study, free amino acids in cooking water could not be analyzed using HPLC because the presence of a large amount of starch in cooking water obstructed the filtration through 0.45 µm filter nylon. Thus, determination of free amino group in cooking water was applied. Heating at cooking level could chemically alter the amino acid residues and partially destroy amino acid. This process might affect the nutrition value of cooked beans. Thus, amino acid composition and chemical score of cooked beans obtaining from different cooking conditions were calculated and presented in Section 4.1.2.

4.1.2 Amino acid profile of cooked adzuki beans and their chemical scores

Figure 4.1 shows type and amount of eighteen amino acids in the soaked beans (the control) and cooked beans from different cooking conditions. Amino acids content was calculated as mg per 30 g raw seeds based on wet basis. Glutamic acid, aspartic acid, phenylalanine and leucine were found as major amino acids present in soaked beans. A small amount of cysteine and tryptophan were found.

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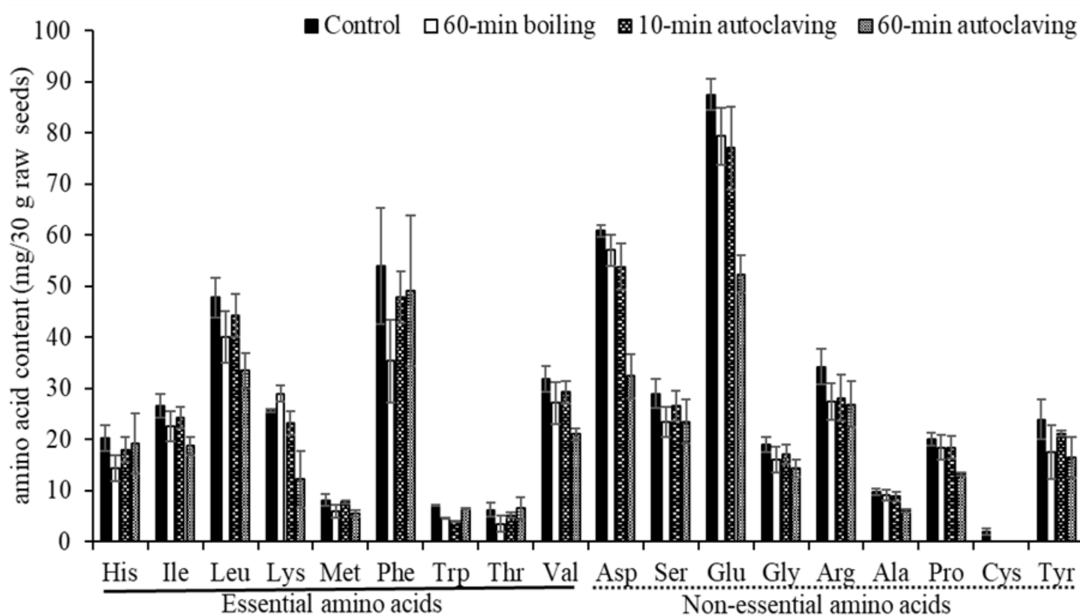

Figure 4. 1 The essential and non-essential amino acids in cooked bean from different cooking conditions

Cooking process caused a significant decrease ($p \leq 0.05$) in the amount of essential amino acids and non-essential amino acids content. Cooking under 60-min autoclaving was found to be the most vigorous condition as the amount of each amino acid was significantly decreased compared to other conditions. Heat sensitivity of amino acid depended on heating pressure of steam and heating temperature. Cystine, lysine, arginine, tryptophan and serine were reported as heat sensitive amino acids and become more destroyed on extreme condition (Taira, Taira, Sugimura, & Sakurai, 2014). Compared to the 10-min autoclaving, 60-min boiling also caused a higher decrease in amino acid content due to leaching of amino acids into cooking water. Cysteine was not detected in cooked beans obtained from all cooking conditions. It might be possible that (1) cysteine, a heat-labile amino acid, is destroyed by thermal process under high pressure (Taira et al., 2014). (2) The disulfide bound of cysteine is easy oxidised under high temperature (Papadopoulos, 1989). Taira et al. (2014) reported that amino acids in defatted soybean flour were significantly decreased after autoclaving. They reported that the unique feature of amino acid (cysteine, lysine and arginine) destruction was influenced by heating pressure at 108 ºC and 115 ºC for 30 min, that of cysteine was the most remarkable while tryptophan and serine were slightly destroyed after heating.

Protein quality of cooked adzuki beans can be explained in terms of chemical score. Chemical score of certain foods such as egg and milk are used as reference protein. Table 4. 2 shows that chemical score of cooked beans was dependent on cooking condition. The result showed that 10-min autoclaving was the appropriate cooking condition to maintain essential amino acids resulting in higher chemical score. The amino acid giving the lowest chemical score is a limiting amino acid. The loss of amino acid content in cooked beans might be due to heat destruction or leaching into cooking water. Thus, both cooked beans and cooking water should be pooled and used in further study. The calculated chemical score of the all cooked beans samples, taken as reference to the essential amino acid composition of whole egg, cow's milk and soybean, showed that methionine and cysteine was presented as the limiting amino acid in the cooked adzuki beans. Although chemical score of cooked beans was quite low compared to whole egg and cow's milk, it is comparable to that of soybean. It has been reported that methionine is the first limiting essential amino acid in legumes (Siddhuraju, Vijayakumari, & Janardhanan, 1996).

Cysteine, non-essential amino acid, is included with methionine, because it has a sparing effect on methionine when added to the diet (Lam & de Lumen, 2003). However, Chemical score of cooked beans only reflects the ratio between essential amino acid in the evaluated protein and the amino acid content in the reference protein without taking in account other quality parameters such as digestibility.

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Table 4. 2 Effect of different cooking conditions on essential amino acids, chemical score and limiting amino acid of cooked adzuki beans

Essential amino acids	Chemical score												Reference patternt[2]
	Compare to whole egg[1]				Compare to cow's milk[1]				Compare to soybean[1]				
	Control (soaked beans)	60-min boiling	10-min autoclaving	60-min autoclaving	Control (soaked beans)	60-min boiling	10-min autoclaving	60-min autoclaving	Control (soaked beans)	60-min boiling	10-min autoclavin g	60-min autoclaving	
His	17.18	8.31	8.66	7.67	27.78	13.45	14.00	12.41	13.97	6.76	7.04	6.24	19
Ile	7.71	5.97	5.21	4.76	13.02	10.08	8.80	8.03	8.34	6.46	5.63	5.14	28
Leu	8.57	6.23	5.52	5.08	12.00	8.72	7.72	7.11	9.72	7.06	6.25	5.76	66
Lys	3.72	5.68	3.73	3.35	4.72	7.22	4.74	4.26	3.99	6.10	4.00	3.60	58
Met + Cys	2.33	1.21	1.22	1.16	5.34	2.78	2.81	2.67	6.79	3.53	3.57	3.39	25
Phe + Tyr	14.22	7.37	8.06	4.91	32.05	16.61	18.16	11.07	15.87	8.23	8.99	5.48	63
Thr	7.07	4.83	4.88	4.30	10.63	7.26	7.33	6.46	8.14	5.56	5.61	4.95	34
Trp	9.88	4.52	5.21	2.85	14.26	6.52	7.52	4.11	10.45	4.78	5.51	3.01	11
Val	7.28	6.04	5.26	4.80	16.44	13.64	11.87	10.83	9.28	7.70	6.70	6.11	35
Limiting amino acid	Met + Cys	Met + Cys	Met + Cys	Met + Cys	Lys	Met + Cys	Met + Cys	Met + Cys	Lys	Met + Cys	Met + Cys	Trp	

[1] Food Data Central Search Results, USDA, 2018

[2] The recommended content of each amino acid for preschool child, FAO/WHO/UNU, 1985

4.2 Cooked adzuki beans and its cooking water: biological activity and compositions

To determine the biological activity of compounds in cooked beans and cooking water, the components in cooked beans and cooking water were determined. There were 3 fractions: (1) the borate buffer-soluble fraction, (2) borate buffer fraction with phenolic removal, (3) phenolic fraction. The borate buffer-soluble fraction was defined as the fraction obtaining from using 20 mM borate buffer (pH 9) containing 2 mM β-mercaptoethanol in cooked beans and its cooking water extraction. This fraction may contain bioactive compounds including small peptides, free amino acids and phenolic compounds. In order to prove whether activity comes from either small peptides or phenolics, polyvinylpyrrolidone (PVPP) was applied to absorb phenolics in the borate buffer-soluble fraction. Thus, borate buffer-soluble fraction was subjected to PVPP precipitation twice in order to remove phenolic compounds. This fraction was called borate buffer-soluble fraction with phenolic removal. The pellets obtaining from PVPP precipitation step was extracted using 95% methanol, the supernatant was collected and defined as phenolic fraction.

The biological activities of each fraction were determined with five different assays: ABTS radical scavenging, FRAP, ferrous chelating, oxygen radical absorbance capacity (ORAC) and ACE-I inhibitory activity. It has been reported that small peptides, free amino acids and phenolic compounds have synergistic effect on showing antioxidant activity. Thus, the antioxidant activity in this experiment may come from the presence of small peptides, free amino acids and phenolic compounds in cooked beans and cooking water. Thus, TCA-soluble peptides, free amino group, total phenolic compounds and total flavonoid in all three fractions obtaining from cooked beans and its cooking water were determined. The diagram of sample fractions obtaining from cooked beans and cooking water and its biological activity and compositions determination is shown in Figure 3.1 (Section 3.3).

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4.2.1 Borate buffer-soluble fraction of cooked adzuki beans and its cooking water

4.2.1.1 Effect of different cooking conditions on antioxidant, ORAC and ACE-I inhibitory activity of borate buffer-soluble fraction

Since pulses are not commonly eaten raw, a variety of thermal processing methods are applied to pulses to achieve desirable sensory characteristics and, sometimes, desirable nutritional and biological activities. It has been reported that cooked beans contain various antioxidant components showing different antioxidant activities. Some antioxidant components show radical scavenging ability or lipid peroxidation inhibitors whereas some shows metal chelating or reducing activities (López-Martínez, Leyva-López, Gutiérrez-Grijalva, & Heredia, 2017). Thus, the borate buffer-soluble fractions obtaining from cooked beans and its cooking water were evaluated for biological activity (ABTS radical scavenging, FARP, and ferrous chelating, ORAC and ACE-I inhibitory assays).

4.2.1.1.1 Antioxidant activity

Different cooking conditions affected antioxidant activity (ABTS, FRAP and ferrous chelating activity) of cooked beans and its cooking water. The results, calculated based on the equivalent weight (30 g) of raw seeds, are shown in Table 4.3. In cooked beans, 10 min-autoclaving showed the highest ABTS scavenging activity and FRAP, however, its FRAP was not significant different compared to 60 min-autoclaving. The 60 min-boiling showed the lowest ABTS scavenging activity, FRAP and ferrous chelating. In cooking water, 60 min-boiling exhibited the highest ABTS scavenging activity, FRAP and ferrous chelating. This was due to leaching effect in heat convection condition. In each antioxidant activity assay, 10 min-autoclaving showed similar value of antioxidant activity in cooked beans and its cooking water. In 60-min boiling condition, it was found that the antioxidant value in cooking water was higher than that in cooked beans. This incident was due to different heat transfer during cooking and leaching effect. The saturated steam under pressure from autoclaving condition rapidly conducts heat into bean structure resulting in resulting in cell wall destruction and diffusion of antioxidant compounds

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into cooking water. However, over-heating for a long time resulted in destruction or/and loss of heat labile antioxidant compounds in both cooked beans and cooking water. While, boiling process is cooking beans in excess water resulting in more leaching of antioxidant chemical species into cooking water. Consequently, antioxidant activity of the borate buffer-soluble fractions obtaining from cooked beans and cooking water from different cooking conditions were different. It has been reported that small peptides, free amino acids and phenolic compounds present synergistic effect on showing antioxidant activity (Fengru Liu, Chen, Shao, Wang, & Zhan, 2017). Thus, the antioxidant activity in this experiment may come from the presence of small peptides, free amino acids and phenolic compounds in cooked beans and cooking water.

Table 4. 3 Effect of different cooking conditions on antioxidant activity of borate buffer-soluble fraction from cooked beans and its cooking water

Sample	**ABTS scavenging activity (mM Trolox/30g raw seeds wb.)**	**FRAP (mM Trolox/30g raw seeds wb.)**	**Ferrous chelating (mM EDTA/30g raw seeds wb.)**
Control (soaked beans)	$18.84^{a} \pm 0.84$	$10.08^{a} \pm 0.47$	$0.75^{a} \pm 0.03$
60-min boiling			
Cooked bean	$29.43^{b} \pm 0.37$	$12.46^{b} \pm 1.92$	$1.39^{b} \pm 0.06$
Cooking water	$44.53^{f} \pm 0.55$	$22.56^{e} \pm 3.28$	$2.12^{f} \pm 0.09$
10-min autoclaving			
Cooked bean	$39.42^{e} \pm 0.54$	$15.91^{c} \pm 1.33$	$1.85^{e} \pm 0.03$
Cooking water	$38.08^{d} \pm 0.25$	$17.94^{d} \pm 1.52$	$1.85^{e} \pm 0.04$
60-min autoclaving			
Cooked bean	$38.26^{d} \pm 1.53$	$15.83^{c} \pm 1.80$	$1.78^{d} \pm 0.09$
Cooking water	$33.90^{c} \pm 0.74$	$12.65^{b} \pm 1.62$	$1.65^{c} \pm 0.06$

wb., wet basis; Mean values ± SD of triplicate determinations. Mean in the same column with different letters are significantly ($p \leq 0.05$).

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Ranilla, Genovese, and Lajolo (2009) reported that both cooked Brazilian beans and its cooking water from autoclaving (100 or 121 ºC) condition, without a soaking stage, would be recommended for consumption in order to retain more antioxidant phenolic compounds. Miglio, Chiavaro, Visconti, Fogliano, and Pellegrini (2008) reported that TEAC, FRAP and TRAP values of carrots, courgettes, and broccoli was increased after cooking by boiling, steaming and frying. It was probably because softening vegetables matrix increased extractability of compounds. Moreover, some phenolics could be partially converted into more antioxidant chemical species. Similar reports were found, both hydrophilic and lipophilic-ORAC values in cooked tomato were reported to be significantly higher compared to raw tomato (Wu, Beecher, et al., 2004; Wu, Gu, et al., 2004).Dewanto, Wu, and Liu (2002) reported that processed tomato and sweet corn exhibited higher antioxidant activities than fresh ones due to the release of bound phenolic compounds from the food matrices.Giusti, Capuano, Sagratini, and Pellegrini (2019) reported that the loss of phenolic compound in various cooked legumes was mainly from soaking and leaching. The cooking process also affected the reduction of heat sensitive compounds such as anthocyanins contributing the beneficial effect of legumes.

4.2.1.1.2 Oxygen radical absorbance capacity (ORAC)

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Antioxidant activity refers to the determination of the kinetics of a reaction between an antioxidant and the free radicals while antioxidant capacity refers to the measurement of the thermodynamic conversion efficiency of reactive oxygen species being scavenged. (Reşat et al., 2013). In this study, the oxygen radical absorbance capacity (ORAC) was also measured. ORAC is defined as an assay combining both inhibition time and degree of inhibition into a single quantity term. The assay relies on the ability of certain antioxidant compound to scavenge synthetic peroxyl radical of 2,2′-azobis [2-amidinopropane] dihydrochloride, AAPH. Thus, fluorescent intensity of a fluorescein sodium salt, fluorescent probe could be retained over certain period of time. Our results showed that bioactive compounds in borate buffer-soluble fraction from both cooked beans and its cooking water played a role against peroxyl radicals ($ROO^{\bullet}$). Data in Table 4.4 show the value ranged from

1.25 to 4.22 mM trolox equivalent/30 g raw seeds (wb). The activity was probably due to the contribution of phenolics and TCA-soluble peptides. Thus, both cooked beans and its cooking water from 10-min autoclaving showed a potent physiological relevance in scavenging peroxyl radicals. This scavenging ability may come from both peptides and phenolic compounds in the borate buffer-soluble fraction.

Table 4. 4 Effect of different cooking conditions on ORAC of borate buffer-soluble fraction from cooked beans and its cooking water

Sample	**ORAC (mM Trolox/30g raw seeds wb.)**
Control (soaked beans)	$1.25^{a} \pm 0.14$
60-min boiling	
Cooked bean	$2.92^{b} \pm 0.30$
Cooking water	$3.75^{c} \pm 0.37$
10-min autoclaving	
Cooked bean	$3.72^{c} \pm 0.11$
Cooking water	$3.83^{c} \pm 0.30$
60-min autoclaving	
Cooked bean	$4.22^{d} \pm 0.38$
Cooking water	$3.82^{c} \pm 0.11$

wb., wet basis; Mean values ± SD of triplicate determinations.

Mean in the same column with different letters are significantly ($p \leq 0.05$).

4.2.1.1.3 ACE-I inhibitory activity

ACE-I inhibitory activity in cooked adzuki beans has not been reported. Thus, this activity in cooked beans and cooking water was studied. All borate buffer-soluble fractions obtaining from different cooking conditions showed a significant increase in ACE-I inhibitory activity in cooked beans and cooking water compared to soaked beans as shown in Table 4. 5. This ACE-I inhibitory activity in soaked beans was very low/ undetectable probably because of because of the presence of intact polypeptides in soaked beans. The cooking water

obtaining from 10 min-autoclaving condition exhibited the highest ACE-I inhibitory activity (95%) while other samples showed activity around 77-85% inhibition. Captopril, a synthetic drug to treat hypertension, was set as the positive control, showing 99 % inhibitory activity. Thus, cooking water obtaining from 10 min-autoclaving could be considered for potential use as functional ingredient for reducing blood pressure.

Table 4. 5 Effect of different cooking conditions on ACE-I inhibitory activity of borate buffer-soluble fraction from cooked beans and its cooking water

Sample	**ACE-I inhibitory activity (%)**
Control (soaked beans)	$0.00^{a} \pm 0.00$
60-min boiling	
Cooked bean	$80.22^{bc} \pm 9.13$
Cooking water	$77.18^{b} \pm 7.31$
10-min autoclaving	
Cooked bean	$82.14^{bc} \pm 13.01$
Cooking water	$94.54^{d} \pm 2.98$
60-min autoclaving	
Cooked bean	$81.25^{bc} \pm 5.87$
Cooking water	$87.80^{cd} \pm 6.65$

wb., wet basis; Mean values ± SD of triplicate determinations.

Mean in the same column with different letters are significantly ($p \leq 0.05$).

4.2.1.2 Effect of different cooking conditions on TCA-soluble peptides, free amino group, total phenolic compounds, total flavonoid and reducing sugar of borate buffer-soluble fraction

The above biological activities of this borate buffer-soluble fraction may come from the presence of small peptides, free amino acids and phenolic compounds in cooked beans and cooking water. Therefore, the amount of TCA-

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soluble peptides, free amino groups, phenolic and flavonoid in this fraction were investigated. The borate buffer-soluble fractions obtaining from cooked beans and cooking water were evaluated for TCA-soluble peptides, free amino group, total phenolic content (TPC), total flavonoid content (TFC) and reducing sugar; the results, calculated based on the equivalent weight (30 g) of raw seeds, are shown in Table 4.6.

Both autoclaving methods, 10-min and 60-min autoclaving, resulted in a significant increase in TCA-soluble peptides content of cooked beans compared to soaked beans (Table 4.6). This was because heating under pressure resulted in more unfolded polypeptides and more releasing small peptides. Among all cooking conditions, cooked beans obtained from the cooking condition “10-min autoclaving” showed the highest TCA-soluble peptides content. In addition, the extended autoclaving time from 10 to 60 min resulted in a significant increase in TCA-soluble peptides content in cooking water. It was possible that the saturated steam under pressure from autoclaving rapidly conducts the heat into bean structure resulting in soluble components diffusion into the cooking water. The extreme condition caused more breakdown of cell wall resulting in more leaching of TCA-soluble peptides and free amino acids into the cooking water.

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Table 4. 6 Effect of different cooking conditions on TCA-soluble peptides, free amino group, TPC, TFC and reducing sugar content in borate buffer-soluble fraction

Sample	TCA-soluble peptides (mg Tyr/30g raw seeds wb.)	Free amino group (mg Leu/30g raw seeds wb.)	TPC (mg GA/30g raw seeds wb.)	TFC (mg CA/30g raw seeds wb.)	Reducing sugar (mg Glu/30g raw seeds wb.)
Control (soaked beans)	$0.26^{d} \pm 0.01$	$7.42^{g} \pm 0.22$	$5.04^{e} \pm 0.11$	$2.19^{f} \pm 0.11$	$3.12^{d} \pm 0.53$
60-min boiling					
Cooked bean	$0.24^{c} \pm 0.01$	$4.21^{a} \pm 0.13$	$2.34^{a} \pm 0.21$	$0.60^{a} \pm 0.02$	$1.34^{a} \pm 0.11$
Cooking water	$0.21^{b} \pm 0.01$	$6.35^{e} \pm 0.14$	$3.34^{d} \pm 0.05$	$0.71^{b} \pm 0.02$	$1.84^{bc} \pm 0.14$
10-min autoclaving					
Cooked bean	$0.34^{g} \pm 0.03$	$4.98^{b} \pm 0.27$	$3.42^{d} \pm 0.04$	$1.23^{e} \pm 0.06$	$1.92^{bc} \pm 0.05$
Cooking water	0.18a ± 0.01	$6.13^{d} \pm 0.13$	$3.04^{b} \pm 0.07$	$0.77^{bc} \pm 0.04$	$2.03^{bc} \pm 0.07$
60-min autoclaving					
Cooked bean	$0.32^{f} \pm 0.02$	$5.72^{c} \pm 0.28$	$3.07^{b} \pm 0.03$	$0.87^{d} \pm 0.06$	$1.88^{bc} \pm 0.29$
Cooking water	$0.29^{e} \pm 0.02$	$6.97^{f} \pm 0.31$	$3.24^{c} \pm 0.05$	$0.85^{c} \pm 0.05$	$1.65^{b} \pm 0.34$

wb., wet basis; Mean values ± SD of triplicate determinations. Mean in the same column with different letters are significantly ($p \leq 0.05$).

4.2.2 Borate buffer-soluble fraction with phenolic removal of cooked adzuki beans and its cooking water

4.2.2.1 Effect of different cooking conditions on antioxidant, ORAC and ACE-I inhibitory activity of borate buffer-soluble fraction with phenolic removal

Phenolic compounds were removed from borate buffer-soluble fractions using PVPP in order to determine biological activities of borate buffer fraction with phenolic removal. The borate buffer-soluble fractions with phenolic removal obtaining from cooked beans and cooking water were evaluated for its biological activity (ABTS radical scavenging, FARP, ferrous chelating, ORAC and ACE-I inhibitory assays).

4.2.2.1.1 Antioxidant activity

Many studies have been reported that small peptides and free amino acids obtaining from beans exhibit different antioxidant activities as well as radical scavenging ability, lipid peroxidation inhibitors, metal chelating and reducing activities. As shown in Table 4.7, antioxidant activity (ABTS, FRAP and ferrous chelating activity) in borate buffer-soluble fraction with phenolic removal obtaining from different cooking conditions were different. In this fraction, cooked beans obtaining from 10 min-autoclaving also showed the highest ABTS scavenging activity and FRAP. Whilst, cooking water from 60 min-boiling exhibited the highest ABTS scavenging activity and FRAP. The extreme cooking condition " 60 min-autoclaving" resulted in a markedly decrease in ABTS scavenging activity and FRAP, but the highest metal chelating activity was obtained. In each antioxidant activity assay, 10 min-autoclaving showed similar value of antioxidant activity in cooked beans and its cooking water. In 60-min boiling, it was found that the antioxidant value in cooking water was higher than that in cooked beans. This was probably due to higher leaching rate in boiling condition.

Table 4. 7 Effect of different cooking conditions on antioxidant activity of borate buffer-soluble fraction with phenolic removal from cooked beans and its cooking water

Sample	ABTS scavenging activity (mM Trolox/30g raw seeds wb.)	FRAP (mM Trolox/30g raw seeds wb.)	Ferrous chelating (mM EDTA/30g raw seeds wb.)
Control (soaked beans)	$11.56^{a} \pm 4.25$	$6.73^{f} \pm 0.23$	$0.88^{a} \pm 0.02$
60-min boiling			
Cooked bean	$25.46^{b} \pm 1.18$	$2.00^{a} \pm 0.15$	$1.39^{b} \pm 1.05$
Cooking water	$36.09^{e} \pm 4.06$	$2.94^{c} \pm 0.30$	$2.19^{f} \pm 0.10$
10-min autoclaving			
Cooked bean	$32.81^{de} \pm 3.67$	$2.66^{b} \pm 0.19$	$1.96^{e} \pm 0.03$
Cooking water	$28.10^{bc} \pm 3.80$	$2.50^{b} \pm 0.13$	$1.87^{e} \pm 0.03$
60-min autoclaving			
Cooked bean	$34.83^{e} \pm 2.88$	$3.76^{e} \pm 0.42$	$1.89^{d} \pm 0.09$
Cooking water	$31.08^{cd} \pm 2.92$	$3.49^{d} \pm 0.24$	$1.63^{c} \pm 0.03$

wb., wet basis; Mean values ± SD of triplicate determinations. Mean in the same column with different letters are significantly ($p \leq 0.05$).

4.2.2.1.2 ORAC

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ORAC values in both cooked beans and cooking water obtaining from different cooking conditions are depicted in Table 4. 8. 10 min-autoclaving showed the highest ORAC value in cooked beans. However, cooking water from 60 min-boiling showed the highest ORAC value. This was due to the leaching effect in boiling condition. The hydrogen atom in the small peptides presence in cooking water showed ability to neutralize free radicals peroxyl radical from AAPH, resulting in the retardation of decreasing fluorescence intensity over time.

Table 4. 8 Effect of different cooking conditions on ORAC of borate buffer-soluble fraction with phenolic removal from cooked beans and its cooking water

Sample	**ORAC (mM Trolox/30g raw seeds wb.)**
Control (soaked beans)	2.96[a] ± 0.08
60-min boiling	
Cooked bean	7.68[c] ± 0.66
Cooking water	10.75[d] ± 1.23
10-min autoclaving	
Cooked bean	14.59[e] ± 1.44
Cooking water	7.04[c] ± 1.63
60-min autoclaving	
Cooked bean	4.96[b] ± 0.57
Cooking water	4.12[b] ± 1.12

wb., wet basis; Mean values ± SD of triplicate determinations.

Mean in the same column with different letters are significantly ($p \leq 0.05$).

4.2.2.1.3 ACE-I inhibitory activity

All samples in borate buffer-soluble fraction with phenolic removal showed ACE-I inhibitory activities as shown in Table 4.9. Cooking caused a significant increase in ACE-I inhibitory activity of cooked beans compared to soaked beans. However, extreme cooking, “60-min autoclaving”, caused a marked decrease in ACE-I inhibitory activity of cooked beans. ACE-I inhibitory activity of each sample could be due to the released peptides occurring during cooking.

Table 4. 9 Effect of different cooking conditions on ACE-I inhibitory activity of borate buffer-soluble fraction with phenolic removal from cooked beans and its cooking water

Sample	ACE-I inhibitory activity (%)
Control (soaked beans)	$55.36^{c} \pm 7.73$
60-min boiling	
Cooked bean	$87.67^{g} \pm 3.05$
Cooking water	$75.89^{f} \pm 8.54$
10-min autoclaving	
Cooked bean	$69.20^{e} \pm 5.50$
Cooking water	$62.72^{d} \pm 2.17$
60-min autoclaving	
Cooked bean	$23.07^{a} \pm 5.44$
Cooking water	$41.52^{b} \pm 3.52$

wb., wet basis; Mean values ± SD of triplicate determinations.

Mean in the same column with different letters are significantly ($p \leq 0.05$).

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4.2.2.2 Effect of different cooking conditions on TCA-soluble peptides, free amino group, total phenolic compounds, total flavonoid and reducing sugar of borate buffer-soluble fraction with phenolic removal

The borate buffer-soluble fraction with phenolic removal obtaining from cooked beans and cooking water were evaluated for TCA-soluble peptides, free amino group, TPC, TFC and reducing sugar content. The results, calculated based on the equivalent weight (30 g) of raw seeds, are shown in Table 4.10.

The results showed that TCA-soluble peptides content considerably decreased compared to that of borate buffer-soluble fraction. Among all cooking conditions, both cooked beans and cooking water obtained from " 60-min autoclaving" showed the highest TCA-soluble peptides content. In the determination of free amino group using the reaction with TNBS, both cooked beans and cooking

water from borate buffer-soluble fraction with phenolic removal showed lower free amino group content than samples from borate buffer-soluble fraction.

In this fraction, phenolics were removed using PVPP. The amount of phenolic compounds was removed up to 57 - 79% compared to borate buffer-soluble fraction. It indicated that PVPP precipitation process was an effective method to remove phenolic compounds.

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Table 4. 10 Effect of different cooking conditions on TCA-soluble peptides, free amino group, TPC, TFC and reducing sugar content in borate buffer-soluble fraction with phenolic removal

Sample	TCA-soluble peptides	Free amino group	TPC	TFC	Reducing sugar
	(mg Tyr/30g raw seeds wb.)	(mg Leu/30g raw seeds wb.)	(mg GA/30g raw seeds wb.)	(mg CA/30g raw seeds wb.)	(mg Glu/30g raw seeds wb.)
Control (soaked beans)	$0.14^{a} \pm 0.02$	$9.15^{e} \pm 1.53$	$2.13^{d} \pm 0.59$	$0.47^{d} \pm 0.11$	$3.94^{d} \pm 0.40$
60-min boiling					
Cooked bean	$0.11^{a} \pm 0.02$	$2.28^{a} \pm 0.57$	$0.60^{a} \pm 0.04$	$0.17^{a} \pm 0.02$	$0.62^{a} \pm 0.01$
Cooking water	$0.09^{a} \pm 0.03$	$3.61^{b} \pm 0.63$	$0.72^{ab} \pm 0.11$	$0.18^{a} \pm 0.04$	$1.04^{b} \pm 0.06$
10-min autoclaving					
Cooked bean	$0.23^{b} \pm 0.03$	$3.56^{b} \pm 0.05$	$0.93^{b} \pm 0.04$	$0.25^{b} \pm 0.05$	$1.00^{b} \pm 0.05$
Cooking water	$0.11^{a} \pm 0.02$	$2.88^{a} \pm 0.19$	$0.64^{a} \pm 0.03$	$0.22^{ab} \pm 0.01$	$1.01^{b} \pm 0.06$
60-min autoclaving					
Cooked bean	$0.27^{bc} \pm 0.06$	$5.09^{d} \pm 0.48$	$1.22^{c} \pm 0.11$	$0.40^{c} \pm 0.03$	$1.34^{c} \pm 0.07$
Cooking water	$0.29^{c} \pm 0.12$	$4.29^{c} \pm 0.36$	$1.31^{c} \pm 0.15$	$0.39^{c} \pm 0.04$	$1.41^{c} \pm 0.04$

wb., wet basis; Mean values ± SD of triplicate determinations. Mean in the same column with different letters are significantly ($p \leq 0.05$).

4.2.3 Phenolic fraction of cooked adzuki beans and its cooking water

The pellets obtaining from PVPP precipitation of the borate buffer-soluble fraction (Section 4.2.1) was extracted using 95% methanol, the supernatant was collected and defined as phenolic fraction. The supernatant mainly contained phenolics. The amount of TCA-soluble peptides was negligible due to the removal of TCA-soluble peptides. The supernatant was determined for antioxidant and ACE-I inhibitory activity. Many studies have been reported that phenolic compounds obtaining from beans exhibit different antioxidant activities as well as radical scavenging ability, lipid peroxidation inhibitors, metal chelating and reducing activities. Thus, phenolic fraction obtaining from cooked beans and cooking water were also evaluated for its antioxidant activity (ABTS radical scavenging, FARP, ferrous chelating, ORAC assays).

4.2.3.1 Effect of different cooking conditions on antioxidant, ORAC and ACE-I inhibitory activity of phenolic fraction

4.2.3.1.1 Antioxidant activity

Different cooking conditions were found to affect antioxidant activity (ABTS, FRAP and ferrous chelating) of cooked beans and cooking water (Table 4.11). The results were calculated based on the equivalent weight (30 g) of raw seeds. In this fraction, cooked beans obtaining from 10 min-autoclaving also showed the highest ABTS scavenging activity and FRAP. Whilst cooking water from 60 min-boiling exhibited the highest ABTS scavenging activity and FRAP. The extreme cooking condition "60 min-autoclaving" resulted in a significant decrease in ABTS scavenging activity and FRAP but the highest ferrous chelating activity was obtained. The antioxidant activity of both cooked beans and cooking water were affected by cooking condition. Cooking condition caused different degree of releasing and leaching of phenolics. In each antioxidant activity assay, 10 min-autoclaving showed similar value of antioxidant activity in cooked beans and its cooking water. In 60-min boiling condition, it was found that the

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antioxidant value in cooking water was higher than that in cooked beans due to higher leaching rate in boiling condition and heat convection effect.

Table 4. 11 Effect of different cooking conditions on antioxidant activity of phenolic fraction

Sample	ABTS scavenging activity	FRAP	Ferrous chelating
	(mM Trolox/30g raw seeds wb.)	(mM Trolox/30g raw seeds wb.)	(mM EDTA/30g raw seeds wb.)
Control (soaked beans)	$16.15^{c} \pm 0.62$	$2.31^{f} \pm 0.11$	$0.95^{a} \pm 0.04$
60-min boiling			
Cooked bean	$7.41^{a} \pm 0.60$	$1.17^{a} \pm 0.14$	$1.42^{b} \pm 0.01$
Cooking water	$11.36^{b} \pm 2.22$	$1.56^{cd} \pm 0.10$	$2.21^{f} \pm 0.10$
10-min autoclaving			
Cooked bean	$12.29^{b} \pm 3.00$	$1.50^{c} \pm 0.07$	$2.00^{e} \pm 0.03$
Cooking water	$10.43^{b} \pm 2.07$	$1.32^{b} \pm 0.10$	$1.89^{d} \pm 0.02$
60-min autoclaving			
Cooked bean	$19.58^{d} \pm 1.08$	$1.96^{e} \pm 0.12$	$1.96^{e} \pm 0.08$
Cooking water	$17.39^{c} \pm 2.14$	$1.64^{d} \pm 0.17$	$1.75^{c} \pm 0.03$

wb., wet basis; Mean values ± SD of triplicate determinations. Mean in the same column with different letters are significantly ($p \leq 0.05$).

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4.2.3.1.2 ORAC

The results supported that phenolic compounds also contributes to the ORAC as shown in Table 4.12. The phenolic fraction showed lower ability in peroxyl scavenging activity because the composition in this fraction was only phenolics. The ORAC of phenolics seemed to be little compared to the contribution of small peptides. Kestwal, Bagal-Kestwal, and Chiang (2012) reported that ferulic acid, *p*-coumaric acid, gallic acid, quercetin and kaempferol can act as potent oxygen radical quenchers. Therefore, the individual phenolic compound was identified in Section 4.3.

Table 4. 12 Effect of different cooking conditions on ORAC of phenolic fraction from cooked beans and its cooking water

Sample	**ORAC (mM Trolox/30g raw seeds wb.)**
Control (soaked beans)	2.48^{d} ± 0.18
60-min boiling	
Cooked bean	1.57^{a} ± 0.20
Cooking water	2.04^{c} ± 0.36
10-min autoclaving	
Cooked bean	1.97^{c} ± 0.27
Cooking water	1.67^{ab} ± 0.19
60-min autoclaving	
Cooked bean	2.39^{d} ± 0.39
Cooking water	1.85^{bc} ± 0.27

wb., wet basis; Mean values ± SD of triplicate determinations.

Mean in the same column with different letters are significantly ($p \leq 0.05$).

4.2.3.1.3 ACE-I inhibitory activity

Cooking water obtaining from 10-min autoclaving and cooked beans obtaining from 60-min autoclaving showed higher ACE-I inhibitory activity (Table 4.13). This might be because both fractions contained certain phenolic showing ACE-I inhibitory activity. It has been reported that quercetin, kaempferol, rutin and epicatechin showed the ACE-I inhibitory activity (Al Shukor et al., 2013). In order to prove, the individual phenolic compound was identified in Section 4.3.

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Table 4. 13 Effect of different cooking conditions on ACE-I inhibitory activity of phenolic fraction from cooked beans and its cooking water

Sample	ACE-I inhibitory activity (%)
Control (soaked beans)	6.92[a] ± 2.13
60-min boiling	
Cooked bean	59.67[c] ± 15.17
Cooking water	47.17[b] ± 2.91
10-min autoclaving	
Cooked bean	12.20[a] ± 0.96
Cooking water	90.59[d] ± 0.64
60-min autoclaving	
Cooked bean	83.88[d] ± 10.26
Cooking water	61.81[c] ± 16.66

wb., wet basis; Mean values ± SD of triplicate determinations.

Mean in the same column with different letters are significantly ($p \leq 0.05$).

4.2.3.2 Effect of different cooking conditions on TCA-soluble peptides, free amino group, total phenolic compounds, total flavonoid and reducing sugar in phenolic fraction

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The phenolic fraction obtaining from cooked beans and cooking water were evaluated for TCA-soluble peptides, free amino group, TPC, TFC and reducing sugar; the results, calculated based on the equivalent weight (30 g) of raw seeds, are shown in Table 4.14. This fraction obtaining from PVPP precipitation step was further extracted using 95% methanol. The results showed that TPC in this fraction was lower than that in borate buffer-soluble fraction. This was possibly due to the higher ability of methanol to extract phenolics in the pellets obtaining after PVPP precipitation.

Borate buffer-soluble fraction of both cooked beans and cooking water provided higher ABTS radical scavenging and FRAP compared to other

fractions. The highest activity in cooked beans was recorded for " 10-min autoclaving". However, ferrous chelating activity of all three fractions exhibited similar value. The activity in cooked beans and cooking water from different cooking conditions appeared to be similar. Antioxidant activity of phenolic fractions was lower than that of borate buffer soluble fraction because TPC and TFC in the former were lower than those in the latter. Further investigation was attempted to identify phenolic composition.

The order of ORAC value of cooked beans and cooking water in 3 fractions were as follows: borate buffer-soluble fraction with phenolic removal > borate buffer-soluble fraction > phenolic fraction. The difference in peroxyl scavenging activity might comprehend the compounds present in each fraction. Both phenolics and TCA-soluble peptides had ability in scavenging peroxyl radicals. However, TCA-soluble peptides appeared to play an important role in this activity. The hydrogen atom in the small peptides and aromatic ring of phenolics shows ability to neutralize free radicals formed by chain-breaking antioxidant reactions of thermally generated peroxyl radical from AAPH.

Borate buffer-soluble fraction possessed the highest ACE-I inhibitory activity compared to other fractions. It was possible that this fraction contained both TCA-soluble peptides and phenolic compounds.

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It was worth noting that 10-min autoclaving cooking condition showed high biological activity compared to other conditions. The activity in both cooked beans and cooking water appeared to be similar in value. Thus, both cooked beans and cooking water from 10-min autoclaving cooking condition should be pooled and used for next experiment (Section part B).

Table 4. 14 Effect of different cooking conditions on TCA-soluble peptides, free amino group, TPC, TFC and reducing sugar content in phenolic fraction

Sample	TCA-soluble peptides (mg Tyr/30g raw seeds wb.)	Free amino group (mg Leu/30g raw seeds wb.)	TPC (mg GA/30g raw seeds wb.)	TFC (mg CA/30g raw seeds wb.)	Reducing sugar (mg Glu/30g raw seeds wb.)
Control (soaked beans)	$0.06^{c} \pm 0.01$	$1.31^{e} \pm 0.06$	$0.63^{e} \pm 0.05$	$0.17^{e} \pm 0.03$	$0.31^{bc} \pm 0.05$
60-min boiling					
Cooked bean	$0.01^{a} \pm 0.00$	$0.59^{a} \pm 0.04$	$0.20^{a} \pm 0.02$	$0.07^{a} \pm 0.01$	$0.25^{ab} \pm 0.03$
Cooking water	$0.02^{b} \pm 0.00$	$0.96^{c} \pm 0.15$	$0.25^{b} \pm 0.03$	$0.10^{b} \pm 0.01$	$0.29^{b} \pm 0.04$
10-min autoclaving					
Cooked bean	$0.02^{b} \pm 0.00$	$0.90^{c} \pm 0.07$	$0.31^{c} \pm 0.08$	$0.10^{b} \pm 0.01$	$0.36^{c} \pm 0.02$
Cooking water	$0.00^{a} \pm 0.00$	$0.72^{b} \pm 0.02$	$0.21^{ab} \pm 0.02$	$0.09^{b} \pm 0.01$	$0.20^{a} \pm 0.03$
60-min autoclaving					
Cooked bean	$0.06^{c} \pm 0.01$	$1.15^{d} \pm 0.06$	$0.43^{d} \pm 0.03$	$0.14^{d} \pm 0.01$	$0.69^{d} \pm 0.16$
Cooking water	$0.06^{c} \pm 0.01$	$1.10^{d} \pm 0.09$	$0.36^{c} \pm 0.07$	$0.12^{c} \pm 0.01$	$0.77^{e} \pm 0.09$

wb., wet basis; Mean values ± SD of triplicate determinations. Mean in the same column with different letters are significantly ($p \leq 0.05$).

4.3 Effect of cooking conditions on phenolics in cooked adzuki beans and its cooking water

Cooked beans and its cooking water obtaining from three different cooking conditions were extracted with two different processes called " methanolic extract" and "phenolics extract". The obtained supernatant from process was determined for antioxidant activity, ORAC, TPC, TFC, reducing sugar content and phenolic composition.

4.3.1 Effect of cooking conditions on antioxidant activity and ORAC in methanolic extract obtaining from cooked adzuki beans and its cooking water

The methanolic extract obtaining from cooked beans and cooking water were determined for its antioxidant activity (ABTS radical scavenging, FRAP, ferrous chelating) and ORAC; the results, calculated based on the equivalent weight (30 g) of raw seeds, are shown in Table 4.15. Both cooked beans and its cooking water obtaining from 60 min-boiling exhibited the highest ABTS scavenging activity. Cooked beans from 60-min autoclaving exhibited the highest FRAP and ferrous chelating activity. In cooking water, 60 min-boiling exhibited the highest FRAP as well as 10-min autoclaving possessed ferrous chelating activity. In each antioxidant activity assay, 60 min-autoclaving showed similar value of antioxidant activity in methanolic extract from cooked beans and cooking water. This was because cooking condition caused different degree of releasing and leaching of phenolics.

The methanolic extract obtaining from cooked adzuki beans and its cooking water contributed to the ORAC as shown in Table 4.15. Cooking water obtaining from 60-min autoclaving showed higher ORAC value than other conditions. The methanolic extract showed higher ability in peroxyl scavenging activity than that of phenolic extract (Table 4.12). These results demonstrated that the influence of extraction solvent on measured ORAC in cooked beans and cooking water extracts was remarkable. Cooking condition showed a significant effect on biological activity of cooked beans and its cooking water. Difference in type and amount of phenolic compounds in cooked beans and cooking water were the main reason in this aspect.

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Table 4. 15 Effect of cooking conditions on antioxidant activity and ORAC in methanolic extract obtaining from cooked adzuki beans and its cooking water

Sample	ABTS scavenging activity (mM Trolox/30g raw seeds wb.)	FRAP (mM Trolox/30g raw seeds wb.)	Ferrous chelating (mM EDTA/30g raw seeds wb.)	ORAC (mM Trolox/30g raw seeds wb.)
Control (soaked beans)	$3.27^{a} \pm 0.89$	$2.57^{a} \pm 0.12$	$0.53^{a} \pm 0.03$	$2.85^{a} \pm 0.17$
60-min boiling				
Cooked bean	$16.31^{e} \pm 1.07$	$2.98^{ab} \pm 0.27$	$0.71^{b} \pm 0.09$	$4.63^{b} \pm 0.23$
Cooking water	$37.90^{f} \pm 2.36$	$16.04^{f} \pm 1.49$	$1.12^{d} \pm 0.10$	$7.37^{f} \pm 0.16$
10-min autoclaving				
Cooked bean	$5.42^{b} \pm 0.54$	$3.38^{b} \pm 0.07$	$0.88^{c} \pm 0.12$	$6.66^{e} \pm 0.10$
Cooking water	$12.11^{d} \pm 0.82$	$7.27^{e} \pm 0.45$	$1.89^{g} \pm 0.02$	$6.70^{e} \pm 0.13$
60-min autoclaving				
Cooked bean	$10.65^{c} \pm 1.20$	$6.45^{d} \pm 0.23$	$1.37^{e} \pm 0.07$	$6.45^{d} \pm 0.16$
Cooking water	$10.17^{c} \pm 0.80$	$5.03^{c} \pm 0.10$	$1.58^{f} \pm 0.06$	$5.91^{c} \pm 0.20$

wb., wet basis; Mean values ± SD of triplicate determinations. Mean in the same column with different letters are significantly ($p \leq 0.05$)

Many studies have reported that pulses contain phenolic compounds including phenolic acids, flavonoids and condensed tannins. These compounds are distributed differently in both seed coat and cotyledon of pulses. Singh et al. (2017) found that phenolic acids such as ferulic acid, vanillic acid, coumaric acid, and caffeic acid were main contributor to total antioxidant activity.

4.3.2 Effect of cooking conditions on TPC, TFC and reducing sugar content in methanolic extract obtaining from cooked adzuki beans and its cooking water

Due to the difference in the moisture content of each sample obtaining from different conditions, data were presented in 2 forms (describe as above). TPC, TFC and reducing sugar content in cooked adzuki beans and its cooking water obtaining from different cooking conditions are presented in Table 4.16.

Based on the equivalent weight (30 g) of raw seeds, TPC of cooked bean significantly decreased ($p \leq 0.05$) compared to raw seeds and soaked beans, respectively. 60-min boiling caused high reduction of TPC. The reduction of TPC in cooked beans was due to the leaching of phenolic compounds in to cooking water (Table 4.16). Among all cooking conditions, cooked beans obtained from the cooking condition “60-min autoclaving” released the highest amount of total phenolic content. However, the cooking water from 60-min boiling contained the highest amount of TPC due to leaching effect. Due to direct heat transfer, convection, during boiling, the leaching of free phenolics occurred during cooking. In this study, reducing sugar content was determined because total phenolic determination using Folin-Ciocalteu assay could be interfered by the presence of reducing sugar (Waterhouse, 2005) The amount of reducing sugar in all samples varied from 0.17 to 0.75 mg glucose/g raw seeds (wb.), less than the limit of 25 mg/g sample. These results showed that a presence of reducing sugar was negligible.

Table 4. 16 Effect of different cooking conditions on TPC, TFC and reducing sugar content of methanolic extract from cooked adzuki beans and its cooking water

Sample	TPC (mg GA)		TFC (mg CA)		Reducing sugar content (mg Glu)	
	(/30g raw seeds wb.)	(/100g cooked portion)	(/30g raw seeds wb.)	(/100g cooked portion)	(/30g raw seeds wb.)	(/100g cooked portion)
Raw seeds (Referent)	93.15 ± 4.61	301.51 ± 15.38	15.29 ± 3.32	50.95 ± 11.05	NA	NA
Control (soaked beans)	40.16 ± 2.14	101.97^{d} ± 4.30	27.61 ± 1.56	70.11^{d} ± 3.03	0.11 ± 0.01	0.21^{e} ± 0.00
60-min boiling						
Cooked beans	32.42 ± 3.52	52.98^{b} ± 5.80	22.68 ± 2.01	37.05^{a} ± 3.19	0.09 ± 0.01	0.07^{a} ± 0.00
Cooking water	556.96 ± 34.78	598.60^{f} ± 32.34	230.90 ± 17.79	248.12^{f} ± 16.98	0.07 ± 0.03	0.10^{c} ± 0.01
10-min autoclaving						
Cooked beans	40.71 ± 1.04	49.34^{a} ± 1.04	37.73 ± 1.41	45.72^{c} ± 1.51	0.05 ± 0.01	0.09^{b} ± 0.00
Cooking water	444.59 ± 15.49	561.50^{f} ± 18.29	313.09 ± 8.24	395.45^{g} ± 9.96	0.06 ± 0.02	0.13^{d} ± 0.00
60-min autoclaving						
Cooked bean	62.60 ± 3.54	75.40^{c} ± 3.79	35.11 ± 1.23	42.30^{b} ± 1.31	0.17 ± 0.01	0.30^{f} ± 0.00
Cooking water	318.68 ± 11.60	437.80^{e} ± 15.94	154.09 ± 6.44	211.62^{e} ± 6.91	0.42 ± 0.01	0.55^{g} ± 0.02

wb., wet basis; NA, not analysis. Mean values ± SD of triplicate determinations. Mean in the same column with different letters are significantly ($p \leq 0.05$).

Based on the equivalent cooked portion (100 g), 60-min boiling condition was an appropriate condition to obtain cooked beans with the highest TPC and TFC (Table 4.16). This was because boiling was done through cooking beans in excess water resulting in higher releasing and leaching of phenolics into cooking water. Dewanto et al. (2002) reported that different cooking conditions (heating at 100, 115 and 121 °C for 25 min) resulted in increasing free phenolic compounds in sweet corn compared to fresh one. On the other hand, Xu and Chang (2008) reported that cooked eclipse black beans (*Phaseolus vulgaris* L.) via boiling and autoclaving exhibited significantly ($p \leq 0.05$) lower TPC compared to raw beans due to leaching effect.

4.3.3 Phenolic composition of the methanolic extract from cooked adzuki beans and its cooking water

Antioxidant activity is dependent on both type and amount of phenolics presence in beans. Thus, phenolics composition in methanolic extract of both cooked beans and cooking water were investigated. The amount of individual phenolic compound in adzuki bean subjected to different cooking conditions is shown in Table 4.16. It was found that cooking process affected both type and amount of phenolics. Cooking processes caused a markedly increase in the amount of catechin, epicatechin and sinapic acid in cooked beans. This may be due to the releasing of bound phytochemicals from the breakdown of cell walls. Moreover, a significant increase in rutin, a high antioxidant compound, was found in cooking water. This may come from the leaching effect. It was also found that cooking also affected the change in the amount of catechin, epicatechin, *p*-coumaric acid and sinapic acid (Table 4.17). It has been purposed that the amount and type of individual phenolic compounds are affected during cooking process because (1) Thermal processing could help releasing bound phytochemicals from the breakdown of cell walls, (2) Thermal processing may cause complex chemical reaction of phenolics transform; ferulic acid → vanillin and vanillic acid, *p*-coumaric acid → *p*-hydroxybenzaldehyde (3). Ferulic acid and p-coumaric acid are susceptible to thermal breakdown (Dewanto et al., 2002; Fengyuan Liu, Chang, Hu, Brennan, & Guo, 2017).

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Table 4. 17 Effect of different cooking conditions on phenolic composition of cooked adzuki bean and its cooking water

Polyphenols*	Retention time (min)	λ_{max} (nm)	Soaking		Cooking condition					
					60-min boiling		10-min autoclaving		60-min autoclaving	
			Soaked bean	Soaking water	Cooked bean	Cooking water	Cooked bean	Cooking water	Cooked bean	Cooking water
Gallic acid	15.529	265	0.09 ± 0.04	1.06 ± 1.77	3.35	0.48 ± 0.08	0.09 ± 0.02	0.43 ± 0.06	0.19 ± 0.04	0.66 ± 0.21
Catechin	25.012	280	2.75 ± 0.13	5.00 ± 1.76	3.56	5.78 ± 0.33	3.22 ± 0.44	8.55 ± 2.58	6.97 ± 1.79	5.43 ± 1.66
4-HBA	28.196	265	0.15 ± 0.10	0.52 ± 0.01	nd	nd	nd	nd	0.45 ± 0.22	nd
Vanillic acid	32.112	265	nd	nd	nd	nd	nd	nd	nd	nd
Epicatechin	32.252	280	nd	nd	6.89	7.20 ± 0.51	7.80 ± 0.29	10.94 ± 0.35	9.60 ± 2.92	4.92 ± 0.85
p-coumaric acid	46.309	280	nd	nd	0.38	nd	0.17 ± 0.06	nd	0.22 ± 0.07	0.32 ± 0.09
Sinapic acid	49.803	280	nd	0.90 ± 0.04	1.04	6.43 ± 0.46	0.80 ± 0.09	4.58 ± 0.06	1.00 ± 0.31	3.59 ± 0.79
Ferulic acid	50.004	280	9.75 ± 0.57	nd	nd	nd	nd	nd	nd	nd
Vitexin	56.281	265	0.44 ± 0.09	0.70 ± 0.40	1.16	1.75 ± 0.09	0.77 ± 0.09	1.30 ± 0.09	0.71 ± 0.16	0.93 ± 26
Rutin	63.640	265	4.88 ± 0.18	8.57 ± 1.17	4.06	12.07 ± 1.16	2.70 ± 0.25	8.11 ± 0.39	2.96 ± 0.51	3.69 ± 1.34
Trans-cinamic acid	73.343	280	0.15 ± 0.01	nd	0.04	nd	0.04 ± 0.01	nd	0.15 ± 0.03	nd
Quercetin	74.336	265	0.89 ± 0.26	nd	1.26	nd	1.00 ± 0.29	nd	1.17 ± 0.73	nd
Naringenin	74.860	280	nd	nd	nd	nd	nd	nd	nd	nd
Genistein	76.173	265	nd	nd	nd	nd	nd	nd	nd	nd

*Individual phenolic content express as mg per 30 g raw seeds; nd, not detected

4.3.4 Phenolic composition in borate buffer-soluble fraction of cooked adzuki beans and its cooking water

The biological activity of borate buffer-soluble fraction is shown in Table 4.3, Table 4.4 and Table 4.5 (Section 4.2.1.1). Borate buffer-soluble fraction contained both small peptides and phenolic compounds. These compounds exhibited biological activity. For phenolic compounds, their biological activity depended on type and amount of individual phenolic. Thus, the amount of individual phenolic compound in borate buffer-soluble fraction of cooked adzuki beans and its cooking water subjected to different cooking conditions were determined and shown in Table 4.18. The main phenolics found in borate buffer-soluble fraction of cooked adzuki beans and its cooking water were 4-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, vitexin, rutin and chrysin. Each phenolics content in adzuki beans attempt to increase after cooking and some phenolic compounds leached into cooking water during cooking.

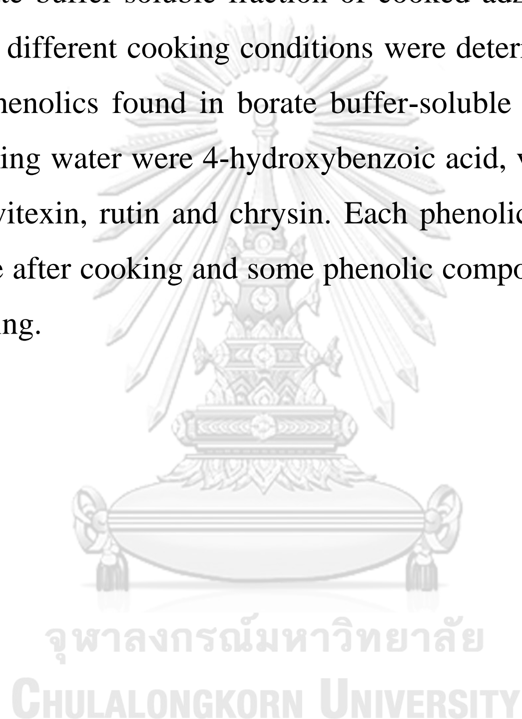
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Table 4. 18 Effect of different cooking conditions on borate buffer-soluble fraction of cooked adzuki beans and its cooking water (per 30 g raw seeds)

Polyphenols*	Retention time (min)	λ_{max} (nm)	Control (Soaked bean)	Cooking condition					
				60-min boiling		10-min autoclaving		60min-autoclaving	
				Cooked bean	Cooking water	Cooked bean	Cooking water	Cooked bean	Cooking water
Gallic acid	4.205	265	13.62 ± 0.36	nd	nd	nd	54.99 ± 11.22	nd	nd
4-HBA	8.279	265	144.89 ± 31.52	1119.81 ± 1.12	nd	nd	nd	1261.98 ± 4.03	86.84 ± 0.61
Vanillic acid	11.795	265	5.40 ± 0.44	7.72 ± 0.86	6.60 ± 0.28	15.25 ± 0.18	5.40 ± 0.37	7.83 ± 1.07	6.65 ± 0.65
Syringic acid	14.111	265	3.27 ± 0.62	4.80 ± 0.22	46.27 ± 0.10	32.64 ± 1.13	46.90 ± 1.10	38.05 ± 10.17	15.08 ± 0.24
Catechin + Epicatechin	15.724	280	4.31 ± 0.35	nd	nd	nd	nd	nd	nd
Caffeic acid	16.046	280	2.30 ± 0.18	nd	46.25 ± 0.48	39.05 ± 0.04	nd	nd	nd
Chlorogenic acid	18.299	280	nd	nd	nd	32.27 ± 0.56	nd	8.10 ± 0.22	nd
Ferulic acid	19.212	280	91.91 ± 0.64	nd	nd	nd	nd	nd	nd
p-coumaric acid	20.963	280	4.66 ± 0.14	8.88 ± 0.51	14.36 ± 3.76	nd	nd	15.27 ± 0.15	nd
Vitein	23.555	265	3.86 ± 0.09	6.71 ± 0.17	12.84 ± 2.52	9.36 ± 0.26	7.82 ± 0.04	25.99 ± 0.37	18.78 ± 2.78
Sinapic acid	24.479	280	nd	nd	nd	nd	nd	nd	nd
Rutin	26.739	265	16.19 ± 0.99	16.27 ± 2.40	29.95 ± 5.50	21.27 ± 0.92	19.71 ± 2.84	51.30 ± 0.11	30.02 ± 0.19
Ellagic acid	30.305	280	31.11 ± 0.03	nd	nd	51.63 ± 0.86	nd	nd	nd
Quercetin	33.832	265	5.36 ± 0.01	nd	nd	nd	nd	nd	nd
Naringenin	34.923	265	57.63 ± 0.06	94.88 ± 0.99	nd	nd	nd	nd	4.21 ± 0.10
Keamferol	37.283	265	2.26 ± 0.17	nd	nd	nd	nd	nd	nd
Trans-cinamic acid	38.614	280	nd	nd	nd	nd	nd	nd	1.44 ± 0.02
Chrysin	49.167	265	2.05 ± 0.08	3.05 ± 0.11	4.07 ± 0.05	5.95 ± 0.04	1.49 ± 0.01	1.67 ± 0.08	nd

*Individual phenolic content expresses as µg per 30 g raw seeds; nd, not detected

4.3.5 Phenolic composition in phenolics extract (phenolic fraction) of cooked adzuki beans and its cooking water

The biological activity of phenolic extract is shown in Table 4.11, 4.12 and 4.13 (Section 4.3.2). The number and position of specific functional group related to activities. Type and amount of individual phenolic compound in phenolics extract (phenolic fraction) of cooked adzuki beans and its cooking water are shown in Table 4.19. 4-Hydroxybenzoic acid, vanillic acid, vitexin, rutin, keamferol and chrysin were found in phenolics extract obtaining from cooked beans and its cooking water.

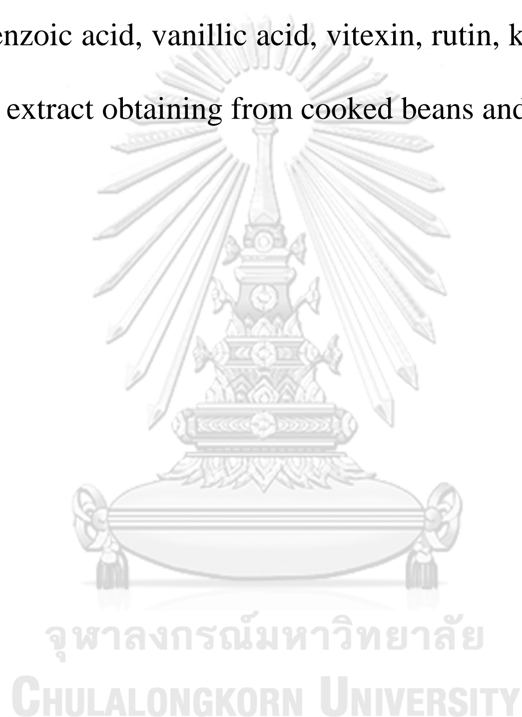
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Table 4. 19 Effect of different cooking conditions on phenolic extract of cooked adzuki beans and its cooking water (per 30 g raw seeds)

Polyphenols*	Retention time (min)	λ_{max} (nm)	Control (Soaked bean)	Cooking condition					
				60-min boiling		10-min autoclaving		60min-autoclaving	
				Cooked bean	Cooking water	Cooked bean	Cooking water	Cooked bean	Cooking water
Gallic acid	4.205	265	nd	52.93 ± 0.12	nd	nd	nd	nd	490.56 ± 0.65
4-HBA	8.279	265	nd	138.52 ± 25.78	22.31 ± 36.78	246.93 ± 0.43	nd	305.59 ± 0.33	300.57 ± 5.70
Vanillic acid	11.795	265	2.90 ± 0.78	5.18 ± 0.20	8.03 ± 0.03	nd	nd	7.04 ± 0.48	7.01 ± 0.08
Syringic acid	14.111	265	25.34 ± 1.62	42.00 ± 1.71	nd	61.22 ± 0.11	nd	nd	nd
Catechin + Epicatechin	15.724	280	nd	17.84 ± 0.38	nd	nd		nd	nd
Caffeic acid	16.046	280	nd	nd	nd	nd	nd	nd	nd
Chlorogenic acid	18.299	280	nd	nd	nd	nd	nd	nd	nd
Ferulic acid	19.212	280	nd	nd	nd	nd	nd	nd	nd
p-coumaric acid	20.963	280	nd	nd	nd	nd	nd	nd	7.69 ± 0.10
Vitein	23.555	265	nd	9.62 ± .87	10.87 ± 0.23	9.40 ± 0.19	nd	nd	nd
Sinapic acid	24.479	280	17.25 ± 1.01	nd	nd	35.52 ± 0.51	nd	nd	nd
Rutin	26.739	265	nd	5.23 ± 0.86	25.20 ± 1.57	21.92 ± 2.03	19.40 ± 2.52	13.60 ± 1.49	9.04 ± 0.28
Ellagic acid	30.305	280	nd	nd	nd	nd	nd	nd	nd
Quercetin	33.832	265	nd	nd	nd	nd	nd	nd	nd
Naringenin	34.923	265	nd	0.12 ± 0.00	nd	nd	nd	nd	nd
Keamferol	37.283	265	2.30 ± 0.21	4.55 ± 0.46	nd	4.83 ± 0.47	4.46 ± 0.05	5.22 ± 0.68	4.34 ± 0.73
Trans-cinamic acid	38.614	280	nd	nd	nd	nd	nd	nd	nd
Chrysin	49.167	265	0.47 ± 0.06	0.72 ± 0.18	1.01 ± 0.26	0.64 ± 0.05	0.89 ± 0.07	1.22 ± 0.02	0.61 ± 0.05

*Individual phenolic content express as µg per 30 g raw seeds; nd, not detected

4.4. Microstructure

Scanning electron micrographs of raw and cooked adzuki bean obtaining from different cooking conditions are presented in Figure 4.2. The starch granules exhibited spherical-oval. Soaked bean sample, starch granules were characterized by smooth surface. For all cooking conditions, their surface was flattened as an effect of heat action. No birefringence was observed as starch gelatinization occurred during cooking. Figure 4.2 shows starch swelling occurred and starch granule ruptured after cooking. Thermal process can modify the complex structure of legume which can affect the release of short-chain peptides, free amino acids, water soluble phenolics and free phenolic from bounded forms.

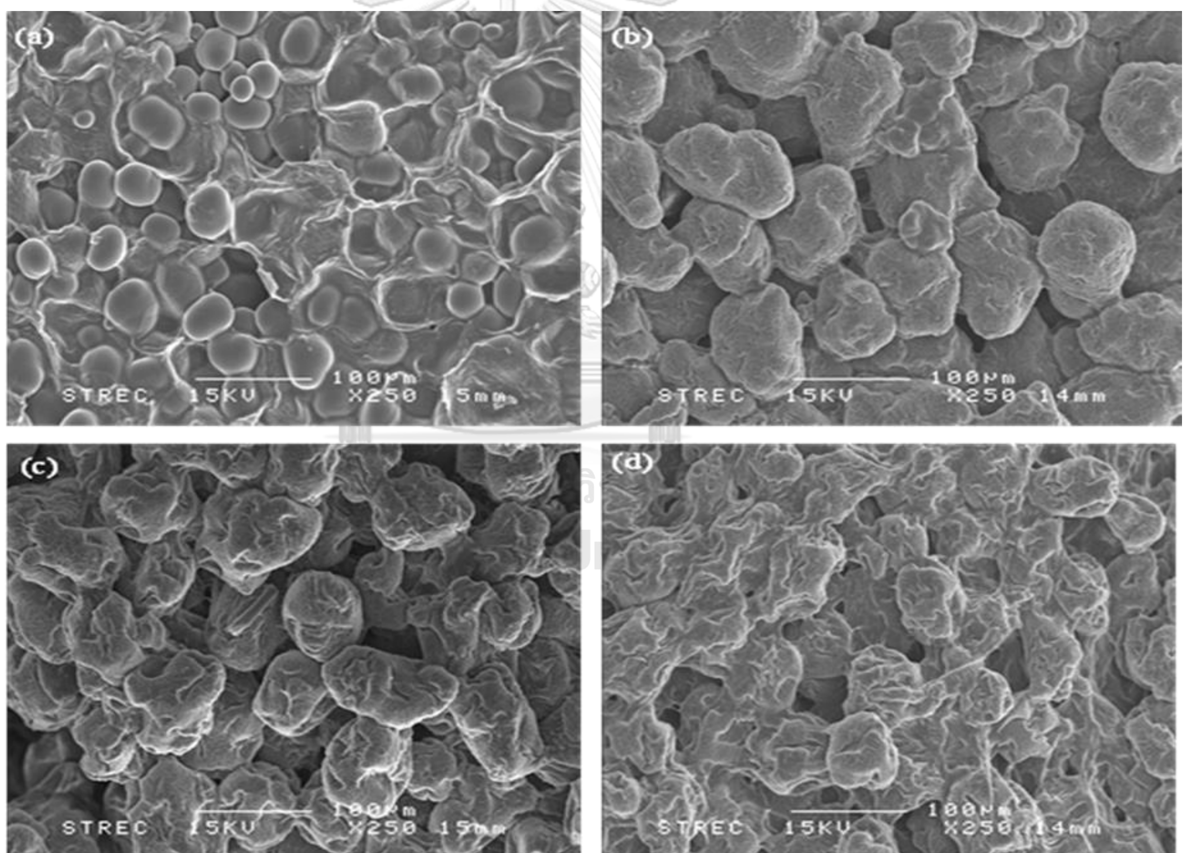

Figure 4. 2 Microstructure of cooked adzuki bean (cross section) at 250× magnification; (a) the control; (b) 60-min boiling; (c) 10-min autoclaving; (d) 60-min autoclaving

Part B: alginate-based encapsulation

4.5 Optimization of encapsulation conditions for producing Ca-alginate beads containing adzuki beans hydrolysate with high encapsulation efficiency

4.5.1 Adzuki beans hydrolysate: preparation and its properties

The autoclaving method at 121 ^{o}C for 10 minutes was selected as a proper cooking condition for adzuki beans hydrolysate preparation. Thermal processing was applied to disrupt a complex structure of adzuki beans. During cooking, some TCA-soluble peptides and some phenolic compounds were released from a complex structure and leached into cooking water. Therefore, cooked adzuki beans and cooking water were mixed and used for adzuki beans hydrolysate production. The adzuki bean hydrolysate was analyzed for free amino group, degree of hydrolysis (DH), TCA-soluble peptides content, released total phenolic content, pH and brix. The results are shown in Table 4.20.

Table 4. 20 Physico-chemical properties of 10-min autoclaving adzuki beans hydrolysate

Parameters	
Free amino group (µmole/g protein)	1139.36 ± 60.98
DH (%)	52.68 ± 3.85
TCA-soluble peptides (mg Tyr/100mL hydrolysate)	7.14 ± 0.47
TPC (mg GA/100 mL hydrolysate)	37.34 ± 2.5
pH	6.41 ± 0.07
Brix (^{o}Brix)	7.67 ± 0.46

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The obtained α-amino group directly related with DH. DH of adzuki beans hydrolysate was 52.68 ± 3.85 %. Our result was higher than DH of azuki beans hydrolysate, around 33 %, reported by (Durak et al., 2013). This might be because denaturation of protein and unfolding of the protein structure during autoclaving

results in increased accessibility of the enzyme to the substrate. Adzuki bean hydrolysate contained TCA-soluble peptides and phenolic compounds. TCA-soluble peptides were from the hydrolysis of protein in adzuki bean and releasing of peptides during cooking. Both free and bound-form phenolics were released from complex structure of adzuki beans. In addition, the hydrolysis can modify the protein-bound polyphenol of the legumes resulting in a releasing of phenolic compounds.

4.5.2 Optimization of Ca-alginate encapsulation condition containing adzuki bean hydrolysate using response surface methodology (RSM)

4.5.2.1 Experimental design

Encapsulation is defined as the process of confining active compounds within a matrix in particulate form in order to achieve one or more desirable effects such as protection sensitive ingredients, extending shelf life and controlling the release of active compounds (Nedovic, Kalusevic, Manojlovic, Levic, & Bugarski, 2011). The formation of cross-linked alginate gel matrices can be achieved with 2 consecutive steps, external gelation and internal gelation, respectively. Initially, the bioactive compounds are added into alginate solution to form the cross-linking network through hydrogen bonding or hydrophobic interaction using extruded or atomized droplets technique. This step is called the external gelation. Then, bioactive compounds-alginate complex was dropped from an air droplet generator into a $CaCl_2$ solution to form a microcapsule. This step is called the internal gelation. The divalent cations form interchain bonding between G-blocks of alginate structure, yielding a 3D network in the form of a gel. An eggbox model has been proposed to explain the nature of this interaction (Ching et al., 2017).

4.5.2.2 Determination of physicochemical properties of response variables

Appropriate encapsulation condition was selected based on the concentration of alginate and $CaCl_2$ in order to obtain high encapsulation efficiency of bioactive compounds (phenolics and TCA-soluble peptides), a key factor showing antioxidant and ACE-I inhibitory activities. The physical properties and release

characteristics of Ca-alginate containing adzuki beans hydrolysate was also investigated to support calcium-alginate bead characteristics. Observed (experiment) values for all responses at different combinations of independent variables are shown in Table 4.21.

Table 4. 21 Ca-alginate encapsulation conditions on encapsulation efficiency (TPCEE and TCA-soluble peptidesEE) of Ca-alginate beads containing adzuki beans hydrolysate

Run	Independents			Responds	
	Sodium alginate (w/v)	$CaCl_2$ (w/v)	TPC (mg gallic acid)	TPCEE (%)	TCA-soluble peptidesEE (%)
1	1	1	15	7.74 ± 0.20	7.37 ± 0.65
2	3	1	15	9.02 ± 0.89	6.91 ± 0.22
3	1	2	15	5.41 ± 0.28	9.08 ± 0.88
4	3	2	15	9.24 ± 0.54	10.67 ±1.14
5	1	1	45	12.54 ± 1.11	10.20 ± 0.40
6	3	1	45	8.64 ± 0.24	7.99 ± 0.31
7	1	2	45	49.79 ± 1.7	10.44 ± 0.65
8	3	2	45	46.38 ± 1.94	4.91 ± 0.29
9	0.32	1.5	30	6.33 ± 0.37	4.27 ± 0.12
10	3.68	1.5	30	3.22 ± 0.18	3.51 ± 0.09
11	2	0.66	30	5.75 ± 1.06	2.86 ± 0.29
12	2	2.34	30	3.99 ± 0.41	3.80 ± 0.06
13	2	1.5	4.77	49.47 ± 6.48	1.05 ± 0.46
14	2	1.5	55.23	11.43 ± 0.81	10.45 ± 0.68
15	2	1.5	30	5.43 ± 0.25	5.38 ± 0.20

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Ca-alginate beads encapsulated using condition 7 (1% sodium alginate, 45 mg gallic acid, and 2% $CaCl_2$) and condition 13 (2% sodium alginate, 4.77 mg gallic acid and 1.5% $CaCl_2$ showed markedly high encapsulation efficiency of total

phenolics content (TPCEE) (49.79 ± 1.7 and 49.47 ± 6.48 %, respectively) compared to other conditions. Ca-alginate beads encapsulated using condition 4 (3% sodium alginate, 15 mg gallic acid, and 2% $CaCl_2$), condition 7 (3% sodium alginate,45mg gallic acid, and 2% $CaCl_2$) and condition 14 (2% sodium alginate, 55.23mg gallic acid and 1.5% $CaCl_2$ yielded markedly high encapsulation efficiency of TCA-soluble peptides content (TCA-soluble peptidesEE) (10.67, 10.44 and 10.45%, respectively) compared to other conditions. The condition 7 (1% sodium alginate, 45 mg gallic acid and 2% $CaCl_2$) showed the highest TPCEE and TCA-soluble peptidesEE. The results showed that encapsulation efficiency of Ca-alginate beads depended on interaction between wall material and core material. An appropriate amount of core and wall material in Ca-alginate beads yielded higher percentage of encapsulation efficiency. In this bead system, alginate-phenolics and alginate-small peptides interaction were formed before the crosslinking of calcium ion with alginate structure. Phenolic compounds found in plants are mainly hydrophilic compounds. The most common type of interactions between the phenolic compounds and the alginate chains was found to be the hydrogen bonding including the carboxyl and hydroxyl groups of carbohydrates (Plazinski & Plazinska, 2011). The interaction of alginate and peptide may be 1) covalent attachment of peptides to the alginate backbone and 2) interaction between proteins and alginate via non-covalent interactions such as hydrogen bonding, van der Waal forces, hydrophobic interaction and ionic bonding.

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4.5.2.3 Modeling and statistical analysis

The experimental data were analysed using Design-Expert software. The software showed several useful statistical tables, linear, 2FI, quadratic and cubic model, which labelled suggested model and aliased model. The optimal Ca-alginate encapsulation condition was obtained by using the desirability function approach on the Design-Expert software after determining the multifactor analysis of variance and the 2nd order model prediction. The fitted polynomial equation was then expressed in the form of three-dimensional surface plots in order to illustrate the relationship between responses and the experimental variables used. RSM was applied to determine the effect of the sodium alginate concentration, $CaCl_2$ concentration and TPC on TPCEE and TCA-soluble peptidesEE of Ca-alginate beads containing adzuki bean hydrolysate. ANOVA for the 2nd order response surface model of TPCEE and TCA-soluble peptidesEE of Ca-alginate beads are shown in Table 4.22.

The results of ANOVA, using Design-Expert software, for encapsulation efficiency of TPC suggested quadratic model due to F-value tests the significance of adding quadratic terms to the 2FI model. The model showed the F-value (98.84). The results implied that the lack of fit was significant. The large of F-value occurred due to noise. Whilst p-value was 1.32 (less than 0.0500) indicating that model terms were significant. There was only 0.01% chance that a large value of the lack of fit could occur due to noise. A small p-value (Prob > F) indicated that adding quadratic terms improved the model fitting.

The results of ANOVA, using Design-Expert software, for encapsulation efficiency of TCA-soluble peptides suggested linear model, the model showed the F-value (31.66). The results implied that the lack of fit was significant. The large of F-value occurred due to noise. Whilst p-value was 0.0007 (less than 0.0500) indicating that model terms were significant.

The three-dimensional representation, cube model plot and perturbation of the response surfaces by models are shown in Figure 4.3, 4.4, 4.5 and 4.6

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Table 4. 22 Analysis of variance (ANOVA) for the 2nd order response surface model of TPCEE and TCA-soluble peptidesEE of Ca-alginate beads

Response	Source	DF	SS	MS	F-value	*P*-value
EETPC	Lack-of-fit	5	1987.44	397.49	98.84	1.32
	Pure error	5	20.11	4.02		
	Total	19	4389.76			
TCA-soluble peptidesEE	Lack-of-fit	11	115.52	10.50	31.66	0.0007
	Pure error	5	1.66	0.3317		
	Total	19	140.22			

$$TPCEE = 7.20-0.5221A + 5.10B + 1.59C + 0.4175\,AB - 1.51\,AC + 9.60BC + 0.1066\,A^2 + 0.1402B^2 + 9.18\,C^2 \quad (R^2 = 0.5427;\ R^2_{Adj} = 0.1311;\ R^2_{pre} = -2.4453)$$

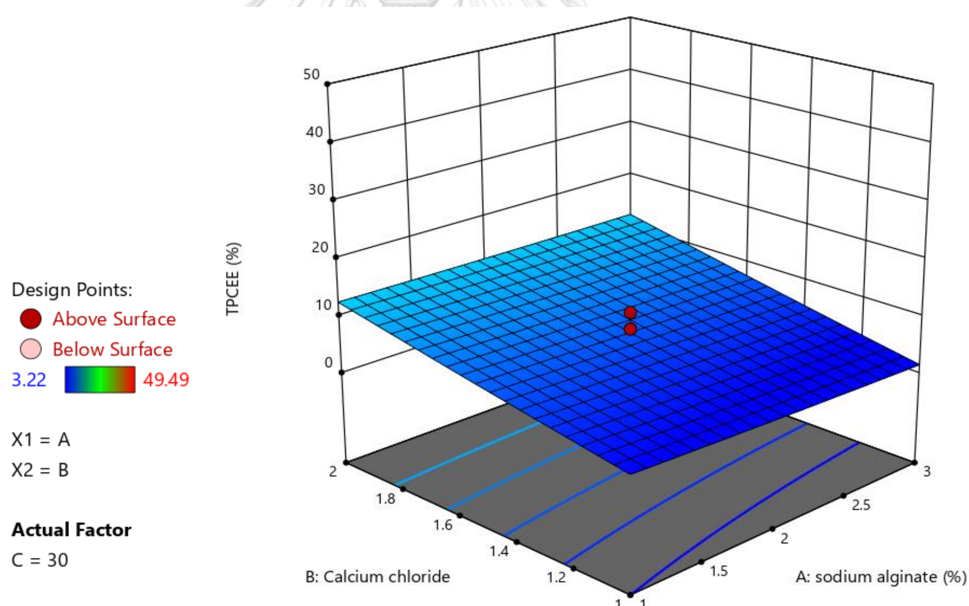

Figure 4. 3 Response surface model plot and regression coefficients of predicted models showing the effects of sodium alginate concentration, $CaCl_2$ concentration and TPC (A, B and C) on TPCEE (%)

TCA-soluble peptidesEE = +6.60 – 0.5776A + 0.3083B +1.12C

(R^2 = 0.1643; R^2_{Adj} = 0.0076; R^2_{pre} = -0.5323)

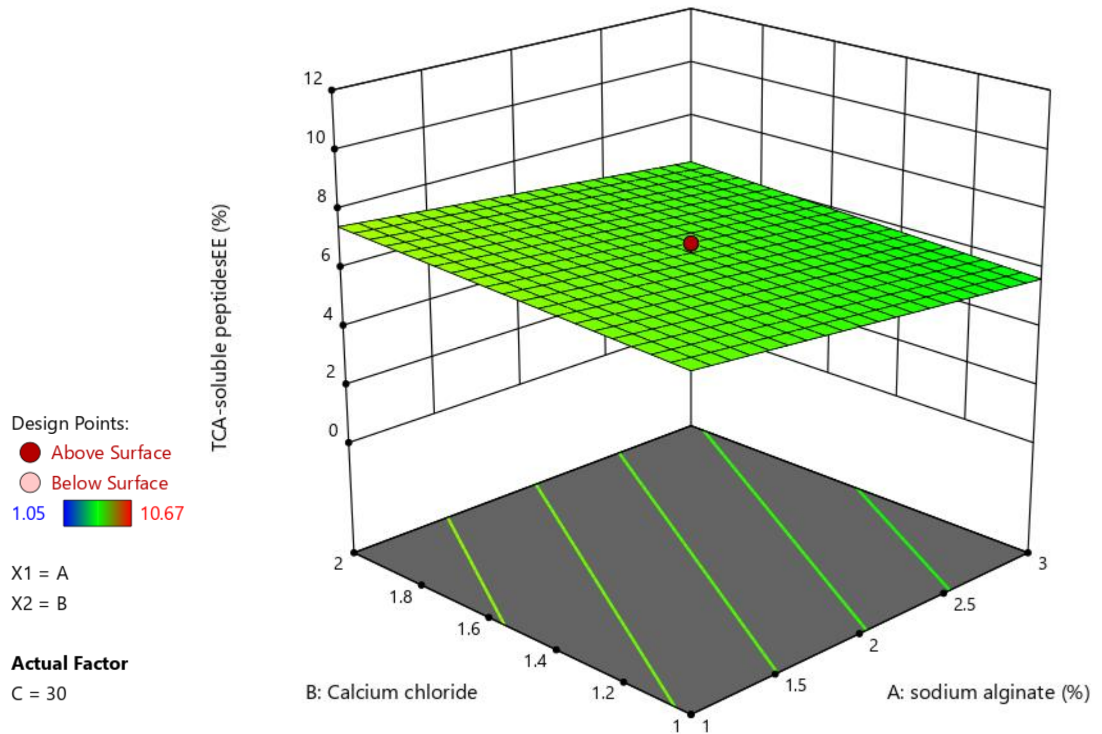

Figure 4. 4 Response surface model plot and regression coefficients of predicted models showing the effects of sodium alginate concentration, $CaCl_2$ concentration and TPC (A, B and C) on TCA-soluble peptidesEE (%)

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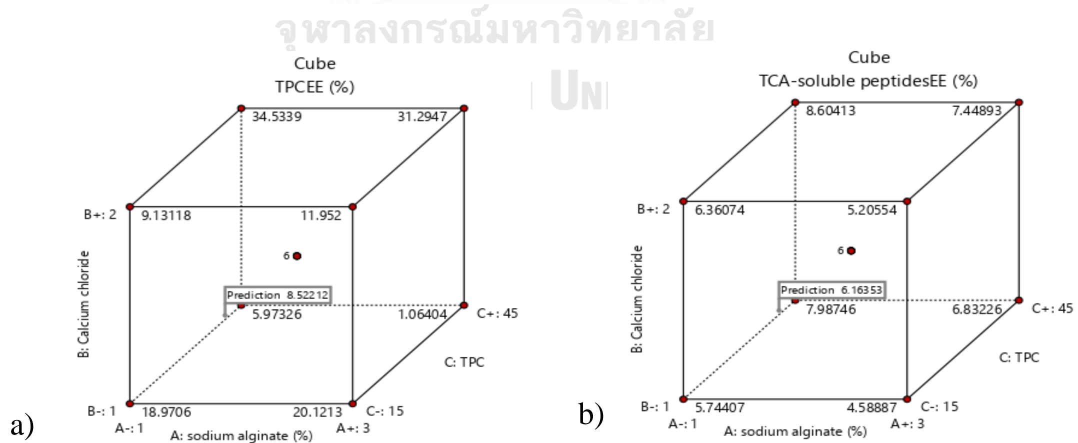

Figure 4. 5 Cube model plot and regression coefficients of predicted models showing the effects of sodium alginate concentration, $CaCl_2$ concentration and TPC on TPCEE (a) and TCA-soluble peptidesEE (b)

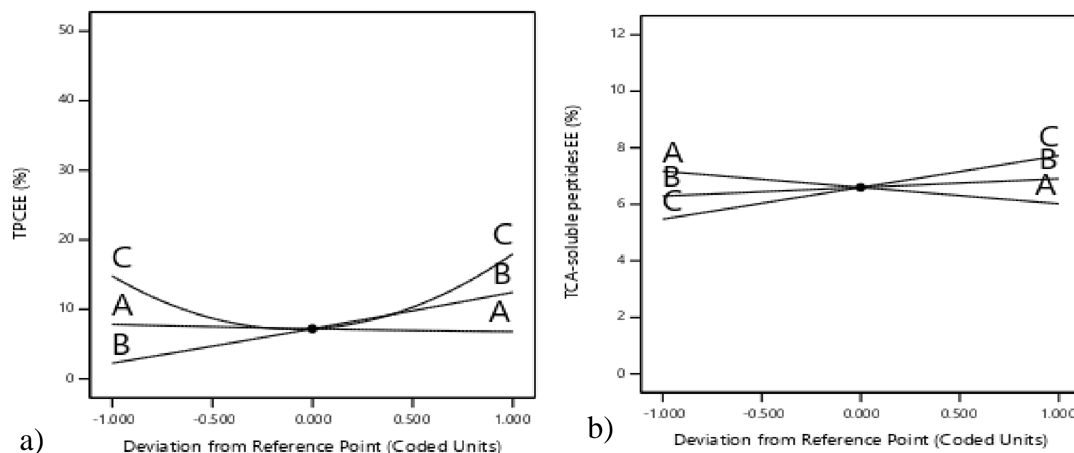

Figure 4. 6 Perturbation of predicted models showing the effects of sodium alginate concentration, $CaCl_2$ concentration and TPC on TPCEE (a) and TCA-soluble peptidesEE (b)

4.5.2.4 Validation

RSM was proved to be effective in estimating the effect of three independent variables, namely sodium alginate, CaCl2 concentration and TPC. The response surface model plot (Figure 4.3 and 4.4) could not show peak of plateau. Thus, validation was not implemented.

4.5.3 Optimization of encapsulation condition of Ca-alginate beads containing adzuki bean hydrolysate using factorial design

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4.5.3.1 Experimental design

An experimental factorial design was employed to investigate the influence of alginate and calcium chloride concentration on encapsulation efficiency of phenolics and encapsulation efficiency of TCA-soluble peptides of Ca-alginate beads containing adzuki bean hydrolysate. These two parameters were selected because preliminary study showed a significant effect of these two parameters on encapsulation efficiency and physico-chemical properties of Ca-alginate beads. Three level of sodium alginate concentrations (1, 2 and 3% w/v) and three different $CaCl_2$ concentrations (1, 1.5 and 2% w/v) were designed. All conditions contained 120 – 125 mL beans hydrolysate (equivalent to 45 mg gallic acid and 8.6 mg tyrosine).

4.5.3.2 Determination of physico-chemical properties of response variables

The gel strength of wet Ca-alginate containing adzuki beans hydrolysate increased when alginate and $CaCl_2$ concentration was increased (Table 4.23). A hydrophilic cavity of Ca-alginate gel structure was formed through multi-coordinated binding of calcium divalent cation with oxygen atoms in the carboxyl groups of L-guluronic acid (G) residues.

Table 4. 23 The effect of different encapsulation condition of wet Ca-alginate beads containing adzuki bean hydrolysate on gel strength of Ca-alginate

Sodium alginate concentration (%)	**Calcium chloride concentration (%)**		
	1	**1.5**	**2**
1	$21.02^{a} \pm 2.20$	$12.02^{a} \pm 2.31$	$19.85^{a} \pm 2.68$
2	$70.07^{c} \pm 25.82$	$51.10^{b} \pm 12.24$	$53.82^{b} \pm 6.26$
3	$109.24^{d} \pm 40.72$	$106.54^{d} \pm 34.87$	$79.88^{c} \pm 17.95$

Mean values ± SD of triplicate determinations. Mean values followed by different superscript letters are significantly different ($p \leq 0.05$).

The mean diameter of various conditions was in a range of 4.39 to 5.06 mm which was found to be within a narrow range (Table 4.24). This was because the bead size was controlled through the method of dripping process and the distance between the syringe and the surface of calcium chloride solution.

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Table 4. 24 The effect of different encapsulation condition of wet Ca-alginate beads containing adzuki bean hydrolysate on diameter of Ca-alginate beads (mm)

Sodium alginate concentration (%)	**Calcium chloride concentration (%)**		
	1	**1.5**	**2**
1	$4.39^{a} \pm 0.19$	$4.55^{b} \pm 0.23$	$4.531^{b} \pm 0.19$
2	$4.98^{de} \pm 0.61$	$4.89^{d} \pm 0.52$	$4.74^{c} \pm 0.43$
3	$5.06^{e} \pm 0.30$	$5.02^{e} \pm 0.28$	$4.97^{de} \pm 0.17$

Mean values ± SD of triplicate determinations. Mean values followed by different superscript letters are significantly different ($p \leq 0.05$).

Figure 4.7 shows the shape of Ca-alginate beads under different encapsulation conditions. The results showed that the concentration of alginate affected bead shape. The condition containing alginate concentration higher than 3% formed bead with tail, a tailed-spherical shape. The tail could be formed due to higher viscosity of high alginate concentration, resulting in higher gravity during dripping process. The smoothness of surface increased when the concentration of alginate and $CaCl_2$ was increased. This phenomenon may be a result of increasing the interaction between alginate and Ca^{2+} ion.

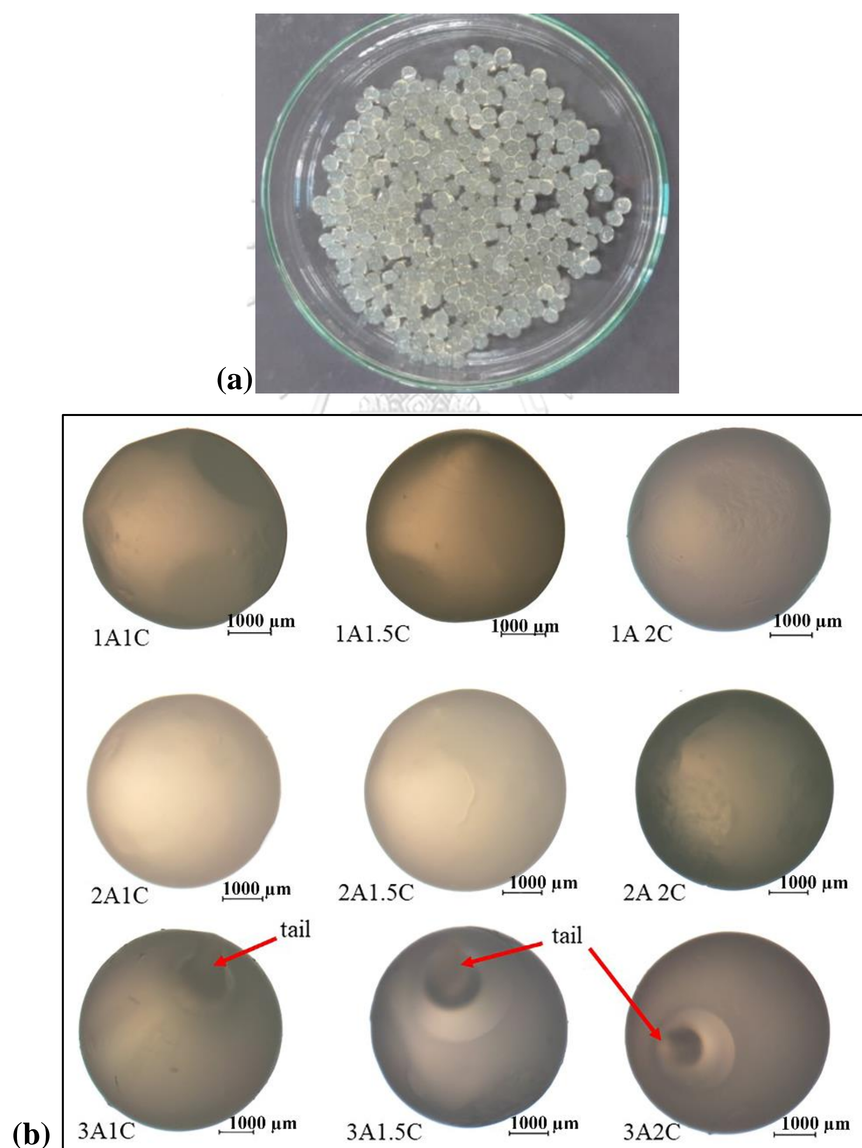

Figure 4. 7 Visual of wet Ca-alginate beads (a) and shape of Ca-alginate beads under different encapsulation condition containing adzuki bean hydrolysate (b), A=alginate concentration and C = $CaCl_2$ concentration

The encapsulation efficiency of wet Ca-alginate beads is shown in Figure 4.8. The percentage of phenolics encapsulation efficiency for all conditions was found to be in the range of 38 to 59%. The interaction between alginate and phenolic compounds may occur through the interaction between polar groups of alginate and hydroxyl groups of phenolic molecules using hydrogen bonding. The percentage of TCA-soluble peptide encapsulation efficiency for all conditions was found to be in the range of 32 to 50%. The interaction of alginate and peptide may occur through 1) covalent attachment of peptides to the alginate backbone and 2) proteins and alginate interact by non-covalent interactions such as hydrogen bonding, van der Waal forces, hydrophobic interaction and ionic bonding. This result indicated that both phenolic compounds and TCA-soluble peptides were entrapped in Ca-alginate network. However, some phenolic compounds and TCA-soluble peptides were lost during the encapsulation process, specifically during immersion of the alginate beads into the calcium chloride collecting solution.

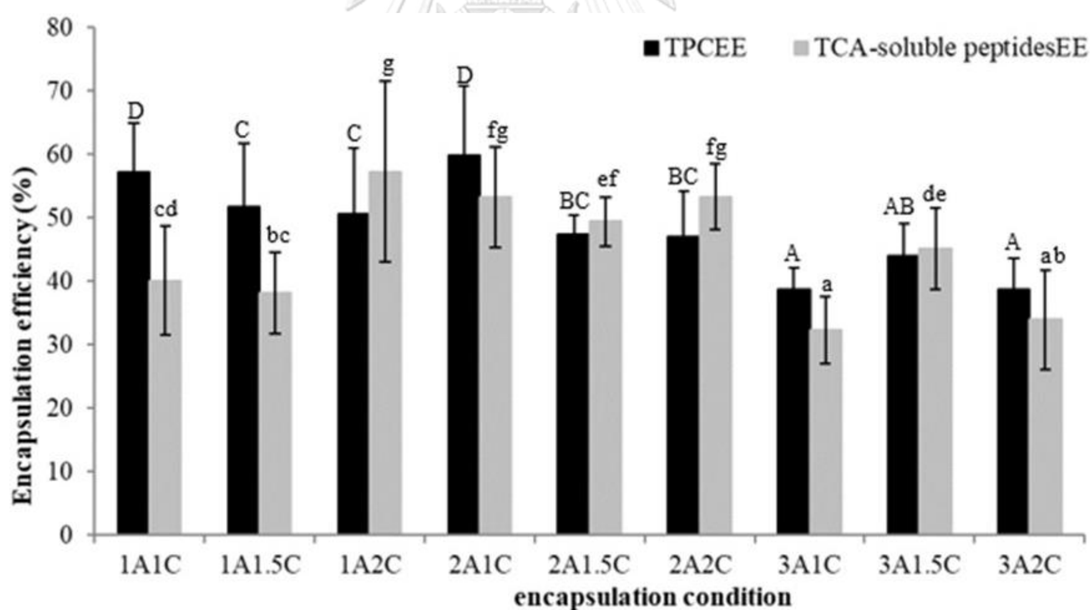

Figure 4. 8 The percentage of encapsulation efficiency of wet Ca-alginate beads under different conditions

The bioactive compounds-release property of these beads was investigated at two different environmental pH (pH 1.2 and 7.4) at 37 ºC for 2 hours. The encapsulated phenolics were able to release from wet beads, around 57.40% - 98.14%

at pH 1.2 and 9.60% - 37.10% at pH 7.4, respectively (Figure 4.9). While, the encapsulated TCA-soluble peptides were able to release from wet beads around 62.29% - 84.64% at pH 1.2 and 55.04% - 93.49% at pH 7.4, respectively (Figure 4.10). The obtained results indicated that Ca-alginate beads could be a vehicle to transport active ingredients.

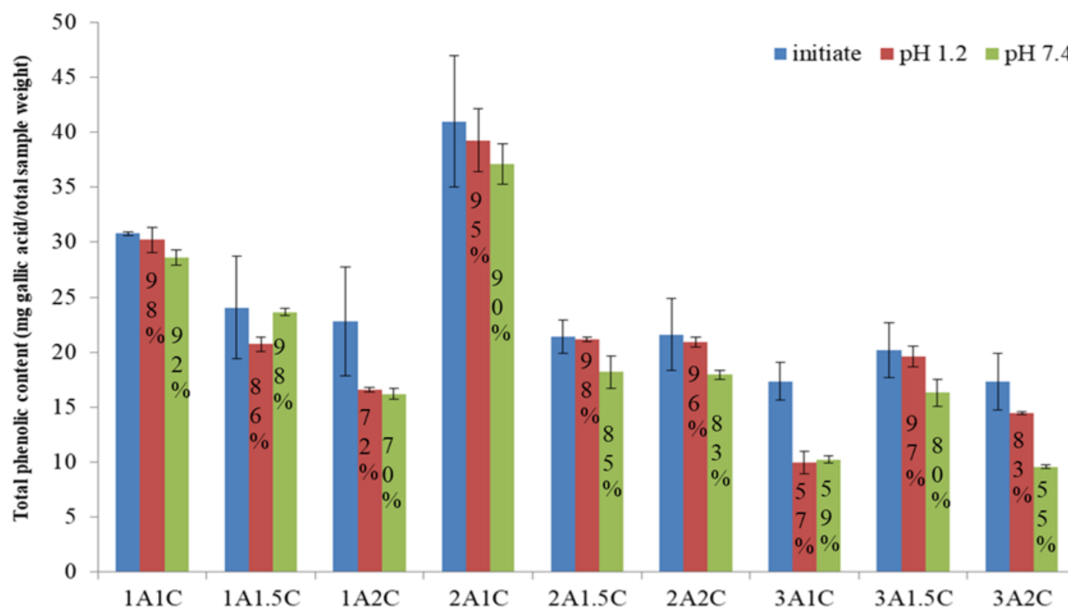

Figure 4. 9 Release of phenolic compound from Ca-alginate beads in pH 1.2 and 7.4 at 37 ºC

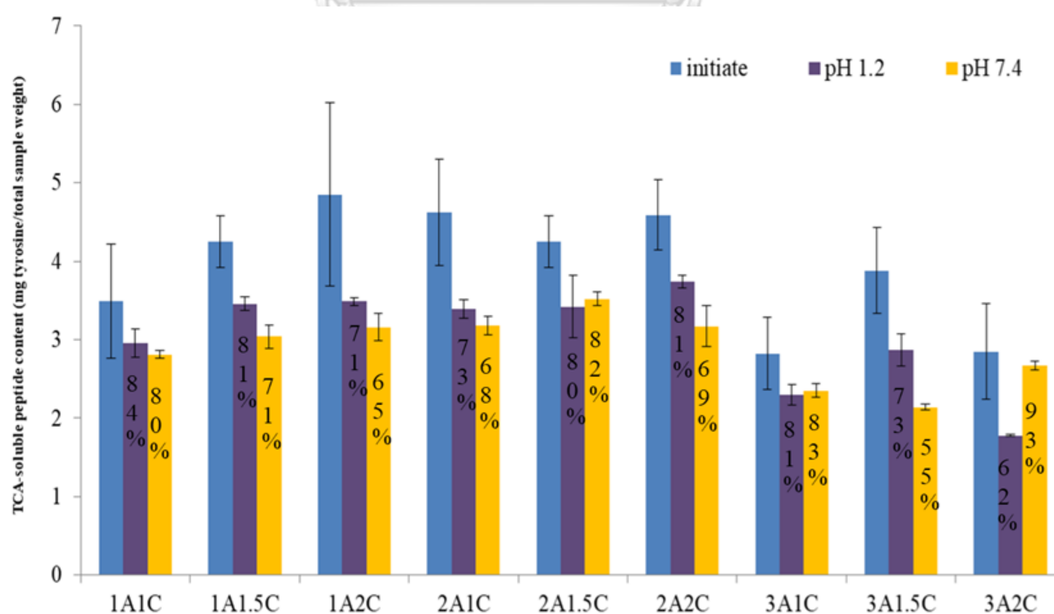

Figure 4. 10 Release of TCA-soluble peptide from Ca-alginate beads in pH 1.2 and 7.4 at 37 ºC

4.5.3.3 Statistical analysis

Ca-alginate beads containing adzuki bean hydrolysate obtaining from different sodium alginate and $CaCl_2$ concentration showed different beads characteristic. Both TPCEE and TCA-soluble peptidesEE of the beads increased with increasing the concentration of sodium alginate. Whilst, encapsulation efficiency was not affected by $CaCl_2$ concentration. Increasing gel strength and size of beads varied with increasing sodium alginate and $CaCl_2$ concentration.

The results showed that the proper encapsulation condition, showing high encapsulation efficiency, of Ca-alginate bead preparation obtained from RSM and factorial design was dependent on calcium chloride and alginate concentrations. In RSM, phenolics content, another independent variable, showed no effect on encapsulation efficiency. This was possibly that adzuki beans hydrolysate as core material containing both phenolics and TCA-soluble peptides affected system interaction and Ca−alginate junction zones. Thus, the controlling of phenolics content was alternative for Ca-alginate beads. The 2% alginate concentration and 1.5% CaCl2 concentration was selected as the selected condition for Ca-alginate beads containing adzuki beans hydrolysate for further study.

4.6 Effect of different encapsulation techniques in alginate-based: Physicochemical properties and release characteristics under simulated gastrointestinal digestion

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4.6.1 Experimental design

Adzuki beans hydrolysate contained both small peptides and phenolic compounds. They possess both antioxidant and health promoting activity. Both small peptides and phenolic compounds can be used as functional food ingredients and supplements. However, few studies have focused on the encapsulation of adzuki beans hydrolysate. Thus, two different encapsulation techniques containing adzuki beans hydrolysate, calcium alginate beads formed via dipping process and sodium alginate entrapment formed via ionic gelation process were investigated. Calcium alginate beads and sodium alginate particles were subjected to freeze drying step. Physico-chemical properties of freeze-dried Calcium alginate beads and sodium

alginate particles were investigated. The release characteristics were also investigated continuously under *in vitro* gastrointestinal digestions. Freeze dried adzuki beans hydrolysate was used as the control.

4.6.2 Determination of physico-chemical properties of different encapsulation systems

The zeta potential provides a quantitative measure of the charge on colloidal particles in liquid suspension. Zeta potential value of 2% sodium alginate solution showed negative charge due to the presence of carboxyl groups of guluronic and mannuronic acids in sodium alginate molecule (Table 4.25). The addition of adzuki bean hydrolysate into 2% sodium alginate solution caused a shift of surface charge, Zeta potential value, of sodium alginate from -54.10 mV to -40.68 mV. This was because the positive charge on the amine group of peptides in bean hydrolysate could interact with carboxyl groups of sodium alginate. Zeta potential value of mixed solution was -40.68 giving good stability of particles in the alginate-hydrolysate suspension. The particles remained stable without aggregation at room temperature for one day. Honary and Zahir (2013) reported that zeta potential value higher than −30 mV yielded stable particles.

Table 4. 25 Zeta potential of sample suspensions

Sample	Zeta potential (mV)
Adzuki beans hydrolysate	-12.87 ± 1.27
2% sodium alginate solution	-54.10 ± 0.44
Mixed hydrolysate and 2% sodium alginate solution	-40.68 ± 4.01

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The infrared spectroscopy is most useful in providing information on the presence of specific functional groups in organic and inorganic compounds. Figure 4.11 depicts the FTIR spectra of Ca-alginate and sodium alginate particles containing adzuki bean hydrolysate compared to that of sodium alginate, calcium alginate, and adzuki beans hydrolysate. Five absorption bands were shown appearance of the vibration and absorptions for functional groups. Strong and sharp bands at wavenumber around 1060, 1420, 1630 and 3445 cm^{-1} were markedly observed, indicating that interaction between S=O, C=O and O-H were formed. The increasing

of the spectra intensity indicates that the degree of molecular bonding at specific functional groups is increased. At wavenumber 1630 cm^{-1} in sodium alginate spectrum presented asymmetric or symmetric stretching vibrations of -COO−. The gel formation ability depends on the amount of carboxyl groups. A broad peak at frequency around 3445 cm^{-1}, represented O-H stretching in sodium alginate, revealed a large amount of water in the sample structure. A sharp band at 1630 cm^{-1}, in adzuki bean hydrolysate was attributed to the stretching vibration of the C=O in primary amine. A stronger and sharper band of the O-H stretching at 3300 - 3500 cm^{-1} in both particle and bead indicated the presence of hydrogen bonds between water and wall material. Spectra at wavenumber around 1603 cm^{-1} and 1439 cm^{-1} was reported to be the interaction between carboxylic group (COO group) and Ca^{2+} (J. Li, Kim, Chen, & Park, 2016).

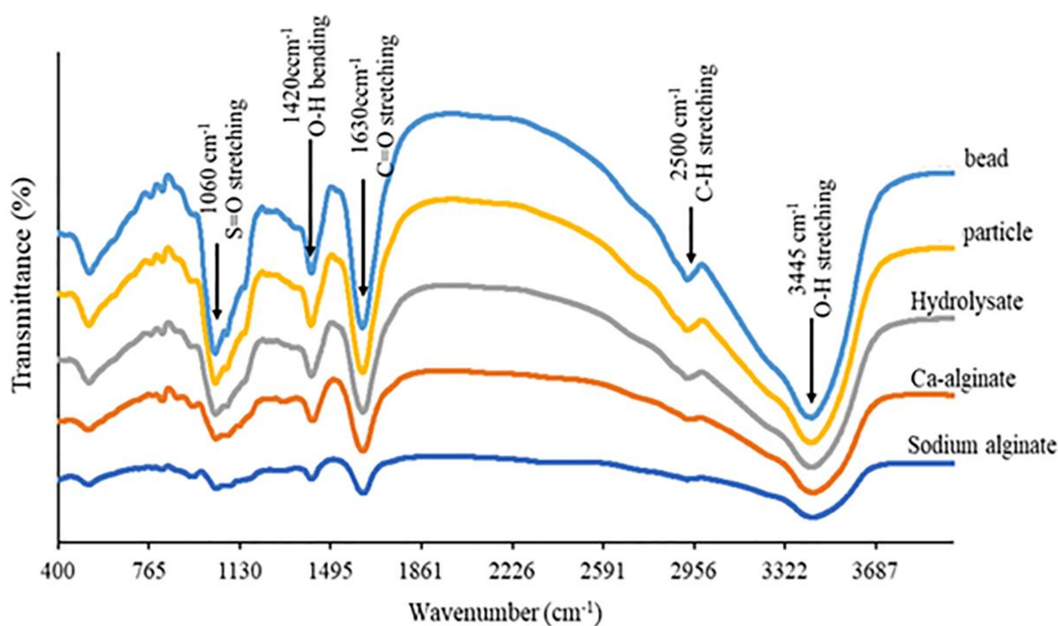

Figure 4. 11 Fourier-transform infrared (FTIR) spectra of sodium alginate, Ca-alginate, adzuki beans hydrolysate, particle containing hydrolysate and beads containing hydrolysate

The microstructure of freeze-dried particles and beads obtaining from different encapsulation methods containing adzuki beans hydrolysate observed under a scanning electron microscope (SEM) is shown in Figure 4.12. The microstructure of freeze-dried particles existed in unorganised pattern with fracture surface while that of freeze-dried beads showed smooth and well-constructed boundary structure. The formation of well-constructed boundary structure could be explained by the fact that calcium ions in the gelation method would firstly cross link to the surface of alginate (Cho et al., 2014).

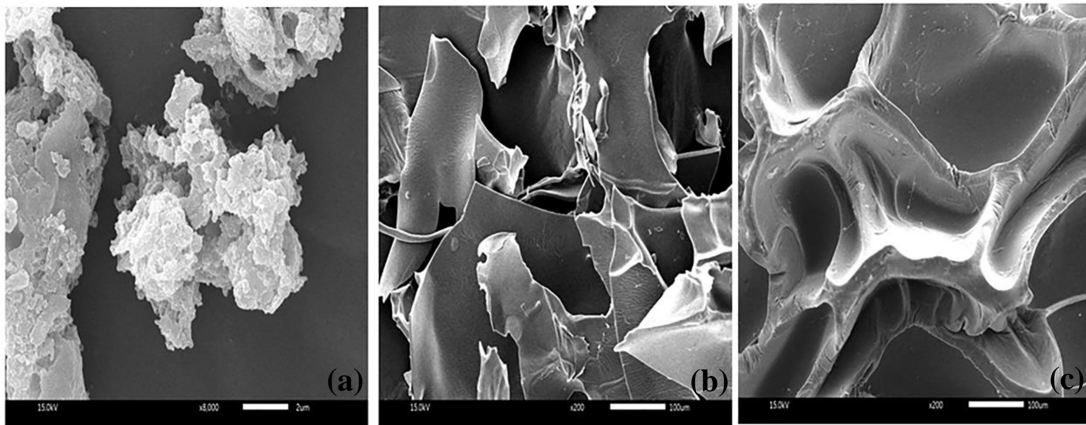

Figure 4. 12 Microstructure of freeze dried adzuki beans hydrolysate (a), particle containing hydrolysate (b) and beads containing hydrolysate (c) at 200× magnification

Since the encapsulation efficiency is also dependent on molecular bonding between specific functional groups in particles formed via encapsulation system. Thus, encapsulation efficiency of particles obtaining from the different encapsulation methods was investigated. The percentage of TPC and TCA-soluble peptides encapsulation efficiency of freeze-dried particles and beads is shown in Figure 4.13. The encapsulation using sodium alginate entrapment (particles) showed a significant higher TPCEE and TCA-soluble peptidesEE (approximately 19% and 8%, respectively) compared to calcium alginate beads (beads). This may be related to the different bonding strength in alginate-based structure.

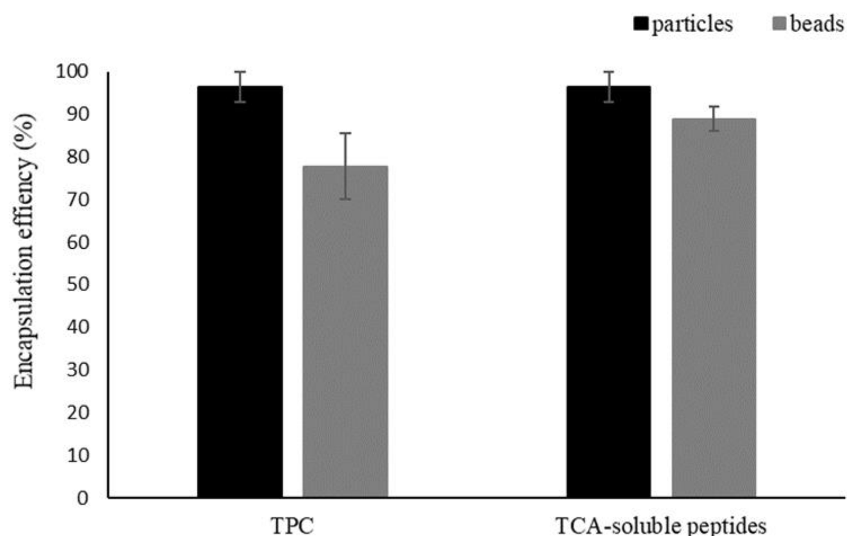

Figure 4. 13 The percentage of TPC and TCA-soluble peptides encapsulation efficiency of freeze-dried particles and beads

4.6.3 Determination of release characteristics under simulated gastrointestinal digestion of different encapsulation systems

The antioxidant activity (ABTS scavenging activity, FRAP and ferrous chelating), ORAC and health promoting (ACE-I inhibitory activity and DPP-IV inhibitory activity) of alginate beads and particles under simulated gastrointestinal digestion are shown in Table 4.25. Antioxidant activity of all freeze-dried samples (adzuki beans hydrolysate, particle containing beans hydrolysate and beads containing beans hydrolysate) under *in vitro* GI tract condition was higher than that of samples without *in vitro* GI tract condition. Freeze-dried hydrolysate (the control) showed the higher ABTS value, FRAP value and health promoting than other conditions. Particle supernatants exhibited the higher FRAP and ferrous chelating value than bead supernatants, however, ABTS value of bead was higher than particles. The cause of this difference was further investigated. μ

Our results showed that particle supernatants obtaining from simulated GI tract had higher ability to scavenge peroxyl radicals (ROO•) compared to bead supernatants. The scavenging ability of particle supernatants and bead supernatants

was calculated as trolox equivalent showing the value around 1006 and 946 mM trolox equivalent/g dry sample, respectively (Table 4.26). The difference in scavenging ability may come from the presence of different peptides and phenolic compounds in digested supernatants.

Table 4. 26 Effect of different encapsulation systems on release characteristics of freeze-dried samples under simulated gastrointestinal digestion

	Adzuki beans hydrolysate (control)	**Particle**	**Bead**
Antioxidant activity			
ABTS scavenging activity (mM trolox/g sample)	$3.35^{b} \pm 0.25$	$0.73^{a} \pm 0.34$	$3.62^{c} \pm 1.07$
FRAP (mM trolox/g sample)	$1494.89^{c} \pm 600.51$	$1031.88^{b} \pm 32.67$	$968.84^{a} \pm 94.18$
Ferrous chelating activity (mM EDTA/g sample)	$524.96^{a} \pm 9.55$	$1006.64^{c} \pm 5.94$	$946.66^{b} \pm 7.51$
ORAC (mM trolox/g sample)	$479.18^{a} \pm 43.74$	$601.68^{c} \pm 16.18$	$526.48^{b} \pm 10.33$
Health promoting activity			
ACE-I inhibitory activity (%)	$93.72^{c} \pm 9.11$	$83.62^{b} \pm 5.89$	$78.12^{a} \pm 12.84$
DPP-IV inhibitory activity (%)	$86.41^{b} \pm 1.53$	$73.48^{a} \pm 10.21$	$72.57^{a} \pm 15.88$
Bioactive compounds			
TCA-soluble peptides (mg Tyr/g sample)	$59.45^{c} \pm 1.83$	$51.19^{b} \pm 8.26$	$30.83^{a} \pm 4.78$
Free amino group (mg Leu/g sample)	$4274.85^{b} \pm 686.78$	$4439.68^{b} \pm 553.17$	$2335.50^{a} \pm 239.66$
TPC (mg GA/g sample)	$307.50^{b} \pm 47.40$	$304.59^{b} \pm 68.75$	$135.82^{a} \pm 24.78$
TFC (mg CA/g sample)	$74.79^{c} \pm 5.41$	$62.26^{b} \pm 4.13$	$45.79^{a} \pm 6.38$

Mean values ± SD of triplicate determinations. Mean in the same row with different letters are significantly ($p \leq 0.05$).

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All digested supernatants obtaining from both particles and beads possessed ACE-I inhibitory and DPP-IV inhibitory activities as shown in Table 4.26. The results showed that particle supernatants showed higher ACE-I inhibitory activity than bead supernatants. It was because particle supernatants contained more TCA-soluble peptides as shown in table 4.26. It was possibly that the entrapped TCA-soluble peptides were ACE inhibitory peptides. Generally, ACE inhibitory peptides contain tryptophan, tyrosine, phenylalanine and proline at carboxyl terminal residue (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980). Both particle supernatants and bead supernatants showed similar value of DPP-IV inhibitory activity, amounting to 70% compared to the positive control, after *in vitro* GI tract digestion. It was because both particles and beads contained the same amount of TCA-soluble peptides in systems; the results are shown in Table 4.26. These peptides may contain similar MW and specific amino acid at N-terminal. Small peptides (MW < 1 kDa) containing Pro or Ala were reported to strongly inhibit the DPP-IV enzyme activity due to competing for the active site with substrate (Castañeda-Pérez et al., 2019). The enzyme dipeptidyl peptidase IV (DPP-IV) is responsible for the degradation of a number of biological peptides substrate including glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) by cleaving proline or alanine at N-terminal residue of the peptide. Consequently, inhibition of DPP-IV has been shown to improve glucose tolerance in diabetic patients by enhancing the insulinotropic effects (Deacon, 2019).

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The releasing of TCA-soluble peptides, free amino group, TPC and TFC from all freeze-dried samples (adzuki beans hydrolysate, particle containing beans hydrolysate and beads containing beans hydrolysate) under *in vitro* GI tract condition was higher than that of samples without *in vitro* GI tract condition. The amount of released bioactive compounds is considered to be available for absorption through the digestion system. According above biological activities and limited information on releasing bioactive compounds from different encapsulation techniques, released bioactive compounds were investigated. The released TCA-soluble peptides, free amino group, TPC, TFC and reducing sugar after *in vitro* GI track digestion are shown in Table 4.26. It has been reported that both small peptides and phenolic compounds act as antioxidant with different mechanism such as radical scavenging

ability, metal chelating or reducing activities (Pérez-Balladares et al., 2019) and potent health promoting such as antihypertensive, immunomodulatory (Durak et al., 2013).

Table 4.26 indicates that different encapsulation techniques resulted in different amount of released TCA soluble peptides, free amino group, TPC and TFC. The particle supernatants showed higher TCA-soluble peptides, free amino group, TPC and TFC than bead supernatants. The difference in release characteristic of encapsulation technique could be ascribed to the difference in bonding strength in alginate-based structure as explained via FT-IR spectra. The above results demonstrated that encapsulation technique had strong effect on release characteristics. A remarkable increase in FRAP, ferrous chelating, ORAC, ACE-I inhibitory and DPP-IV inhibitory activity after *in vitro* GI tract digestion was observed with the particles while bead supernatants exhibited the higher ABTS scavenging activity. Thus, both interactions between the specific groups in alginate structure and hydrolysate and structure of particle yielded different entrapped bioactive compounds in the structure providing a specific release characteristic. This information could apply to a control-release of specific compounds at intestinal phase. It can also apply for bioactive compounds-specific release characteristic

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CHAPTER V
CONCLUSIONS AND SUGGESTIONS

5.1 Conclusions

Cooking led to a decrease in crude protein, TCA-soluble peptides, free amino groups, total phenolic and total flavonoid content in cooked adzuki beans. Leaching effect resulted in an increase in TCA-soluble peptides, free amino groups, total phenolic and total flavonoid content in cooking water. The application of cooking on adzuki beans resulted in more unfolded polypeptides and releasing phenolics from complex structure of adzuki beans, causing an increase in releasing small peptides and phenolic compounds. Both small peptides and phenolic compounds in cooked beans and its cooking water presented synergistic effect in antioxidant activity.

The 10-min autoclaving was found to be the appropriate cooking condition. It was not only for retaining amino acids and releasing bound phenolics but also lowering the cooking time. The condition "10-min autoclaving" showed high biological activity compared to other conditions. Thus, both cooked beans and cooking water from 10-min autoclaving cooking condition were pooled and used as substrate for protein hydrolysis. The obtained adzuki beans hydrolysate was entrapped in alginate-based encapsulation via two different encapsulation methods (calcium alginate beads and sodium alginate particles). The sodium alginate particles exhibited higher encapsulation efficiency. The specific functional group interactions (S=O, C=O and O-H) were formed, confirmed via Fourier-transform infrared analysis.

In vitro simulated gastrointestinal digestion study indicated that a remarkable increase in FRAP, ferrous chelating, ORAC value and ACE-I inhibitory after in vitro GI tract digestion was observed with the particles. Whilst beads exhibited the higher ABTS scavenging activity after in vitro GI tract digestion than particles. DPP-IV inhibitory activity of particles and beads were not significantly different. This might be because encapsulation technique had a significant effect on the entrapped bioactive compounds and control-release characteristics.

5.2 Suggestions

In this study, the antioxidant activity come from the presence of small peptides, free amino acids and phenolic compounds in adzuki bean samples. The phenolic composition in cooked beans and its cooking water were already determined. Thus, further research should focus on:

1) Identification of interesting bioactive peptide sequence such as ACE inhibitory peptide, DPP-I inhibitory peptide

2) The bioavailability of phytochemicals in beads and particles containing adzuki beans hydrolysate under simulated gastrointestinal digestion

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REFERENCES

Abbas, M., & Shah, H. U. (2007). Proximate and mineral composition of mung bean. *Sarhad Journal of Agriculture (Pakistan).*

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of agricultural and food chemistry,* 27(6), 1256-1262.

Al Shukor, N., Van Camp, J., Gonzales, G. B., Staljanssens, D., Struijs, K., Zotti, M. J., . . . Smagghe, G. (2013). Angiotensin-converting enzyme inhibitory effects by plant phenolic compounds: a study of structure activity relationships. *J Agric Food Chem,* 61(48), 11832-11839. doi:10.1021/jf404641v

Alashi, A. M., Blanchard, C. L., Mailer, R. J., Agboola, S. O., Mawson, A. J., He, R., . . . Aluko, R. E. (2014). Antioxidant properties of Australian canola meal protein hydrolysates. *Food Chem,* 146, 500-506. doi:10.1016/j.foodchem.2013.09.081

Almeida, M. M. B., de Sousa, P. H. M., Arriaga, Â. M. C., do Prado, G. M., Magalhães, C. E. d. C., Maia, G. A., & de Lemos, T. L. G. (2011). Bioactive compounds and antioxidant activity of fresh exotic fruits from northeastern Brazil. *Food research international,* 44(7), 2155-2159. doi:10.1016/j.foodres.2011.03.051

Amarowicz, R., Estrella, I., Hernandez, T., & TROSZYŃSKA, A. (2008). Antioxidant activity of extract of adzuki bean and its fractions. *Journal of Food Lipids,* 15(1), 119-136.

AOAC. (2019). *Official Methods of Analysis (21th ed.)* (21 ed.). Gaithersburg, Maryland: AOAC International, 2019.

Balanč, B., Trifković, K., Đorđević, V., Marković, S., Pjanović, R., Nedović, V., & Bugarski, B. (2016). Novel resveratrol delivery systems based on alginate-sucrose and alginate-chitosan microbeads containing liposomes. *Food Hydrocolloids,* 61, 832-842. doi:https://doi.org/10.1016/j.foodhyd.2016.07.005

Betancur-Ancona, D., Sosa-Espinoza, T., Ruiz-Ruiz, J., Segura-Campos, M., & Chel-Guerrero, L. (2014). Enzymatic hydrolysis of hard-to-cook bean (Phaseolus vulgaris L.) protein concentrates and its effects on biological and functional properties. *International Journal of Food Science & Technology,* 49(1), 2-8. doi:10.1111/ijfs.12267

Bisht, I. S., & Singh, M. (2013). Asian vigna. In *Genetic and Genomic Resources of Grain Legume Improvement* (pp. 237-267): Elsevier.

Caló, E., & Khutoryanskiy, V. V. (2015). Biomedical applications of hydrogels: A review of patents and commercial products. *European Polymer Journal,* 65, 252-267. doi:https://doi.org/10.1016/j.eurpolymj.2014.11.024

Castañeda-Pérez, E., Jiménez-Morales, K., Quintal-Novelo, C., Moo-Puc, R., Chel-

Guerrero, L., & Betancur-Ancona, D. (2019). Enzymatic protein hydrolysates and ultrafiltered peptide fractions from Cowpea Vigna unguiculata L bean with in vitro antidiabetic potential. *Journal of the Iranian Chemical Society, 16*(8), 1773-1781. doi:10.1007/s13738-019-01651-0

Chan, E.-S., Yim, Z.-H., Phan, S.-H., Mansa, R. F., & Ravindra, P. (2010). Encapsulation of herbal aqueous extract through absorption with ca-alginate hydrogel beads. *Food and Bioproducts Processing, 88*(2), 195-201. doi:https://doi.org/10.1016/j.fbp.2009.09.005

Cheng, L. K., O'Grady, G., Du, P., Egbuji, J. U., Windsor, J. A., & Pullan, A. J. (2010). Gastrointestinal system. *WIREs Systems Biology and Medicine, 2*(1), 65-79. doi:10.1002/wsbm.19

Cheung, H.-S., Wang, F.-L., Ondetti, M. A., Sabo, E. F., & Cushman, D. W. (1980). Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the COOH-terminal dipeptide sequence. *Journal of biological chemistry, 255*(2), 401-407.

Ching, S. H., Bansal, N., & Bhandari, B. (2017). Alginate gel particles-A review of production techniques and physical properties. *Crit Rev Food Sci Nutr, 57*(6), 1133-1152. doi:10.1080/10408398.2014.965773

Cho, A. R., Chun, Y. G., Kim, B. K., & Park, D. J. (2014). Preparation of alginate–CaCl2 microspheres as resveratrol carriers. *Journal of Materials Science, 49*(13), 4612-4619. doi:10.1007/s10853-014-8163-x

Clemente, A., & Olias, R. (2017). Beneficial effects of legumes in gut health. *Current Opinion in Food Science, 14*, 32-36.

Clemente, A., Vioque, J., Sánchez-Vioque, R., Pedroche, J., Bautista, J., & Millán, F. (1999). Protein quality of chickpea (Cicer arietinum L.) protein hydrolysates. *Food Chemistry, 67*(3), 269-274. doi:https://doi.org/10.1016/S0308-8146(99)00130-2

Cumby, N., Zhong, Y., Naczk, M., & Shahidi, F. (2008). Antioxidant activity and water-holding capacity of canola protein hydrolysates. *Food Chemistry, 109*(1), 144-148. doi:https://doi.org/10.1016/j.foodchem.2007.12.039

Deacon, C. F. (2019). Physiology and Pharmacology of DPP-4 in Glucose Homeostasis and the Treatment of Type 2 Diabetes. *Frontiers in endocrinology, 10*, 80-80. doi:10.3389/fendo.2019.00080

Dewanto, V., Wu, X., & Liu, R. H. (2002). Processed sweet corn has higher antioxidant activity. *Journal of agricultural and food chemistry, 50*(17), 4959-4964.

Durak, A., Baraniak, B., Jakubczyk, A., & Świeca, M. (2013). Biologically active peptides obtained by enzymatic hydrolysis of Adzuki bean seeds. *Food*

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Chemistry, 141(3), 2177-2183.

Duranti, M. (2006). Grain legume proteins and nutraceutical properties. *Fitoterapia, 77*(2), 67-82.

Dutson, T. R., & Orcutt, M. W. (1984). Chemical changes in proteins produced by thermal processing. *Journal of Chemical Education, 61*(4), 303. doi:10.1021/ed061p303

Frota, K. d. M. G., Lopes, L. A. R., Silva, I. C. V., & Arêas, J. A. G. (2017). Nutritional quality of the protein of Vigna unguiculata L. Walp and its protein isolate. *Revista Ciência Agronômica, 48*(5SPE), 792-798.

Giusti, F., Capuano, E., Sagratini, G., & Pellegrini, N. (2019). A comprehensive investigation of the behaviour of phenolic compounds in legumes during domestic cooking and in vitro digestion. *Food Chem, 285*, 458-467. doi:10.1016/j.foodchem.2019.01.148

Gonzalez, E., Gomez-Caravaca, A. M., Gimenez, B., Cebrian, R., Maqueda, M., Martinez-Ferez, A., . . . Robert, P. (2019). Evolution of the phenolic compounds profile of olive leaf extract encapsulated by spray-drying during in vitro gastrointestinal digestion. *Food Chem, 279*, 40-48. doi:10.1016/j.foodchem.2018.11.127

Guillon, F., & Champ, M.-J. (2002). Carbohydrate fractions of legumes: uses in human nutrition and potential for health. *British Journal of Nutrition, 88*(S3), 293-306.

Hayakari, M., Kondo, Y., & Izumi, H. (1978). A rapid and simple spectrophotometric assay of angiotensin-converting enzyme. *Analytical Biochemistry, 84*(2), 361-369. doi:https://doi.org/10.1016/0003-2697(78)90053-2

Hedley, C. L. (2001). *Carbohydrates in grain legume seeds: improving nutritional quality and agronomic characteristics*: CABI.

Honary, S., & Zahir, F. (2013). Effect of zeta potential on the properties of nano-drug delivery systems-a review (Part 2). *Tropical Journal of Pharmaceutical Research, 12*(2), 265-273.

Jamdar, S. N., Rajalakshmi, V., Pednekar, M. D., Juan, F., Yardi, V., & Sharma, A. (2010). Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. *Food chemistry, 121*(1), 178-184. doi:https://doi.org/10.1016/j.foodchem.2009.12.027

Karppinen, K., Taulavuori, E., & Hohtola, A. (2010). Optimization of protein extraction from Hypericum perforatum tissues and immunoblotting detection of Hyp-1 at different stages of leaf development. *Mol Biotechnol, 46*(3), 219-226. doi:10.1007/s12033-010-9299-9

Katoch, R. (2013). Nutritional evaluation, protein digestibility and profiling of different

Vigna species. *Indian Journal of Agricultural Biochemistry,* 26(1), 32-35.

Kestwal, R. M., Bagal-Kestwal, D., & Chiang, B. H. (2012). Analysis and enhancement of nutritional and antioxidant properties of Vigna aconitifolia sprouts. *Plant Foods Hum Nutr,* 67(2), 136-141. doi:10.1007/s11130-012-0284-2

Khandelwal, S., Udipi, S. A., & Ghugre, P. (2010). Polyphenols and tannins in Indian pulses: Effect of soaking, germination and pressure cooking. *Food research international,* 43(2), 526-530.

Kopeček, J., & Yang, J. (2012). Smart Self-Assembled Hybrid Hydrogel Biomaterials. *Angewandte Chemie International Edition,* 51(30), 7396-7417. doi:10.1002/anie.201201040

Lam, Y., & de Lumen, B. O. (2003). LEGUMES | Dietary Importance. In B. Caballero (Ed.), *Encyclopedia of Food Sciences and Nutrition (Second Edition)* (pp. 3529-3534). Oxford: Academic Press.

Lee, K. Y., & Mooney, D. J. (2012). Alginate: Properties and biomedical applications. *Progress in Polymer Science,* 37(1), 106-126. doi:https://doi.org/10.1016/j.progpolymsci.2011.06.003

Li-Chan, E. C. Y., & Lacroix, I. M. E. (2018). Properties of proteins in food systems: An introduction. In *Proteins in Food Processing* (pp. 1-25).

Li, G. H., Wan, J. Z., Le, G. W., & Shi, Y. H. (2006). Novel angiotensin I-converting enzyme inhibitory peptides isolated from Alcalase hydrolysate of mung bean protein. *Journal of peptide science: an official publication of the European Peptide Society,* 12(8), 509-514.

Li, J., Kim, S. Y., Chen, X., & Park, H. J. (2016). Calcium-alginate beads loaded with gallic acid: Preparation and characterization. *LWT - Food Science and Technology,* 68, 667-673. doi:10.1016/j.lwt.2016.01.012

Lin, P.-Y., & Lai, H.-M. (2006). Bioactive compounds in legumes and their germinated products. *Journal of agricultural and food chemistry,* 54(11), 3807-3814.

Liu, F., Chang, X., Hu, X., Brennan, C. S., & Guo, X. (2017). Effect of thermal processing on phenolic profiles and antioxidant activities inCastanea mollissima. *International Journal of Food Science & Technology,* 52(2), 439-447. doi:10.1111/ijfs.13299

Liu, F., Chen, Z., Shao, J., Wang, C., & Zhan, C. (2017). Effect of fermentation on the peptide content, phenolics and antioxidant activity of defatted wheat germ. *Food Bioscience,* 20, 141-148. doi:https://doi.org/10.1016/j.fbio.2017.10.002

López-Martínez, L. X., Leyva-López, N., Gutiérrez-Grijalva, E. P., & Heredia, J. B. (2017). Effect of cooking and germination on bioactive compounds in pulses and their health benefits. *Journal of Functional Foods,* 38, 624-634.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

doi:https://doi.org/10.1016/j.jff.2017.03.002

López-Barrios, L., Gutiérrez-Uribe, J. A., & Serna-Saldívar, S. O. (2014). Bioactive peptides and hydrolysates from pulses and their potential use as functional ingredients. *Journal of food science,* 79(3), R273-R283.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of biological chemistry,* 193, 265-275.

Luna-Vital, D. A., Mojica, L., de Mejía, E. G., Mendoza, S., & Loarca-Piña, G. (2015). Biological potential of protein hydrolysates and peptides from common bean (Phaseolus vulgaris L.): A review. *Food research international,* 76, 39-50.

Luo, J., Cai, W., Wu, T., & Xu, B. (2016). Phytochemical distribution in hull and cotyledon of adzuki bean (Vigna angularis L.) and mung bean (Vigna radiate L.), and their contribution to antioxidant, anti-inflammatory and anti-diabetic activities. *Food Chemistry,* 201, 350-360.

Mamilla, R. K., & Mishra, V. K. (2017). Effect of germination on antioxidant and ACE inhibitory activities of legumes. *LWT,* 75, 51-58.

McCrory, M. A., Hamaker, B. R., Lovejoy, J. C., & Eichelsdoerfer, P. E. (2010). Pulse consumption, satiety, and weight management. *Advances in Nutrition,* 1(1), 17-30.

Miglio, C., Chiavaro, E., Visconti, A., Fogliano, V., & Pellegrini, N. (2008). Effects of Different Cooking Methods on Nutritional and Physicochemical Characteristics of Selected Vegetables. *Journal of agricultural and food chemistry,* 56(1), 139-147. doi:10.1021/jf072304b

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., . . . Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food – an international consensus. *Food & Function,* 5(6), 1113-1124. doi:10.1039/C3FO60702J

Mubarak, A. (2005). Nutritional composition and antinutritional factors of mung bean seeds (Phaseolus aureus) as affected by some home traditional processes. *Food Chemistry,* 89(4), 489-495.

Nedovic, V., Kalusevic, A., Manojlovic, V., Levic, S., & Bugarski, B. (2011). An overview of encapsulation technologies for food applications. *Procedia Food Science,* 1, 1806-1815.

Nongonierma, A. B., & FitzGerald, R. J. (2013). Dipeptidyl peptidase IV inhibitory and antioxidative properties of milk protein-derived dipeptides and hydrolysates. *Peptides,* 39, 157-163. doi:10.1016/j.peptides.2012.11.016

Olaleye, A., Adeyeye, E., & Adesina, A. (2013). Chemical composition of bambara groundnut (V. subterranea L. Verdc) seed parts. *Bangladesh Journal of Scientific*

and Industrial Research, 48(3), 167-178.

Ozkan, G., Franco, P., De Marco, I., Xiao, J., & Capanoglu, E. (2019). A review of microencapsulation methods for food antioxidants: Principles, advantages, drawbacks and applications. *Food Chemistry,* 272, 494-506. doi:https://doi.org/10.1016/j.foodchem.2018.07.205

Panyam, D., & Kilara, A. (1996). Enhancing the functionality of food proteins by enzymatic modification. *Trends in food science & technology,* 7(4), 120-125.

Papadopoulos, M. C. (1989). Effect of processing on high-protein feedstuffs: A review. *Biological Wastes,* 29(2), 123-138. doi:https://doi.org/10.1016/0269-7483(89)90092-X

Pasukamonset, P., Kwon, O., & Adisakwattana, S. (2016). Alginate-based encapsulation of polyphenols from Clitoria ternatea petal flower extract enhances stability and biological activity under simulated gastrointestinal conditions. *Food Hydrocolloids,* 61, 772-779. doi:10.1016/j.foodhyd.2016.06.039

Pérez-Balladares, D., Castañeda-Terán, M., Granda-Albuja, M. G., Tejera, E., Iturralde, G., Granda-Albuja, S., . . . Alvarez-Suarez, J. M. (2019). Chemical composition and antioxidant activity of the main fruits, tubers and legumes traditionally consumed in the Andean regions of Ecuador as a source of health-promoting compounds. *Plant foods for human nutrition,* 74(3), 350-357.

Plazinski, W., & Plazinska, A. (2011). Molecular dynamics study of the interactions between phenolic compounds and alginate/alginic acid chains. *New Journal of Chemistry,* 35(8), 1607-1614.

Ranilla, L. G., Genovese, M. I., & Lajolo, F. M. (2009). Effect of Different Cooking Conditions on Phenolic Compounds and Antioxidant Capacity of Some Selected Brazilian Bean (Phaseolus vulgaris L.) Cultivars. *Journal of agricultural and food chemistry,* 57(13), 5734-5742. doi:10.1021/jf900527v

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine,* 26(9), 1231-1237. doi:https://doi.org/10.1016/S0891-5849(98)00315-3

Rehman, Z.-u., & Shah, W. (2005). Thermal heat processing effects on antinutrients, protein and starch digestibility of food legumes. *Food Chemistry,* 91(2), 327-331.

Reşat, A., Shela, G., Volker, B., Karen, M. S., Mustafa, Ö., & Kubilay, G. (2013). Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). *Pure and Applied Chemistry,* 85(5), 957-998. doi:https://doi.org/10.1351/PAC-REP-12-07-15

Rui, X., Boye, J. I., Simpson, B. K., & Prasher, S. O. (2013). Purification and characterization of angiotensin I-converting enzyme inhibitory peptides of small red bean (Phaseolus vulgaris) hydrolysates. *Journal of Functional Foods,* 5(3),

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1116-1124.

Saltveit, M. E. (2010). Synthesis and metabolism of phenolic compounds. *Fruit and vegetable phytochemicals: chemistry, nutritional value and stability. Wiley-Blackwell, Ames*, 89-100.

Sangsukiam, T., & Duangmal, K. (2017). A comparative study of physico-chemical properties and antioxidant activity of freeze-dried mung bean (Vigna radiata) and adzuki bean (Vigna angularis) sprout hydrolysate powders. *International Journal of Food Science & Technology,* 52(9), 1971-1982.

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: a review. *Peptides,* 31(10), 1949-1956.

Shi, Z., Yao, Y., Zhu, Y., & Ren, G. (2016). Nutritional composition and antioxidant activity of twenty mung bean cultivars in China. *The Crop Journal,* 4(5), 398-406.

Siddhuraju, P., Vijayakumari, K., & Janardhanan, K. (1996). Chemical composition and nutritional evaluation of an underexploited legume, Acacia nilotica (L.) Del. *Food chemistry,* 57(3), 385-391. doi:https://doi.org/10.1016/0308-8146(95)00238-3

Singh, B., Singh, J. P., Kaur, A., & Singh, N. (2017). Phenolic composition and antioxidant potential of grain legume seeds: A review. *Food research international,* 101, 1-16.

Sritongtae, B., Sangsukiam, T., Morgan, M. R., & Duangmal, K. (2017). Effect of acid pretreatment and the germination period on the composition and antioxidant activity of rice bean (Vigna umbellata). *Food Chemistry,* 227, 280-288.

Taira, H., Taira, H., Sugimura, K.-i., & Sakurai, Y. (2014). Studies on Amino Acid Contents of Processed Soybean. *Agricultural and Biological Chemistry,* 29(12), 1074-1083. doi:10.1080/00021369.1965.10858517

Torres, J., Rutherfurd, S. M., Muñoz, L. S., Peters, M., & Montoya, C. A. (2016). The impact of heating and soaking on the in vitro enzymatic hydrolysis of protein varies in different species of tropical legumes. *Food Chemistry,* 194, 377-382.

Tsai, F.-H., Chiang, P.-Y., Kitamura, Y., Kokawa, M., & Islam, M. Z. (2017). Producing liquid-core hydrogel beads by reverse spherification: Effect of secondary gelation on physical properties and release characteristics. *Food Hydrocolloids,* 62, 140-148. doi:10.1016/j.foodhyd.2016.07.002

Verma, A. K., Kumar, S., Das, M., & Dwivedi, P. D. (2012). Impact of thermal processing on legume allergens. *Plant foods for human nutrition,* 67(4), 430-441.

Wang, N., Hatcher, D. W., Tyler, R. T., Toews, R., & Gawalko, E. J. (2010). Effect of cooking on the composition of beans (Phaseolus vulgaris L.) and chickpeas (Cicer arietinum L.). *Food research international,* 43(2), 589-594. doi:https://doi.org/10.1016/j.foodres.2009.07.012

Waterhouse, A. L. (2005). Determination of Total Phenolics. *Current Protocols in Food Analytical Chemistry, 6*(1), I1.1.1-I1.1.8. doi:10.1002/0471142913.fai0101s06

Wong, T. W. (2011). Alginate graft copolymers and alginate-co-excipient physical mixture in oral drug delivery. *J Pharm Pharmacol, 63*(12), 1497-1512. doi:10.1111/j.2042-7158.2011.01347.x

Wongsiri, S., Ohshima, T., & Duangmal, K. (2015). Chemical Composition, Amino Acid Profile and Antioxidant Activities of Germinated Mung Beans (V igna radiata). *Journal of Food Processing and Preservation, 39*(6), 1956-1964.

Wu, X., Beecher, G. R., Holden, J. M., Haytowitz, D. B., Gebhardt, S. E., & Prior, R. L. (2004). Lipophilic and Hydrophilic Antioxidant Capacities of Common Foods in the United States. *Journal of agricultural and food chemistry, 52*(12), 4026-4037. doi:10.1021/jf049696w

Wu, X., Gu, L., Holden, J., Haytowitz, D. B., Gebhardt, S. E., Beecher, G., & Prior, R. L. (2004). Development of a database for total antioxidant capacity in foods: a preliminary study. *Journal of Food Composition and Analysis, 17*(3), 407-422. doi:https://doi.org/10.1016/j.jfca.2004.03.001

Xu, B. J., & Chang, S. K. C. (2008). Total Phenolic Content and Antioxidant Properties of Eclipse Black Beans (Phaseolus vulgaris L.) as Affected by Processing Methods. *Journal of food science, 73*(2), H19-H27. doi:10.1111/j.1750-3841.2007.00625.x

Yao, Y., Cheng, X., & Ren, G. (2014). alpha-Glucosidase inhibitory activity of protein-rich extracts from extruded adzuki bean in diabetic KK-Ay mice. *Food Funct, 5*(5), 966-971. doi:10.1039/c3fo60521c

Yao, Y., Cheng, X., Wang, L., Wang, S., & Ren, G. (2011). Biological potential of sixteen legumes in China. *Int J Mol Sci, 12*(10), 7048-7058. doi:10.3390/ijms12107048

Yao, Y., Xue, P., Zhu, Y., Gao, Y., & Ren, G. (2015). Antioxidant and immunoregulatory activity of polysaccharides from adzuki beans (Vigna angularis). *Food research international, 77*, 251-256. doi:10.1016/j.foodres.2015.05.029

Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry, 64*(4), 555-559.

Zia-Ul-Haq, M., Ahmad, S., Bukhari, S. A., Amarowicz, R., Ercisli, S., & Jaafar, H. Z. (2014). Compositional studies and biological activities of some mash bean (Vigna mungo (L.) Hepper) cultivars commonly consumed in Pakistan. *Biological Research, 47*(1), 23.

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APPENDIX A
ASSAY TECHNIQUES

Appendix A.1 Determination of TCA-soluble peptides content using Lowry method

1.1 Preparation of reagent

Reagent A: 2 g of sodium carbonate was mixed with 100 mL of 0.1 M sodium hydroxide.

Reagent B: dissolve 0.5 g of copper sulfate and 1.0 g of sodium potassium tartrate in 100 mL of distilled water.

Reagent C: mix 50 mL of reagent A and 1 mL of reagent B (prepare daily).

Reagent D: dilute the commercial Folin-Cocalteu reagent (2N) with distilled water at the ratio of 1:1

1.2 TCA-soluble peptides content

1.2.1 Added 0.2 ml of sample solution, tyrosine standard solutions, or blank (deionized or distilled water) and 1 mL of reagent C in a glass tube. Mixture was slowly mixed and incubated at room temperature for 20 minutes.

1.2.2 Added 0.1 ml Reagent D, mixed immediately and incubated at room temperature for 30 minutes.

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1.2.3. Transferred 2 ml of the obtained solution to a plastic or glass cuvette and measure its absorbance at 750 nm in a spectrophotometer.

1.2.4. Subtracted the absorbance of the blank from all reading and created a calibration curve from the standard. The standard curve is shown is Figure A.1.

1.2.5. Used this standard curve to determine the corresponding tyrosine concentration in the sample. The results were reported as tyrosine equivalents.

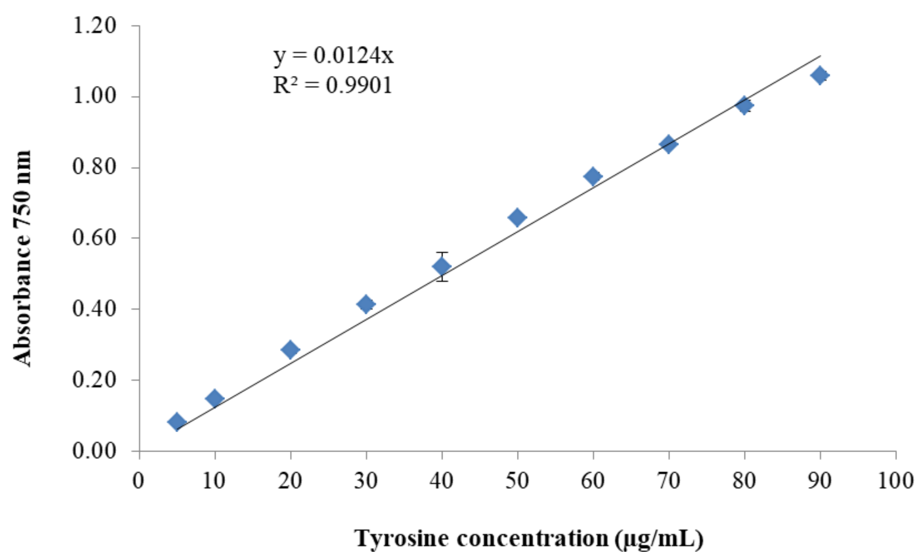

Figure A.1 Tyrosine standard curve for TCA-soluble peptides content determination

Appendix A.2 Free amino group determination

2.1 0.125 mL of cooked beans extracted and cooking water with an appropriate dilution was added with 0.2 M phosphate buffer (pH 8.2) and 2 mL of 0.05% of TNBS.

2.2 After mixing, the solution was incubated at 50 °C for 60 minutes in a temperature-controlled water bath.

2.3 The reaction was terminated by adding 4 mL of 0.1 N hydrochloric acid. After cooling down to 30 °C, the absorbance of obtaining solution was measured at 420 nm.

2.4 The standard curve prepared from leucine was shown in Figure A.2. This standard curve was used for estimation of free amino group content (leucine equivalents) in the sample.

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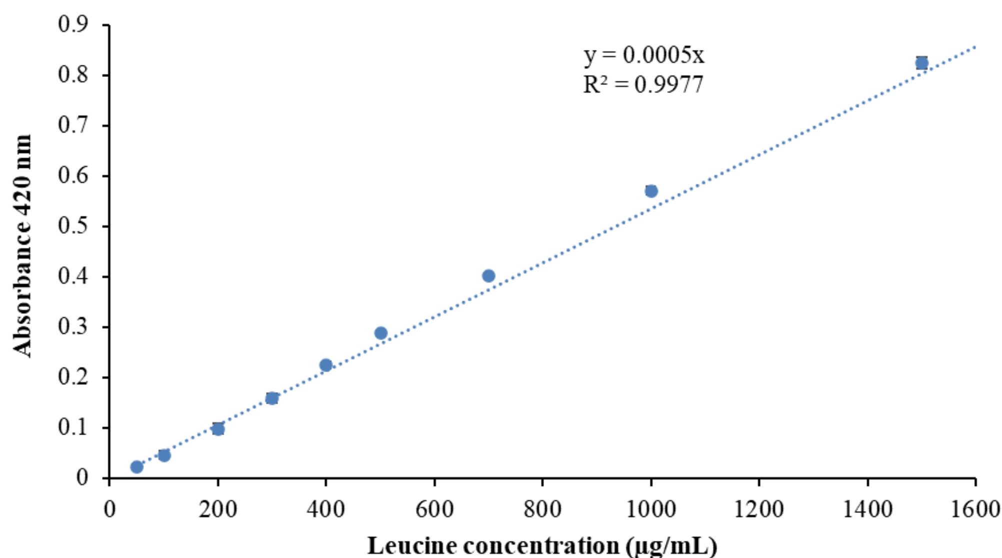

Figure A.2 Leucine standard curve for free amino group content determination

Appendix A.3 ABTS radical scavenging activity determination

3.1 Preparation of reagent

ABTS cation radical ($ABTS^{•+}$) reagent was generated by reaction of 5 mL of 7mM of ABTS solution and 88 µL of 140 mM of potassium persulfate solution. The mixture was kept in dark at 30 °C for 16 h before being used. Then, ABTS•+ reagent was diluted with ethanol in order to obtain an absorbance of 0.7 ± 0.05 at 734 nm using spectrophotometer.

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3.2 ABTS radical scavenging activity

To initiate the reaction, 30 µl of the sample extracts was mixed with 3 ml of diluted $ABTS^{•+}$ solution. The mixture was vigorously vortexed and left to stand at room temperature in the dark for 6 minutes. Distilled water was used instead of the sample and prepared in the same manner to obtain the control.

Trolox standard curve (0–2.5 mM) was prepared. Distilled water was used instead of the sample and prepared in the same manner to obtain the control. The standard curve prepared from standard solutions of Trolox is shown in Figure A.3.

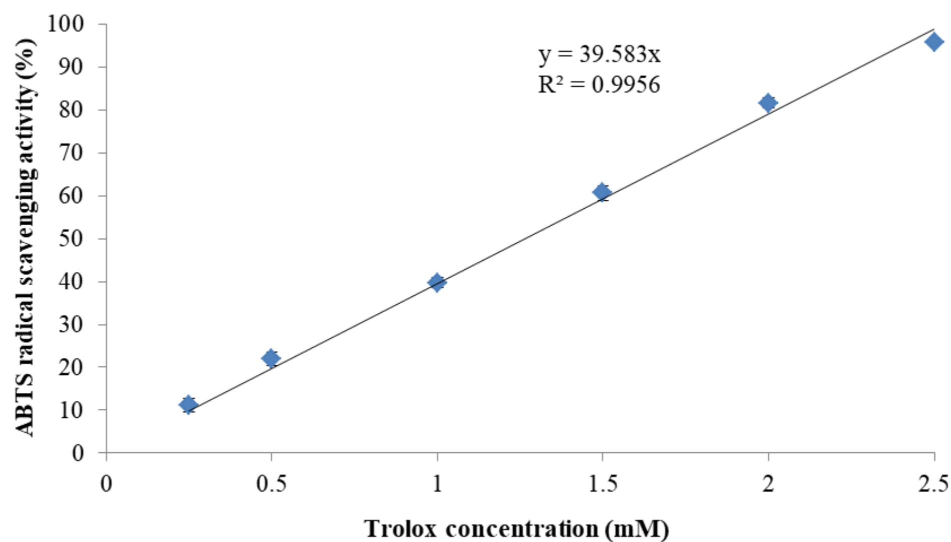

Figure A.3 Trolox standard curve for $ABTS^{\bullet+}$ radical scavenging activity determination

Appendix A.4 FRAP determination

The working FRAP reagent was prepared by mixing 25 ml of 300 mM acetate buffer (pH 3.6) with 2.5 ml of 10 mM 2,4,6-tripyridyl-*s*-triazine (TPTZ) in 40 mM hydrochloric acid solution and with 2.5 ml of 20 mM ferric chloride solution. The working FRAP reagent was incubated at 37°C for 30 minutes.

A 100 µl of the sample extracts was mixed with 1900 µl of FRAP solution and kept in the dark at room temperature (30 ± 2 °C) for 30 min. The ferrous tripyridyltriazine complex (colored product) was measured by reading absorbance at 593 nm.

Trolox standard curve (0–500 µM) was prepared. Distilled water was used instead of the sample and prepared in the same manner to obtain the control. The standard curve prepared from standard solutions of Trolox is shown in Figure A.4.

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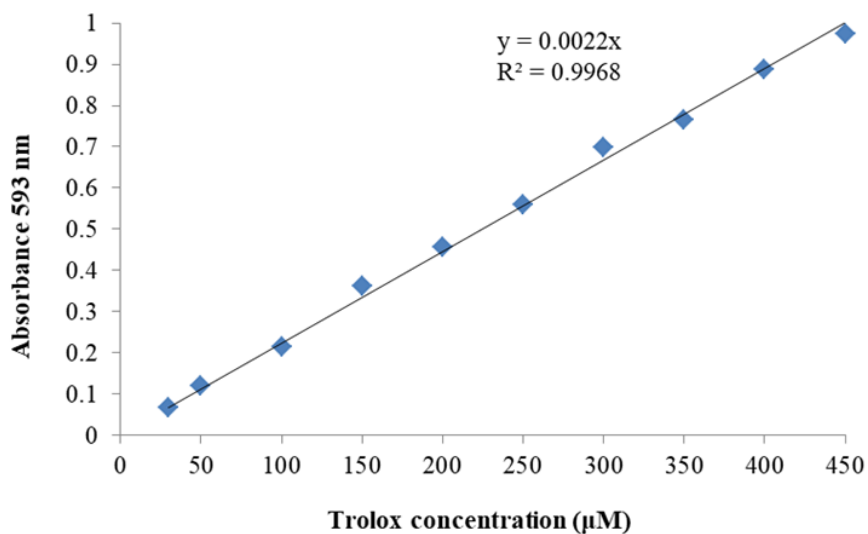

Figure A.4 Trolox standard curve for FRAP determination

Appendix A.5 Ferrous chelating determination

A 1 mL of sample was premixed with 0.05 ml of 2 mM iron dichloride solution and 1.85 ml of double distilled water. Thereafter, 0.1 ml of 5 mM ferrozine solution was added and mixed vigorously. The absorbance was determined at 562 nm after the mixture was allowed to stand for 10 min at room temperature. Double distilled water was used as the control.

EDTA standard curve (0–100 µM) was prepared. Distilled water was used instead of the sample and prepared in the same manner to obtain the control. The standard curve prepared from standard solutions of EDTA is shown in Figure A.5.

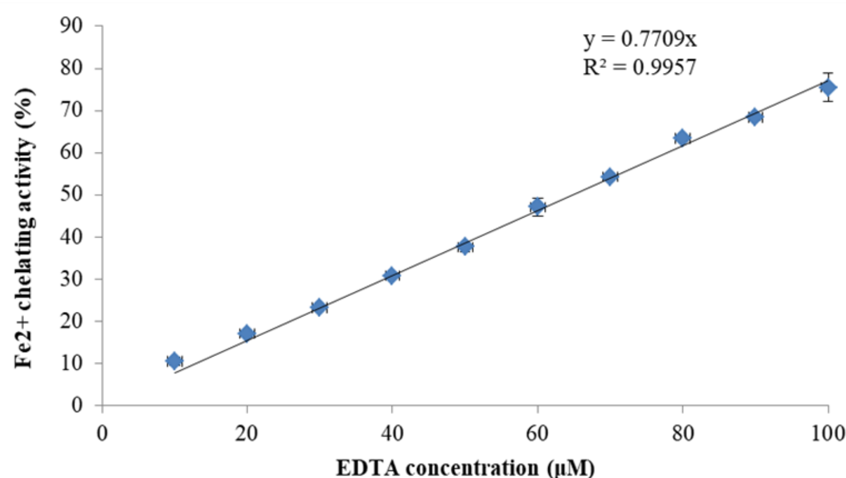

Figure A.5 EDTA standard curve for metal chelating activity determination

Appendix A.6 ORAC determination

A 20 µL of extracted sample with an appropriate dilution using 75 mM phosphate buffer (pH 7.4) was transferred into 96-well microplate wells. A 120 µL of 0.008 µM fluorescein was added and incubated at 37°C for 15 min in microplate reader. Then, 150 mM of 2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH) was added. The intensity of fluorescence was recorded at excitation/emission wavelength of 495/528 nm. Area under curve was calculated by MARS data analysis software.

Trolox standard curve (12–70 µM) was prepared. 75 mM phosphate buffer (pH 7.4) was used instead of the sample and prepared in the same manner to obtain the control. The standard curve prepared from standard solutions of Trolox is shown in Figure A.6.

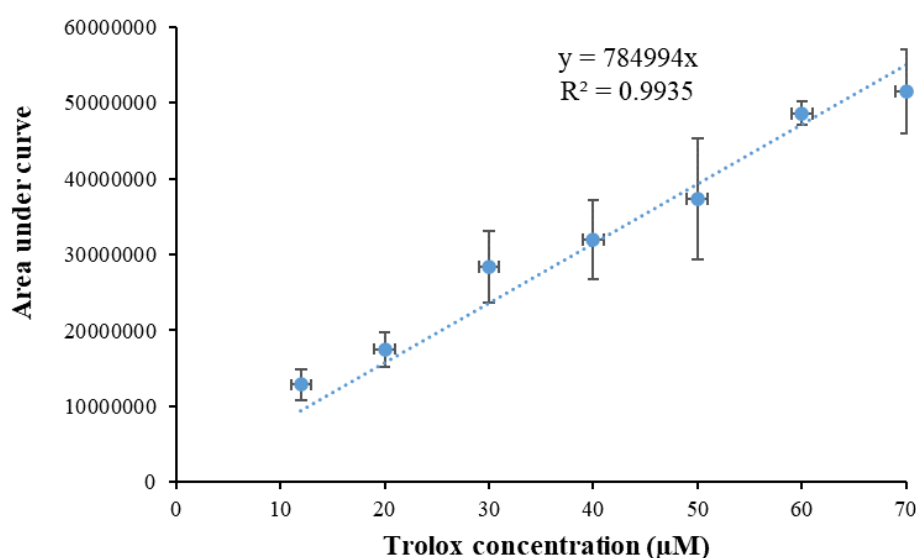

Figure A.6 Trolox standard curve for ORAC determination

Appendix A.7 ACE-I inhibitory activity determination

Twenty µL of sample was mixed with 200 µL of 5 mM HHL and 60 µL of Sodium borate buffer (pH 8.3). The mixture was pre-incubated at 37 °C for 5 minutes. The reaction was initiated by addition of 20 µL of ACE solution followed by incubation at 37 °C for 30 minutes. The reaction terminated by addition of 2,4,6-

trichloro-S-triazine (3% v/v) in dioxane and 3 mL of 0.2 M potassium phosphate buffer (pH 8.3). The mixture was left for 10 minutes until the solution was clear, and it was subsequently measured for absorbance at 382 nm. The sample blank was prepared in the same manner except that HHL was added after the reaction mixture was terminated. The control was prepared by using distilled water.

Appendix A.8 Total phenolic content determination

Ten gram of ground cooked adzuki bean or cooking water were blended with 95% ethanol at a ratio of 1:10 (w/v) and extracted at 30 ± 2 ºC in a water bath with continuous shaking for 2 hours. The obtained slurry was centrifuged at 10,000 ×g for 10 min and the supernatant was filtered through Whatman no. 1 filter paper.

Gallic acid standard curve was prepared. Distilled water was used instead of the sample and prepared in the same manner to obtain the control. The standard curve prepared from standard solutions of gallic acid is shown in Figure A.7.

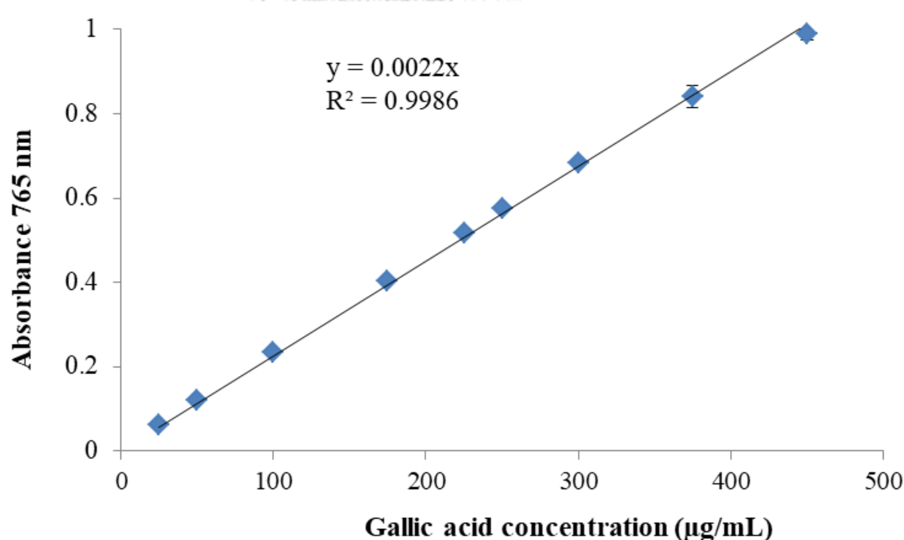

Figure A.7 Gallic acid standard curve for TPC determination

Appendix A.9 Total flavonoid content determination

Placed 1 ml of sample solution, catechin standard solutions, or blank (deionized or distilled water) in a 10 ml volumetric flask. Added 4 ml of distilled water followed by 0.3 ml of 5% (w/v) sodium nitrite. Swirled to mix and incubate 5 min at room

temperature. Then, 0.3 mL of 10% (w/v) aluminum chloride was added into the mixture. After 1 min, 2 ml of 1 M sodium hydroxide was added into the mixture and added water to the 10 ml line, mixed, and incubated 30 min at room temperature. The absorbance of obtained solution was measure at 510 nm in a spectrophotometer.

Catechin standard curve was prepared. Distilled water was used instead of the sample and prepared in the same manner to obtain the control. The standard curve prepared from standard solutions of catechin is shown in Figure A.8.

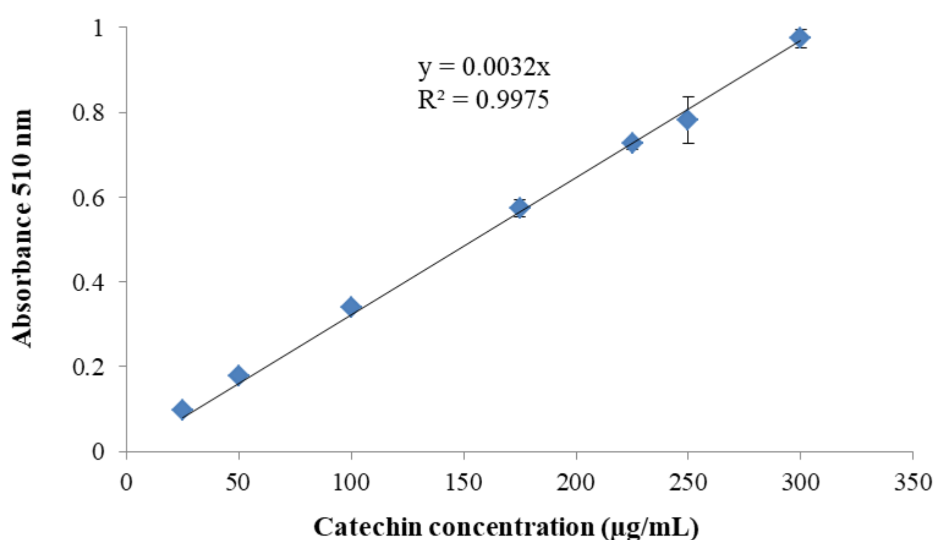

Figure A. 8 Catechin standard curve for TFC determination

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Appendix A.10 Reducing sugar content determination

10.1 Preparation of reagent

Low-alkalinity copper reagent: the first solution was prepared by dissolving 12 grams of sodium potassium tartrate, 24 grams of anhydrous sodium carbonate, 4 grams of copper sulfate, and 16 grams of sodium hydrogen carbonate in 450 mL of distilled water. The second solution was prepared by dissolving 180 grams of anhydrous sodium sulfate in 500 mL of boiling water. These two solutions were added to 1000-mL volumetric flask and made up the volume to 1000 mL with distilled water.

Arsenomolybdate reagent: dissolving 25 grams of ammonium molybdate in 450 mL distilled water, then 21 mL of concentrated sulfuric acid and 25 mL of distilled water containing 3 grams of disodium hydrogen arsenate heptahydrate were added. The mixture was stirred at room temperature for 24 hours.

10.2 Reducing sugar content

A 1 ml of extracted sample, standard glucose solutions, or blank (deionized or distilled water) was transferred to a glass tube and mixed with 1 ml of low-alkalinity copper reagent. Heated the glass tube containing reaction mixture in boiling water for 10 min. Then cool down and added 1 ml of arsenomolybolic acid reagent. The obtained solution was measured its absorbance at 500 nm. Subtracted the absorbance of the blank from all reading and created a calibration curve from the standard. The standard curve is shown is Figure A.9.

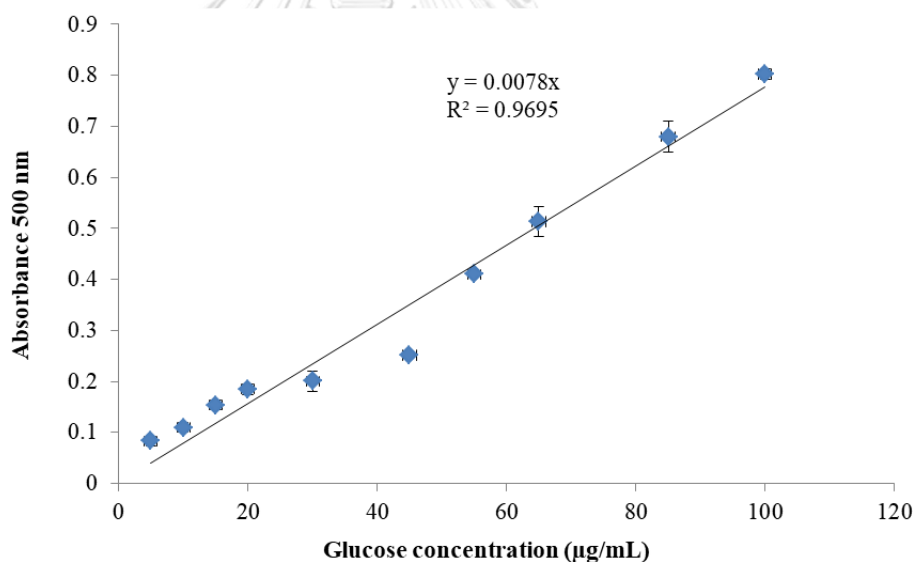

Figure A. 9 Glucose standard curve for reducing sugar determination

APPENDIX B
CHROMATOGARM

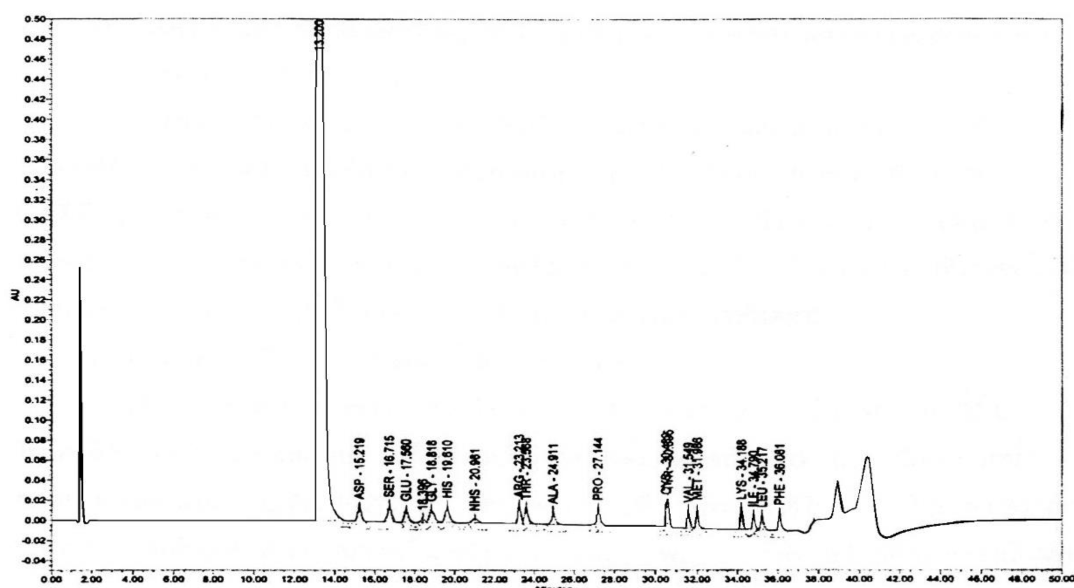

Figure B.1 Chromatogram of standard amino acids mixture

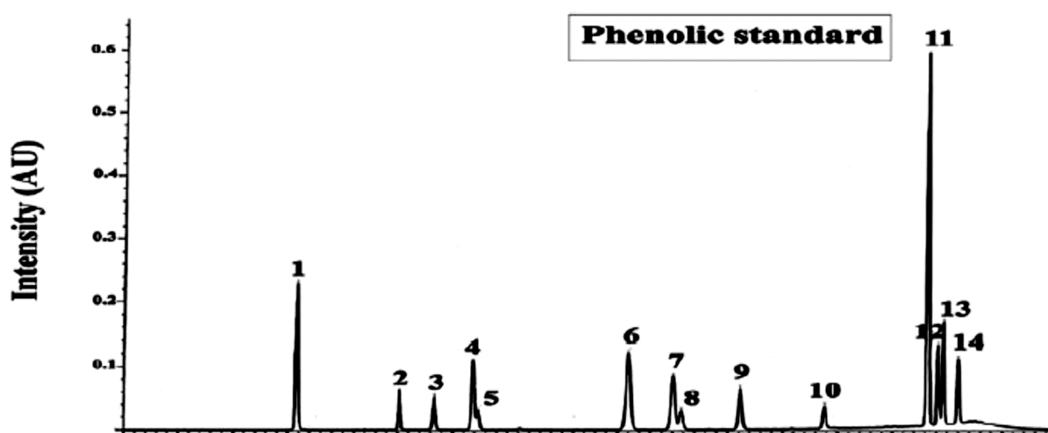

Figure B.2 HPLC profile of standard phenolic; (1) gallic acid, (2) catechin, (3) 4-HBA, (4) vanillic acid, (5) epicatechin, (6) p-coumaric acid, (7) sinapic acid, (8) ferulic acid, (9) vitexin, (10) rutin, (11) trans-cinnamic acid, (12) quercetin, (13) naringenin and (14) genistein.

APPENDIX C
STATISTIC ANALYSIS

Table C.1 The ANOVA table showing the effect of different cooking conditions on weight and moisture content of cooked adzuki beans and its cooking water

	SOV	df	MS	F	Sig
Weight	Treatment	7	4760.76	6358.43	0.000
	Error	64	0.749		
	Total	72			
Moisture	Treatment	7	12434.69	4284.46	0.000
	Error	64	2.902		
	Total	72			

Table C.2 The ANOVA table showing the effect of different cooking conditions on crude protein, TCA-soluble peptides, free amino group and total amino acid of cooked adzuki beans and its cooking water (based on 30 g raw seed, wb)

	SOV	df	MS	F	Sig
Crude protein	Treatment	7	56.558	958.88	0.000
	Error	64	0.059		
	Total	72			
TCA-soluble peptides	Treatment	7	2362.281	1358.505	0.000
	Error	64	1.739		
	Total	72			
Free amino group	Treatment	7	461965.93	42.268	0.000
	Error	64	10929.44		
	Total	72			
Total amino acid	Treatment	7	15936.342	8.884	0.002
	Error	64	1793.851		
	Total	72			

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Table C.3 The ANOVA table showing the effect of different cooking conditions on crude protein, TCA-soluble peptides, free amino group and total amino acid of cooked adzuki beans and its cooking water (based on 100 g cooked portion)

	SOV	df	MS	F	Sig
Crude protein	Treatment	7	476.592	3471.25	0.000
	Error	64	0.14		
	Total	72			
TCA-soluble peptides	Treatment	7	1827.666	115.787	0.000
	Error	64	15.785		
	Total	72			
Free amino group	Treatment	7	610430.024	31.097	0.000
	Error	64	19629.596		
	Total	72			
Total amino acid	Treatment	7	99411.133	21.100	0.000
	Error	64	4711.346		
	Total	72			

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Table C.4 The ANOVA table showing the effect of different cooking conditions on biological activity of borate buffer-soluble fraction from cooked adzuki beans and its cooking water

	SOV	df	MS	F	Sig
ABTS scavenging activity	Treatment	6	634.287	1007.550	0.000
	Error	56	0.630		
	Total	63			
FRAP	Treatment	6	154.105	43.767	0.000
	Error	56	3.521		
	Total	63			
Ferrous chelating activity	Treatment	6	1.790	459.720	0.000
	Error	56	0.004		
	Total	63			
ORAC	Treatment	6	9.140	127.480	0.000
	Error	56	0.072		
	Total	63			
ACE-I inhibitory activity	Treatment	6	9336.772	166.031	0.000
	Error	56	56.235		
	Total	63			

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Table C.5 The ANOVA table showing the effect of different cooking conditions on TCA-soluble peptides, free amino group, TPC, TFC and reducing sugar of borate buffer-soluble fraction from cooked adzuki beans and its cooking water

	SOV	df	MS	F	Sig
TCA-soluble peptides	Treatment	6	0.031	103.673	0.000
	Error	56	0.000		
	Total	63			
Free amino group	Treatment	6	11.128	222.112	0.000
	Error	56	0.050		
	Total	63			
TPC	Treatment	6	6.126	634.913	0.000
	Error	56	0.010		
	Total	63			
TFC	Treatment	6	2.735	815.716	0.000
	Error	56	0.003		
	Total	63			
Reducing sugar	Treatment	6	2.779	37.348	0.000
	Error	56	0.074		
	Total	63			

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Table C.6 The ANOVA table showing the effect of different cooking conditions on biological activity of borate buffer-soluble fraction with phenolic removal from cooked adzuki beans and its cooking water

	SOV	df	MS	F	Sig
ABTS scavenging activity	Treatment	6	22.126	342.801	0.000
	Error	56	0.065		
	Total	63			
FRAP	Treatment	6	628.925	54.564	0.000
	Error	56	11.526		
	Total	63			
Ferrous chelating activity	Treatment	6	1.718	609.165	0.000
	Error	56	0.003		
	Total	63			
ORAC	Treatment	6	149.463	126.600	0.000
	Error	56	1.181		
	Total	63			
ACE-I inhibitory activity	Treatment	6	4251.304	135.856	0.000
	Error	56	31.293		
	Total	63			

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Table C.7 The ANOVA table showing the effect of different cooking conditions on TCA-soluble peptides, free amino group, TPC, TFC and reducing sugar of borate buffer-soluble fraction with removal from cooked adzuki beans and its cooking water

	SOV	df	MS	F	Sig
TCA-soluble peptides	Treatment	6	0.06	21.817	0.000
	Error	56	0.003		
	Total	63			
Free amino group	Treatment	6	46.848	94.243	0.000
	Error	56	0.497		
	Total	63			
TPC	Treatment	6	2.615	45.274	0.000
	Error	56	0.058		
	Total	63			
TFC	Treatment	6	0.125	45.483	0.000
	Error	56	0.003		
	Total	63			
Reducing sugar	Treatment	6	11.195	448.033	0.000
	Error	56	0.025		
	Total	63			

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Table C.8 The ANOVA table showing the effect of different cooking conditions on biological activity of phenolic fraction from cooked adzuki beans and its cooking water

	SOV	df	MS	F	Sig
ABTS scavenging activity	Treatment	6	167.459	47.494	0.000
	Error	56	3.526		
	Total	63			
FRAP	Treatment	6	1.348	94.043	0.000
	Error	56	0.014		
	Total	63			
Ferrous chelating activity	Treatment	6	1.633	554.707	0.000
	Error	56	0.003		
	Total	63			
ORAC	Treatment	6	1.053	13.871	0.000
	Error	56	0.076		
	Total	63			
ACE-I inhibitory activity	Treatment	6	9448.949	105.425	0.000
	Error	56	89.627		
	Total	63			

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Table C.9 The ANOVA table showing the effect of different cooking conditions on TCA-soluble peptides, free amino group, TPC, TFC and reducing sugar of phenolic fraction from cooked adzuki beans and its cooking water

	SOV	df	MS	F	Sig
TCA-soluble peptides	Treatment	6	0.005	141.348	0.000
	Error	56	3.823×10^{-5}		
	Total	63			
Free amino group	Treatment	6	0.555	89.320	0.000
	Error	56	0.006		
	Total	63			
TPC	Treatment	6	0.205	86.356	0.000
	Error	56	0.002		
	Total	63			
TFC	Treatment	6	0.011	56.890	0.000
	Error	56	0.000		
	Total	63			
Reducing sugar	Treatment	6	0.459	82.508	0.000
	Error	56	0.006		
	Total	63			

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CHULALONGKORN UNIVERSITY

VITA

NAME	Thasanporn Sangsukiam
DATE OF BIRTH	11 March 1987
PLACE OF BIRTH	BANGKOK, THAILAND
INSTITUTIONS ATTENDED	Bachelor of Science (Food Science), Faculty of Applied Science, King Mongkut's University of Technology North Bangkok in academic year 2008. In 2009, she went to study at Department of Food Technology, Faculty of Science, Chulalongkorn University and received Master of Science (Food Technology) in 2012. In 2014, she enrolled in the doctoral degree program in Food Technology at Department of Food Technology, Faculty of Science, Chulalongkorn University.
HOME ADDRESS	69 Moo 5, Sanam Chan Sub-district, Mueang Nakhon Pathom District, Nakhon Pathom
PUBLICATION	1. Duangmal, K. and Sangsukiam, T. 2015. Effect of germination period on changes in mungbean and azuki bean protein and amino acid profiles. Acta Horticulturae. 1088: 563-568. 2. Sritongtae B., Sangsukiam T., Morgan, MR. and Duangmal K. 2017. Effect of acid pretreatment and the germination period on the composition and antioxidant activity of rice bean (Vigna umbellata). Food Chemistry. 227: 280-288. 3. Sangsukiam T. and Duangmal K. 2017. A comparative study of physico-chemical properties and antioxidanta activity of freeze-dried mung bean (Vigna radiata) and adzuki bean (Vigna angularis) sprouts hydrolysate powders. International Journal of Food Science and Technology. 52: 1971-1982.

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