PHYSICOCHEMICAL PROPERTIES OF MODIFIED CASSAVA FLOUR FORTIFIED WITH MICROBIAL POLY-γ-GLUTAMIC ACID

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FACULTY OF FOOD SCIENCE AND NUTRITION
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DECLARATION

I hereby declare that the work presented in this thesis is my own, except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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ABSTRACT

Cassava (*Manihot esculenta* Crantz) is one of the most important staple crops worldwide. It has been processed into many products, and there are still emerging new products from cassava. However, cassava flour needs to meet the high-quality requirements in terms of physicochemical characteristics to be widely accepted by the food industry. In the present study, the enzymatic modification was carried out on cassava flours using crude enzyme of α-amylase and cellulase derived from *Bacillus subtilis* var. *natto* culture, at 37℃ over 24 hours. The enzyme-modified cassava flours were further investigated for their physicochemical properties after the addition of crude poly-γ-glutamic acid (γ-PGA) at different levels (10%, 20%, 30%, 40%, and 50%; v/w). The chemical, functional, and pasting properties of flours were performed using standard methods, and native cassava flour was used as control. Increasing γ-PGA content resulted in a significant ($p<0.05$) increase in protein (ranged from 1.19 to 2.32%) and fat (ranged from 0.11 to 0.40%) content. In contrast, there were decreases in moisture (varied from 5.91 to 6.80%), ash (ranged from 1.25 to 1.36%), and amylose (ranged from 20.28 to 21.51%) content. All flour samples showed no significant ($p>0.05$) differences in terms of lightness (L*), while the greenness to redness (a*) of native flours were significantly ($p<0.05$) higher than modified flours with γ-PGA. There were significant ($p<0.05$) differences in the swelling power and solubility measured at various temperatures. Water and oil absorption capacities of native flour were significantly ($p<0.05$) higher than cassava flour with γ-PGA blends. From scanning electron microscopy (SEM), after the modification process of native cassava flours, the surfaces of hydrolysed starch granules were extensively eroded indicates of enzyme attack. From the pasting profiles, there were significant ($p<0.05$) increases in peak viscosity, final viscosity, and pasting temperature of cassava flours due to the addition of γ-PGA. High pasting values were recorded for the modified cassava flour fortified with 30% γ-PGA compared to other flour blends. In conclusion, up to 30% of γ-PGA addition in modified cassava flour is considered desirable as pasting properties is essential in determining the texture, digestibility, and end-use of starch-based food products. This information can be utilised in the preparation of textured products with a potential for industrialisation.

ABSTRAK

SIFAT FIZIKOKIMIA TEPUNG UBI KAYU TERUBAHSUAI DIPERKUAT DENGAN ASID POLIGLUTAMIK

Ubi kayu (Manihot esculenta Crantz*) merupakan salah satu tanaman ruji yang penting. Ia telah diproses kepada pelbagai jenis produk dan masih terdapat produk baharu yang terhasil daripada ubi kayu. Walau bagaimanapun, tepung ubi haruslah memenuhi kehendak industri makanan seperti kualiti yang tinggi dari segi ciri-ciri fizikokimia supaya dapat diterima secara meluas dalam industri makanan. Dalam kajian ini, ubahsuai secara enzimatik telah dijalankan ke atas tepung ubi kayu dengan menggunakan enzim kasar amilase dan selulase daripada kultur Bacillus subtilis var. natto pada suhu 37°C selama 24 jam. Tepung ubi kayu terubahsuai dikaji secara mendalam terhadap sifat-sifat fizikokimia setelah ditambah dengan asid poliglutamik (γ-PGA) pada tahap yang berbeza (10%, 20%, 30%, 40% dan 50%; v/w). Sifat-sifat kimia, berfungsi dan pempesan tepung dijalankan menggunakan kaedah piawai dan tepung ubi kayu asli digunakan sebagai kawalan. Peningkatan keatas tahap kandungan γ-PGA menyebabkan penambahan yang signifikan ($p<0.05$) dalam kandungan protein (1.19 to 2.32%) dan lemak (0.11 to 0.40%). Manakala, terdapat penurunan yang signifikan ($p<0.05$)* dalam *kandungan kelembapan (5.91 to 6.80%), abu (1.25 to 1.36%) dan amilosa (20.28 to 21.51%). Semua sampel tepung tidak menunjukkan perbezaaan yang signifikan ($p>0.05$) dari segi kecerahan (L*), manakala kehijauan ke kemerahan (a*) dalam tepung ubi kayu asli adalah lebih tinggi secara signifikan ($p<0.05$) berbanding tepung ubi kayu terubahsuai ditambah dengan γ-PGA. Terdapat perbezaan yang signifikan ($p<0.05$) dalam kuasa pembengkakan dan keterlarutan pada suhu yang berbeza. Tepung ubi kayu asli menunjukkan kapasiti penyerapan air and minyak yang lebih tinggi ($p<0.05$) berbanding tepung ubi terubahsuai yang ditambah dengan γ-PGA. Pemerhatian menggunakan mikroskop pengimbasan elektron (SEM) mendapati, selepas proses terubahsuai ke atas tepung ubi kayu asli, retakan yang ketara pada permukaan granul kanji dapat diperhatikan akibat daripada tindakan enzim. Profil pempesan menunjukkan terdapat peningkatan yang signifikan ($p<0.05$) dalam kelikatan puncak, kelikatan akhir dan suhu pempesan kesan daripada penambahan γ-PGA. Tepung ubi kayu terubahsuai yang ditambah dengan 30% γ-PGA menunjukkan profil pempesan yang lebih tinggi berbanding sampel yang lain. Kesimpulannya, penambahan sebanyak 30% γ-PGA ke dalam tepung ubi kayu terubahsuai adalah dianggap wajar kerana sifat-sifat pempesan adalah penting bagi menentukan tekstur, penghadaman dan penggunaan akhir sesuatu produk makanan berasaskan kanji. Maklumat ini dapat digunakan dalam penyediaan produk tekstur yang berpotensi untuk dipasarkan.*

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LIST OF ABBREVIATIONS/SYMBOLS

ANOVA	-	Analysis of variance
AOAC	-	Association of Official Analytical Chemists
CFU	-	Colony forming units
DE	-	Dextrose equivalent
DNS	-	3, 5-dinitrosalicylic acid
FAO	-	Food and Agriculture Organization
HCN	-	Hydrogen cyanide
HPLC	-	High performance liquid chromatography
kDa	-	Kilodalton
NDA	-	National Department of Agency
PGA	-	Polyglutamic acid
RVA	-	Rapid Visco Analyser
RVU	-	Rapid visco unit
SEM	-	Scanning electron microscopy
SmF	-	Submerged fermentation
SSF	-	Solid-state fermentation
γ-PGA	-	Poly-γ-glutamic acid
α	-	Alpha
β	-	Beta
γ	-	Gamma

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cassava (*Manihot esculenta* Crantz), also known as tapioca, *yucca*, or *manioc*, is a woody shrub with tuberous roots belonging to the family Euphorbiaceae (Alves, 2002). During the 16th century, the Portuguese distributed this perennial woody shrub to Africa, which was believed to be originated from South America (El-Sharkawy, 2012). Now, the cultivation of cassava is widely practiced in tropical and subtropical regions of Africa, Asia, and Latin America. Cassava is considered the third most crucial carbohydrate source in tropics, after maize and rice (FAO, 2014). While cassava was once considered as the "food for the poor," now it has become significant world agriculture and provides a multipurpose utilisation in developing countries, become a global trend economically, and a challenge towards climate change (Howeler *et al.*, 2013). The outstanding characteristics of the cassava crop, such as its tolerance and high resistance to soil with a lack of nutrients make cassava one of the crops that contribute to the economic importance (Poonsrisawat *et al.*, 2014). The main nutritional values in cassava are water (60 g 100 g^{-1}) and carbohydrates (38 g 100 g^{-1}). Meanwhile, the contents of fibre, protein, and fat are limited (1.4, 1.8, and 0.28 g 100 g^{-1}, respectively) (NDA, 2013). The values mentioned above are only approximations as varieties of cassava cultivars exists (Aryee *et al.*, 2006).

Currently, cassava has been produced globally over 103 countries, with a total production of approximately 270 million tons (FAOstat, 2014), which covered

25 million hectares worldwide (Parmar *et al*., 2017). Major producing countries of cassava are Thailand, Central and West Africa (Nigeria and Ghana), Indonesia, and northern Brazil, where half of the world production is from Africa. It is estimated that 800 million people consumed cassava as a primary foodstuff, especially those that live in the least industrialised regions (Parmar *et al*., 2017). In terms of cassava consumption, approximately 65% of its productions were made for human consumptions, while 25% is for utilisation in various industries, and finally 10% is considered waste (Fish & Trim, 1993). Cassava is a traditional food security crop, often processed into a variety of traditional food products. Extensive efforts were made to utilise the uses of cassava fully. The most common way is by processing the fresh cassava tubers into the flour and applied in a broad range of new products to these developing countries with rapid urbanising societies. Due to its limited functionalities, perishable, and bulky, proper strategies and technologies are required to overcome these limitations. Besides, although native flour is demanded industrial applications, the industries require modified flour with improved functionality as direct application of native flour is quite challenging. In the last few decades, various methods have been developed for starch modification to achieve suitable functional properties for utilisation in various industries. Generally, there are four modification methods, namely physical, chemical, enzymatic and genetically, or their combinations (Kaur *et al*., 2012).

Modification of starch has been extensively studied to overcome the functional limitations of native starch and increase the importance of starch for industrial applications (Kaur *et al*., 2012). Apart from reducing gelling tendencies, retrogradation of gel/paste texture, film formation, and adhesion of native starches (Kaur *et al*., 2012), stabilisation of starch granule can be achieved through modification (Ashogbon & Akintayo, 2014). Briefly, modification often leads to few changes towards the starch polymer, thus increasing its flexibility and change its physical, functional, and structural properties to enhance its usefulness for food and non-food industries (Lopez *et al*., 2010). Enzymatic modifications have been studied, partly replacing the chemical and physical methods over recent decades. This is due to enzymes that are safer, healthier, and mild than the chemical method to the environment and food consumers (Park *et al*., 2018). In an enzymatic modification, hydrolysing enzymes are mainly used for starch modification, and its products are either high fructose corn syrup or glucose syrup (Kaur *et al.*, 2012). Enzymatic modification has several advantages, such as more specific hydrolysis products, fewer by-products, and high yield. Moreover, the process and its end products can be controlled by selecting an individual enzyme to modify a specific or particular property of the starch or flour (Dura *et al.*, 2014).

Starch is a polysaccharide consists of two fractions – amylose, composed of α-(1-4) D-glucopyranosyl units and amylopectin, composed of many short chains linked together by a α-(1-6) linkage (Biliaderis, 1998). Amylose constitutes about 20-25% of the starch molecule, while amylopectin constitutes about 75-80%. There are abundant enzymes used to modify starch structure and achieve the desired functional properties, such as α-amylase and cellulase. α-Amylase is one of the hydrolase enzymes that catalyse the hydrolysis of α-1, 4-glycosidic linkages in starch to produce products such as maltose, glucose, and dextrin while retaining the α-anomeric configuration of the products (Gupta *et al.*, 2003). Hence, the importance of α-amylase is crucial due to its starch hydrolysis activity (Sundarram & Murthy, 2014). Meanwhile, cellulases are grouped into glycoside hydrolases (GH) family and often used to catalyse the hydrolysis of glycosidic linkages depolymerising cellulose to fermentable sugars (Juturu & Wu, 2014). Moreover, cellulose can be broken down by employing cellulases to hydrolyse β-1,4 glycosidic bonds of the cellulose polymer (Behera *et al.*, 2017). Bacteria have been widely explored for α-amylase and cellulase production. Among them, *Bacillus sp.* has become the dominant bacteria due to its ability to produce and secrete large quantities of extracellular enzymes (Rastogi *et al.*, 2009). In this research, cassava flours are hydrolysed by enzymatic culture containing α-amylase and cellulase synthesised from non-pathogenic microorganisms of bacterial species, which is *Bacillus subtilis* var. natto.

Poly-γ-glutamic acid (γ-PGA) is a water-soluble and biodegradable biopolymer consisting of D and L-glutamic acid units, connected by amide linkages between α-amino and γ-carboxyl groups produced by bacteria for various applications. γ-PGA is edible and is present abundantly in the traditional Japanese dish called *natto,* made by fermenting soybean with *Bacillus* strains. To date, there are four methods to produce γ-PGA, which are chemical synthesis, peptide synthesis, biotransformation, and microbial fermentation (Sanda *et al.*, 2001). Among these methods, microbial fermentation is deemed the most cost-effective, including inexpensive raw materials, minimal environmental pollution, mild reaction conditions, and high natural product purity (Luo *et al.*, 2016). In microbial fermentation, submerged fermentation (SmF) technology remains challenging due to limited oxygen supply during the fermentation process, high expenditure of raw materials, and rigorous laboratory equipment (Xu *et al.*, 2014). Unlike SmF, solid-state fermentation (SSF) is more advantageous, including lower production costs, more straightforward equipment, and reduced contamination risks (Pandey, 2003). Thus, SSF was chosen for γ-PGA production in this study. The γ-PGA characteristics, which are being edible and non-toxic to humans and environments, allow its application to several industries (Bajai & Singhal, 2011). In the food industry, γ-PGA is used as a thickener, bitterness relieving agent, cryoprotectant, encapsulation, water adsorbent, and as a nutrition supplement (Shih & Van, 2001). Therefore, it may be suggested that γ-PGA as a food additive can improve the physicochemical properties of native flour. In this research, microbial γ-PGA is chosen to overcome the limitation of physicochemical properties in cassava.

To date, there is only one research regarding the utilisation of γ-PGA in modified cassava flour (Soetikno *et al.*, 2017). However, the previous study is different from the current study in terms of cassava flour modification, and γ-PGA production and application. The study was undertaken to modify cassava flour using microbial starter culture derived from the culture of *A. Oryzae* and *B. natto* and further fortified with fermented product containing γ-PGA. In this study, γ-PGA was isolated and extracted from the solid-state fermentation of soybean to produce an aqueous γ-PGA solution. After that, different levels (10 – 50%) of γ-PGA was incorporated into modified cassava flour. Therefore, this current study was the first attempt to utilise γ-PGA in cassava flour to improve its nutritional composition and physicochemical properties.

1.2 Problem Statement

Recently, the increasing population and urbanisation, as well as changes in food habits, have led to an increased demand for wheat-based foods in many developing countries (Eriksson *et al.*, 2014). However, the local climate condition in Malaysia is not suitable for profitable wheat production. Thus, cassava flour has been one way of addressing this need as cassava flour has been extensively studied as a local alternative to wheat flour (Alvarenga *et al.*, 2011). However, the utilisation of starchy tubers instead of wheat flour in food depends on their physical and chemical properties. For example, the properties of starch granules influence the behaviour of flour in food systems, such as viscosity and gelatinisation, which affect the texture of the end product (Eriksson *et al.*, 2014). Most native starches are limited for direct application because they are unstable to changes in temperature and shear forces. Besides, native starches have a strong tendency for decomposition and retrogradation (Berski *et al.*, 2011). Moreover, some starch granules are inert, insoluble in water at room temperature, highly resistant to enzymatic hydrolysis and thus lack functional properties (Alcazar-Alay & Meireles, 2015). Therefore, native starch needs to undergo a modification of its starches to meet the high-quality requirements in terms of physicochemical characteristics so that these flours can be accepted widely by the food and non-food industries (Eriksson *et al.*, 2014).

1.3 Justification of Study

Specific properties of cassava flour, such as physical, chemical, and pasting parameters are essential to be considered as useful in food industries. Moreover, some functional characteristics, such as swelling power and viscosity, are positively correlated with the qualities of the products (Ponzio *et al.*, 2008; Linlaud *et al.*, 2009). For example, starch granules characteristics of milled flours may affect the rate of hydration and swelling capacity during food processing (Hatcher *et al.*, 2009). Besides, the quality of colour determines the visual appearance and eye appeal of the finished product (MacDougall, 2002), while water binding and absorption capacities, swelling power, and solubility have a strong influence on carbohydrate quality, which affect the viscosity and gelling ability of flour/starch (Oladunmoye *et al.*, 2004). Therefore, with the increasing interest in utilising cassava flour and starch in the development of food products, mostly new or novel products, the availability of their physicochemical properties would ease the processing protocol for the development of various value-added food products. Besides, through this research, fundamental knowledge can be obtained regarding the effect of γ-PGA fortification on the physicochemical properties of cassava. At present, there are very few studies documentation on the application of γ-PGA either in flour or its products. Some of the previous studies investigated the effect of γ-PGA on rheology and thermal properties of wheat dough (Shyu *et al.*, 2008), emulsion and foam activity of sponge cake paste (Shun & Sung, 2010) as well as oil uptake and moisture loss in doughnut products (Lim *et al.*, 2012). Hence, this study is expected to provide new information on the addition of γ-PGA in cassava flour so that other scientific studies may be done in the future. Hopefully, this research could increase the utilisation of local cassava flour with value-added ingredients.

1.4 Objectives of Study

The objective of this study was to fortify enzyme-modified cassava flour by incorporating microbial poly-γ-glutamic acid (γ-PGA). Studies on physicochemical properties of the modified flour were carried out using instrumental methods and chemical analysis.

The specific objectives of this study were:

1.4.1 To determine the capability of microbial enzymes dealing with modification of cassava flour.

1.4.2 To produce microbial poly-γ-glutamic acid through solid-state fermentation of soybean using *Bacillus subtilis* var. natto.

1.4.3 To determine the proximate composition and physicochemical properties of modified cassava flour fortified with poly-γ-glutamic acid.

CHAPTER 2

LITERATURE REVIEW

2.1 Cassava (*Manihot esculenta* Crantz)

2.1.1 Origin and Morphology

Cassava (*Manihot esculenta* Crantz) is one of some 100 species of trees, shrubs, and herbs belonging to the genus *Manihot* (Howeler *et al*., 2013). The word cassava comes from the word *Casavi* or *Cazabi*, which means bread as translated from *Arawak* (the language of the first indigenous people who lived in the Greater Antilles). It is also known as *yucca* (Spanish), *manioc* (French), *mandioc* (Portuguese), *cassave* (Dutch) and *manioc* (German) in certain regions of the world. After the discovery of this crop by the Europeans, cassava was taken to Africa and eventually become one of the useful food crops. These crops were later taken to Asia and cultivated as food security crops and starch extraction (Balgbrough *et al*., 2010; Akinpelu *et al*., 2011). Cassava was first introduced into Malaysia in 1836 through Jakarta, Indonesia, as a potential replacement of sago (Burkill, 1936).

Cassava is a dicotyledonous plant belonging to the family Euphorbiaceae that can grow for years. It has lateral subterranean storage organs in the form of starchy roots (Parmar *et al*., 2017). Botanically, cassava is a woody perennial shrub, with a mature height of 2 to 4 meters, and is mainly cultivated for its starchy roots. The cassava root is long and tapered (Figure 2.1), with a firm homogenous flesh covered in a tough outer skin of about 1 mm thick, brown and coarse on the outside. The roots can be 5 to 10 cm in diameter at the middle, and 50 to 80 cm long. A woody cordon runs along the root’s axis, while the flesh can be chalk-white or yellowish (Anyanwu *et al*., 2015). The cassava shoot consists of stems, leaves, inflorescences and root systems made up of fibrous and tuberous roots. The edible starchy root (the internal white/yellow flesh) can be harvested within 8 to 24 months of planting, depending on cultivar and climate. Mature cassava roots can be measured between 15 and 100 cm in length and 0.5 to 2.0 kg in weight, depends on the species and growing conditions. The edible root is circular in cross-section, where it is fattest at the centre and narrowed gently towards the end. Crosswise a cassava root consists of three principal areas namely, the periderm, which comprises the outermost layer of the root, the cortex that is located below the periderm and the starchy flesh located at the middle portion of the root (Anyanwu *et al*., 2015).

Figure 2.1 : Cassava roots
Source : Anyanwu *et al*. (2015)

In terms of the morphological characteristics of cassava, a high degree of interspecific hybridisation can be observed, which indicates the crossing between different species of the same genus (Jovanka, 2004). The morphological and agronomic properties are usually used to determine the type of cassava. From the morphology of cassava, the following was defined as the minimum or fundamental factors that should be considered during identification of the cultivar: apical leaf colour, apical leaf pubescence, central lobe shape, petiole colour, stem exterior colour, phyllotaxis length, root peduncle presence, external root colour, root cortex colour, root pulp colour, root epidermis texture and flowering. However, morphological descriptors become difficult due to the wide range of cassava genotypes and the environmental conditions that affect the morphology of cassava (Hillocks *et al.*, 2002).

2.1.2 Nutritional Value

The nutrient composition of cassava depends on its specific tissue, such as the root and leaf, and factors such as geographic location, variety, age of the plant, and growing conditions. The roots and leaves, which constitute 50% and 6% of the mature cassava plant, are the nutritionally valuable parts of cassava (Tewe & Lutaladio, 2004). The nutritional values of cassava roots are essential as they are the central part of the plant consumed in developing countries (Montagnac *et al.*, 2009). In general, cassava is often considered inferior to maize and wheat because of low protein levels, vitamins and minerals.

Table 2.1: Nutritional composition of raw cassava tubers

Nutrient	**Per 100g**
Proximate composition (100 g)	
Water (g)	59.68
Energy (Kcal)	160
Protein (g)	1.36
Total lipid (fat) (g)	0.28
Fibre, total dietary (g)	1.8
Sugars, total (g)	1.7
Carbohydrate (by difference) (g)	38.06
Vitamins	
Vitamin C (mg)	20.6
Thiamine (mg)	0.087
Riboflavin (mg)	0.048
Niacin (mg)	0.854
Vitamin B6 (mg)	0.088
Beta-carotene (mg)	Not detected
Vitamin B12 (μg)	27

(continued)