Development and characterization of peptide antioxidants from sorghum proteins

by

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Abstract

Antioxidants are widely used in food industries to delay lipid oxidation and prevent oxidative deterioration. In recent years, growing interests in developing safe and efficient antioxidants from natural sources due to the health-related risks associated with synthetic antioxidants. Recently, peptide antioxidants have drawn growing interests as since proteins are a macronutrient with various functionalities and high consumer acceptability. A lot of dietary proteins have been validated for their antioxidant potentials especially those obtained from animal proteins, nuts and pulses. Relatively less information is available on characterizing the antioxidant profile of cereal protein, and even less for sorghum protein. Sorghum is the fifth largest crop worldwide and is the third in United States. U.S. is leading in global sorghum production and distribution, and the state of Kansas is producing nearly half of U.S. sorghum. Currently, about one third of the U.S. sorghum is being used for ethanol production, resulting in more than 450 kilotons of by-products (e.g., DDGS) annually, which were often discarded or underutilized. DDGS is a premium protein source (~ 30% protein) that could be potentially modified into value-added products such as peptide antioxidants.

In this study, relevant literatures detailing the extraction of cereal proteins, enzymatic hydrolysis of proteins, purification and characterization of hydrolysates, and evaluation of antioxidant profiles were extensively reviewed in Chapter 1. As preliminary experiments, sorghum kafirin protein was extracted from defatted sorghum white flour and hydrolyzed by 10 different types of enzymes from microbial, plant and animal sources. Hydrolysates prepared with Neutrase, Alcalase, and Papain displayed the most promising antioxidant activities as well as total protein recovery were primarily selected and investigated in depth described in Chapter 2, Chapter 3, and Chapter 4. The reaction conditions including substrate content, enzyme-to-

substrate ratio, and hydrolysis time are critical parameters in producing peptides with desired activity and consistency, were therefore examined and optimized for each case of kafirin hydrolysates. The antioxidant capacity of the resulting hydrolysates was measured for antioxidant capacity through *in vitro* assays (DPPH, ABTS, ORAC, reducing power, and metal chelating) and then demonstrated in model systems (oil-in-water emulsion and ground meat). The fractions of hydrolysates possessing strongest activities were further fractionated by gel filtration and HPLC. Peaks representing the largest areas from HPLC were identified for major sequences by MALDI-TOF-MS.

The experiment results indicated that all the three selected fractions of kafirin hydrolysates revealed excellent inhibition effects against oil and fat oxidations, which could be employed as tools to predict their performances in real food products. In addition, the structure studies showed that medium-sized hydrolysates of Neutrase (3 – 10 kDa) and Alcalase (5 – 10 kDa), and small-sized hydrolysates of Papain (1 – 3 kDa) exhibited relatively stronger activities.

This study provided a workable processing method and critical reaction parameters for the production of peptide antioxidants from sorghum protein. The experiment results revealed that the sorghum peptide antioxidant could act through multiple mechanisms including free radical scavenging, metal ion chelation, hydrogen donating, and forming physical barriers to minimize the contact of oxidative agents to targets. These antioxidative peptides are a promising ingredient that can be potentially incorporated to food and feed products as alternatives to synthetic antioxidants or synergetic elements to nonpeptic antioxidants for protection of susceptible food ingredients. This study also made a positive impact to sorghum ethanol industry by guiding the conversion of sorghum protein-rich by-products into value-added antioxidant products as an additional revenue stream.

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Chapter 1 - Literature Review: Grain Sorghum as a Potential Source for Peptide Antioxidant

Abstract

Many food proteins contain antioxidative peptide sequences and structural domains, however, they are mostly buried within the proteins' hydrophobic core and inaccessible to prooxidants, radical species, and transition metal ions to exert their antioxidant functions. Enzymatic hydrolysis could be a feasible approach to release these functional peptide fragments and produce antioxidative peptides with high selectivity and efficacy. Peptides antioxidants are naturally derived and generally recognized as safe therefore are considered as a more preferable option to substitute synthetic antioxidants. In addition, the peptide antioxidants could act as surfactants at oil–water interface due to the surface amphiphilicity while providing profiles of amino acids and serving as a nutrient and energy source. All these features make antioxidative peptides attractive and applicable to food and feed industry as functional ingredients or additives.

Sorghum is one of the oldest known ancient grains and is the third largest cereal grain in United States. Sorghum was proven to possess health-beneficial values and various bioactive potentials confirmed by numerous studies. Currently, about one third of sorghum is used for bioenergy in U.S. and leaving a substantial amount of protein-rich by-products underutilized. The transformation of these by-products into high-valued goods will make a positive impact to the comprehensive economy of sorghum biofuel industry. Special properties of sorghum protein determined by its grain kernel structures and chemical compositions makes it a potential source for production of antioxidant peptides.

This work built the foundation for further studies by providing systematic information for the development of a practical processing method to convert sorghum proteins to peptide

hydrolysates with superior antioxidant activities. The action modes of peptide antioxidants were researched and better understood. The potential of transforming sorghum protein into peptide antioxidants was discussed, and relevant literatures describing the processing procedure for the production of peptide antioxidants were studied. Specific technologies including cereal protein extraction, precursor protein hydrolysis, fractionation and purification of subsequent hydrolysates, and evaluation assays of antioxidant profiles were summarized for future reference.

1. Introduction

Deterioration and failures of food quality induced by oxidative stress is a projecting problem in food industry, which leads to the alterations and failures of functionalities (e.g., water/oil binding capacity), nutritional profile, quality attributes (e.g., flavor, aroma, texture, color), and formation of toxic products. Antioxidants such as BHA, BHT, TBHQ and ETDA was commonly used in a wide variety of food products to retard the oxidative deterioration. However, due to the impelling safety concerns, the use of these synthetic antioxidants has been restricted or prohibited. The interests of research have focused on the development of novel antioxidants from natural sources, which is projected to reach 3-billion-dollar market value in 2020.

Recent studies revealed that peptides and proteins hydrolysates from dietary proteins hold promise to retard or inhibit the oxidative deterioration of lipid and oil. These studies built the foundation of development of peptide antioxidants. Compared to synthetic antioxidants, peptide antioxidants are naturally-derived, generally recognized as safe, effective, nutritious, multi-functional, and low-cost. Thus, peptide antioxidants are of great potential to substitute the synthetic antioxidants or as synergetic antioxidants as food additives or functional ingredients to improve their oxidative-stability and maintain the food quality.

Among the literatures reviewed, dietary proteins from various sources have been validated for their potentials in producing peptide antioxidants, including wheat (Zhu, Zhou, & Qian, 2006; Tang et al., 2012; Zhu, Guo, Guo, Peng, & Zhou, 2013), rice (Tang & Zhuang, 2014; Thamnarathip, Jangchud, Nitisinprasert, & Vardhanabhuti, 2016; Phongthai, D'Amico, Schoenlechner, Homthawornchoo, & Rawdkuen, 2018), barley (Bamdad, Wu, & Chen, 2011; Xia, Bamdad, Gänzle, & Chen, 2012), corn (Li et al., 2010; Liu et al., 2015; Wang et al., 2014; Zhuang, Tang, & Yuan, 2013), canola (Cumby, Zhong, Naczk, & Shahidi, 2008; Alashi et al.,

2014), millet (Agrawal, Joshi, & Gupta, 2016), rapeseed (Zhang, Wang, Xu, & Gao, 2009; Pan, Jiang, & Pan, 2011; He, Girgih, Malomo, Ju, & Aluko, 2013), quinoa (Aluko & Monu, 2003), soybean (Chen, Muramoto, & Yamauchi, 1995; Park, Lee, Baek, & Lee, 2010), chickpea (Li, Jiang, Zhang, Mu, & Liu, 2007; Kou et al., 2013), peanut (Su et al., 2011), walnut (Gu et al., 2015), pumpkin (Bučko et al., 2016), sweet potato (Zhang & Mu, 2017; Zhang, Mu, & Sun, 2014) and so on. Several comprehensive reviews of food-derived bioactive peptides (Samaranayaka & Li-Chan, 2011; Elias, Kellerby, & Decker, 2008; Sarmadi & Ismail, 2010; Shahidi & Li, 2014; Udenigwe & Aluko, 2012) extensively categorized the production process, characterization techniques, evaluation assays, and potential applications of bioactive proteins and peptides antioxidants. A handful of reviews on specified topics such as cereal storage proteins (Cavazos & Gonzalez de Mejia, 2013), pulses (López- Barrios et al., 2014), fish protein (Chalamaiah, Hemalatha, & Jyothirmayi, 2012), marine (Kim & Wijesekara, 2010), marine by-products (Sila & Bougatef, 2016) were also available.

To the best of our knowledge, relatively less studies were found to exploit cereal grain proteins. Especially, characterization of the antioxidant potential of sorghum protein was not adequately reported, the development of peptide antioxidants from sorghum kafirin was principally absent. Sorghum is the fifth leading cereal crop worldwide and ranks the third largest crop in United States. It is a versatile and sustainable cereal grain which plays an important role in food security especially for tropical and semi-arid regions, where the other cultivars could barely survive the harsh climate conditions. A lot of studies have proposed the health-beneficial values and chronic-diseases-preventing potentials of sorghum, which were attributed to its micronutrients, phytochemicals, phenolic content, and recently discovered, bioactive peptides. For a long time, the use of sorghum proteins had been limited due to inferior functional qualities

and poor digestibility in comparison to animal proteins. Enzymatic hydrolysis is a useful tool for protein modification for various applications including the production of peptide antioxidants. Kafirin is the main storage protein of sorghum grain and was proven to be bioactive by several *in vitro* and *in vivo* studies. Thus, it is expected that sorghum kafirin hold antioxidative capacity which can be enhanced through certain modifications including enzymatic hydrolysis.

Right now, one third of U.S. sorghum is used as for bioethanol production, leaving more than 450 kilo tons of byproducts (e.g., DDGS) annually. DDGS is a premium protein source (> 30%) that can be potentially modified into value-added goods such as peptide antioxidants. The direction of research interest goes into the development of peptide antioxidant from sorghum protein, and further, adding an additional revenue stream to sorghum ethanol industry by utilizing the protein-rich sorghum by-products.

This work is aimed to provide a workable processing procedure as well as optimized reaction parameters in production of peptide antioxidants from sorghum proteins, which will guide the transformation of protein-rich sorghum bioethanol industry by-products into value-added products as a long-term goal.

2. Sorghum

2.1. Background of sorghum

Sorghum (*Sorghum bicolor* L. Moench) is one of the oldest known ancient grains (Mahasukhonthachat el al., 2010), which appeared to be domesticated in Ethiopia about 5000 years ago (FAO, 1995). Sorghum is a member of the subfamily Panicoideae that is belonged to the family Poaceae. As the fifth leading cereal crop in total world production, sorghum is succeeded only by corn, wheat, rice and barley (Table 1-1). Sorghum shares many similarities to other cereals especially maize and millets although sorghum has several unique attributes in

composition and structure (Bean et al., 2016). Different varieties of sorghum such as grain sorghum, forage sorghum, biomass sorghum, and sweet sorghum were available for different uses.

2.1.1. Sorghum production and cultivation area

In the World Agriculture Production Report released in May 2018 by Office of Global Analysis, United States Department of Agriculture, the global production of sorghum from 2017 to 2018 is 57.43 million metric tons (MMT) and is projected to increase by 1.79% (1.03 MMT) in 2019. The current cultivation area takes up to 39.93 million hectares and yields a production of 1.44 metric tons per hectare. Meanwhile, the regional production of sorghum varies. The top five producing countries of sorghum in 2017/2018 (Table 1-2) are United States, Nigeria, Mexico, India and Ethiopia. Together these countries accounted for more than half of the global production of sorghum. In total, over 80 percent of the cultivation area of sorghum is in developing countries (FAO, 1995). In Asia, sorghum is extensively cultivated in India, China, Yemen, Pakistan and Thailand. At the same time, production of sorghum in Europe is limited to only a few areas in France, Italy, Spain and the southeastern countries. Other major countries contribute to the global sorghum production include South Americans countries such as Argentina and Brazil; African countries including Sudan, Burkina, Mali, Niger, and Cameroon; Asian countries including India and China (USDA, 2018).

In U.S., sorghum ranks the third largest cereal crop only behind wheat and corn (Table 1-3). U.S. is currently dominant in the global sorghum production and distribution, accounting for 20% of the world's supply and 70-80% of the world's exportation (Lee et al., 2011). Cultivation of sorghum in U.S. is spread throughout the Sorghum Belt, which runs from South Dakota to Southern Texas, primarily on dryland acres. The top five sorghum-producing states in 2017 were

Kansas, Texas, Colorado, Oklahoma, and South Dakota and the state of Kansas planted nearly half of the U.S. sorghum acreage. The acreage is increasing in non-traditional areas such as the Delta and Southeast regions (All about Sorghum, n.d.).

2.1.2. A sustainable crop

Compared to other cereals, sorghum is known as an environmental-friendly and biological-efficient crop due to a high biomass yield, efficient utilization of nutrients and water from soil, and short production cycle (Ragaee, Abdel-Aal, & Noaman, 2006). Sorghum is one of the most efficient crops in conversion of solar energy, whose energy output is higher than many other economical crops such as sugarcane, sugar beet, corn, and wheat (Dar, Dar, Kaur, & Phutela, 2017). Typically, sorghum is harvested after a growing cycle of 90 to 130 days and planted 1–2 times a year (FAO, 1995).

Sorghum possesses substantial tolerance to a lot of adverse growing factors such as heat, pests, drought, flood, hail, severe temperature fluctuations; besides, sorghum also withstands a wide range of soil pH, salinity, alkalinity, soil textures and fertility (Wu, Johnson, Bornman, Bennett, & Fang, 2017). Sorghum is believed to be the best option of crop in regions under hot and dry climatic conditions (Dar et al., 2017), it is also a good rescue option where the growing conditions are not successful for other cereal cultivars (e.g., maize). Therefore, sorghum plays an important role in global sustainable grain production and food security especially for the countries in tropical and semi-arid regions of the world (Anglani, 1994; Badi et al., 1990).

The adaption of sorghum to the dry climates is reflected in several aspects: a low transpiration ratio; large area of fibrous roots (twice that of maize) and up to 2.5m in length for moisture absorption; waxy coating of leaves and stalks preventing moisture evaporation; and ability of grains to remain dormant during drought (FAO, 1995). Sorghums can withstand

temperatures above 38 °C with a reduced yield when coupled with dry winds during pollination. Optimal growing temperatures are 24-27 °C when best yields are realized. The water requirements for sorghum vary within the range of 350-700 mm. Within many semi-arid areas, typical temperatures range from 20-38 °C with annual rainfall ranging from 300-750 mm. In U.S., hybrid grain sorghums are grown where annual rainfall ranges from 380-640 mm (FAO, 1995).

Because of its wide use and good adaption to a lot of growing conditions, sorghum is known as one of the indispensable crops for the survival of mankind (All about Sorghum, n.d.).

2.1.3. Sorghum varieties

Depending on the species and variety selected, sorghum can be grown as a grain, forage, biomass, or sweet crop for different purposes, which made it known as a versatile crop.

Grain sorghum is the major type of sorghum grown throughout Kansas, which is commonly applied for food, feed, and industrial applications for the use of its grain kernels. Forage sorghum is typically used for grazing pasture, hay production, silage and green-chop. It typically grows 8 – 15 feet tall and is the most popular variety for livestock feeds. Hybrids of biomass sorghum have been bred to yield non-grain biomass such as leave and stalks. Biomass sorghum is primarily used to produce biofuel and bioenergy and reaches a height of 20 feet. Sweet sorghum contains high levels of sugar in the stalk and have been predominantly grown for the extraction of juice to produce sugar syrup. Sweet sorghum used predominate the table sweetener in the U.S. Recently, it has been used to produce whiskey and rum type products, and for biofuel production (All about Sorghum, n.d.).

2.2. Current uses of sorghum

Sorghum is widely used as human food, animal feed, and biofuel all over the world (Figure 1-2). However, the use can vary widely among different regions. In some semiarid tropical areas, sorghum is a primary crop and a basic food staple for large numbers of population (Bean et al., 2016). In other areas, such as U.S. and western countries, sorghum is mainly used for animal feeds and bioenergy. Due to the unique chemical and physical properties of sorghum (e.g., low-digestibility of sorghum protein), novel applications such as biomaterials including biofilms and bioplastic have been exploited accordingly.

2.2.1. Human consumption

About 50% of total sorghum production is used for human consumption (Awika, 2011). More than 95% of the food use of sorghum occurs in Africa, Asia, and other semi-arid regions of the world (FAO, 1995). In these countries, sorghum is one of the most important cereal staples (Ragaee et al., 2006). In contrast, in western societies such as United States, Australia, Brazil, and European countries, sorghum is better known as an animal feed instead of a human food source. Due to the long history of using sorghum as an animal feed, customers in these regions considered sorghum as an inferior grain crop, which constrained its desirability and acceptability of inclusion in commercial food products (Anunciação et al., 2017). Other unpleasant organoleptic properties of sorghum such as color, aroma, mouthfeel, aftertaste and stomach-feel also limited its use as an ingredient for food products.

In recent years, the use of sorghum for human consumption in western countries has increased due to its functional properties and health-beneficial elements including multi-minerals, dietary fiber, vitamin E, phenolic content, bioactive compounds, and antioxidative components (Anunciação et al., 2017). It is worth noting that, sorghum is free from gluten.

Therefore, it has drawn keen interest to be considered as a substitute ingredient to gluten-containing cereals for the growing market of people suffering from celiac disease and other adverse gluten-related health concerns (Lee et al., 2011).

Sorghum grain and its flour has been used in a wide variety of traditional and non-traditional food products such as bakeries (e.g., cookies, bread, tortilla, pizza crust), porridge and couscous, opaque beer, extruded products, sugar syrup, and so on. Lately, the research of interest has focused on development of food and snack products using sorghum flour, alone or in blends, with acceptable to excellent attributes such as color, flavor and texture. Anunciação et al. (2017) compared sensorial acceptance and bioactive compound of extruded whole grain breakfast cereals made with sorghum and wheat. It was impressive to find that sorghum breakfast cereal had better sensory acceptance (70.6%) than wheat breakfast cereal (41.18%); and higher levels of bioactive compounds including 3-deoxyanthocyanidin content (100% higher), total phenolic compounds (98.2% higher) and antioxidant activity (87.9% higher). Bedolla, de Palacios, & Rooney (1983) found that tortillas prepared with un-pearled white sorghum were softer and darker than those prepared with white maize. Bangu, Mtebe, & Nzallawahe, (1994) studied the acceptability of stiff porridge made with different proportions of sorghum flour composite and concluded that up to 30% incorporation of sorghum did not produce a significant decrease in acceptability as evaluated by a panelist. The incorporation of sorghum flour may also improve the processing procedure. Choto, Morad, & Rooney (1985) replaced the yellow maize with whole white sorghum flour at ratios of 0%, 25%, 50% and 100% in making tortillas and observed a decreased cooking time when the percentage of whole white sorghum flour increased in the blend.

In general, sorghum is still underutilized as a human food source. Sorghum demand for direct human food use has been declining in India and Africa. In East and Southern Africa, sorghum fields have been largely replaced with maize as the staple in these regions. Maize requires even moisture distribution throughout growing season and cannot withstand drought stress. This has contributed to food insecurity in the regions where farmers rely on rainfall (Awika, 2011).

2.2.2. Animal feed

The use for animal feed has been a dynamic element in stimulation of global sorghum production and international trade since the early 1960s (FAO, 1995). This demand is heavily concentrated in the developed countries and other higher-income developing countries such as Latin America, where animal feeds accounts for over 80% of total use of sorghum (FAO, 1995). In U.S., one third of sorghum grain is used as cattle feed (Lee et al., 2011). Broilers, swine, aquaculture and other animals have also been found amenable to a diet contain some form of sorghum grain. Sorghum has a lower energy density and protein digestibility compared to maize. However, the steamed pelleted sorghum was found to have a higher total digestible energy (12.4 kJ/g) than similarly treated maize (10.7 kJ/g) in the study of Sklan, Prag, & Lupatsch (2004). The feed and fecal analysis indicated that sorghum carbohydrates are more digestible than those of maize or soybean meal at inclusion 25-50% of total feed weight. Red drum can utilize 50% of the available carbohydrates and 77% protein in pelleted 30% sorghum grain (McGoogan & Reigh, 1996).

The use of sorghum grain has also been incorporated to pet food markets. Carciofi, Sakomura, Kawauchi, & Vasconcellos (2010) studied several carbohydrate sources for dogs and discovered that sorghum had a better digestibility and higher amount of metabolizable energy for

dogs than several other commonly used feedstuffs such as wheat bran, maize germ and rice bran. Sorghum grain or flour was also combined to some cat foods as a source of carbohydrate, antioxidants, or as a binding agent to hold the rest of formula (Bean et al., 2016).

2.2.3. Biofuels production

Sorghum is a promising prospective bioenergy crop. Especially, sweet sorghum is regarded as the most promising feedstock source for renewable bioethanol production due to its many advantages including rich germplasm resource, high biomass yield, rapid growth, wide adaptability, rich sugar content in stalk which yields a relatively low production cost (Zhang, Xie, Li, Ge, & He, 2010). Sorghum also has a highly efficient photosynthetic pathway, which provides the most efficient use of land, water, nitrogen, sold nutrients, and energy source (Cifuentes, Bressani, & Rolz, 2014). In 2016, more than 20% of the U.S. sorghum was used for ethanol and biofuel production (All about Sorghum, n.d.).

The flow path of bioethanol production from sorghum is similar to that of corn. One of the two most critical steps in this process is liquefaction/saccharification by amylase and glucoamylase enzymes, which decomposes the polysaccharide into simple sugars; and fermentation with yeast, which converts sugars into ethanol, carbon dioxide and other minor products. The procedures in practice may differ determined by using the whole or different part of sorghum such as sorghum grain, sorghum stalk, bagasse and straw, sorghum juice, etc. The production from sorghum stalks include extraction of juice, distillation and dehydration of the ethanol. The production using grain sorghum involves grain cleaning and milling; gelatinization, liquefaction, and saccharification for glucose production; and ethanol distillation and dehydration. DDGS (dried distillers' grains with solubles) is an important by-product produced

from this process, which is a high-valued protein source. A flow chart showing the comprehensive processing scheme was drawn by Dar et al. (2017) (Figure 1-3).

The ethanol yield and net energy output in terms of hydrogen and methane from sorghum is significantly higher than other feedstocks such as sugarcane, sugar beet, corn, and wheat (Dar et al., 2017). The potential food versus fuel conflict from the diversion agricultural land for energy crop is compensated as sorghum meets the diverse requirements of food, fuels, and feeds (Dar et al., 2017). Meanwhile, advanced biotechnology and genetic engineering provided feasible techniques for the biofuel industry, for example, the creatures of genetically modified cultivars for efficient ethanol production as well as development of ethanol-tolerant yeast and bacteria strains (Dar et al., 2017).

2.2.4. Other uses

Some novel industry-based applications have been developed from sorghum as well as sorghum products.

The gelling and pasting properties of sorghum starch makes it an appropriate substrate to synthesize novel starch films and biomaterials for various applications. For example, in pharmaceutical industry, sorghum starch is used for production of capsules as a substitute for collagen or modified cellulose (Bean et al., 2016). Pregelatinized sorghum starch with satisfying compressibility and degradability was also utilized as a binding agent for tablets (Alebiowu & Itiola, 2001).

The unique attributes of sorghum protein including such as low digestibility limit its nutritional quality as human food or feed but contribute to other potential utilizations. Sorghum DDGS with a high protein content has been modified for many bio-industrial applications such as adhesives, resins, biofilms, bioplastic and other packaging materials (Wang et al., 2009).

These novel biomaterials have incomparable advantages as they are completely safe, biodegradable, reproducible, environmental-friendly, and edible if accidentally consumed by kids or pets (Bean et al., 2016).

2.3. Sorghum grain

2.3.1. Kernel structures

The grain structure and composition are governing its physiochemical attributes, end-use qualities, and potential applications. The sorghum grain or seed is a naked caryopsis which is generally spherical but varies in physical attributes including shape, size, color, and hardness determined by the genetic diversity (Bean et al., 2016). The thousand-kernel weight of sorghum grain was reported to vary from 30 to 80 grams, although the majority falls between 25 and 30 grams (FAO, 1995). Sorghum grain is composed of three main components, the pericarp (the outer layer), germ (the embryo), and endosperm (main nutrition storage tissue) (Bean et al., 2016). Testa separates the pericarp from the endosperm which may or may not be present.

The germ accounts for 5 to 10% of the grain and contains majority of all lipid, protein, vitamins, and minerals found in sorghum (Waniska & Rooney, 2000). The two major parts of the germ are embryonic axis and scutellum (FAO, 1995). The scutellum is a storage tissue that is rich in lipids, protein, enzymes and minerals. The pericarp composes 3 to 6% of the grain and is consisted of multiple layers including the epicarp, mesocarp, and endocarp, (Waniska & Rooney, 2000) with the outer layer covered with wax. Sorghum bran contained highest content of protein, lipid, ash, β-glucan, total and insoluble dietary fiber; and the lowest content of non-resistant and total starch contents (Moraes et al., 2015). Sorghum is the only cereal that has starch granule in the pericarp which is directly related to the thickness of pericarp (Bean et al., 2016).

The endosperm composes 84 to 90% of the grain and contains the aleurone layer and starchy endosperm which can be divided to three categories: the peripheral endosperm, the vitreous (corneous) endosperm, and opaque (floury) areas (Waniska & Rooney, 2000). The aleurone layer is a single layer of cells lies below the seed-coat or testa containing both protein and lipid bodies along with inclusion bodies and some hydrolyzing enzymes. The aleurone cells are rich in minerals, B-complex vitamins and oil. The peripheral endosperm lies below the aleurone layer and is dense layers of cells with high protein concentration and small starch granules. The less content of peripheral endosperm in waxy genotypes of sorghum was inferred to associate with its improved starch digestibility. The vitreous endosperm is tightly packed with protein bodies covered with continuous protein matrix. Starch granules were shown to have indentations on the surfaces due to the pression of protein bodies. Otherwise, the floury endosperm is loosely packed with a discontinuous protein matrix and round starch granules in the center of the kernel. The relative proportions of vitreous to floury endosperm is correlated to the endosperm type and overall grain hardness (Bean et al., 2016). The structure of vitreous and floury endosperm is shown in the Figure 1-1. Large grains with vitreous endosperm are usually preferred for human consumption. The endosperm color can be either yellow or white dependent on the presence of carotenoids; yellow endosperm with carotene and xanthophyll has an increased nutritive value (FAO, 1995).

The color of sorghum grain can be white, yellow, pink, red, orange, brown, or black, which is decided by the pericarp color and thickness, the endosperm color, the presence of testa layer, and can be influenced by biotic factors such as molds infection causes discoloration of grain (Bean et al., 2016).

2.3.2. Proximate composition

The compositional profile of sorghum is comparable to that of corn and millet. Anglani (1994) reported that, maize products, such as raw maize, nixtamal and masa contained lower protein and insoluble fiber and a higher fat content than the corresponding sorghum products.

Starch is the largest portion in the sorghum grain which accounts for around 75% weight. It is mainly deposited in the endosperm as a storage tissue of energy for reproduction and development. The ratio of amylose to amylopectin contributes to functional and nutritional properties of the flour and its products such as starch gelatinization and retrogradation properties, pasting properties, glycemic index, and so on. The amylose content of sorghum has been reported to vary from 0 to 50%, and majority has 20 to 30% amylose.

The total protein content of sorghum grain was reported to range from 7 to 15% according to agronomic conditions (Waniska & Rooney, 2000) which is lower than that of corn which is 17 to 20%. The level of oil and lipids in sorghum (2 to 4%) is slightly lower than corn (about 4.5%). Sorghum lipids are highly unsaturated, and the major lipid classes in sorghum is triacylglycerols which made up about 90% of total lipids. Almost all of lipid found in sorghum grain is in the germ. Triacylglycerols including most of the esterified fatty acids are used as a storage for energy and carbon source during seed germination and development. Linoleic acid and linoleic acids accounts for at least 76% of the total fatty acids (Ragaee et al., 2006) followed with palmitic acids, stearic acid, and linolenic acid. The fiber fraction of sorghum grain is primarily insoluble and 86% of total insoluble fiber is contained in the pericarp, which provides protection for the kernel (Waniska & Rooney, 2000). The major fiber components are cellulose and pentosane (Ragaee et al., 2006). Soluble fibers such as β-glucans and 4-hemicellulose are present in the corneous and floury endosperm. (Bean et al., 2016)

Sorghum was reported to be a good source of B vitamin families including thiamin, riboflavin, vitamin B6, biotin and niacin. However, the refining process causes loss of part or all B vitamins (Anglani, 1994). The mineral composition of sorghum is similar to that of millet (Hulse, Laing, & Pearson, 1980). The chief minerals present in sorghum grain include potassium, phosphorus, iron, zinc, copper, and magnesium whilst calcium content is low (Khalil, Sawaya, Safi, & Al-Mohammad, 1984; Bean et al., 2016). The presence of other components (e.g., phytate) as well as processing procedures such as milling, cooking, fermenting and germinating can impact the composition and bioavailability of minerals (Bean et al., 2016). Biotechnology has been identified to be a potential tool for breeding of sorghum lines with improved mineral content.

Sorghum contains a group of high molecular mass polyphenolic compounds called tannins, which are found in grains with a brown pericarp and pigmented testa (Obizoba, 1988) and are absent in white and colored sorghums without a pigmented testa (Anglani, 1994). Certain tannins known as condensed tannins are antinutritional factors, which can form complexes with proteins, alimentary tract proteases, minerals (e.g., iron), and B vitamins (e.g., thiamin, vitamin B6), which ultimately reduces the protein digestibility in the grain. Due to this property, manufactures of livestock feed prefer to use grains from white sorghums or low-tannin pigmented sorghums (FAO, 1995). On the other hand, high-tannin varieties possess bird and insect resistance and higher malting potential than white grain varieties. It was confirmed that there was a strong correlation between tannins and phenolic compounds with antioxidant activity (Moraes et al., 2015; Awika et al., 2003).

2.3.3. Nutritional qualities

A lot of studies have revealed the nutritional qualities of sorghum is comparable or superior to many other major cereals (Ragaee et al., 2006) with a wide spectrum of functional and health promoting ingredients such as dietary fiber, resistant starch, minerals, unsaturated fatty acids, phenolic compounds, antioxidant substances and so on (Awika & Rooney, 2004; Mahasukhonthachat el al., 2010; Lee et al., 2011). There are demographic evidences suggesting that, populations in developing countries consuming more frequently whole grains, particularly sorghum and millet, have lowered incidence of cancer and other chronic diseases (e.g., cardiovascular disease) than populations primarily consuming refined grains like wheat (Van Rensburg, 1981).

Protein plays a key role in the nutritional quality of sorghum for both human foods and animal feed (Bean et al., 2011). Like other cereal proteins, the protein quality of sorghum is considered poor due to low content of essential amino acids such as lysine, tryptophan, and threonine (Badi et al., 1990). Particularly, the lysine amino acid score of sorghum is the lowest among several commonly used cereal grains (Table 1-4) (WHO, 1985).

The content of resistant starch in sorghum (1.8%) is higher than wheat flours and other cereal whole grains (Ragaee et al., 2006), which is associated with a healthier glycemic response in digestive tract. Sorghum lipids were proven to be associated with various health-beneficial potentials such as regulating gastrointestinal health, lowering risks of cardiovascular diseases and cancer and so on (Lee et al., 2011). Besides, sorghum contains elevated phenolic content compared to many other cereals including wheat, rice, and maize. The presence of phenolic compounds, proanthocyanidins, and other bioactive phytochemicals provides a cholesterol-

lowering effect, blood thinning ability, red blood cell protection and potentials in obesity treatment (Awika & Rooney, 2004).

Cooking of sorghum may reduce the insoluble fiber due to the loss of pericarp tissue (Serna-Saldivar et al., 1988). However, the soluble fiber content was increased after cooking attributed to the physical disruption of the cell walls released soluble polysaccharides such as pectin and gums.

2.4. Sorghum protein

Sorghum proteins possessed special structures and unique properties that determined the nutritional quality as well as potential applications of sorghum products.

Endosperm contained the highest proportion (80-85%) of protein followed with the germ (9.4-16%) and the pericarp (3-6.5%) (Bean et al., 2016). Based on the theory of Osborne and Voorhees, sorghum proteins are categorized into fractions as albumins, globulins, prolamins (known as kafirins for sorghum), and glutelins, according to their solubilities in different solvent extracting systems. Despite the overlap among the four classes, the solubility-based method provided a useful tool for characterization of cereal proteins. Addition of reducing agents such as sodium dodecyl sulfate (SDS) allowed further refinement in the isolation of protein fractions exhibiting more specific properties within given protein classes (Bean et al., 2011).

In previous studies, sorghum proteins have been proven to be biologically active *in vitro* and *in vivo*, including angiotensin-converting enzyme (ACE) inhibitory activity (Wu et al., 2016); antioxidant activity disclosed by DPPH, ABTS, Fe^{2+} chelating, oxygen radical absorbance capacity (ORAC), and reducing power assays (Agrawal, Joshi, & Gupta, 2017; Moraes et al.,2015); positive influence in hyperlipidemic rats (Ortíz-Cruz et al., 2015); and impact on

reduction of intracellular reactive oxygen species (ROS) in cultured THP-1 human cells (Sullivan et al., 2018).

2.4.1. Kafirin

Kafirin is the main storage protein of sorghum localized almost exclusively within the kernel endosperm in the forms of protein bodies and function as a storage agent of nitrogen and amino acids for plant germination and reproduction (Bean et al., 2011). Kafirin represents roughly 48% to over 70% of the total protein on a whole sorghum grain basis (Bean et al., 2011). It is a prolamin typically obtained by isolating and purifying the alcoholic extractant during sorghum protein fractionation. Due to the high proportion of nonpolar amino acid residues and high hydrophobic amino acid content, kafirin possess many unique properties including hydrophobicity and indigestibility, which has led to special applications such as edible biofilms for encapsulation (Sullivan, Pangloli, & Dia, 2018) and fruit and nut coating (Taylor, Taylor, Dutton, & de Kock, 2005). Kafirin contains a low level of lysine, and is rich in proline and glutamine, which contribute to over 30% of the total amino acids (Anglani, 1994; Bean et al., 2016). The amino acid composition of kafirins revealed a higher hydrophobic amino acid content such as alanine, isoleucine, leucine, tyrosine and phenylalanine than sorghum flour extract (Ortíz-Cruz et al., 2015).

Three kafirin subclass proteins were named α-, β-, and γ-kafirin based on the existing zein prolamin nomenclature system, considering the similarities regarding molecular weight, solubility, and structural characteristics that were confirmed by SDS-PAGE and immunological cross-reactivity (Shull, Watterson, & Kirleis, 1991; Bean et al., 2016) A fourth kafirin subclass protein known as δ-kafirin was identified by using homology similarities of DNA sequences to δ-zein (Bean et al., 2016). α-kafirin is the major subclass and make up 70 to 80% of total kafirin.

At gene level, α-kafirin was characterized to have two bands of M_w at 19 kDa and 22 kDa. β-kafirin were identified to have three components with molecular weight at 16 kDa, 18 kDa, and 20 kDa (Shull et al., 1991). γ-kafirin is the most hydrophobic kafirin and composed of fractions with molecular weight at 20 kDa and 49 kDa, which comprises 9 to 12% of vitreous endosperm and 19 to 21% of opaque endosperm (Bean et al., 2016). Proteins in the interior of the protein bodies are predominantly α-kafirin, and the out-layers contain more β- and γ-kafirins, which may form a cross-linked shell around the α-kafirins (Bean et al., 2016). δ-kafirin is a minor protein component that comprises less than 1% of total storage protein in mature sorghum grain (Bean et al., 2016).

Factors determining the characteristic of sorghum protein include physical grain structure, protein body structure, protein cross-linking, starch properties, and phenolic content/composition of the grain (Bean et al., 2011). Wang, Tilley, Bean, Sun, & Wang (2009) studied structural characteristics and physical thermal properties of kafirin, and found that the bands of α-1, α-2, and β-kafirins were present in all kafirin samples extracted by different methods. Moreover, α-helix dominated in all samples and only a small portion of β-sheet present as revealed by FTIR and Electrophoresis. The authors also revealed that glass transition peaks of the extracts were approximately 230 °C, and kafirin degraded at 270−290 °C as shown by a DSC meter.

2.4.2. Non-kafirin protein

The albumins (water-soluble fractions) and globulins (saline-soluble fraction) are found primarily in greatest abundance in outer layers of the grain and the germ, which comprises 10 to over 30% of total protein content (Bean et al., 2016; Bean et al., 2011) The molecular weight range of these proteins were found between 14 and 67 kDa. The albumin and globulin fractions

contain significantly higher levels of lysine and tryptophan than the prolamins and were believed to offer a more desirable nutritional balance of amino acids (Bean et al., 2011; Bean et al., 2016) Some recent studies revealed that albumin and globulin contain heat-shock proteins, chaperones, enzymes, and proteins involved with metabolic processes of the maturing plant and plant defense compounds (Bean et al., 2016).

Glutelin (acid or alkali fraction) was reported to contain 25% to 50% of the total protein. The molecular weight ranges were shown to be 20 to 67 kDa by SDS-PAGE (Bean et al., 2011). The glutelin protein of sorghum is a structural element within the matrix of the peripheral and inner endosperm of the sorghum kernel as well as a possible source of heat shock proteins, chaperones, luminal binding proteins, enzymes, vicilin- and legumin-like storage proteins (Bean et al., 2016; Bean et al., 2011). The endosperm matrix proteins provide a connecting structure of proteins bodies to starch granules, which is the second most important component of endosperm.

2.4.3. By-products proteins

More than 20% of the U.S. sorghum is used for ethanol production (All about Sorghum, n.d.), leaving more than 450 kilotons of distillers dried grains with solubles (DDGS) as a major co-product (Wang et al., 2009). DDGS is generated during the starch-based ethanol production, and the material remaining after fermentation and distillation are combined and dried to be known as DDGS (Bean et al., 2016). Sorghum DDGS typically contains less fat and higher protein content than corn DDGS (Dar et al., 2017), which is a premium protein source that is currently discarded or used as a high-protein, non-animal-based livestock feed supplement.

In order to harvest the maximum potential of sorghum, concept with zero wastage urged the development of value-added products from these co-products. Sorghum DDGS has been modified for many bio-industrial applications such as adhesives, resins, biofilms, bioplastic and

other biomaterials (Wang et al., 2009). The utilization of by-products and co-products and transformation into high-valued goods will be an important factor boosting the overall economics of biofuel production

2.4.4. Protein qualities

Overall, the protein quality of sorghum is considered low to poor due to the low content of essential amino acids (Badi et al., 1990). The protein quality of sorghum grain is associated with different protein fractions. Glutenins contained higher quantities of the essential amino acids, such as lysine and tryptophan, while prolamins contained the lowest quantities of lysine and the highest quantity of non-essential amino acids such as proline and glutamine (Anglani, 1994). Serna-Saldivar et al. (1988) found that decortication of sorghum grain decreased the quantity of protein and essential amino acids such as lysine, arginine, isoleucine and leucine. Malting can improve the protein quality of cereals in regard of an increased amount of lysine (Dalby & Tsai, 1976).

As reported by FAO, the digestibility of sorghum (46%) is significantly lower than other cereals such as wheat (81%), maize (73%) and rice (66%). Cooking substantially reduces the digestibility of prolamins such as α- and β- kafirins due to the formation of disulfide bonds while the presence of reducing agents restores the protein digestibility close to raw protein (Vivas, Waniska, & Rooney, 1987; Oria, Hamaker, & Shull, 1995; Bean et al., 2016). As suggested by Badi et al. (1990), the brown bread made up with more than 10% of sorghum bran is not suitable for infants as it affects the digestibility in a negative way. This unique property of sorghum proteins has been utilized to develop edible bioplastic applications such as protein films, micro-encapsulation agents and adhesives (Bean et al., 2016). Sorghum lines with improved cooked and uncooked protein digestibility has been recently discovered. A mutation in signal peptide of

one α-kafirin gene was reported to be responsible for the misshaped protein bodies and consequent improved digestibility (Bean et al., 2016).

3. Antioxidant potentials of sorghum

3.1. Antioxidants

3.1.1. Antioxidants in food system

In food industry, processing operations such as grinding, mixing, homogenizing, and comminuting could incorporate molecular oxygen into food components (Tang et al., 2012). Other processing techniques also introduce oxidation stress, for example, thermal treatment releases protein-bound transition metals, lightening exposure leads to generation of singlet oxygen. Some natural antioxidants and/or endogenous antioxidant systems could be removed or destructed during processing such as thermal inactivation and separation of oil refining.

Once the oxidant-antioxidant balance is destabilized, oxidative reaction will be initiated and lead to the alteration and failure of quality attributes and nutritional profile, and formation of toxic products (Tang & Zhuang, 2014; Wang, Zhao, Zhao, & Jiang, 2007). The oxidation of lipids results in the decomposition of fatty acid and formation of lipid hydroperoxides that can eventually decompose to low M_w carbonyls associated with potential rancid aroma, discoloration, and altered texture (Elias et al., 2008). Protein oxidation in food products often lead to alterations in protein functionalities such as gelation, emulsification, viscosity, solubility, and oil/water-holding capacity that will impose adverse effects to food quality (Tang et al., 2012). The oxidation of foods also generates toxic products that can potentially harm human health by consuming those food products (Chen, Chi, Zhao, & Lv, 2012; Kim, Je, & Kim, 2007).

To protect the susceptible food components from oxidative deterioration and to prevent oxidation-associated qualities failures and health-challenging diseases, antioxidants have been

developed to improve the oxidative stability of foods. Antioxidants can be added to a wide variety of food products such as oil, fat, meat, fish, baked goods, fried products, snacks foods, meat products, salad dressings, breakfast cereals, confectionery, dairy products, condiments and sauces, beverages, and many other livestock feeds and pet food. Antioxidants can be used directly as additives or indirectly through diffusion from packaging materials (Sila & Bougatef, 2016). The antioxidants used for food products or food processing must be inexpensive, nontoxic, effective at low concentrations, capable of surviving processing procedures, stable in the finished products and compatible with other food ingredients, and devoid of undesirable color, flavor, and odor (Sila & Bougatef, 2016).

Some chemically synthetic antioxidants include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ), ethylenediaminetetraacetic acid (EDTA), citric acid, parabens, propyl gallate have been widely used in food industry during processing and storage of food products (Wang et al., 2007). Others naturally existed compounds such as ascorbic acid (vitamin C), vitamin A, tocopherols especially vitamin E, flavonoids particularly quercetin, and polyphenols were also found to be antioxidant and can be chemically synthesized. Synthetic antioxidants are cheap to produce at industrial scale and are effective at a low dosage. However, due to the potential risks to human health such as induction of DNA damage and toxicity (Ito et al., 1986; Sohaib et al, 2017), the use of synthetic antioxidants has been restricted or prohibited.

Ingredients derived from various natural herbal plants such as green tea, rosemary, and acerola were found to be antioxidative as well as antimicrobial due to the redox properties of constituent phenolic compounds, phytochemicals and other bioactive components. Rosemary (*Rosmarinus officinalis L.*) extract is a well-known antioxidant on account of its phenolic

diterpenes, carnosic acid, and carnosol, that terminates the cycle of free-radical chain reactions by hydrogen donation and ROS scavenging (Schilling et al., 2018). Rosemary extract was found to exhibit greater inhibition on lipid oxidation in fresh pork sausage at 2500 ppm compared to BHA and BHT at 200 ppm (Sebranek, Sewalt, Robbins, & Houser, 2005). There are some commercialized antioxidants using rosemary extract as a major effective component (e.g., Herbalox® Rosemary Extract, Kalsec). Green tea (*Camellia sinensis L.*) extract is another commonly used natural antioxidant with its catechin gallates, particularly, its main polyphenolic constituent epigallocatechin gallate, as major functional components (Schilling et al., 2018). The mechanisms of its activities have been attributed to the chelation of free iron ions released from meat hemoproteins during processing and storage as well as quenching activities of superoxide, hydroxyl, and peroxyl free radicals. These naturally-sourced antioxidants avoid "chemical sounding" names are label-clean and consumer-friendly. The addition of these ingredients could enhance the sensory properties of certain food products or may cause annoyance in others. On the other hand, the utilization of these naturally derived antioxidants can be cost-cutting as they are much more expensive due to the inefficient extraction process, and the extremely low yield. Besides, the active components of these extracts are enormously complex, and the nature of the action mechanisms is not completely understood, which made industrial synthesis less feasible.

3.1.2. Antioxidants and human health

In healthy biological systems, the prooxidants-antioxidants balance is normally under control. For example, free radicals are continuously generated in human body through metabolic activities and exert physiological actions such as sending signals (Tang & Zhuang, 2014; Jin et al., 2016), which can be quenched by radical scavenger or antioxidants (e.g., ascorbate and tocopherols) to reduce accumulations. Some endogenous antioxidants within biological systems

include uric acid, glutathione, enzymes can inactivate reactive oxygen species (e.g., catalase, superoxide dismutase, and glutathione peroxidase). Exogenous antioxidants such as vitamin A, vitamin C, and vitamin E can be absorbed to bodies through dietary intake (Elias et al., 2008). When there is an excessive accumulation of prooxidants such as oxygen reactive species, they could cause structural damages to biomolecules including DNA mutation, membrane phospholipids alteration, protein damages, etc. As a result, various pathological conditions and human diseases including aging, cancer, multiple sclerosis, inflammation, coronary heat, diabetes, arthritis, atherosclerosis, neurodegenerative disorders, cardiovascular diseases, senile dementia will occur (Tang & Zhuang, 2014; Wang et al., 2007; Elias et al., 2008). Therefore, it is important to have balanced antioxidants in human diet in assisting body to diminish oxidative damages (López- Barrios et al., 2014).

Dietary antioxidants can be obtained from bio-tissues from foods or pharmaceutical products (e.g., GNC® grapeseed extract). A lot of plants contain various types of natural antioxidants including polyphenols, vitamin A, vitamin C, vitamin E, especially those green-leaf vegetables and fruits. Some synthetic antioxidants such as BHT and BHA were also used in biological systems (Tang & Zhuang, 2014). Natural antioxidants from plant or animal origins were more preferable as they may have potential beneficial effects to human body with little or no side effects (Jin et al., 2016).

Greater interest has been paid to develop novel antioxidants from natural sources that are efficient, cost-effective, and are generally recognized as safe at large dosage for long term use (Xia et al., 2012).

3.1.3. Peptide antioxidants

In general, all 20 amino acids can interact with free radicals with high energy (Samaranayaka & Li-Chan, 2011), but they are not solely used as antioxidants in food or biological systems. Peptides with unique structures and properties conferred by their amino acid sequences have higher antioxidant activities than free amino acids (Samaranayaka & Li-Chan, 2011). The antioxidant capacity of these peptides is affected by the amino acid composition, position, configuration, and peptide sequences (Chen et al., 1996).

Possible mechanisms of peptide antioxidants have been attributed to three main categories: chelation of transition metal ions, scavenging free radicals (e.g., singlet oxygen, hydroxyl radical, lipid peroxyl radical, reactive oxygen species, etc.), and forming a physical barrier at the oil–water interface to minimize the contact with prooxidative agents due to the surface amphiphilicity (Zhao et al., 2012; Kong & Xiong, 2006; Samaranayaka & Li-Chan, 2011; Xia et al., 2012; Jin et al., 2016; Sohaib et al., 2017; Elias et al., 2008).

Glutathione (γ-Glu-Cys-Gly), also known as GSH, is a naturally existing antioxidant peptides present in various plants, animals, and microorganisms (Sila & Bougatef, 2016). Carnosine (β-alanyl-L-histidine) and anserine (β-alanyl-N-methylhistidine) are both dipeptide antioxidant found in skeletal muscle. Endogenous synthesis or dietary intake of these peptides are important to biological tissues in preventing damages to cellular components caused by reactive oxygen species, lipid peroxidation, and myoglobin discoloration (Samaranayaka & Li-Chan, 2011; Elias et al., 2008). For example, blood proteins of plasma were estimated to provide 10-50% of the peroxyl radical trapping activity (Elias et al., 2008).

In addition to the naturally present peptide antioxidants, peptides from hydrolyzed dietary proteins also possess antioxidant activities. Milk containing trypsin was reported to be

antioxidative dated back to 1940s. In recent years, peptide antioxidants obtained from various food proteins have been extensively reported, especially those from animal proteins, nuts and pulses. Some literatures reported the antioxidant activities of cereal protein hydrolysates such as those obtained from wheat, rice, barley, and corn. Meantime, few studies were found to characterize the antioxidative profile of obtained from sorghum protein.

The purified fraction of antioxidative hydrolysates possess comparable or superior efficacy the commonly known antioxidants. Moure et al. (2006) reported that an ultrafiltrated fraction of hydrolysates from soy protein presented a similar antioxidant activity in emulsion to tea extracts at the same concentration (5 g/L), and synthetic antioxidants (BHA and BHT) at 10 times lower concentration (0.5 g/L). Li et al. (2008) isolated chickpea protein hydrolysates by Sephadex G-25 gel filtration, and the Fraction IV presented an antioxidant activity (81.13%) close to that of α-tocopherol (83.66%) and lower than that of BHT (99.71%) in the linoleic acid oxidation system. Xia et al. (2012) determined that several ultrafiltrated fractions of barley glutelin hydrolysate possessed a comparable DPPH and superoxide radical scavenging activity to BHT, a very close metal chelating ability to EDTA, and a higher reducing power than ascorbic acid.

Besides, these peptide antioxidants may also serve as functional ingredients when incorporated to food products such as texture enhancers, emulsifiers, biding agents, and so on (Tang et al., 2012) due to the surface properties of proteins and peptides.

3.2. Antioxidant potential of sorghum

3.2.1. Bio-active compounds of sorghum as potential antioxidants

There are a broad array of natural phytochemicals and bioactive compounds present in sorghum grain. Especially phenolics, carotenoids, and tocopherols are natural antioxidants can

exert health-beneficial actions and prevention of chronic and degenerative diseases as a healthy diet. Some phytochemicals also provide many other biological activities such as enzyme inhibitory, antimicrobial, anti-inflammation, anticancer and so on.

Dietary phytochemicals with antioxidant properties in sorghum are minerals, trace elements, vitamins, folates, carotenoids, betaine, tocotrienols, tocopherols, oryzanols; and other minor components carotenoids, polyphenols, alkylresorcinols, betaine, choline, sulfur amino acids, phytic acids, lignans, avenanthramides are primarily located in the bran, pericarp, and germ fractions. Some of the bioactive compounds concentrations were quantified by Shen et al. (2013) (Table 1-5). These water- and fat-soluble dietary antioxidants contribute unevenly to the antioxidant profile of sorghum and provide protection through the entire digestive tract, where the total antioxidant potentials may be improved by *in vivo* GI digestion. Mechanisms of these antioxidant capacities include but not limited to: prevention of oxidation of polyunsaturated lipids (vitamin E); reduction of the concentration of plasma homocysteine (vitamin B9, betaine, choline); acting as cofactor of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and thioredoxine (Zn, Fe, Se, Cu, Mn); or stabilization and delocalization of unpaired electrons (vitamin E, polyphenols, alkylresorcinols). Phenolic acids (ρ-coumaric acid, ferulic, syringic, sinapic and vanillic) are believed to chelate transition metals as well as activate or repress particular genes. Sulfur-containing amino acids (cysteine and methionine) contribute to the synthesis of a major endogenous antioxidant glutathione (Masisi et al., 2016).

Phenolic compounds include derivatives (benzoic and cinnamic acids, anthocyanidins, quinines, flavanols, chalcones, flavones, flavanones, and amino phenolic compounds) are a group of natural antioxidants present in many vegetables, fruits and seeds. It was suggested to consume up to 1.0 g of daily intake of phenolic compounds from a diet to support health-

beneficial implications in human and prevent many diseases including cancer, cardiovascular diseases, mutagenesis, atherosclerosis, etc. (Duh, Tu, & Yen, 1999; Anunciação et al., 2017). Sorghum possesses exceptional higher levels of phenolic compounds compared to other widely consumed cereals such as wheat, rice, barley, millet, etc. (Ragaee et al., 2006). Polyphenols are believed to be the main contributor in antioxidant properties of sorghum (Wu et al., 2017). They were thought to act as direct free radical scavengers, cofactors of antioxidant enzymes or as indirect antioxidants to provide oxidative stress reducing and to provide antioxidative, anti-inflammatory and anticarcinogenic effects (Masisi et al., 2016; Wu et al., 2017). A wide array of phenolic compounds and its derivatives were found in sorghum such as phenolic acids, flavonoids, condensed tannins. Sorghum genotypes with pigmented testa have been found to have the highest phenolic content (Awika & Rooney, 2004). The profile of phenolic content is related to the pericarp color, pericarp thickness, and presence of testa (Wu et al., 2017) as most of phenolic compounds are present in the out-layers. This significant amount of phenolic content is significantly beneficial to health effects in the human body after liberation by colonic bacteria and enzymes from the intestinal wall, making sorghum a competitive substitute to other cereals.

Nevertheless, the content of these bioactive compounds is extremely low (< 0.1%), which makes it difficult or infeasible to extract for commercial use. Therefore, the research of interested turned to the macronutrients (e.g., proteins) for bioactive potentials.

3.2.2. Protein and peptides of sorghum as potential antioxidants

Proteins are a group of important macronutrients in sorghum which has been proven to be bioactive *in vivo* and *in vitro*.

Moraes et al. (2015) indicated both whole sorghum flour and decorticated sorghum flour exhibited antioxidant activity as revealed by DPPH%, ABTS%, ORAC, and reducing power

assays. Agrawal, Joshi, & Gupta (2017) identified 7 antioxidant peptides from the hydrolysates of green tender sorghum protein prepared with Alcalase after fractionation by ultrafiltration and gel filtration. The antioxidant capacity was confirmed through multiple assays.

Ortíz-Cruz et al. (2015) observed that the hyperlipidemic rats fed with sorghum kafirin as a diet supplement have improved lipid metabolism and increased serum antioxidant potential (67%), especially in rats fed with added cholesterol, which was due to the release of bioactive peptides generated from kafirin *in vivo* hydrolysis. Apparently, this finding supports the use of sorghum kafirin for prevention of atherosclerosis and other chronic diseases (Ortíz-Cruz et al., 2015).

Sullivan et al. (2018) reported that, THP-1 human cells treated with kafirin at concentration of 100 μg/mL possessed anti-inflammatory property, which was linked to its potential to reduce the production of intracellular ROS, as measured by a reduced production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α by 28.3%, 74.0%, and 81.4%, respectively.

Both Vasudeva, Sajeeda, Chandrashekar, & Rajini (2006) and Wu et al. (2016) revealed that kafirin hydrolysates possessed angiotensin converting enzyme (ACE) inhibitory activity, which can be potentially developed to functional food ingredients or pharmaceuticals for prevention of hypertension (Wu et al., 2016).

Based on the results of these *in vivo* and *in vitro* studies, it is anticipated that kafirin is a potential protein source for production of antioxidant peptides. Upon hydrolysis with proper enzymes, the solubility of kafirin could be increased and peptide sequences with antioxidant potentials will be exposed, therefore, an extended application of sorghum protein and sorghum protein-rich products could be developed.

4. Production, fractionation, and identification of antioxidant peptides

Typical steps in producing bioactive proteins and peptides involves food source selection, protein extraction, hydrolysis or other modification of protein, fractionation and purification of resulting hydrolysates, and final identification of peptides. Further modification and design of novel peptides ground on the identified sequences are also concerned research topics. A schematic diagram exemplifying the processing steps of producing bioactive peptides from proteins was drawn by Udenigwe and Aluko (2012) (Figure 1-4). Methods and treatment for production of antioxidant peptides as well as identified peptide sequences in reviewed literatures were summarized in Table 1-6. Due to the different assays employed and variations of the same assay, it is difficult to compare the antioxidant potency of identified sequences among literatures, thus, the published results were not included.

4.1. Protein isolation and extraction

Fractional extraction is one of the oldest techniques that was broadly used in cereal chemistry for protein extraction (Ewart, 1968). The extraction methods, solvent, substrate, and extraction conditions greatly affect the yield, purity and properties of the outcome proteins (Wang et al., 2009). For example, the saline extraction is affected by the pH and molarity of the solution; the extraction of prolamin could be affected by operational temperature and the type of the alcohol used. Other impacting factors include fineness of flour, type and concentration of solvent, solvent to substrate ratio, and so on. Besides, the addition of reducing agents such as SDS assists the extraction process by breaking down the S-S bonds. Literatures indicated that the presence of lipids caused considerable interference to the subsequent fractionations attempts. Thus, lipids were typically removed by using butanol, hexane, or other fat-soluble organic solvent (Coates & Simmonds, 1961). At the same time, some protein fraction might be reduced if

defatted before extraction. One of the main drawbacks of this extraction method is the presence of overlap among different fractions.

Ewart (1968) proposed practical methods for the extraction of four categories of proteins from several cereal flours. The flours were extracted three times with NaCl solution (0.04 M), three times with water, three times with ethanol-water (70:30 w/w), and once with acetic acid (0.1 N). At each stage, extractant were mixed with substrate on a magnetic stirrer for 1 to 2.5 hours, and then the extractant along with the soluble part of the protein were separated by centrifugation. The extracted protein could be recovered by distilling the solvent (alcoholic extractant) or dialysis against water (saline extractant) followed with freeze-drying. As a result, the saline extraction mainly removed the albumins and globulins, which can be further separated by changing the solution salinity. The prolamins appeared mostly in the alcoholic extracts, and the acid-soluble fraction was correlated with glutenin.

Kafirin can be extracted from sorghum flour or by-products of sorghum grain processing like DDGS and bran (Taylor et al., 2005). Effective extraction of kafirin can be achieved by using aqueous alcohols at elevated or ambient temperature, or, extracting total grain proteins in basic buffer containing SDS followed by precipitation of non-kafirin proteins with aqueous *tert*-butanol. Ortíz-Cruz et al. (2015) extracted kafirin by mixing defatted sorghum flour with 5 volumes of 60% *tert*-butanol/water at 37 °C and vigorously stirred for 6 hours. The dried kafirin obtained from the supernatant portion after centrifugation had a protein content of 40%. There are potential problems of this processing method in industrial production and food use application such as inflammability of aqueous ethanol at high temperature and the potential harm of some organic solvents including *tert*-butanol.

Taylor et al. (2005) indicated that glacial acetic acid is a very effective ambient temperature solvent for kafirin extraction appears due to its low dielectric constant which enables it to dissolve hydrophobic kafirin proteins.

Wang et al. (2009) compared different methods of kafirin extraction from sorghum DDGS including using acetic acid, HCl−ethanol and NaOH−ethanol under reducing conditions. It was found that kafirin extracted using acetic acid possessed highest purity as 98.9%, and second highest yield as 44.2%. As the authors described, sorghum DDGS was milled through a screen and then presoaked in four volumes of 0.5% (w/w) sodium metabisulfite ($Na_2S_2O_5$) for 16 hours in an incubator shaker. After removal of $Na_2S_2O_5$ solution by sieve filtration combined with vacuum, the precipitate was mixed with five volumes of glacial acetic acid and stirred for 1 hour. After centrifuging the mixture, the supernatant was collected and slowly adjusted to pH of 5.0 in an ice water bath. After keeping the mixture overnight at 4 °C, the precipitate was collected and rinsed three times with distilled water. The obtained protein was freeze-dried and defatted three times by mixing with 5-fold weight of petroleum ether. In addition, the authors characterized the chemical composition and structural characteristics of extracted kafirins and found that kafirin extracted with acetic acid and HCl−ethanol contain more large M_w protein than NaOH−ethanol method as revealed by size exclusion chromatography, and γ- kafirins were found only in NaOH−ethanol extracted kafirin from HPLC.

Since the acetic acid-based method resulted in favorable yield and protein purity, it was adopted as the extraction methods for future studies.

4.2. Production of antioxidative peptides

Typically, the hydrolyzed protein possesses superior functional properties and antioxidant activities than the original protein. Proteins are typically large organic matrix with complex

three-dimension structures with M_w varying from several thousands to millions. Intact proteins had minimal bioactivity because the critical structures responsible for the antioxidant activities (e.g., amino acid sequence, functional groups and structural domains) are buried inside the hydrophobic core and are inaccessible to prooxidants. After undergoing certain degree of hydrolysis, critical structures and sequences can be exposed and released, which ultimately increase the bioactivities of proteins. It was also reported that hydrolysis developed hydrophobicity due to the exposure of hydrophobic groups in protein chains. Therefore, the increased solubility of peptides in lipid agents increased the availability of antioxidants to targets (Xia et al., 2012). The hydrolysis also facilitates the dissolution of proteins and increases overall adsorption to target molecules caused by protein unfolding (Suetsuna & Chen, 2002), which results in enhanced antioxidant activities. The functional features such as emulsifying and foaming capacities could be improved in this process, and the nutritional profiles may also be upgraded through an increased digestibility and diminished allergenic agents (Sarmadi & Ismail, 2010).

Nevertheless, extensive hydrolysis could adversely affect functional properties and activities of peptides due to the excessive amount of free amino acids and loss of essential construction of amino acid sequences. Thus, the process of hydrolysis is preferred to be handled under practicable control to produce consistent peptides with desirable properties.

4.2.1. Enzymatic hydrolysis

There are multiple ways to produce biologically active peptides from the precursor proteins including enzymatic hydrolysis, microbial fermentation, and other processing such as thermal treatment (Sarmadi & Ismail, 2010). Hydrolysis with strong acid is hard to control the

reaction conditions and degree of hydrolysis, while fermentation is considered less efficient (Jin et al., 2016).

Nowadays, hydrolysis using industrial food-grade enzymes has become a predominant approach in producing bioactive hydrolysates and peptides. Compared to other proteolysis methods, hydrolysis using enzymes is a mild process which was generally conducted at moderate temperature and neutral pH. Enzymes cleave peptide bonds and release various sizes of peptides and amino acids with minimized side reactions and rare irreversible damages to the hydrolysates (Jin et al., 2016). Most importantly, the specificity of enzymes enables production of hydrolysates with high selectivity and unique property. As a result, bioactive peptides with special functions and desired features can be produced and screened. Enzymes usually required less energy and produced a more efficient yield compare to other methods. Also, there are less by-products and pollution generated.

A wide range of commercial enzymes has been used for the hydrolysis of proteins from different sources. Industrial proteases such as Alcalase, Flavourzyme, Neutrase, Protamex were derived from microorganisms. Others such as Papain (from papaya), Bromelain (from pineapple) and Ficin (from fig) were obtained from plant sources. Some animal originated enzymes such as for example, pepsin (in gastric juice) and trypsin (in pancreatic juice). Exogenous enzymes are preferred in most cases of protein hydrolysis due to shorter reaction time to attain certain degree of hydrolysis (Samaranayaka & Li-Chan, 2011), and they tend to produce hydrolysates with more consistent M_w profiles and peptide compositions.

Enzyme specificity determines the selective cleavage spot therefore affects size, yield, composition, peptide sequences, and final properties of hydrolysates. Peñta-Ramos and Xiong (2002) hydrolyzed native and heated soy protein with six different types of enzymes and found

that the resulting hydrolysates all had different degrees of hydrolysis and antioxidant activities in a liposomal system. It is notable to understand that the selection of enzyme, reaction conditions such as temperature and pH, as well as the control of hydrolysis progress including reaction time and enzyme-to-substrate ratio are crucial in producing protein hydrolysates or peptides with desired functional and/or bioactive properties.

4.2.2. Fermentation

Fermentation is an old technique commonly used in a lot of Southeast Asian countries such as China, Japan and Korea for centuries to preserve foods and to improve their nutritional values. Fermentation could also be used as an alternative method to produce bioactive peptides by action of microbes and endogenous proteolytic enzyme and therefore increase the antioxidant potentials (Masisi et al., 2016). Antioxidative properties have been found in a wide variety of fermented products such as soybean, black bean, buckwheat, barley and rye (Liu, Chen, Shao, Wang & Zhan, 2017).

Fermentation was characterized as an extensive method of hydrolysis of protein into amino acids, peptides and ammonia resulting in the improved functionality, utilization and bioactivities of the products (Niu, Jiang & Pan, 2013; Liu et al., 2017). Hydrolysis using fermentation avoids using high cost of commercial enzyme and prevents potential bitterness of hydrolysates. It is believed that fermented foods possess improved nutraceutical values and extended storage stability; one of the possible contributors is the fragmentation of protein ingredients into bioactive peptides owing to the hydrolysis effect by microorganism-derived proteases (Sarmadi & Ismail, 2010).

Niu, Jiang & Pan (2013), and Liu et al. (2017) both studied the antioxidant activity of fermented defatted wheat germ (DWG) by Bacillus subtilis. They both found that the antioxidant

activity was positively correlated to the peptide content during the fermentation, which was especially significant in the early stage of fermentation (Liu et al., 2017). The both observed a significantly higher total phenolic content and antioxidant activity of fermented hydrolysate than non-fermented DWG. The degree of hydrolysis both increased with prolongation of the fermentation time, and more low M_w peptides related to antioxidant activities were produced.

4.2.3. *In vitro* GI digestion

In vitro gastrointestinal (GI) digestion simulated sequential gastric and intestinal digestion by digestive or microbial enzymes from gut microflora that releases antioxidative peptides directly in the gut (Möller, ScholzAhrens, Roos, & Schrezenmeir, 2008). Antioxidative oligopeptides, especially di- and tri-peptides could survive the digestion and permeate the epithelial cell membranes through the upper GI tract, and directly enter the blood circulation and reach the target sites via receptors or cell signaling (Samaranayaka & Li-Chan, 2011). Larger sizes of protein may also release bioactive peptides in GI tracks and upon degradation by GI enzymes or brush border peptidases (Samaranayaka & Li-Chan, 2011).

The activities of pre-formed antioxidative peptides can be decreased, improved or not altered during passage through the GI tract, depending on the type of enzymes used and hydrolysis conditions applied. Peptides isolated from zein hydrolysate by *in vitro* GI digestion exhibited excellent antioxidant activity in aqueous solutions and in food systems reported by Zhu, Chen, Tang, and Xiong (2008). Phongthai et al. (2018) used a pepsin-trypsin system to hydrolyze rice bran protein and the hydrolysates produced exhibited antioxidant activities revealed by ABTS radical scavenging activity, reducing power and metal chelating activity. In other cases, Sheih, Wu, and Fang (2009) reported that the antioxidative peptide VECYGPNRPQF isolated from microalgae was resistant to GI digestion. Toledo, de Mejia,

Sivaguru, and Amaya-Llano (2016) isolated peptides from bean proteins via hydrolysis with Alcalase and bromelain followed with *in vitro* GI digestion with pepsin-pancreatin. The resulting peptides were found to increase glucose stimulated insulin secretion and glucose uptake (67%), reduce oxygen species (70%) and inhibited lipid accumulation.

Therefore, the possibility of modification or breakdown of peptides during the GI-digestion is one of the most important factors to be considered when evaluate the potential antioxidative peptides for promotion of human health (Samaranayaka & Li-Chan, 2011).

4.2.4. Other modification methods

Food processing procedures such as heating, grinding, and extrusion can cause alteration and modification of proteins and impact their potential bioactivities. Thus, these modification methods have also been used to generate or modify bioactive proteins and their hydrolysates, such as alkali treatment, heating treatment (Tang et al., 2012), sonication (Liang et al., 2017), and extrusion (Zheng et al., 2006; Wang et al., 2014). Other techniques such as starch removal with amylase (Wang et al., 2014; Liu et al., 2015; Zheng et al., 2006) and defatting (Liu, Chen, Shao, Wang, & Zhan, 2017; Gu et al., 2015) were also applied as pretreatment or assisting procedures for better extraction or higher yield of bioactive proteins and peptides.

The dipeptide His-Pro (cyclo) is an antioxidative peptide that can be produced during thermal processing and was found to be present in various food products such as dried shrimp, fish sauce, tuna, ham, noodles, potted meat, non-dairy creamer, white bread, and nondairy creamer (Prasad, Hilton, Lohr, & Robertson, 1991; Sohaib et al., 2017). It is readily absorbed through the GI tract due to the simple structure and therefore leads to reduction of oxidative stress.

Zheng et al. (2006) produced antioxidant peptides from enzymatic hydrolysates of extruded and non-extruded corn gluten. They found that the hydrolysis of extruded corn gluten was obviously more efficient than the non-extruded, and after hydrolysis of 2 hours, degree of hydrolysis of extruded was higher than non-extruded corn gluten. The authors explained that the high temperature, pressure, and shear forces that protein underwent during extrusion altered the structures by weakening the hydrogen bonds, electrostatic and hydrophobic interaction, disulfide bonds, and other covalent bonds. Thus, extrusion provided a useful tool to pretreat the substrate proteins for more efficient enzymatic hydrolysis (Zheng et al., 2006).

Another technique used was low-frequency ultrasonication assisted enzymatic hydrolysis reported by Liang et al. (2017). Under the optimal conditions of ultrasound pretreatment, the authors observed that degree of hydrolysis, conversion rate of protein, and DPPH scavenging capacity of corn protein were significantly increased, which was due to the fact that ultrasound pretreatment enhanced the formation of short-chain peptides with M_w of 200–3000 Da, especially those containing hydrophobic amino acids.

Tang et al. (2012) treated the wheat peptides with heating and lipid oxidation by malandialdehyde, separately, and the antioxidative activities of the modified peptides were evaluated through *in vivo* and *in vitro* assays. They found that, with prolonged treatment time from 30min to 90min, peptides treated with heating and MDA lost their hydrophobicity and reducing power and exhibited a relatively lower DPPH radical scavenging (Figure 1-8). The authors also found that heating and MDA induces more reactive oxygen species *in vivo* hence indicated that these treatments could influence the functional properties and directly alter the structure of peptides.

4.3. Isolation and purification of antioxidative peptides

During hydrolysis, the molecular weight of protein was decreased through cleavage of peptide linkage and a wide variety of peptides with different chain length, M_w, and amino acid compositions were generated, depending on enzyme specificity and reaction conditions. The crude protein hydrolysate is a mixture of various lengths of peptides and free amino acids including both active and inactive antioxidants. After undergoing a series of fractionation and purification steps, the fraction with higher activities and more uniform compositions can be concentrated and isolated. The purification steps could also eliminate of other non-peptic interfering components such as phenolic compounds (Thamnarathip et al., 2016). The characterization of antioxidant peptides' amino acid composition, structure characteristics, and peptide sequences will provide vital information to understand the structure-activity relationship. The sequence identification of peptide antioxidants also makes it feasible to synthesize and amplify at an industrial level.

4.3.1. Membrane filtration

Molecular weight distribution is one of the most important properties in determination of the functional properties and bioactivity of hydrolysates. It is decisive to characterize the hydrolysates according to their different M_w ranges. Membrane filtration is one of the most commonly used technology for fractionation of protein based on M_w with advantages including fast, convenient, reusable, easily-handled, and cost-effective.

Molecular weight cut-off (MWCO) penetration is commonly performed in a stir-cell applied with nitrogen pressure to accelerate the penetration process across the membrane. Other filtration devices include centrifugal tubes coupled with M_w cut-off membranes. When the solution of protein hydrolysates passes through a series of membranes with different MWCO

sizes (e.g., 100, 50, 30, 20, 10, 5, 3, and 1 kDa), hydrolysate fractions with different ranges of M_w could be obtained. Analysis and comparison among the obtained fractions from filtration assist the selection of the fraction with desired M_w ranges as well as specific activities. Microfiltration separates sample with a M_w larger than 100 kDa while ultrafiltration can retain particles with a M_w larger than 1 kDa, and nanofiltration is suitable for sample with a M_w at 100-1000 Da. Low M_w membranes such as ultrafiltration and nanofiltrations are also used to desalt the sample, concentrate and purify the hydrolysates, and separate the peptides from the remaining reactant components such as undigested proteins and enzymes. Dialysis bag is a semipermeable membrane with porosity that allows the smaller-sized particles such as salt to penetrate through the bag and keeps the larger-sized molecules within the bag. The solution is stored within the bag and then sealed with clips. The bag filled with sample is put inside a large container against water. Desalting of sample with a higher salt content happens spontaneously. Typically, the whole process takes 1 to 2 days with water changed at least 2 to 3 times a day. In addition, an electro-membrane filtration process using ion-exchange membranes and ultrafiltration membranes to achieve successful separation called electrodialysis was developed and patented by Bazinet, Amiot, Poulin, Tremblay, and Labbé (2008).

In large scale production of protein hydrolysates, membrane filtration could be coupled with enzymatic hydrolysis in a continuous process to reduce the cost of inactivating enzymes by heat treatment and pH adjustment at the end of hydrolysis (Samaranayaka & Li-Chan, 2011).

Many studies indicated that hydrolysates with relatively low M_w generally possessed higher antioxidant potentials (Jin et al., 2016; Liu et al., 2015; Wang et al., 2014; Zhuang, Tang, Dong, Sun, & Liu, 2013; Agrawal et al., 2017; Tang & Zhuang, 2014; Alashi et al., 2014). It was proposed that the smaller-sized peptides are more accessible and easily adsorbed to the oxidant

agents compared to the larger peptides. However, other studies found that medium- or large sized hydrolysate possessed stronger activities (Phongthai et al., 2018; Wang et al., 2014; Zhang, Li, & Zhou, 2010; Park, Jung, Nam, Shahidi, & Kim, 2001).

Jin et al. (2016) and Wang et al. (2014) reported peptides size below 6 kDa has relatively higher radicals intervening activities in oxidizing process than other fractions. Moure et al. (2006) evaluated the antioxidant activities of soy protein concentrate hydrolysates and observed that fraction with M_w < 10 kDa fraction possessed the highest antioxidant activity in all considered assays than 30 – 50 kDa and > 50 kDa fractions.

Xia et al. (2012) found that large-sized peptides (M_w > 10 kDa fraction) of barley glutelin hydrolysate exhibited much stronger DPPH% and reducing power, whereas small-sized peptides (< 1 kDa fractions) demonstrated significantly greater hydroxyl scavenging and Fe^{2+} chelating ability. Zhang et al. (2010) identified that higher M_w >10 kDa fraction of soy protein hydrolysates exhibited better DPPH% and Fe^{2+} chelating ability, while lower M_w especially 1 – 3 kDa fraction possessed higher ORAC. Tentative reasons explained by the authors was that specific hydrophobic clusters of hydrolysates with bulky and aromatic side chains may act as hydrogen donors and as direct radical scavengers (Xia et al., 2012).

4.3.2. Gel filtration chromatography

The further isolation of hydrolysates mixture based on difference in molecular size can be achieved by gel filtration or size exclusion chromatography. The separation depends on different affinity to either stationary or mobile phase (Sila & Bougatef, 2016).

Gel filtration chromatography separates protein hydrolysates by the different sizes of peptides. As the peptides pass through a gel packed column, peptides with a larger molecular sized will be eluted out first since smaller sized proteins are retained by the pored beads for a

longer time in the gel. In a practical procedure, a sample is dissolved in minimal amount of buffer and transferred to the top of a vertical slim column filled with resin. The samples can be eluted out with either buffer agent or distilled water since peptides do not bind to the medium and therefore the elution agent does not affect resolution or biological activity (Sila & Bougatef, 2016). The heights or length of gel medium is a critical factor determining the resolution of separation and the spread of chromatogram. Different lengths of packed gel medium can also be used to desalt or concentrate the protein or peptide samples. Gel filtration is often combined with other separation techniques based on differences in acidity, basicity, charge, hydrophobicity, metal chelating and adsorption affinities (Sila & Bougatef, 2016).

Suetsuna and Chen (2002) purified wheat gluten pepsin hydrolysate using gel filtration chromatography on a Sephadex G-25 column (2.6 × 140 cm) equilibrated with deionized water (Figure 1-5 (A)) and estimated the M_w of the eluent peptides using the retention volume relative to the standard bovine serum albumin standard. The fraction having a M_w between 300 and 5000 Da was freeze-dried and further fractionated by ion exchange chromatography on an SP Sephadex C-25 column (2 × 50 cm) equilibrated with deionized water (Figure 1-5 (B)). The WP-3 fraction separated by the ion exchanger was found to possess stronger activity than α-tocopherol with an induction period of 7.5 days using ferrous thiocyanate method.

4.3.3. Ion exchange chromatography

Ion exchange chromatography is another chromatographic technique uses ion exchangers to separate a mixture of sample based on the net charge. The sample has a positive net charge can bind to cation exchanger through adsorption and/or electrostatic interaction, and the negatively charged sample binds to the anion exchanger. Separation is achieved through increasing ionic strength of the mobile phase, for example, increasing the concentration of

elution buffer, or changing the pH of the binding resin. The difference in the net charges of separated fractions was related to the presence of the distinct amino acid composition in the peptide sequences that affected the specific activities of the peptide fractions (Thamnarathip et al., 2016). This chromatography can be achieved through stepwise elution or a continuous phase.

Thamnarathip et al. (2016) used anion exchange chromatography to purify the rice bran protein Alcalase hydrolysates and the eluent profile is shown in Figure 1-6.

4.3.4. SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used by many studies to characterize different protein fractions based on their M_w distributions as it yields a distinct observation patterns compared with standard marks.

A research work by Zheng et al. (2006) produced antioxidant peptides from enzymatic hydrolysates of extruded and non-extruded corn gluten exemplifying the observation of M_w distribution change from unhydrolyzed proteins to hydrolysate by SDS-PAGE (Figure 1-7). Upon hydrolysis, some large-sized protein band almost totally disappeared as the macroproteins were digested into smaller peptide fragments. The smaller peptides were shown in the lower part of gel indicated a smaller M_w (Phongthai et al., 2018).

Ortiz-Martinez et al. (2017) isolated four protein factions from quality protein maize (a white hybrid maize) and normal whole maize kernels using SDS-PAGE. It was found that quality protein maize presented both α- and β- zeins, while the normal variety contains mainly α-zein. In the antioxidant potential analysis, hydrolysates of each protein fraction from these two varieties of corn shows very different antioxidant activities. This result concluded that the varieties of corn impacted the protein composition and the bioactivity of the proteins.

Thamnarathip et al. (2016) conducted SDS-PAGE to determine the M_w distribution of rice bran protein hydrolysates with wide range M_w standards between 4 and 250 kDa. Then, tricine-SDS-PAGE with low range of M_w standard between 2 and 40 kDa was used to characterize the M_w distribution of the hydrolysate prepared at optimal conditions. All three isolated peptide fractions showed a clear band at 6 kDa, which assisted the authors conclusion that peptides with M_w lower than 6 kDa react easily with radicals and therefore showed a higher antioxidant activity.

Waga and Zientarski (2007) used preparative polyacrylamide gel electrophoresis in acidic conditions (A-PAGE) was to fractionate individual group of gliadins in order to assess the isolated fractions by ELISA to compare their immuno-reactions causing celiac disease. A-PAGE was used in conjunction with analytical electrophoresis in the presence of sodium dodecyl sulfate, which eliminates most non-covalent protein-protein interactions. Accordingly, A PAGE isolated several highly purified gliadins, including A-gliadins (a specific group of α-gliadins), the well-known most toxic in causing celiac disease. It was also found that, α- and β-gliadins were stronger in binding antigliadin antibodies than γ- and ω- gliadins. It was therefore concluded that A-PAGE was an effective method for purifying gliadins for their immune-reactive and allergenic properties (Waga & Zientarski, 2007).

4.3.5. Other chromatographic techniques

There are many variations of chromatographic techniques with different stationary phase and separation mechanisms.

Reverse phase chromatography (RPC) employs polar mobile phase that eluted molecules with higher hydrophilic first since the nonpolar or hydrophobic molecules tend to be adsorbed to the hydrophobic stationary phase. Jin et al. (2015) used an RPC column to separate the active

fraction of corn gluten hydrolysate from Sephadex G-25 gel filtration; and the fraction showed highest DPPH% was further separated using an analytical RPC column.

High performance liquid chromatography (HPLC) is a quick separation technique used for preparative and/or analytical purposes. There are has a wide variety of stationary phase, with improved resolution, making it easy for sample recovery. HPLC was also used by some studies to purify the synthesized peptides (Jin et al., 2015; Suetsuna & Chen, 2002).

Gel permeation chromatography (GPC) was often coupled with HPLC (Wu et al., 2016; Zhu, Zhou & Qian, 2006; Wang et al., 2007) to determine the molecular weight distribution of hydrolysate mixtures.

4.4. Identification of bioactive peptides

4.4.1. Amino acid analysis

Amino acids are basic building blocks of peptides and proteins. Both amino acid composition and the amino acid sequence determined the functional and bioactive properties of peptides and protein hydrolysates (Xia et al., 2012). The presence of favorable amino acids as well as their positions in peptides chain (C-/N-terminal) could also influence the overall antioxidant activity (Jin et al., 2016; Gu et al., 2012).

Some amino acids are generally accepted as antioxidants or important constituent amino acid in antioxidant peptides despite prooxidative effects in some cases (Guo, Kouzuma, & Yonekura, 2009; Kawashima, Itoh, Miyoshi, & Chibata, 1979). The phenolic group of aromatic peptides (Trp, Tyr, His, and Phe) were able to convert radicals to stable molecules by donating electrons or protons which contributes to the antioxidant activity of peptide (Jin et al., 2016). Especially, histidine (His) exhibited strong radical scavenging activity due to the decomposition of its imidazole ring (Wang et al., 2007). Nucleophilic sulfur-containing amino acids (Cys and

Met) in sequence have the ability to donate protons to free radicals while the basic amino acids could prevent oxidation as the carboxyl and amino groups in their side chains can bind metal ions (López- Barrios et al., 2014). Amino acids (Val, Leu, and Ile) with bulky and branched side chains showed higher antioxidant activities than those did not as they may act as hydrogen donor antioxidants (Kawashima et al., 1979; Kou et al., 2013; Suetsuna & Chen, 2002). Hydrophobic amino acids (Pro, Phe, Leu, Ile, and Val) (Samaranayaka & Li-Chan, 2011) could also increase the presence of peptides at water-lipid interface (Thamnarathip et al., 2016), which is important for their protective performances in lipid peroxidation by interacting with hydrophobic molecules and scavenging the lipid derived free radicals in a heterogeneous lipid phase.

Chen et al. (1996) designed 28 peptides based on an identified antioxidative peptide from soybean protein hydrolysates to investigate the residue-activity relationship. The authors found that His and Pro played important roles in the antioxidative activity of the peptide sequence. It was also observed that, the deletion of the C-terminal His decreased the antioxidant activity of LLPHH, whereas the deletion of the N-terminal Leu of LLPHH made no effect. The substitution for the second L-His with D-His of PHH diminished the activity, which indicated that configuration of the amino acids was also important for the activity. Besides, the introduction of Tyr (a known antioxidant amino acid itself) to the positions of Pro or His of PHH did not increase the activities of the corresponding peptides YHH and PYY. Other Tyr-containing peptides, such as LPYY, LYPY, and YLYP, were also less active than the corresponding His peptides (Chen et al., 1996).

Gu et al. (2012) identified that the presence of with His located at C-terminal position of (Glu-Cys-His) demonstrated better electrons donating capacity; with Tyr at N-terminal position, YECG (Tyr-Glu-Cys-Gly) exhibited strong oxygen radical absorbance capacity. Branched amino

acids (Val, Leu, Ile) at N-terminals were preferable for higher antioxidant activity as well as Try and Tyr at C-terminal (Kawashima et al., 1979; López- Barrios et al., 2014). A free N-terminal amino group was also found to be important for the appearance of antioxidant activity determined by Kawashima et al. (1979). Blocking the N-terminal and C-terminal of branched-chain amino acid residues will greatly decrease the antioxidant activity of peptides (Kong & Xiong, 2006). Suetsuna and Chen (2002) claimed that dipeptides consisting of Ala, Tyr, His, and Met at the N-terminal had a higher antioxidant activity than the constituent amino acid mixtures in an aqueous system. Zhuang et al. (2013) reported a thermal stable Leu exhibited a critical association with the antioxidant activity of corn protein peptides.

The amino acid analysis typically involves two steps, the complete hydrolysis of protein and peptides with highly-concentrated HCl followed by the determination of amino acid composition using analytical technique such as automatic amino acid analyzer or HPLC. The quantification was based on external standards where tryptophan was not determined. In the work of Liu et al., 2017, the fermented wheat gluten protein sample was hydrolyzed with 6 N HCl at 110 °C for 22 hours before re-dissolving in loading buffer for determination of amino acid composition by an automatic amino acid analyzer (835-50, Hitachi). Suetsuna and Chen (2002) hydrolyzed the peptides with 6 N HCl containing 0.1% phenol at 110 °C for 24 hours and then analyzed amino acid profile using a PICO-TAGTM (Milford, MA) amino acid analyzer.

4.4.2. Identification of the peptide sequence

Not only the amino acid composition but also the position of certain amino acids in the peptide sequences makes an impact to the overall activity. Consequently, after a serial fractionation and concentration steps, it is necessary to identify the major peptides sequences present in the purified fraction accounting for higher antioxidant activities.

Liquid chromatography followed by mass spectrometry (LC-MS) is a mostly commonly used method for identification of peptide sequences and their molecular mass (Chen, Xiong & Chen, 2009; Xia et al., 2012; Zhuang, Tang, & Yuan, 2013). Other techniques including Matrix-Assisted Laser Desorption Ionization – Time-of-Flight Mass Spectrometry (MALDI-TOF MS) (Agrawal et al., 2017; Phongthai et al., 2018). Quadrupole Time-of-Flight Mass spectrometer (Q-TOF (Zheng et al., 2006; Wang et al., 2014; Jin et al, 2015; Chi et al., 2014), Ion trap mass spectrometer (IT-MS) (Wu et al., 2016), protein sequencer (Suetsuna & Chen, 2002; Kumrungsee et al., 2016) were also used for sequence identification.

In the study of Suetsuna and Chen (2002), two peptides, LQPGQGQQG and AQIPQQ, with promising antioxidant activity were determined from wheat gluten pepsin hydrolysates by stepwise Edman's degradation using a 477A gas-phase automatic sequencer (Applied Biosystem, CA) coupled to HPLC. The M_w of peptides was confirmed by atom bombardment mass spectrum (FAB-MS). Zhuang et al. (2013) analyzed the fraction with M_w below 10 kDa of corn gluten meal hydrolysates which showed higher antioxidant activities and found that all peptides contained the sequence of Leu-Pro-Phe. Hence, this sequence may be related to the high antioxidant activity. In this study, the peptide FLPF shown the highest DPPH radical scavenging. Tang and Zhuang (2014) identified two peptides, Pro-Phe and Leu-Pro-Phe, that exhibited extraordinary scavenging activity and inhibition of linoleic acid peroxidation from zein hydrolysates. The peptide Leu-Pro-Phe showed the higher DPPH and ABTS scavenging activity than Pro-Phe and had an inhibition effect (72.84%) of linoleic acid peroxidation comparable to that of GSH (80.8%). Jin et al. (2016) determined that APLC plays a critical role in good antioxidant activity of peptide CSQAPLA. Kou et al. (2013) determined the peptide sequences

Arg-Gln-Ser-His-Phe-Ala-Asn-Ala-Gln-Pro to be responsible for the radical scavenging activity and reducing power of chickpea albumin hydrolysates.

4.4.3. Synthesis and modification of peptides

Based on the identified sequences, peptides with desired activities and functionalities can be amplified for large-scaled use. Novel techniques also made it feasible to modify the known peptides or re-design the novel peptides.

Suetsuna and Chen (2002) synthesized two previously determined peptides, LQPGQGQQG and AQIPQQ, by using a solid-phase method on a 443A automated peptide synthesizer. The obtained peptides underwent a hydrogen fluoride treatment to cut off the support resin and to remove the protecting groups followed by a series of purification including HPLC. The induction periods for these peptides are 16 and 14 days in a linoleic acid system and oxidation was measurement with the ferric thiocyanate method.

Tang and Zhuang (2014) synthesized two previously identified peptides obtained from zein hydrolysates, Pro-Phe and Leu-Pro-Phe, by using the solid phase procedure on a peptide synthesizer. The synthesized peptide was further purified by HPLC, and the M_w was identified by MS. The final purity of theses peptides is 99.7% and 98.18%, respectively. These two peptides exhibited excellent radical scavenging activity and inhibition of linoleic acid peroxidation. At concentration of 500 μg/ml, Leu-Pro-Phe exhibited inhibition of linoleic acid peroxidation (72.84%) comparable to that of GSH (80.8%) (Figure 1-9).

Both Chen et al. (1996) and Gu et al. (2012) used the fluorenylmethoxycarbonyl (Fmoc)-strategy on an automated simultaneous multiple peptide synthesizer. Two novel peptides ECH (Glu-Cys-His) and YECG (Tyr-Glu-Cys-Gly) based on the structure of glutathione (GSH) were designed by Gu et al. (2012). The obtained peptides were evaluated by their chemical and

cellular antioxidant activity and It was found that ECH displayed an excellent DPPH radical scavenging activity, reducing power, and inhibition activity toward linoleic acid peroxidation with inhibition rate 98.25% at 7th day. Meantime, YECG showed extraordinary oxygen radical absorption capacity and ABTS radical scavenging ability. Chen et al. (1996) designed 28 peptides based on an identified antioxidative peptide (Leu-Leu-Pro-His-His) isolated from soybean protein hydrolysates. The antioxidant activity of the resulting peptides was evaluated by the inhibition against the peroxidation of linoleic acid in an aqueous system measured by ferric thiocyanate method to study the residue-activity relationship. The sequence Pro-His-His was found to be the most potent peptide.

4.5. Assessment of antioxidative activities of peptides

Official methods or standard bio-marks have not been established for measuring the antioxidant activity of proteins or peptides. As a result, assays that were used to measure antioxidative capacity of other non-peptide substances have been used to assess the antioxidative potentials of peptides (Samaranayaka & Li-Chan, 2011). For example, ORAC and ABTS, are assays originally used to analyze phenolic compounds and phytochemicals and have been used to analyze peptides. Still, development of theses existing methods provides useful tools for assessment of peptide antioxidants.

Due to the complexity of oxidative process occurring in food or biological systems, it is not possible to use one single method to characterize the overall antioxidative potential of an antioxidants in food products (Sila & Bougatef, 2016). The measurement results of an antioxidant sample depend largely on the type of assays applied, technologies employed to measure, and type of free radical generator or oxidant is used. The same test sample may exhibit different results of abilities depending on the assay system used (Samaranayaka & Li-Chan,

2011). For example, a good radial scavenger may not be a good metal chelator. What's more, the reaction condition and sample's solubility in different solvent system media also caused variability in antioxidant activity results among different assays. For example, the results of both ORAC and DPPH assays are sensitive to pH (Awika et al., 2003). The FRAP assay requires acidic conditions when measuring the antioxidative capacity of a sample, however, ABTS and Folin-Ciocalteu reagent assay need to be conducted under neutral and basic conditions, respectively.

Because of these reasons, it is recommended that more than one analytical method and variation of oxidation conditions should be considered in understanding the comprehensive antioxidative capacity of a potential antioxidant (Sarmadi & Ismail, 2010). Using model system that simulating the oxidation environments expected in real food products is also needed for evaluation of antioxidants efficacy (Decker, Warner, Richards, & Shahidi, 2005). These combined results will provide a comprehensive understanding of the antioxidant efficiency and action mechanisms (Zulueta, Esteve, & Frígola, 2009).

4.5.1. *In vitro* chemical assays

The *in vitro* assays based on chemical reactions are widely used for quantifying antioxidative potential of a protein and peptides (Samaranayaka & Li-Chan, 2011). Generally, they can be classified to two groups based on chemical reaction mechanisms: the electron transfer (ET) and hydrogen atom transfer (HAT) (Zulueta et al., 2009; Sarmadi & Ismail, 2010).

The ET assays measure the capacity of an antioxidant in reduction of an oxidative agent which will change color when reduced (Masisi et al., 2016) due to the participation in single electron transfer reaction (Sila & Bougatef, 2016). The change of color acts as a probe for monitoring the process and endpoint of reaction (Sarmadi & Ismail, 2010). The electron transfer

reaction of ET-based assays can be described as followed: Probe (oxidant) + e^- (from antioxidant) → reduced probe + oxidized antioxidant (Sarmadi & Ismail, 2010). ET assays include Trolox equivalent antioxidant capacity (TEAC), a.k.a., 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) assay (ABTS), ferric iron reducing power (FRAP), copper reduction assay (CUPRAC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity (Masisi et al., 2016). DPPH assay can be used as a primary screening assay to characterize the scavenging potential of antioxidants (Wang et al., 2007). It was also one of the most commonly used assays among the reviewed literatures as it is fast, simple, stable, and consistent.

HAT assays involve competition between the antioxidant and substrate for thermally generated peroxyl radicals through the decomposition of azo-compounds (Zulueta et al., 2009). It was believed that HAT-based reaction is a key step in the radical chain reaction; therefore, it is more relevant to the radical chain breaking antioxidant capacity (Sarmadi & Ismail, 2010). HAT assay includes the oxygen radical absorbance capacity (ORAC), total peroxyl radical trapping antioxidant parameter (TRAP), β-carotene bleaching assay, crocin bleaching assay, and so on (Masisi et al., 2016; Sarmadi & Ismail, 2010).

Awika et al. (2003) compared several screening methods (DPPH, ABTS, ORAC) for evaluation of antioxidant activity for sorghum and sorghum products. The authors pointed that, DPPH and ABTS assays are rapid, simple to perform, inexpensive, can be used in both aqueous and organic environments, and have good repeatability. They have a good correlation to ORAC. However, unlike ORAC method which used biologically relevant free radicals, DPPH and ABTS are both foreign radicals to biological systems. Thus, their relevance to in vivo antioxidant

efficacy is unknown. ORAC assay allows for data comparison across labs, that normally requires use of expensive equipment.

In addition to the aforementioned assays, there are other methods involving the measurement of reactive oxygen species (ROS) and reactive nitrogen species (RNS) quenching abilities, including superoxide-, hydroxyl-, hydrogen peroxide-, single oxygen- and peroxynitrite scavenging activities (Sarmadi & Ismail, 2010). It is notable to mention that the *in vitro* measurement of antioxidant capacity cannot be directly related to their actual efficacy for *in vivo* instances or potency in real food products. The bioavailability after digestive absorption, reactivity *in vivo*, storage in tissue, are important factors to be taken into considerations when evaluating their capacity under specific circumstance (Sarmadi & Ismail, 2010).

4.5.2. Model systems

Due to the complexity of oxidations in real food products with various components, the antioxidant capacity of a food component depends on a lot of factors including the colloidal properties of the substrates, the conditions and stages of oxidation, and the localization of antioxidants in different phases (Frankel & Meyer, 2000; Zulueta et al., 2009). The *in vitro* methods do not take count of factors such as physical location of the antioxidant, interaction with other food components, environmental conditions, thus, it is important to use model system in evaluating the antioxidants to inhibit oxidative deterioration (Decker et al., 2005). By using model systems, the potential of antioxidants can be accurately evaluated in chemical, physical and environmental (e.g., pH and Ionic strength) conditions expected in food products. Decker et al. (2005) outlined three model systems for food lipids: bulk oil, oil-in-water emulsion, and muscle foods.

In food emulsion systems, due to the surface properties of proteins and peptides, the protein hydrolysates act as surfactants at the oil–water interface and form a physical barrier to minimize the contact of lipids with oxidizing agents therefore contribute to reducing lipid peroxidation (Samaranayaka & Li-Chan, 2011). Donnelly, Decker, and McClements (1998) reported that the oxidative stability of emulsified Menhaden oil was improved by the formation of a thick protein membrane made with whey proteins instead of Tween 20 as the emulsifier. The meat products, especially ground meat products such as fresh pork sausages are easily deteriorated by oxidative reactions due to the damaged muscle membrane systems during the grinding and exposing of unsaturated fats and proteins to oxidative agents. As a result, it serves as another ideal model widely used to evaluate the inhibition of lipid oxidation and peroxidation.

The lipid deterioration in meat products involved three stages including initiation, propagation, and termination. The lipid deterioration is slow at initiation stage; however, it proceeds rapidly after induction. During the initiation stage, in the presence of pro-oxidants, a hydrogen atom is abstracted from the methylene group from the hydrocarbon chain of the lipid especially unsaturated fatty acids molecule (RH) and produced free radicals (R·) which tend to be stabilized by the rearrangement of methylene-interrupted double bond in polyunsaturated fatty acids to generate conjugated dienes. Conjugated dienes readily react with oxygen to form lipid peroxyl radicals (ROO·) under aerobic conditions, once these highly reactive radicals are generated, they will attack new lipid molecule sites, and initiate in the propagation of chain reaction. During the propagation stage, (ROO·) remove (H·) from the lipid molecule to form lipid hydroperoxides (ROOH), which are the primary oxidation products. Secondary oxidation products produced from lipid hydroperoxides include aldehydes (hexanal, 4-hydroxynonenal, malondialdehyde), ketones, alcohols, hydrocarbons, volatile organic acids, and epoxy

compounds varied by the different fatty acid substrates as well as reaction conditions. Some of these compounds such as alcohols have rancid odor, which can be detected at low levels and caused undesired deterioration. (Sohaib et al., 2017)

In addition, other model systems such as liposome system (Kong & Xiong, 2006; Peñta-Ramos & Xiong, 2002) and unsaturated free fatty acid system (e.g., linoleic acid autoxidation inhibition) (Zhu et al., 2006; Suetsuna & Chen, 2002; Park et al., 2001) were also widely used among reviewed literatures.

4.5.3. *In vitro* biological assays

Compared to the expensive and time-consuming animal studies and human clinical trials, the *in vitro* cultured cell model systems yield rapid inexpensive screening of antioxidants for their bioavailability, metabolism as well as bioactivity. The well-established cell-based model has the potential to demonstrate the *in vivo* mechanisms of the action of antioxidants in human body (Samaranayaka & Li-Chan, 2011; Zhu et al., 2013).

The *in vitro* antioxidant model based on rat adrenal pheochromocytoma (PC12) cells is an important method to assess potential health benefits of antioxidant peptides (Gu et al., 2012). Meantime, THP-1 human monocytic leukemia cells (THP-1) is one of the most extensively utilized cell lines as a useful surrogate for *in vitro* studies by simulating specific cellular signaling pathways (Sullivan et al., 2018).

Zhu et al. (2013) pre-treated the cultured PC12 cells with wheat germ Alcalase hydrolysate isolates before exposure to H_2O_2-induced oxidative stress. It was found that the pre-treated cells possessed significantly stronger resistance against oxidative stress as reflected by cell viability. Increased activities of catalase (CAT) and superoxide dismutase (SOD), and reduced levels of lactate dehydrogenase (LDH) release and malondialdehyde (MDA) production

for was also observed in pre-treated cells. It was concluded that the presence of hydrolysate isolates provided protection effects of cells as a neuroprotective agent.

Gu et al. (2012) also used *in vitro* cultured PC12 cell model to evaluate the protection effect of two synthesized peptides ECH (Glu-Cys-His) and YECG (Tyr-Glu-Cys-Gly) to resist hydroxyl-treated necrosis. It was found the presence of Tyr at N-terminal position of YECG exhibited stronger protection for PC12 cell to resist hydroxyl-treated oxidative stress.

Toledo et al. (2016) isolated peptides with M_w < 1 kDa from bean protein hydrolysates. They were found able to increase glucose stimulated insulin secretion from rat insulinoma INS-1E cells, reduce the expression of dipeptidyl peptidase and receptor for advanced glycation end products. As a result, these novel peptides significantly increased the glucose uptake (67%), reduced oxygen species (up to 70%) and inhibited lipid accumulation.

Duh, Tu and Yen (1999) used rat liver tissue homogenate as substrate to evaluate the antioxidative action of Harng Jyur extracts in biological system. The authors observed remarkable inhibition effects on $FeCl_2$-ascorbic acid-stimulated lipid peroxidation measured by TBA method, which were comparable to BHA.

Park et al. (2016) identified three peptides from Mytilus edulis peptic hydrolysate with excellent DPPH and ORAC activities. Peptide PIIVYWK and FSVVPSPK exhibited hepatoprotective effects of cultured human liver cells against H_2O_2-induced toxic damage observed by a significantly increased cell viability. It was concluded that these antioxidant peptides act through upregulating heme oxygenase-1 (HO-1) and activating HO-1 expression under normal and oxidative stress conditions.

4.5.4. *In vivo* assays

Even though *in vitro* based assays could provide a vision of the potential antioxidant activities of peptide antioxidants, further corresponding *in vivo* study is required to support the claimed antioxidative functions and efficacy in biological systems (Samaranayaka & Li-Chan, 2011; Sarmadi & Ismail, 2010). The results of the *in vivo* studies, including animal studies and human clinical trials, will provide essential evidence in gaining approval from FDA or USDA to use the developed antioxidants in food additives, dietary supplements, functional ingredients, and nutraceuticals formulations (Sarmadi & Ismail, 2010).

Using biomarkers is a common way to measure the ability of dietary antioxidants to protect lipids, proteins, and DNA from oxidative damages through investigating the fate of peptides during GI digestion, permeability through cellular membranes, as well as their *in vivo* stability and reactivity and so on (Samaranayaka & Li-Chan, 2011).

Up to now there are only a few studies evaluated the efficacy of antioxidant peptides. In a study of Ortíz-Cruz et al. (2015) it was found that, rats fed with sorghum kafirin as a diet supplement had an improved lipid metabolism and increased serum antioxidant activity (67%). The authors associated this positive effect on serum lipids with the release of bioactive peptides generated from kafirin *in vivo* hydrolysis, which was in consistency with the *in vitro* results of kafirin hydrolysates measured by the DPPH and ABTS assays. These results served as important evidence of sorghum kafirin in prevention of atherosclerosis and other chronic diseases.

Tang et al. (2012) determined the effects of heating and malandialdehyde (MDA) oxidation on the wheat peptides by an *in vivo* assay with rats. In this study, rats were randomly assigned to different groups and orally fed with modified peptides. The blood samples were collected and for determination of reactive oxygen substances (ROS) levels, and higher ROS

levels were observed among the group treated with modified peptides. This result was also supported by *in vitro* assays. The authors concluded that oxidation treatments of peptides altered the functional properties and structure of peptides which directly lead to loss of antioxidant potency and imbalance of redox status, as a result, cellular macro-molecules was damaged by ROS. Based on these results, the authors proposed that oxidized foods in human diet may impose risks to human health.

5. Applications and Challenges

Despite the excellent antioxidant activities of protein hydrolysates and peptides, there are some potential problems impacting their practical use in food applications. One of the major limitations is suspicious allergenicity, especially those obtained from previously identified allergens such as wheat, soy, dairy, nuts and eggs (Elias et al., 2008). It remains as an unresolved problem due to lacking clinical trials to confirm the bioactivity, efficacy, and safety (Samaranayaka & Li-Chan, 2011).

Bitterness and other off-flavors of protein hydrolysates is a common issue influencing food quality in enzymatically modified proteins (Elias et al., 2008). This feature can be used as a flavor enhancer for certain food products such as cheese and fermented bakeries, in other circumstances, it might be an undesired taste that need to be masked off. Cho et al. (2004) characterized the bitterness and molecular mass relationship of two commercial soy protein hydrolysates. The authors reported that, the peptides with a M_w less than 1 kDa showed a much less bitterness than other fractions, and the bitterness decreased as the peptide M_w decreased.

Further, since the protein hydrolysates are derived from the supernatant portion after hydrolysis, the protein-based antioxidants are mostly water-soluble, which can be a pro or a con for future processing or applications. Finally, proteins and peptides could cause alterations in

texture and functional properties such as viscosity, water/oil solubility, water/oil holding capacity, gelation, foaming, and/or emulsification ability. The protein could also initiate Maillard reactions that may cause color formation and generation of toxic materials (e.g., acrylamide) during food processing (Elias et al., 2008).

6. Conclusions

This chapter provides an overview of sorghum agronomic characteristics, grain kernel structures, chemical compositions as well as its consequent properties and potential applications. Literatures available about production, purification, and characterization of antioxidative peptides and protein hydrolysates were reviewed. Meantime, the processing techniques and analytical methods are not limited to the ones described in this study.

Enzymatic hydrolysis is an effective approach to obtain antioxidative peptides from parent proteins. Type of enzyme for hydrolysis is a critical factor in defining the properties and antioxidant activities of the hydrolysates as the selectivity of enzymes regulates the cleavage cite and further decides the features of subsequent peptides. Hydrolysis using industrial food-grade enzymes is a predominant approach to produce peptides and hydrolysates with enhanced antioxidant activities. It is a mild but effective process that releases peptides with varied chain lengths, diverse amino acid compositions, and selective peptide sequences which determined the consequent functionalities. Reaction parameters of enzymatic hydrolysis include type of enzyme employed for hydrolysis, enzyme to substrate ratio, duration of hydrolysis process, and other conditions such as pH and temperature. By selecting the specific type of enzyme and controlling crucial reaction variables, hydrolysates with certain characteristics such as the extent of hydrolysis, water and/or oil solubility, and desired antioxidant activity can be produced. Purification and fractionation techniques such as membrane filtration and size exclusion

chromatography are commonly used steps in isolation and concentration of the most active fraction from the hydrolysate mixture. Identification of amino sequences responsible for antioxidant activities enables peptide synthesis and amplification for large-scaled applications, which was commonly achieved by high performance liquid chromatography followed with mass spectrometry. Besides, critical peptide sequences accounted for higher activities will be determined by serial purification procedures and identification techniques.

Information in this study will greatly assist future studies in utilizing sorghum protein as well as protein-rich sorghum by-products for production of antioxidant peptides.

They can provide protection effect of food products against oxidation through multiple pathways including inactivation of reactive oxygen species, scavenging free radicals, chelation of catalytic metals, reduction of hydroperoxides and alteration of the physical properties of food systems such as forming physical barriers to hinder the access of lipids to prooxidants.

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Table 0-1 World major cereal grain area, yield, and production of 2017/2018.

Commodity	Area (Mha)	Yield* (MT/ha)	Production* (MMT)	Change from last year (%)
Corn	184.32	5.62	1036.66	1.87
Wheat	219.42	3.46	758.38	-1.40
Rice	160.75	4.53	488.23	0.26
Barley	47.91	2.98	142.97	3.22
Sorghum	39.93	1.44	57.43	1.79
Oats	9.54	2.46	23.49	0.63
Rye	4.03	3.07	12.38	1.28

*Yield is on a rough basis, before the milling process. Production is on a milled basis, after the milling process.
Ref: USDA, 2018

Table 0-2 World area, yield, and production of sorghum of 2017/2018.

Country/Region	Area (Mha)	Yield* (MT/ha)	Production* (MMT)	Change from last year (%)
World	39.93	1.44	57.43	1.79
United States	2.04	4.53	9.24	-5.72
Nigeria	5.80	1.13	6.55	3.82
Mexico	1.48	3.21	4.75	1.05
India	4.83	0.97	4.66	-1.27
Ethiopia	1.82	2.23	4.05	1.23
Sudan	6.30	0.59	3.74	6.87
China	0.68	4.74	3.20	7.81
Argentina	0.70	4.29	3.00	5.00
Niger	3.70	0.53	1.94	-12.55
Brazil	0.66	2.85	1.87	1.07
Mali	1.35	1.26	1.71	-23.75
Cameroon	0.85	1.65	1.40	0.00
Burkina	1.67	0.82	1.37	31.77
Australia	0.50	2.60	1.30	53.85
Tanzania	0.80	1.00	0.80	0.00
Egypt	0.14	5.36	0.75	0.00
EU	0.12	5.42	0.67	-4.05
Uganda	0.35	0.94	0.33	3.03
Ghana	0.25	1.12	0.28	0.00
Mozambique	0.30	0.50	0.15	0.00
Pakistan	0.20	0.65	0.13	7.69
South Africa	0.03	2.83	0.09	76.47
Others	5.37	1.02	5.46	0.88

*Yield is on a rough basis, before the milling process. Production is on a milled basis, after the milling process.
Ref: USDA, 2018

Table 0-3 U.S. major cereal grain area, yield, and production of 2017/2018.

Commodity	Area (Mha)	Yield* (MT/ha)	Production* (MMT)	Change from last year (%)
Corn	33.47	11.08	370.96	-3.86
Wheat	15.21	3.11	47.37	4.64
Sorghum	2.04	4.53	9.24	-5.72
Rice	0.96	8.41	5.66	14.01
Barley	0.79	3.91	3.09	3.59
Oats	0.40	2.21	0.72	33.61
Rye	0.12	2.12	0.25	21.14

*Yield is on a rough basis, before the milling process. Production is on a milled basis, after the milling process.
Ref: USDA, 2018

Table 0-4 Lysine amino acid scores for different age groups.

Grain	Infant (< 1 year)	Preschool child (2 - 5 years)	Schoolchild (10 - 12 years)	Adult
Wheat	43	46	62	100+
Rice (hushed)	57	61	82	100+
Maize	41	43	58	100+
Sorghum	17-51	18-55	25-74	71-100+
Pearl millet	26-69	28-74	38-100+	100+

Ref: WHO, 1985

Table 0-5 Concentrations of bioactive components in sweet sorghum millets.

Bioactive component	Concentration (μg/g)
ferulic acid	107.6 ± 10.1
p-couramic acid	17.9 ± 1.4
cinnamic acid	8.9 ± 0.5
catechin	144.9 ± 3.7
gallic acid	130.6 ± 8.1
syringic acid	38.8 ± 4.0
kaempferol	133.7 ± 6.7
quercetin	22.1 ± 0.9
apigeninidin	1570.0 ± 9.3
lutein	4.8 ± 0.2
β-carotene	18.8 ± 1.1
α-tocopherol	7.7 ± 0.7
γ-tocopherol	145.7 ± 12.7

Ref: Shen et al., 2013

Table 0-6 Literature review of bioactive peptides.

Research substrates	Enzymes or treatments	Evaluation assays	Fractionation and identification	Identified peptide sequences	References
Kafirin		THP-1 human cells			Sullivan et al., 2018
a-Kafirin	Chymotrypsin	ACE inhibitory activity	Sephadex G-25 gel filtration		Vasudeva et al., 2006
Sroghum flour; extracted kafirin		ABTS; DPPH; ORAC; In vivo blood serum ORAC	SDS-PAGE; Amino acids analysis		Ortíz-Cruz et al., 2015
Green tender sorghum protein isolate	Alcalase	DPPH; ABTS; Fe chelating; Reducing power	SDS-PAGE; Ultrafiltration: 10, 3 kDa; Sephadex G-25 gel filtration; RP-UFLC; Amino acids analysis; MALDI-TOF-MS/MS	VPPSKLSP; VAITLTMK; GLLGKNFTSK; LDSCKDYVME; HQTSEFK; VSKSVLVK; TSVEIITSSK	Agrawal, Joshi, & Gupta, 2017
Sweet sorghum grain protein	Alcalase	ACE inhibitory activity	Ultrafiltration: 10, 5, 1 kDa; 732 cation-exchange column; Sephadex G-50 gel filtration; RP-HPLC; IT-MS; Peptide synthesis	Thr–Leu–Ser	Wu et al., 2016
Wheat germ	Alcalase	Calcium-binding	Ultrafiltration: 3, 1 kDa; HiPrep DEAE-FF AEX; DEAE gel filtration; RP-HPLC; LC-ESI/MS; Peptide synthesis	Phe-Val-Asp-Val-Thr	Wang et al., 2018

Wheat germ	Fermentation	DPPH; Reducing power; Fe chelating	Amino acids analysis; GPC-HPLC		Liu et al., 2017
Wheat germ	Neutrase; Alcalase; Protamex; Thermolysin;	In vitro CaMK II inhibitory; Ex vivo CaMK II inhibitory	RP-HPLC; Protein sequencer	Trp-Val; Trp-Ile	Kumrungsee et al., 2016
Wheat germ protein	Alcalase	Linoleic acid; DPPH; Superoxide; Hydroxyl; Reducing power; Fe chelating	Amino acids analysis; GPC-HPLC		Zhu, Zhou & Qian, 2006
Wheat germ protein isolate	Alcalase	In vitro oxidative stress in PC12 cells	GPC-HPLC		Zhu et al., 2013
Wheat gluten	Papain	DPPH; TBA	Ultrafiltration: 5k; GPC-HPLC; Amino acids analysis		Wang et al., 2007
Wheat gluten	Pepsin	Linoleic acid	Sephadex G-25 gel filtration; C-25; RP-HPLC; Amino acids analysis; Gas-phase sequencer-HPLC; FAB-MS; Peptide synthesis	Ala-Gln-Ile-Pro-Gln-Gln; Leu-GlnPro-Gly-Gln-Gly-Gln-Gln-Gly	Suetsuna & Chen, 2002
Wheat gluten	Protex	ACE I inhibitory; ABTS; Reducing power; Copper-chelating; β-carotene-linoleic acid	RP-HPLC; SDS-PAGE; FTIR; FPLC		Cian, Vioque & Drago, 2015

Wheat peptides	Heating; MDA	Reducing power; Hydroxyl; DPPH; In vivo blood ROS level	GPC-HPLC		Tang et al., 2012
Rice bran protein	Pepsin; Trypsin	DPPH; ABTS; Fe chelating; Reducing power	Ultrafiltration: 10, 5, 3 kDa; SDS-PAGE; Free amino acids content; MALDI-TOF MS	A octapeptide	Phongthai et al., 2017
Rice bran protein	Neutrase; Alcalase; Flavourzyme	ABTS; Reducing power	SDS-PAGE; Tricine-SDS-PAGE; AEX-FPLC		Thamnarathip et al., 2016
Rice dreg protein	Trypsin	Emulsion turbidity; Emulsion stability; POV; TBARS			Zhao et al., 2012
Corn gluten	Alcalase; Extrusion	Pyrogallol inhibition	SDS-PAGE; Superdex gel filtration-HPLC; Q-Sepharose AEX; Sephadex G-25 gel filtration; Q-TOF2-MS	Phe-ProLeu-Glu-Met-Met-Pro-Phe	Zheng et al., 2006
Corn protein	Alcalase; Protamex; Extrusion	DPPH; ABTS; Hydroxyl; Superoxide; Reducing power; Fe chelating	Ultrafiltration: 50, 20, 10, 6 kDa; Q-Sepharose AEX; Sephadex G-25 gel filtration; RP-HPLC; Q-TOF2-MS/ESI; Peptide synthesis	Gln-Gln-Pro-Gln-Pro-Trp	Wang et al., 2014
Corn gluten meal	Alcalase; Protamex; Flavourzyme	Pyrogallol inhibition; In vivo blood SOD, GSH-Px, TBARS	Superdex gel filtration		Liu et al., 2015

Corn gluten meal	Alcalase; Flavourzyme; Alcalase + Flavourzyme; Flavourzyme + Alcalase	DPPH; Fe chelating; Reducing power; Hydroxyl; Superoxide	Ultrafiltration: 6 kDa; Q-Sepharose AEX; Sephadex G-25 gel filtration; RPC; Analytical RPC; Q-TOF2-MS/ESI; Peptide synthesis	Cys-Ser-Gln-Ala-Pro-Leu-Ala; Tyr-Pro-Lys-Leu-Ala-Pro-Asn-Glu; Tyr-Pro-Gln-Leu-Leu-Pro-Asn-Glu	Jin et al., 2015
Corn gluten meal	Alkaline protease; Flavourzyme	DPPH; ABTS; Hydroxyl; Superoxide; Fe chelating; Cu chelating; Linoleic acid	Sephadex G-25 gel filtration; LC-MS/MS; Peptide synthesis	Leu-Pro-Phe; Leu-Leu-Pro-Phe; Phe-Leu-Pro-Phe	Zhuang, Tang, & Yuan, 2013
Corn gluten meal	Alcalase	Hydroxyl; DPPH; Reducing power; Linoleic acid	Amino acids analysis; Sephadex G-15 gel filtration		Li, Han, & Chen, 2008
Corn gluten meal	Alkaline protease; Trypsin; Papain; Flavourzyme	DPPH; Reducing power; ABTS; Hydroxyl; Superoxide; Fe chelating; Linoleic acid	Sephadex G-25 gel filtration; LC-MS/MS	Gly-His-Lys-Pro-Ser	Zhuang et al., 2013
Corn gluten meal zein	Alcalase	DPPH; Superoxide; Hydroxyl; Linoleic acid; Reducing power	MS; Amino acids analysis		Li et al., 2010

Corn gluten meal protein	Alcalase; Sonication	DPPH	Gel filtration; Amino acids analysis; C18 semipreparative column; UPLC-ESI/MS	40 potential peptides	Liang et al., 2017
Corn gluten meal zein	Trypsin; Alkaline protease; Flavourzyme; Papain;	DPPH; ABTS; Fe chelating; Cu chelating; Reducing power; Linoleic acid	Ultrafiltration: 3 kDa; Sephadex G-15 gel filtration; Sephadex G-25 gel filtration; LC-MS/MS; Peptide synthesis; HPLC	Pro-Phe; Leu-Pro-Phe	Tang & Zhuang, 2014
Zein	Alcalase; Papain	Liposome POV; Liposome TBARS; Reducing power; ABTS; Fe chelating Cu chelating	SDS-PAGE; Amino acids analysis		Kong & Xiong, 2006
Barley glutelin hydrolysate	Alcalase; Flavourzyme	DPPH; Superoxide; Hydroxyl; Reducing power; Fe chelating	Ultrafiltration: 10, 1 kDa; RP-HPLC; LC-MS/MS;	Gln-Lys-Pro-Phe-Pro-Gln-Gln-Pro-Pro-Phe; Pro-Gln-Ile-Pro-Glu-Gln-Phe; Ser-Val-Asn-Val-Pro-Leu; Leu-Arg-Thr-LeuPro-Met	Xia et al., 2012
Canola meal protein	Alcalase; Pepsin; Trypsin; Pancreatin; Chymotrypsin	ABTS; DPPH; Superoxide; Linoleic acid; ORAC	Ultrafiltration: 10, 5, 3, 1 kDa		Alashi et al., 2014
Rapeseed protein isolate	Alcalase	Reducing power; DPPH; Superoxide; Hydroxyl	Amino acids analysis		Pan, Jiang, & Pan, 2011

Rapeseed	Fermentation	DPPH; Reducing power; Fe chelating; Linoleic acid	Amino acids analysis; Superdex gel filtration-UV		He et al., 2013
Soy protein	Neutrase; Validase; Alkaline protease	ORAC; DPPH; Fe chelating; Ground beef TBARS	Ultrafiltration: 10, 5, 3, 1 kDa		Zhang, Li, & Zhou, 2010
Soy protein	Alcalase	Linoleic acid POV; Linoleic acid TBARS	Ultrafiltration: 30, 10, 3 kDa; FPLC with Superdex column HPLC Amino acids analysis		Park et al., 2010
Soy protein concentrates	Flavourzyme	Hydroxyl ; ABTS; Superoxide; β-Carotene; FRAP	Ultrafiltration: 50, 30, 10, 5 kDa; Biologic Chromatography System coupled with BioGel columns		Moure et al., 2006
Soy protein isolate	Papain; Pepsin; Chymotrypsin	Liposome TBARS	SDS-PAGE		Peñta-Ramos & Xiong, 2002
Chickpea protein	Alcalase	Reducing power; Linoleic acid; DPPH; Hydroxyl; Superoxide	Sephadex G-25 gel filtration; Amino acids analysis		Li et al., 2008
Chickpea albumin isolate	Alcalase; Flavorzyme	DPPH; Hydroxyl; ABTS; Reducing power	Sephadex G-25 gel filtration; RP-HPLC; LC–ESI–MS/MS	Arg-Gln-Ser-His-Phe-Ala-Asn-Ala-Gln-Pro	Kou et al., 2013

Potato protein concentrate	Alcalase	Emulsion turbidity; Emulsion stability; POV; TBARS	Sephadex G-15 gel filtration; RP-HPLC; LC Q-TOF-MS/UPLC/ESI	Thr–Tyr; Ser–Tyr–Thr–Ala; Asn–Tyr–Lys–Gln–Met	Chen, Xiong & Chen, 2009
Lecithin-free egg yolk	Alcalase	Linoleic acid POV; Linoleic acid TBARS; TBARS in cultured human liver cells	Ultrafiltration: 10, 5, 1 kDa; GPC-HPLC; SP-Sephadex C-25; Sephadex G-25 gel filtration; RP-HPLC		Park et al., 2001
Sardinelle	Alcalase; Alkaline protease; Enzyme extract	DPPH; Linoleic acid; Reducing power	Sephadex G-25 gel filtration; RP-HPLC; MS/ESI	Leu-His-Tyr; Leu-Ala-Arg-Leu; Gly-Gly-Glu; Gly-Ala-His; Gly-Ala-Trp-Ala; Pro-His-Tyr-Leu; Gly-Ala-Leu-Ala-Ala-His	Bougatef el al., 2010
Monkfish	Trypsin	Hydroxyl; DPPH; Superoxide; Linoleic acid	Ultrafiltration: 3, 1 kDa; Sephadex G-15 gel filtration; RP-HPLC; Protein sequencer; Q-TOF-MS/ESI	Glu-Trp-Pro-Ala-Gln; Phe-Leu-His-Arg-Pro; Leu-Met-Gly-Gln-Trp	Chi et al., 2014
Mackerel	Protease N	linoleic acid; DPPH; Reducing power	Ultrafiltration: 5 kDa; Sephadex G-25 gel filtration; Free amino acids content; Amino acids analysis		Wu, Chen, & Shiau, 2003
Mytilus edulis	Pepsin	DPPH; ORAC; Hepatoprotective effects of human liver cells	Ultrafiltration: 5, 1 kDa; SP-Sephadex C-25; RP-HPLC; LC-MS/MS; Peptide synthesis	PIIVYWK TTANIEDRR FSVVPSPK	Park et al., 2016

Designed peptides		DPPH; Reducing power; Linoleic acid; ORAC; ABTS; PC12 cell study	Peptide synthesizer	Glu-Cys-His; Tyr-Glu-Cys-Gly	Gu et al., 2012
Designed peptides		Linoleic acid ferric thiocyanate	Peptide synthesizer	28 peptides	Chen et al., 1996

Abbreviations:

ABTS	ABTS radical scavenging activity
AEX	Anion exchange chromatography
CaMK II	Calmodulin-dependent protein kinase II
Cu chelating	Copper ion chelating activity
DPPH	DPPH radical scavenging activity
ESI	Electrospray ionization source
FAB-MS	Atom bombardment mass spectrum
Fe chelating	Ferrous iron chelating activity
FPLC	Fast performance liquid chromatography
FRAP	Ferric iron reducing power
FTIR	Fourier transform infrared spectroscopy
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
Hydroxyl	Hydroxyl radical scavenging activity
IT-MS	Ion trap mass spectrometer
LC	Liquid chromatography
Linoleic acid	Linoleic acid autoxidation model system
MALDI-TOF	Matrix assisted laser desorption ionization time-of-flight
MS	Mass spectrometry
ORAC	Oxygen radical absorbance capacity
POV	Lipid hydroperoxide values
Q-TOF	Quadrupole Time-of-Flight
RPC	Reverse phase chromatography
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Superoxide	Superoxide anion radical scavenging activity
TBARS	TBA-reactive substances assay
UFLC	Ultra-fast liquid chromatography
β-Carotene	β-Carotene bleaching method

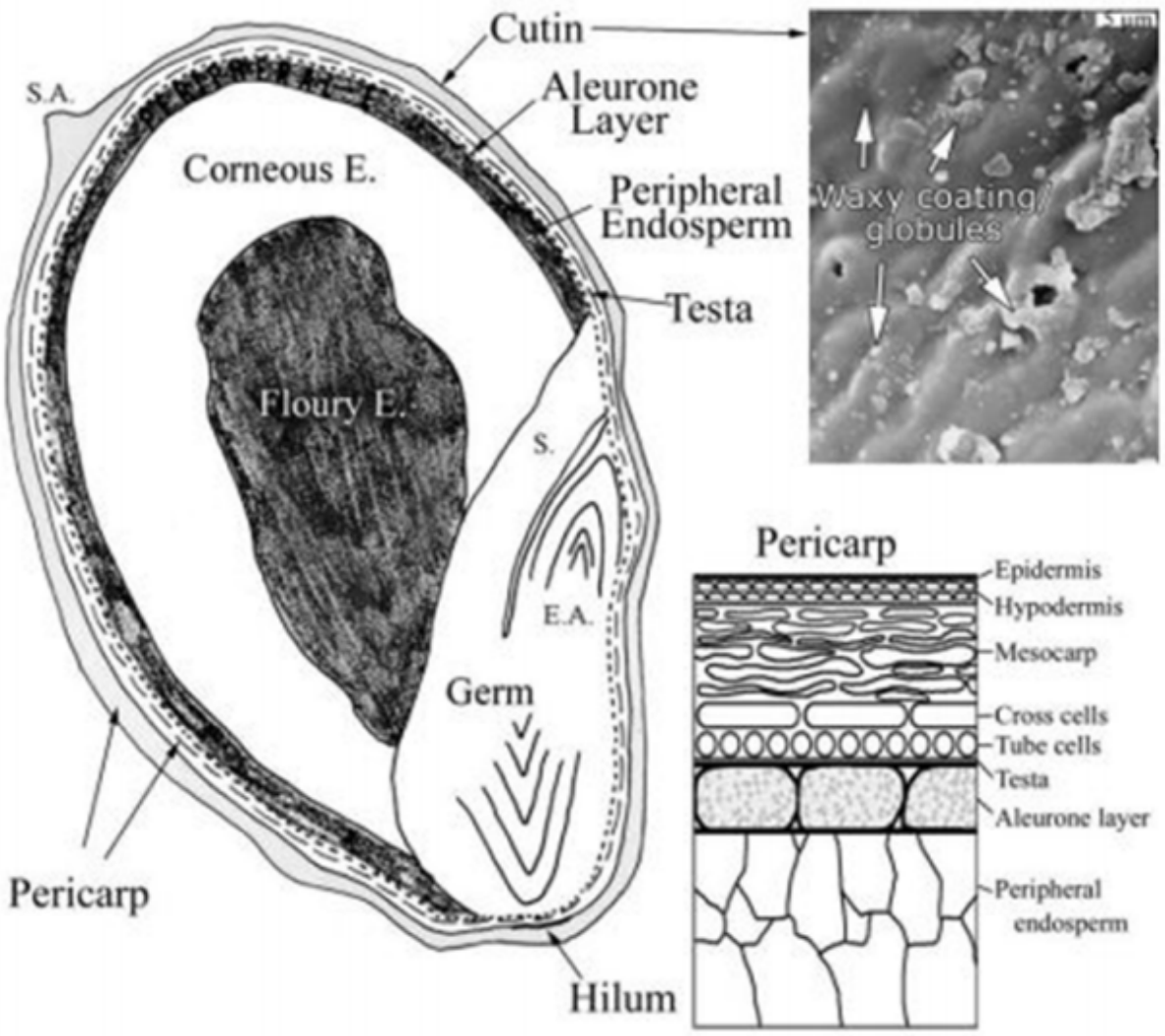

Figure 0-1 Structure of sorghum grain S.A., stylar area; E.A., embryonic axis; S., scutellum; E., endosperm.

Ref: Earp, McDonough, & Rooney, 2004

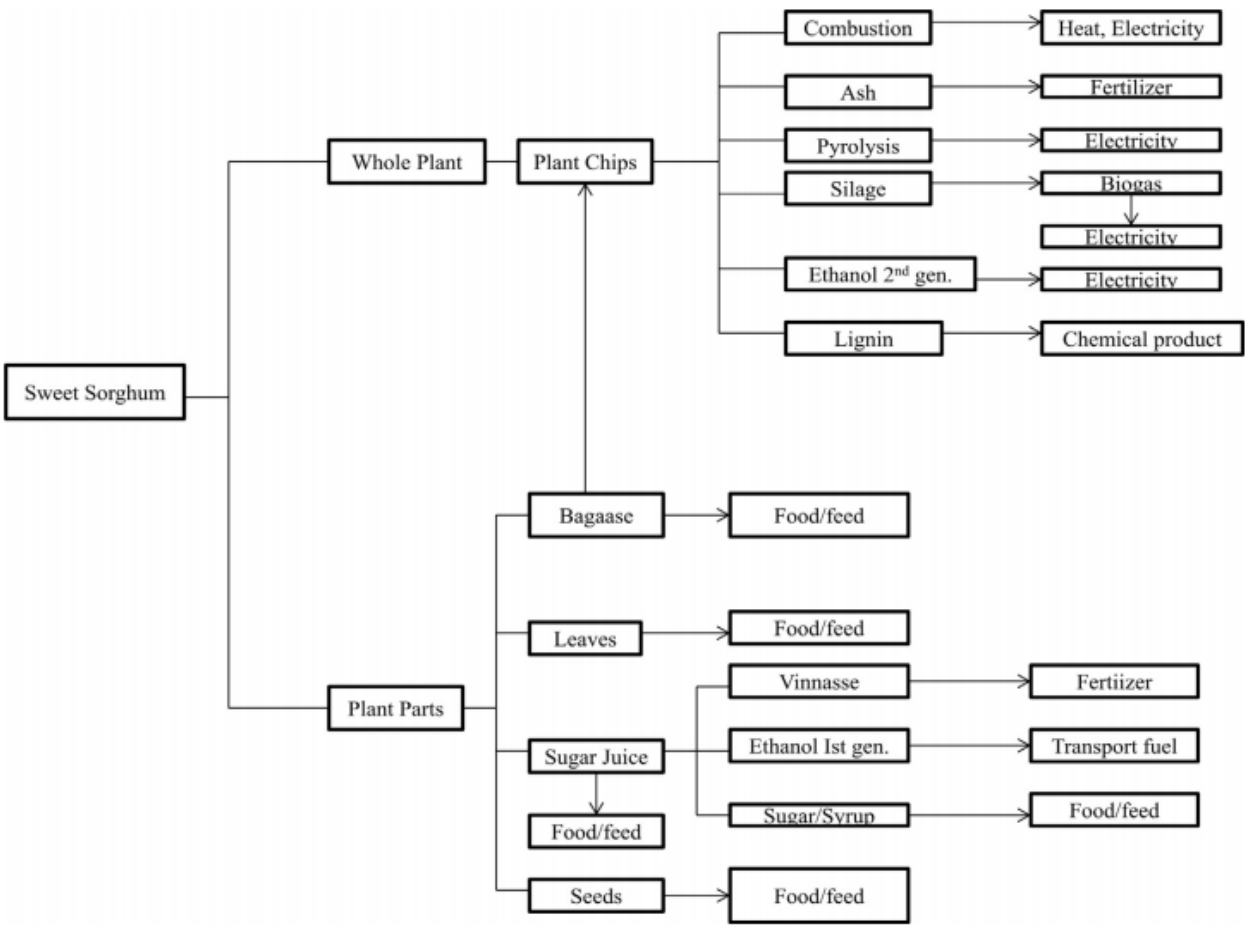

Figure 0-2 Different uses of sweet sorghum.

Ref: Dar et al., 2017

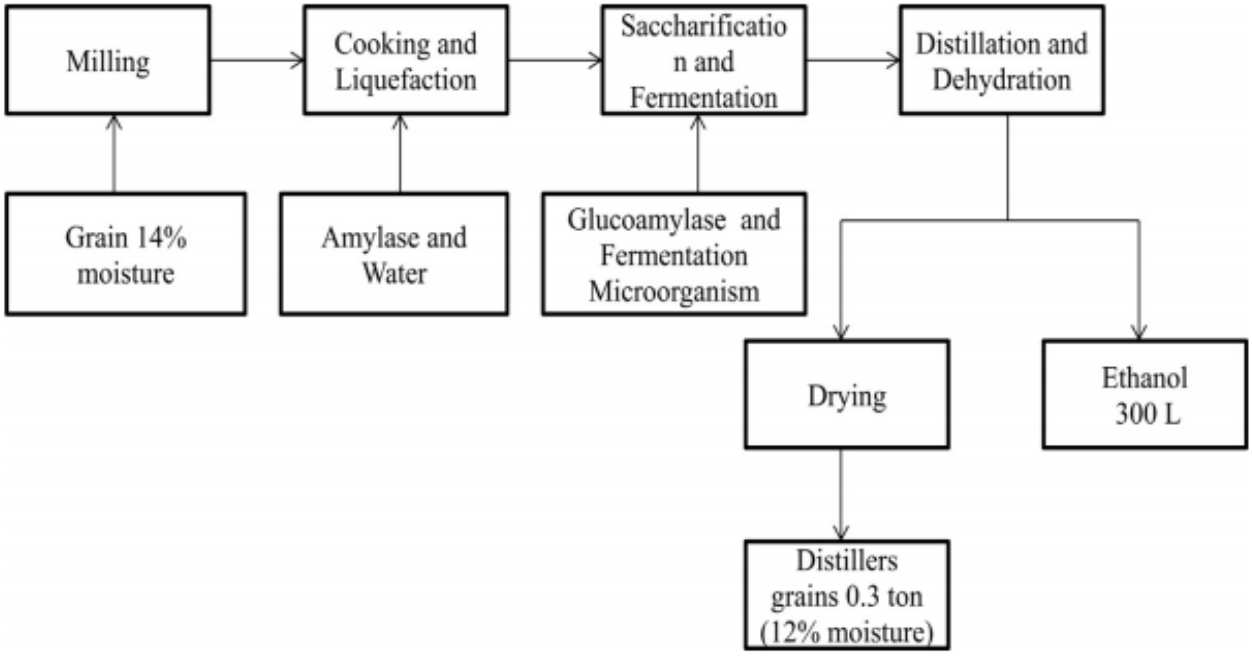

Figure 0-3 Process flow chart of ethanol production from sorghum grain.
Ref: Dar et al., 2017

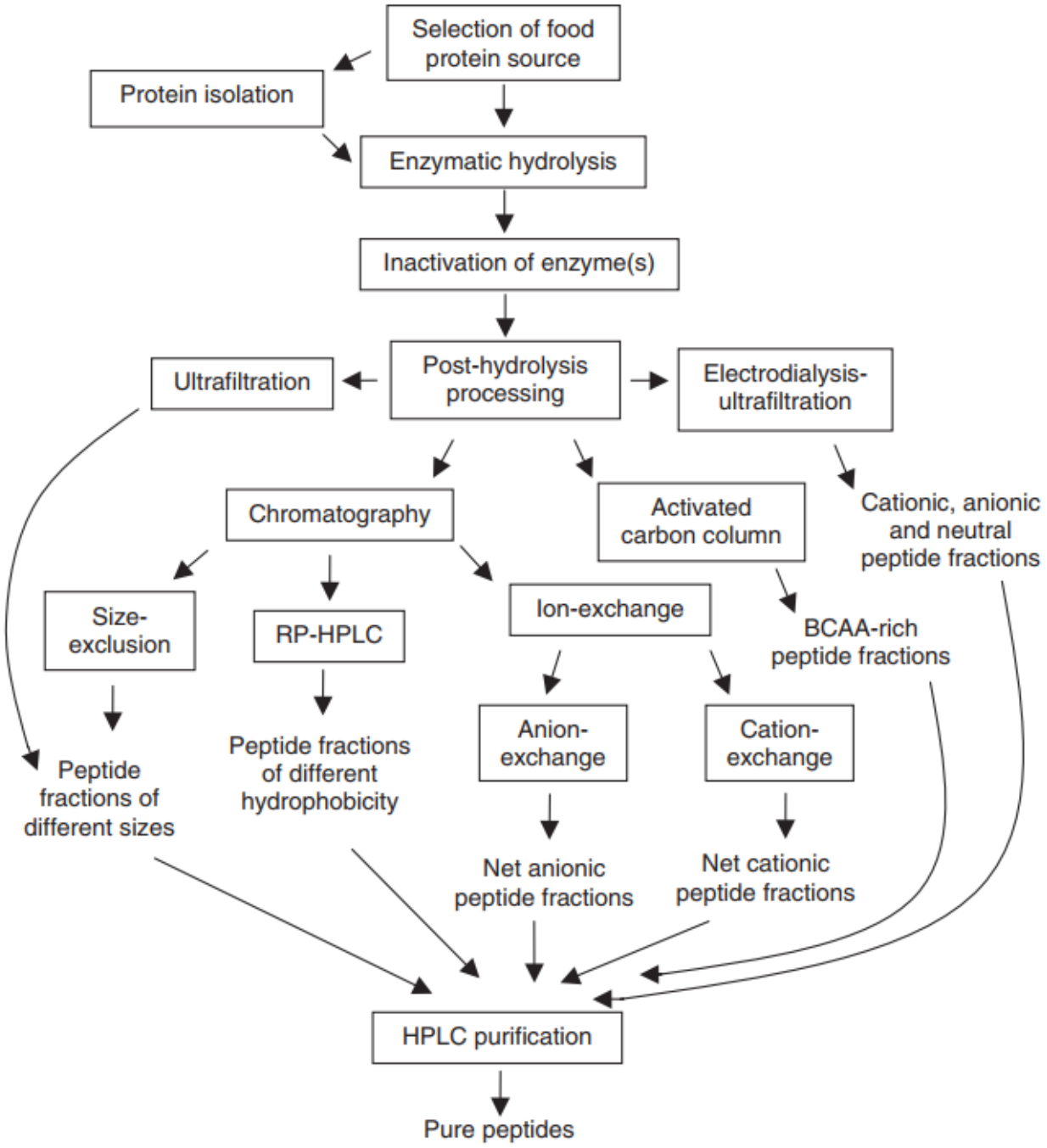

Figure 0-4 Schematic diagram of the production and processing of food protein-derived bioactive peptides.

Ref: Udenigwe & Aluko, 2012

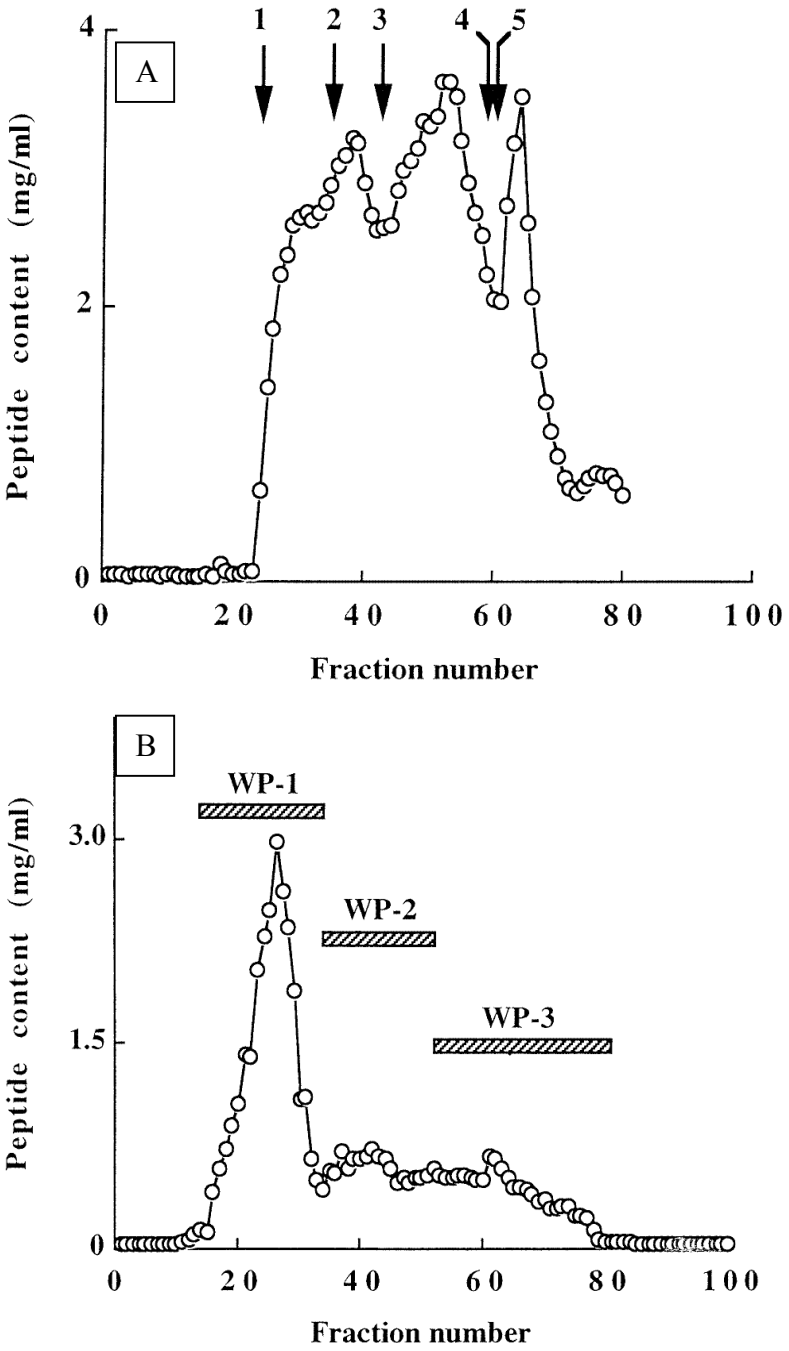

Figure 0-5 A, gel filtration chromatogram of wheat gluten peptic hydrolysate on Sephadex G-25 with molecular size markers: 1, insulin (M_w 6000); 2, insulin B chain (M_w 3500); 3, insulin A chain (M_w 2550); 4, cyanocobalamin (M_w 1355); 5. Glycine (M_w 75); B, Ion exchange chromatogram of wheat peptic hydrolysate on SP Sephadex C-25 column.

Ref: Suetsuna & Chen, 2002

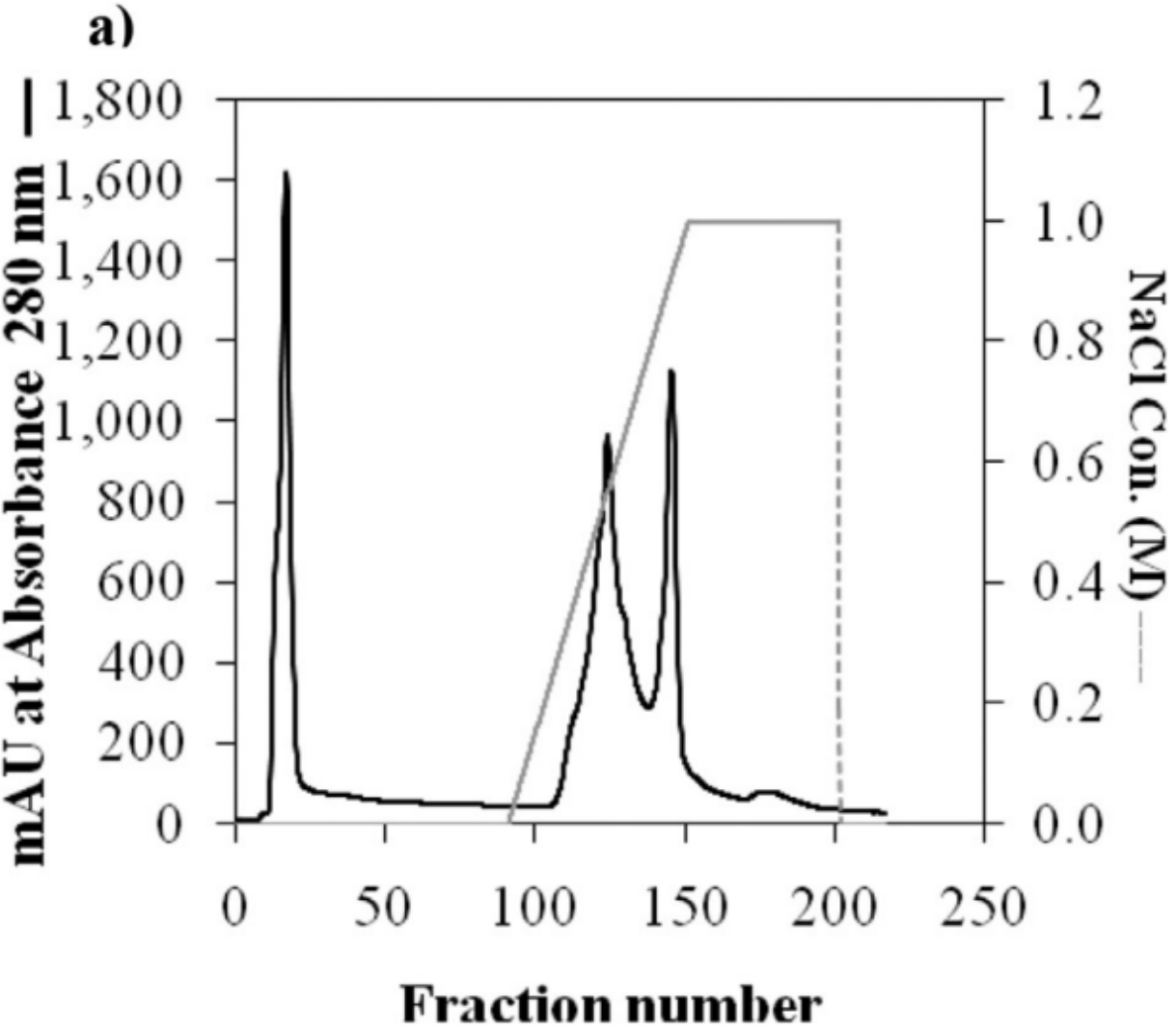

Figure 0-6 Chromatogram of anion exchange fast performance liquid chromatography.
Ref: Thamnarathip et al., 2016

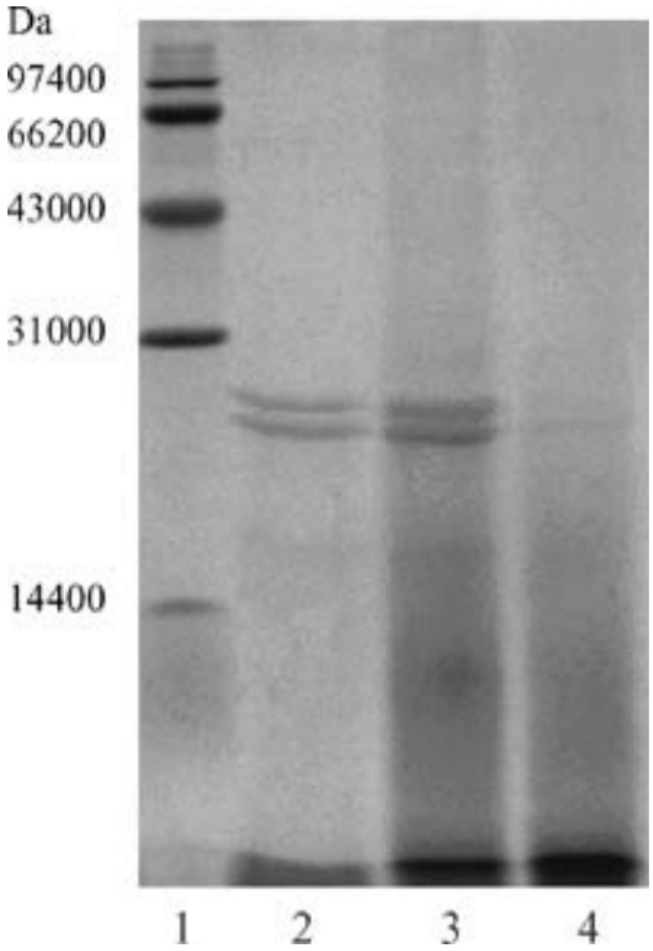

Figure 0-7 SDS-PAGE electropherograms of Alcalase hydrolysates of extruded and non-extruded corn gluten. 1, Molecular weight standards; 2, corn gluten; 3, non-extruded corn gluten hydrolyzed with Alcalase, 4, extruded corn gluten hydrolyzed with Alcalase.

Ref: Zheng et al., 2006

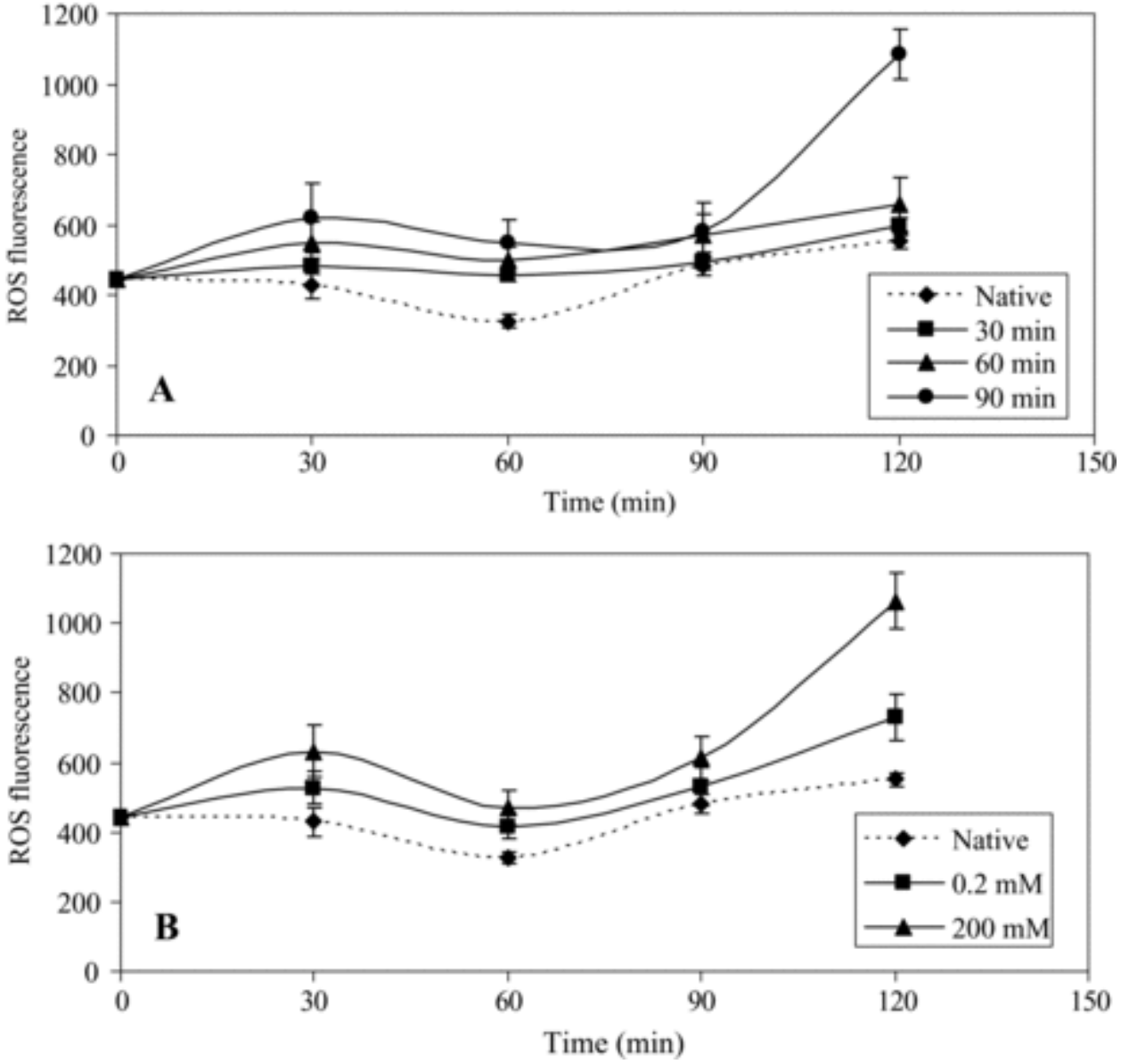

Figure 0-8 The dynamic changes of blood ROS level induced by native, heated (A), and MDA oxidized (B), wheat peptides in male mice.

Ref: Tang et al., 2011

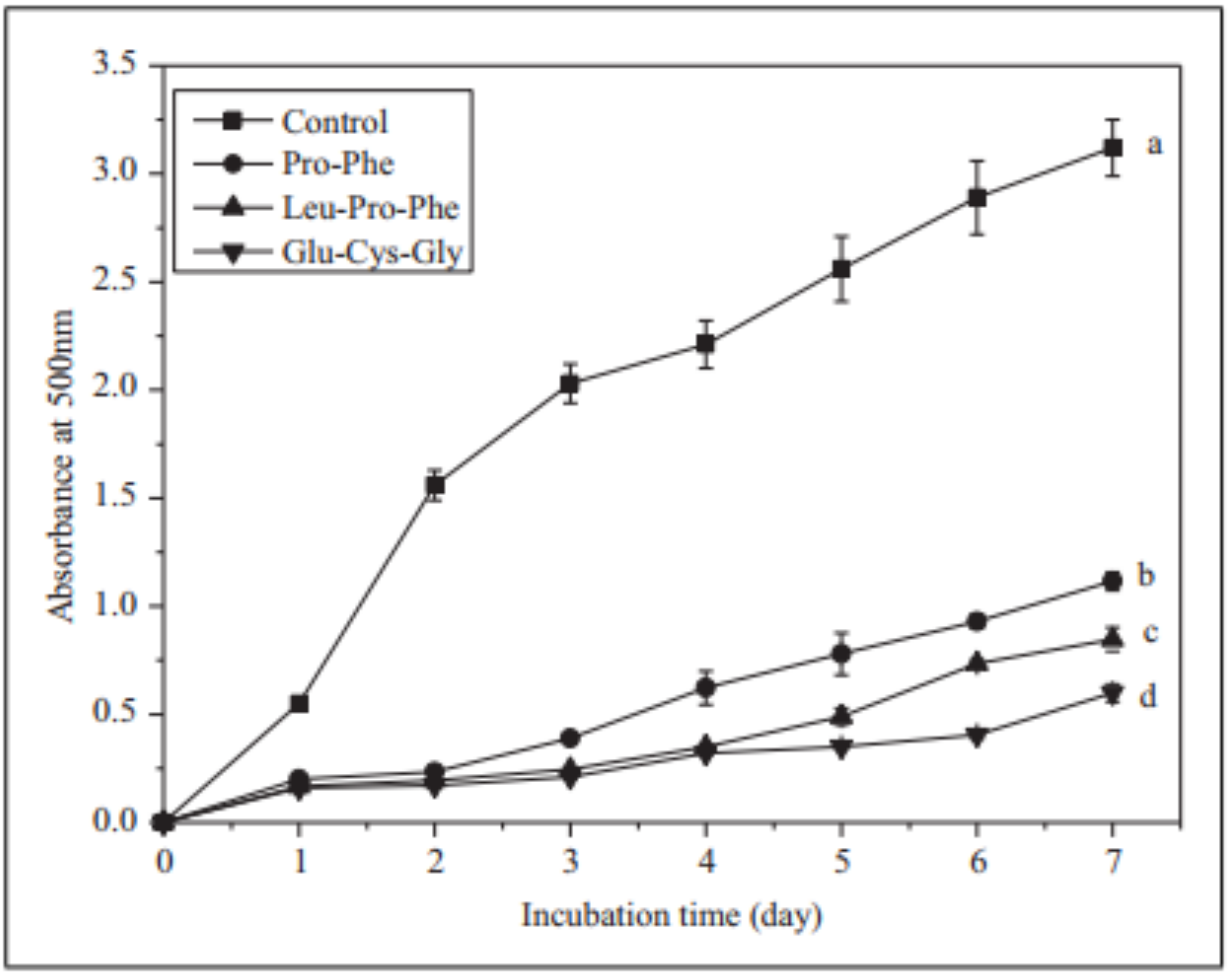

Figure 0-9 Linoleic acid peroxidation inhibition activity of synthesized peptides at 500 μg/mL. Ref: Tang & Zhuang, 2014

Chapter 2 - Reaction Optimization, Antioxidant Activity Characterization, and Peptides Identification of Sorghum Kafirin Hydrolysates Prepared with Neutrase

Abstract

Sorghum kafirin protein was extracted from defatted white sorghum flour and hydrolyzed with different types of enzymes from bacterial, plant and animal origins. Hydrolysate prepared with Neutrase displayed excellent antioxidant activities as well as total protein recovery yield. Therefore, it was primarily selected for further optimization and analysis. Essential reaction parameters in producing these antioxidant peptides include substrate content, enzyme-to-substrate ratio, and hydrolysis time, which will make impacts on the molecular weight distribution, amino acid composition, structural characteristics and functional properties of the resulting peptides. Hydrolysates obtained at optimized conditions (substrate content of 4%, enzyme-to-substrate ratio of 0.4 Au/g, and hydrolysis time of 17 hours) were fractionated by ultrafiltration. Medium-sized hydrolysates (3 – 10 kDa) were found to possess relatively higher total phenolic content and stronger antioxidative activities with regard to free radical scavenging activity, metal ion chelating activity, reducing power, and oxygen radical absorbance capacity. In an oil-in-water emulsion model system, the selected fraction of hydrolysates incorporated at 50 mg/mL inhibited the formation of primary and secondary oxidation products by 77.14% and 54.34%, respectively, during a 14-day incubation period. In the ground meat model system, the addition of peptides at 0.5 mg/g decreased the lipid peroxidation by 24.16% during a 12-day storage. The selected fraction of hydrolysate with strongest activities was further fractionated by gel filtration chromatography, and the most potent fraction of peptides (F2) was collected and

identified for its major peptide compositions and sequences using RP-HPLC and MALDI-TOF/TOF MS. Glutamine and alanine were the top two amino acids found in identified peptide sequences whilst MDMQ and VAQ the most frequently appeared peptide sequences, which could be vital constituent peptides and/or amino acids for the antioxidant activity of kafirin hydrolysates.

These combined results revealed that our selected sorghum peptides obtained through enzymatic hydrolysis and fractionation can act as efficient antioxidants in real food products to protect oil and fat from oxidative stress. Possible antioxidative mechanisms include free radical scavenging, metal ion chelation, hydrogen donating, reducing hydroperoxides, and forming physical barriers to deter the contact between oxidative agents and susceptible targets. Positive correlation between total phenolic content and antioxidant activities indicated that the peptides with phenolic amino acid residues and phenolic compounds released during hydrolysis contributed largely to their antioxidant activities.

1. Introduction

Oxidation of oils and fats in food products results in the deterioration of many quality features such as color, flavor, aroma, and texture, which will ultimately shorten the shelf-life and decrease the sensory and nutritional quality (Zhao et al., 2012). In biological systems, oxidation could cause structure alteration and biological function failures such as destabilization and disintegration of cell membranes, DNA mutation, protein damage, and many age-related diseases including cancer (Suetsuna & Chen, 2002; Tang & Zhuang, 2014). Antioxidants play an important role in neutralizing and reducing oxidative stress. In food industry, antioxidants are widely used to preserve various food products from oxidation and deterioration, hence, maintain the stability and integrity of qualities, and extend their shelf-life (Kim et al., 2001). In human body, antioxidants provide prevention effect of oxidative reactions induced diseases (Shahidi & Zhong, 2010). Chemically synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and *tert*-Butylhydroquinone (TBHQ) are cheap and effective at low dosage thus have been widely used in food industry. However, due to the potential risks to human health such as induction of DNA damage and toxicity (Ito et al., 1986), safety concerns over synthetic antioxidants have restricted the use of these compounds. Naturally extracted antioxidants such as green tea extracts and rosemary extracts are found to be effective antioxidants, but they are much more expensive due to the complex manufacturing process and relatively low yield.

In recent years, the interest of research is growing in developing safe and effective natural antioxidants from protein sources. Protein sourced antioxidants can serve as energy source and provide profile of essential amino acids while exerting antioxidative effects in food products (Sloan, 2014). In addition, the unique amphiphilicity of antioxidant peptides allow them

to perform oxidation inhibition functions in both aqueous and lipid systems as well as many other functionalities such as surface-active agents, emulsifiers, and foaming agents (Xia et al., 2012). All these properties make antioxidative peptides very applicable to food industry. Enzymatic hydrolysis is a predominant approach in producing peptide sequences with good selectivity and high antioxidant activity (Thamnarathip et al., 2016; Jin et al., 2016). Many studies have reported the antioxidative activities of protein hydrolysates. However, few reports have been found on characterizing the antioxidative activities of sorghum protein hydrolysates. Kafirin, the main storage protein of sorghum endosperm, can be obtained by isolating the alcoholic extractant in sorghum protein fractionation which has been proven to be biologically active by several studies (Ortíz-Cruz et al., 2015; Wu et al., 2016; Sullivan et al., 2018). Thus, kafirin is being an attractive source for production of antioxidant peptides with various human health promoting benefits and quality-enhancing functional properties.

This study was designed to utilize the sorghum kafirin as a protein source to produce hydrolysates with antioxidative activities. Reaction parameters involved in hydrolysis will be optimized in turns using single factor methodology, and more than ten types of enzymes of different origins will be screened. The hydrolysates obtained with promising enzymes at optimal conditions will be further fractionated by ultrafiltration and gel filtration chromatography. Peptides in the fraction that showed strongest activities will be identified using mass spectrometry. The antioxidative capacities of the obtained hydrolysates will be evaluated by *in vitro* assays and will be applied into two different model systems. To summarize, the objectives of this study are to: 1) evaluate the antioxidative performances of the sorghum kafirin enzymatic hydrolysates; 2) optimize the reaction parameters affecting the hydrolysate yield and

antioxidative activity for hydrolysis with Neutrase; and 3) study the peptide sequences and molecular structures related to the antioxidative activities of kafirin hydrolysates.

2. Materials and Methods

2.1. Materials and chemicals

The Harvest Pearl white sorghum flour was kindly provided by ADM Milling Co. (Overland Park, KS, USA).

Alcalase® 2.4L (Proteinase from *Bacillus licheniformis Subtilisin A*), Flavourzyme® (Protease from Aspergillus oryzae), Neutrase® 0.8L (Protease from *Bacillus amyloliquefaciens*), Everlase 16.0L (Protease from *Bacillus sp.*), Protamex® (Protease from *Bacillus sp.*), Papain (Papaya latex), and Bromelain (Pineapple stem) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Another brand of Papain (Carica papaya) was bought from EMD Millipore Corporation (Billerica, MA, USA). Ficin (Fig tree latex) was received from TCI America Co. (Portland, OR. USA). Trypsin (Bovine pancreas) was received form Alfa Aesar (Haverhill, MA. USA). Pepsin (Porcine) was obtained from Acros Organics (New Jersey, USA).

Rosemary leaf extract powder was acquired from Z Natural Foods (West Palm Beach, FL, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2′-Azobis (2-methylpropionamide) dihydrochloride (AAPH), fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylicacid (Trolox), Folin & Ciocalteu phenol reagent, sodium tetraborate decahydrate ($Na_2B_4O_7$), and cumene hydroperoxide were from Sigma-Aldrich (St. Louis, MO, USA). Potassium ferricyanide ($K_3[Fe(CN)_6]$), and L-serine, were purchased from Acros Organics (New Jersey, USA). Sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA), o-phthaldialdehyde (OPA), and dithiothreitol (DTT), were acquired from Thermo Fisher Scientific Inc. (Ottawa, ON, USA).

Unless otherwise specified, all chemicals and solvents were of analytical grade.

2.2. Preparation of sorghum protein hydrolysates

2.2.1. Defat of sorghum flour

The sorghum flour was extracted three times by mixing with twice volumes of chloroform to remove the oil and crude fat. The flour suspension was mixed on a magnetic stirrer at room temperature. The chloroform was then removed by paper-filtration with vacuum. The filtrate from the three extractant was combined and transferred to a rotary evaporator (IKA®, Wilmington, NC, USA) to recycle the chloroform. The total crude fat content was determined by measuring the weight difference of flask after distilling chloroform extractant. The flour residue and the flask containing residual fat were kept in a fume hood for 48 hours to evaporate the solvent and then stored in a zippered bag at -4 °C before use.

2.2.2. Kafirin extraction

Kafirin was isolated from the defatted sorghum flour according to the method described by Wang et al. (2009) with modifications. Defatted sorghum flour was presoaked in four volumes of 0.5% (w/w) sodium metabisulfite for 16 hours on a magnetic stirrer at 4 °C. The suspension was centrifuged (Avanti® J-E high-speed centrifuge, Beckman Coulter Inc., Brea, CA, USA) at 8200 xg, 4 °C for 10 minutes to remove the sodium metabisulfite solutions. The precipitated residue was transferred to another beaker and added with five volumes of glacial acetic acid and stirred for 1 hour at room temperature. After centrifugation at 8200 xg, 20 °C for 15 minutes, the supernatant was collected and adjusted to pH of 5.0 with 3 M NaOH in an ice water bath. The resulting suspension was left on benchtop overnight at 4 °C. After centrifuging at 8200 xg, 4 °C for 20 minutes, the precipitate was collected and rinsed with distilled water three times by centrifuging at 8200 xg for 10 min. The obtained protein was lyophilized using a

freeze-drier (Freezone 4.5, Labconco Corporation, Corneous City, MO. USA) and stored in zippered bags at -4 °C for later use. Glutelin can be further extracted from the flour residue by using the sodium borate/ sodium hydroxide buffer, which will not be discussed in detail here.

2.2.3. Enzymatic hydrolysis of kafirin

Extracted sorghum kafirin protein was dispersed into distilled water in a conical flask on a magnetic stirrer to form a protein suspension. pH of the suspension was adjusted in accordance with enzymes requirements with 1 N HCl and/or 1 M NaOH, and then, enzyme was added to the protein solution. The flask sealed with a rubber stopper was transferred to a water bath shaker (Shel Lab SWBR27, VWR International, LLC., Radnor PA, USA) and incubated with shaking force. Table 2-2 summarized the preliminary recommended reaction parameters for different enzymes used in this study. After reaching the predetermined hydrolysis time, the reaction was stopped by heating the reaction mixture in a boiling water bath for 15-20 minutes. After cooling down, the resulting solution was adjusted to pH of 7.0 with 1 M NaOH and centrifuged at 3500 xg, 4 °C for 25 minutes. The supernatant was collected and freeze-dried and was referred to as the protein hydrolysates. The precipitate was freeze-dried and weighed for calculation of total protein recovery.

2.3. Fractionation and identification of antioxidative peptides

2.3.1. Ultrafiltration with centrifugal tubes

Peptide fractions with different molecular size ranges were separated from the hydrolysate mixture through centrifugation by sequentially loading into Amicon® Ultra-15 Centrifugal Filter Devices (EMD Millipore Corporation, Billerica, MA, USA) at molecular weight cut-off of 3 kDa and 10 kDa. The processing time for 10 kDa and 3 kDa centrifugal filters is 23 minutes and 55 minutes, respectively, at 4 °C, 3500xg. Permeates and retentate with

different M_w were obtained as followed: < 3 kDa fraction, 3 – 10 kDa fraction, and > 10 kDa fraction. Along with the initial hydrolysates that did not go through ultrafiltration, the four fractions of hydrolysates were collected and lyophilized respectively for later analysis.

2.3.2. Gel filtration chromatography

The aforementioned hydrolysates (100 mg) prepared at optimized reaction conditions were dissolved in 3 mL deionized water and loaded onto a Sephadex G-25 (medium) gel filtration column (26 mm × 850 mm) which had been previously equilibrated with deionized water. A total portion of 600 mL deionized water was used to elute the sample, and aliquots of 3 mL were collected at a gravity-driven flow. The absorbance of collected fractions was measured at 280 nm to determine the elution profile. Fractions of eluent making up major peaks were combined into several fractions and lyophilized for analysis.

2.3.3. Identification of representative peptide sequences from gel filtration

2.3.3.1. Trypsin digestion and MALDI-TOF/TOF MS analysis of kafirin

Crude extracted kafirin was dissolved in 100 μL DTT (15 μg/mL) for 30 minutes at 80 °C. 100 μL iodoacetamide (18 μg/mL) was then added. The mixture was placed in the dark and incubated for 1 hour at room temperature. The proteins were then digested with 10 μL of trypsin (1 μg/30 μL, Trypsin Gold, mass spectrometry grade; Promega Corp., Madison, WI) overnight at 37 °C. Digested kafirin solutions were spotted in a 2,5-dihydroxybenzoic acid (DHB) matrix (Sigma-Aldrich, St. Louis, MO) on a Bruker Ultraflex III Matrix-Assisted Laser Desorption Ionization – Time of Flight/Time of Flight Mass Spectrometry (MALDI-TOF/TOF MS) (Bruker Daltonik GmbH, Bremen, Germany). Spectra were obtained in positive ion reflection mode at 66.7 Hz with 1000 laser shots per spectrum. Spectra were analyzed using FlexAnalysis (version 3.3, Bruker Daltonik GmbH) and internally calibrated with DHB matrix peaks. Known alpha,

beta, and gamma kafirin protein sequences were obtained from http://www.uniprot.org. Using mMass (http://www.mmass.org) software, these sequences were analyzed allowing for up to 4 missed cleavages, a peptide mass tolerance of 0.55 Da, and variable modifications of carbamidomethyl (C) and oxidation (W, M). The spectrum from the trypsin cleavage of the proteins was compared to the known theoretical sequence cleavage results.

2.3.3.2. HPLC peak collecting and sequence analysis of gel filtration sample

Samples of lyophilized fractions from gel filtration were analyzed using reverse phase high performance liquid chromatography (RP-HPLC) on a Beckman machine running 32 Karat (version 8.0) software with a C8 column (Buffer A: 99.9% water and 0.1% TFA; Solvent B: 90% Acetonitrile, 9.9% water, and 0.1% TFA) with a 10% to 50% Buffer B gradient over 30 minutes. Peaks from the RP-HPLC runs were manually collected and analyzed using the same MALDI-TOF/TOF MS procedure as described for crude kafirin. The spectra obtained were compared to beta kafirin protein sequences (http://www.uniprot.org). These protein sequences were cleaved using non-specific cleavage sites that resulted in peptides between 500 and 3,000 Da, and masses were compared to the spectra from the HPLC peaks using a peptide mass tolerance of 0.55 Da.

2.4. Evaluation of hydrolysis process

2.4.1. Total protein recovery

Total protein recovery was an indicator of the yield of hydrolyzed water-soluble peptides released from water-insoluble intact proteins. Total protein recovery was determined by the percentage of the hydrolysates to the initial protein by excluding the unhydrolyzed protein obtained through centrifuging the resulting reaction mixture:

$$\text{Total protein recovery} = \frac{W_i - W_p}{W_i} \times 100\%$$

where W_i was the weight of initial protein, and W_p was the weight of lyophilized precipitate from centrifuging the resulting mixture at the end of hydrolysis.

2.4.2. Degree of hydrolysis

Degree of hydrolysis (DH) is defined as the percentage of hydrolyzed peptide bonds to the number of total bonds per unit weight of the substrate protein, which is a typical indicator of the extent of hydrolysis degradation. The value of DH can also be used for comparison among different protein hydrolysates (Bougatef et al., 2010). DH in this study was determined using OPA method described by Nielsen, Petersen, & Dambmann (2001). Basically, OPA reagent was prepared by dissolving 7.62 g $Na_2B_4O_7$ and 200 mg SDS in 150 mL deionized water and then added with 160 mg OPA dissolved in 4 mL ethanol. 176 mg DTT was added to the above solution and the total volume was equilibrated to 200 mL with deionized water. The serine standard solution was prepared by dissolving 50 mg serine in 500 mL deionized water (0.9516 meqv/L). 400 µL of the hydrolysate sample at 1.2 mg/mL was added to 3 mL of OPA reagent and vigorously mixed for 5 seconds. The absorbance of the mixture was measured at 340 nm on a spectrophotometer (UV-6300PC, VWR International, LLC., Radnor PA, USA) after standing for exactly 2 minutes at room temperature. 400 µL of serine standard or deionized water instead of sample was measured using the same procedure and the means of triplicates denoted as standard and blank, respectively, were used for calculations. DH (%) was calculated as followed:

$$\mathrm{SerineNH_2} = \frac{\mathrm{A_{sample}} - \mathrm{A_{blank}}}{\mathrm{A_{standard}} - \mathrm{A_{blank}}} \times 0.9516 \,/\, (\mathrm{X} \times \mathrm{P})$$

where A_{sample} was the absorbance of sample, $A_{standard}$ was the absorbance of serine standard, A_{blank} was the absorbance of deionized water, X = 1.2 mg/mL sample concentration; P = 100% protein purity; $SerineNH_2$ represents meqv serine NH_2/g protein;

$$h = \frac{\text{SerineNH}_2 - \beta}{\alpha}$$

where α = 1, β = 0.4, h represents meqv/g protein; degree of hydrolysis was calculated as:

$$DH = \frac{h}{h_{tot}} \times 100\%$$

where h_{tot} = 8.3 mmol/g (Nielsen, Petersen, & Dambmann, 2001).

2.4.3. Total phenolic content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu procedure according to the method described by Thamnarathip et al. (2016). Generally, 1 mL 1:10 (v/v) Folin-Ciocalteu reagent and 3 mL 7.0% (w/w) Na_2CO_3 was sequentially added to 1 mL of sample solution at 1.0 mg/mL. The absorbance of the reaction mixture was measured at 760 nm after incubated for 30 minutes in darkness at room temperature. Distilled water was used as a blank control, and gallic acid was used in generation of a standard curve. The total phenolic content in sample was expressed as mg gallic acid equivalents per gram of sample (mg GAE/g).

2.5. Assessment of antioxidative activity

2.5.1. DPPH radical scavenging activity

DPPH radical scavenging activity (DPPH%) was determined by the percentage of decrease in DPPH radical concentration. Briefly, 0.02 mM DPPH reagent was prepared freshly by dissolving 7.88 mg of DPPH in 100 mL 95% (v/v) ethanol. 4.8 mL of sample dissolved in with deionized water at varied concentration (1 – 10 mg/mL) was mixed with equal volume of DPPH reagent. The reaction mixture was incubated in darkness for 30 minutes at room temperature before reading absorbance at 517 nm. Deionized water without sample was set as a blank control. DPPH is a free radical that has an intrinsic purple color which can be detected at 517 nm. The reduction of DPPH radicals by samples was expressed as percentage of decrease in

absorbance at 517 nm as compared to a blank control (Bougatef et al., 2010). DPPH radical scavenging activity was calculated as:

$$\text{DPPH\%} = \frac{A_b - A_s}{A_b} \times 100\%$$

where A_b was the absorbance of blank and A_s was the absorbance of sample.

2.5.2. Oxygen radical absorbance capacity

Oxygen radical absorbance capacity (ORAC) assay measures the effect of antioxidant on delaying the decline of fluorescence induced by a peroxyl radical generator, AAPH. The assay was performed according to a protocol described by Huang et al. (2002) using a Biotek® Synergy H1 Hybrid Microplate Reader (Winooski, VT, USA). Fluorescein was used as the fluorescent probe. Except for hydrolysate samples and Trolox standards, which were dissolved in deionized water, all other reagents were prepared with 75 mM phosphate buffer (pH = 7.4). 100 µL 6 nM fluorescein solution was added to 50 µL sample solution. The mixture was incubated at 37 °C for 30 minutes. Then, 50 µL 76.5 mM AAPH solution was added to the previous mixture. The fluorescence of the reaction mixture was recorded every minute for 2 h at 37 °C with excitation and emission wavelengths were 485 and 528 nm, respectively. Trolox was used as standards to generate a standard curve under the same experimental conditions. ORAC values for samples were expressed as gram of Trolox equivalent per gram of sample (g Trolox equiv./g) by comparing the relative area under the sample curve to the Trolox standard curve.

2.5.3. Ferric ion reducing power

The reducing power assay measures the ability of the antioxidant to reduce ferric ion to ferrous ion, which indicates the antioxidant's capacity in donating an electron or hydrogen (Xia et al., 2012; Duh, Tu, & Yen, 1999). Briefly, hydrolysates dissolved in 4 mL 0.2 M phosphate buffer (pH = 6.6) were added with 4 mL 1% (w/v) potassium ferricyanide ($K_3[Fe(CN)_6]$). The

mixture was incubated at 50 °C for 20 minutes, after which 4 mL of 10% (w/v) trichloroacetic acid was added. The reaction mixture was centrifuged at 3500 xg, 20 °C for 10-15 minutes and 4 mL of the supernatant was transferred. Finally, 4 mL of deionized water and 0.8 mL of 0.1% (w/v) ferric chloride were added to the reactant supernatant. After 10 minutes incubation at room temperature, the absorbance of the resultant mixture was measured at 700 nm. Distilled water was set as blank. A larger increased absorbance of the sample over blank indicates a stronger reducing power (Xia et al., 2012; Bougatef et al., 2010).

2.5.4. Metal chelating capacity

The metal chelating capacity was determined according to the method described by Decker and Welch (1990) with modifications. Generally, 1 mL of sample solution at different concentrations (1 – 10 mg/mL) was pre-mixed with 0.05 mL of 2 mM $FeCl_2$ solution, then, 2 mL of distilled water was added to the mixture and the solution was mixed vigorously on a Vortex mixer (Vortex-Genie 2, Scientific Industries, Inc., Bohemia NY, USA). Then, 0.1 mL ferrozine solution at 5 mM was added to the previous reaction mixture. The absorbance of the final solution was measured at 562 nm after 10 minutes' incubation at room temperature. Distilled water was used as a blank control. The metal chelating ability was calculated as:

$$\text{Metal Chelating } \% = \frac{A_c - A_s}{A_c} \times 100\%$$

where A_s and A_c represent the absorbance of sample and control, respectively (Jin et al., 2016)

2.5.5. Inhibition of lipid oxidation in an oil-in-water emulsion system

2.5.5.1. Emulsion preparation and incubation

The edible oils are easily oxidized during processing, storage, and cooking. An oil-in-water emulsion system is suitable to mutate the chemical, physical and environmental conditions

in real food products, and the lipid autoxidation and peroxidation can be therefore monitored. The oil-in-water emulsion samples were prepared according to the methods described by Cheng, Xiong & Chen (2010). Briefly, 250 mg and 500 mg of kafirin hydrolysates were suspended in 45 mL of 0.1 M phosphate buffer (pH = 7.0) in 100 mL cap-screwed bottles. The protein solutions were sequentially added with 5 mL soy oil and 0.45 mL Tween 20. The final concentrations of hydrolysates were 50 mg and 100 mg per mL of soy oil (50 mg/mL & 100 mg/mL), respectively. The mixture was blended with a homogenizer (PowerGen 700, Fisher Scientific Inc., Ottawa ON, USA) for 2 minutes followed by passing through a high-pressure microfluidizer (Microfluidics Corp, MA, USA) twice at 30,000 psi to obtain final fine emulsions. For comparison, a blank control containing all other reagents at same emulsifying conditions except for hydrolysates was also prepared. The three obtained emulsions (blank, 50 mg/mL, and 100 mg/mL) were transferred to 50 mL cap-screwed tubes and were incubated in a dark oven (Gravity Convection General Incubator, VWR International, LLC., Radnor PA, USA) set at 37 °C for autoxidation. A few drops of 3 mM sodium azide were added as a microbial preservative, and the oxidative stabilities were evaluated by measuring the accumulation of hydroperoxide values (POV) and thiobarbituric acid reactive substances (TBARS) at 0, 2, 4, 6, 8, 10, 12, and 14 days of incubation, respectively.

2.5.5.2. Emulsion turbidity and stability

The emulsion turbidity and stability were determined as described by Zhao el al. (2012) with slight modifications. 25 µL of the obtained fine emulsion was transferred to 7 mL of 0.1% SDS solution immediately after the emulsion was formed, and the absorbance of the mixture was monitored at 500 nm on a spectrophotometer. After 180 minutes, this value was monitored again using the same procedure. The absorbance at time 0 minute was interpreted as emulsion

turbidity. The emulsion stability was defined as the percentage of turbidity ratio of 180 minutes to 0 minute.

2.5.5.3. Lipid hydroperoxide values

Lipid hydroperoxide values (POV) were determined using a ferric thiocyanate method (Faraji, McClements, & Decker, 2004; Zhao el al., 2012). 0.3 mL of the incubated emulsion was added with 1.5 mL isooctane/2-propanol (3:1, v/v) and the slurry was vigorously swirled on a vortex three time for 10 seconds. The organic solvent phase (supernatant) was separated by centrifugation at 2000 xg for 2 minutes. 200 μL of the supernatant was transferred to 2.8 mL of methanol/1-butanol (2:1, v/v). Meanwhile, a ferrous chloride solution was freshly prepared by mixing equal amount of 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$ and excluding the precipitate through centrifugation at 3000 xg for 3 minutes. The ferrous chloride solution was mixed with equal amount of 3.97 M ammonium thiocyanate, and 30 μL of this mixture was added to the methanol/1-butanol solution containing emulsion extractant. The absorbance of the resulting mixture was monitored at 510 nm after 20 minutes of incubation at room temperature on spectrophotometer. POV was determined using a standard curve made from cumene hydroperoxide and was expressed as mM cumene hydroperoxide equivalent. The inhibition rate of emulsion sample added with hydrolysates was calculated by percentage decrease in POV concentration from blank control.

2.5.5.4. Thiobarbituric acid reactive substances

The accumulation of secondary reaction products thiobarbituric acid reactive substances (TBARS) was determined by mixing 0.3 mL of the incubated emulsion sample with 0.7 mL of deionized water and 2 mL of TBA reagent containing 15 g of trichloroacetic acid (TCA), 0.375 g of 2-thiobarbituric acid (TBA), and 1.76 mL of 12 N HCl in 82.9 mL of distilled water. The

mixture was heated in a boiling water bath for 15 minutes and then cooled to room temperature for 10 minutes in a cool water bath. After cooled down, the mixture was centrifuged at 3500 xg for 15 minutes and the absorbance of upper layer was determined at 532 nm after 10 minutes standing at room temperature. The concentration of TBARS was calculated against a standard curve prepared with 1,1,3,3-tetramethoxypropane and expressed as μM tetramethoxypropan equivalent. The inhibition rate of emulsion sample added with hydrolysates was calculated by percentage decrease in TBARS concentration from blank control.

2.5.6. Inhibition of lipid oxidation in a meat system

2.5.6.1. Meat preparation

The kafirin hydrolysates fraction from ultrafiltration exhibited stronger antioxidative activities in previous assays were further evaluated for their performances against lipid peroxidation in a ground pork model system according to a protocol described by Zhang et al. (2010). Fresh ground pork was purchased from Dillion's and was stored at 4 °C before use. Kafirin hydrolysates were dispersed in 5 mL distilled water and then transferred to 50 g of ground pork to reach final concentrations of 0.5 mg and 1.0 mg hydrolysate per gram of meat. The homogenized samples were added with a few drops of 3 mM sodium azide before transferred to 100 mL glass bottles with screwed caps and centrifuged at 100 xg, 4 °C for 10 minutes to eliminate trapped air. The meat samples were stored at 4 °C refrigerator until analysis. Distilled water was incorporated to the ground meat as a blank control, and rosemary extracts at 0.5 mg/g was used as a positive control. The extent of lipid peroxidation was quantified by measuring TBARS on 0, 1, 2, 4, 6, 8, 10, and 12 days during incubation period.

2.5.6.2. Thiobarbituric acid reactive substances

The concentration of secondary reaction products thiobarbituric acid reactive substances (TBARS) was measured as an indicator of lipid peroxidation extent. Briefly, the meat sample at certain days of incubation was gently blended with a glass rod before weighting out 5 g of sample. The sample was added with 50ml of distilled water, 0.1 mL of 10% sodium dodecyl sulfate (SDS) solution, and 10 mL of reducing solution containing 0.01% propyl gallate and 0.02% ethylenediaminetetraacetic acid (EDTA). The meat sample containing above reagents was thoroughly homogenized in a blender (Oster®, Boca Raton, FL, USA) at high speed for 60 seconds. 1 mL of the resulting homogenate, in triplicate, was immediately transferred into a 15 mL centrifuge tube and was added with 4 mL of TBA buffer containing 0.4% TBA, 0.5% SDS, and 9.3% acetic acid. The mixture was vigorously stirred on a vortex for 15 seconds and then heated in a boiling water bath for 60 min. After cooling, 5 mL of pyridine/n-butanol (1:15, v/v) was added and mixed on a vortex. After centrifugation at 3500 xg, 4 °C for 15 minutes, the upper organic layer was collected and monitored for absorbance at 532 nm on a spectrophotometer. 1,1,3,3-teramethoxypropane (TMP) solutions were prepared at 0.0, 2.5, 5.0, 7.5, and 10.0 μM to generate a standard curve, and the coefficient was used to convert absorbance values into TBARS (Duh, Tu, & Yen, 1999). The final TBARS value was expressed as mg malonaldehyde equivalents per kg of sample (mg MDA equiv./kg). The inhibition rate of meat sample added with hydrolysates or rosemary extract was calculated by percentage decrease in TBARS concentration from blank control.

2.6. Statistical analysis

The data were analyzed using SAS software version 9.3 (SAS Institute, Cary NC, USA). Results were evaluated by one-way analysis of variance (ANOVA). Tukey's post-hoc test was

used to assess the significant differences among individual data set. The results were illustrated as means ± standard deviation (n = 3) and were considered as significant at $P < 0.05$.

3. Results and Discussion

3.1. Kafirin extraction

The extracted kafirin obtained from glacial acetic acid extractant along with other protein samples from fractional extraction was analyzed for their protein content using nitrogen combustion analysis with LECO FP-2000 nitrogen analyzer (St. Joseph, MI) (Table 2-1). The protein content was calculated based on nitrogen content using a conversion factor of 6.25. The total protein content of the white sorghum flour was 9.33%, which fell into the protein content range of 7 – 15% reported by Waniska and Rooney (2000). The defatted sorghum flour was found to have a lower protein content than original flour as 8.27%, which might be due to the partial loss of protein removed by the organic solvent during defatting. Crude kafirin obtained from different batches of extraction were combined and had a protein content of 97.31%. This result is very close to that reported by Wang et al. (2009) using the same extraction method (98.94%). The end-up extraction rate of kafirin from the sorghum flour was 4.95 ± 0.111% on a weight basis. The total crude fat content was determined to be 0.5125% on a weight basis percentage.

3.2. Reaction optimization of kafirin enzymatic hydrolysis

Enzyme, protein substrate, and the hydrolysis conditions (time, pH, temperature, etc.) altogether imposed a synthetic impact on the yield and bioactivity of protein hydrolysates. Five bacterial originated enzymes, two animal originated enzyme and three plant originated enzymes were preliminary screened in obtaining antioxidative hydrolysates. Further, the substrate content,

enzyme-to-substrate ratio, and hydrolysis time were optimized individually using single factor methodology.

Total protein recovery rate and degree of hydrolysis (DH) were measured as key indicators in evaluating the total yield of the water-soluble hydrolysates as well as the production of short-chained polypeptides. DPPH radical scavenging activity assay has been widely used in evaluating the antioxidant capacities, which can be used as a primary screening method in evaluating the abilities of hydrolysates to act as electron donors in antioxidative performances (Wang et al., 2007).

3.2.1. Enzyme screening

The type of enzyme applied to the substrate plays a dominant role in determination of the properties of hydrolysates. Due to the specificity of enzyme, different cleavage positions on polypeptides chain resulted in uniqueness of hydrolysates in amino acid sequences, peptide lengths, structural characteristics that influencing the antioxidative properties of hydrolysates.

Five bacterial originated enzymes Alcalase, Flavourzyme, Neutrase, Everlase, and Protamex were primarily selected to hydrolyze kafirin at substrate content of 2%, enzyme-to-substrate ratio of 0.4 Anson Units per gram of protein (Au/g). The hydrolysis performed with each enzyme was carried out at the optimal pH and temperature conditions (Table 2-2). The reaction was stopped after 5 hours by deactivating enzyme at 92 °C for 15 – 20 minutes.

Figure 2-1 showed the protein recovery yield, DH, and DPPH% of the hydrolysates obtained by treatment with five different bacterial originated enzymes. Protein recovery percentage is the indicator of total yield of the water-soluble peptides, which was obtained by centrifuging the resulting reactant mixture and collecting the supernatant, which was freeze-dried and was generally regarded as hydrolysates. The protein recovery percentage tended to increase

with prolonged hydrolysis time. Hydrolysate prepared with Alcalase, Neutrase, and Protamex led to higher yield, while Flavorzyme and Everlase resulted in extremely low yield, which was due to the different of protease. For example, endo-protease nature of Neutrase that it randomly cleaves the internal peptide bonds. During hydrolysis, the molecular weight of protein was decreased to short-chained peptides with more charged groups (NH_3^+ and COO^-) exposed through cleavage of peptide linkage, which improve the solubility and the consequent recovery yield (Thamnarathip et al., 2016).

DH indicates the percentage of cleaved peptide bonds to total peptide bonds and is associated with lots of properties including peptide size, amino acid composition, and biological activities of peptides. Thus, it is important to control the DH for preparation of reproducible hydrolysates with desired functional and biological properties. Hydrolysates prepared with Flavourzyme had the highest DH (25.88 ± 0.21%) and hydrolysates prepared with Everlase had the lowest DH (12.17 ± 0.01%) for 5 hours of hydrolysis. Flavourzyme is a fungal protease complex that contains both endo-peptidase and exo-peptidase, which might be responsible for the higher DH compared to other endo-protease only proteases (Thamnarathip et al., 2016).

The ability of an antioxidant in donating electron to free radicals and further interrupt the radical mediated lipid chain oxidation is an important indicator of antioxidant capacity (Xia et al., 2012). DPPH is a radical that was commonly used to imitate the free radicals present in biological tissue and therefore used to evaluate the electron donating ability of a sample. All five kafirin hydrolysates exhibited some extent of DPPH radical scavenging abilities, which indicated that some peptides within the hydrolysates were potential radical scavengers. Hydrolysates prepared with Neutrase and hydrolyzed for 21 hours exhibited the highest radical scavenging activity (42.57 ± 2.13%), followed with hydrolysates prepared with Everlase (39.35 ± 1.92) and

Flavourzyme (31.25 ± 2.00%). The activities of unhydrolyzed kafirin was not tested as it formed a turbid solution even at low concentration due to low solubility in water.

Trypsin and pepsin are animal-originated enzymes which were typically used to hydrolyze animal proteins. It was found that kafirin hydrolyzed with pepsin and trypsin had relatively lower protein recovery rates as compared to microbial-originated proteases (Figure 2-2). Especially for hydrolysate prepared with trypsin, it was observed that with ten times of enlargement in enzyme use, no significant improvement on protein recovery was detected. The increased amount of trypsin also largely decreased the DPPH% of hydrolysates. Alashi et al. (2014) also found that canola meal proteins hydrolyzed with pepsin has a low yield compared to that of several other enzymes. This might be imputable to the specificity nature of the pepsin as it mainly breaks the peptide bonds in macro-proteins between hydrophobic and cleaves aromatic amino acids (Alashi et al., 2014). Meanwhile, trypsin not only hydrolyzed the peptide chains mainly at the carboxyl side of the amino acid into oligopeptides, but they also produced more free amino acids due to its greater hydrolysis activities (Phongthai et al., 2018). Thus, these specific properties might lower their yield of kafirin hydrolysate.

Bromelain, Ficin, and Papain are plant-originated protein enzymes. Due to the inconsistency of enzyme units, the efficacy of different enzyme was compared on a weight basis given the same time of hydrolysis (Figure 2-3). It was found that Papain purchased from EMD yielded highest protein recovery rate with minimum amount of enzyme applied. The DPPH% of hydrolysate prepared with Papain EMD were also competitive. Thus, it was selected for future study as described in Chapter 4.

The results above demonstrated that type of enzyme used is a key factor in determining total yield and antioxidative activities of resulting hydrolysates due to the specificity of enzyme.

Overall, microbial-originated and plant-originated proteases are more preferred than animal-originated enzymes. Microbial-originated proteases are most efficient regarding total protein recovery with minimal amount of incorporation. Hydrolysates prepared with Neutrase possessed a good balance in total protein recovery, degree of hydrolysis, and antioxidative activities compared to those prepared with other enzymes. Thus, Neutrase was primarily selected as a promising enzyme for future study in reaction optimization and isolation for antioxidant peptides.

3.2.2. Protein content

In order to study the effect of substrate content on the yield of hydrolysates and their activities, kafirin was hydrolyzed with Neutrase at different protein content from 2% to 10% while keeping the other parameters constant. The results in Figure 2-4 clearly specified that the total soluble protein recovered from hydrolysis persistently decreased (from 95.56% to 71.1%) as protein content increased from 2% to 10%. Degree of hydrolysis initially increased from 16.59 ± 0.91% to 17.84 ± 0.24% when protein content increased from 2% to 4%. However, after reaching the maximum value at 4% protein content, DH decreased afterwards. Similarly, DPPH% initially increased and then decreased after reached its maximum at 4%. Zheng et al. (2006) conveyed that more intensive hydrolysis was observed in more diluted suspensions for enzymatic hydrolysates of corn gluten. Surowka, & Fik (1994) detected an increased reaction homogeneity and proteolysis rate of the diluted reaction mixture when studying the recovery of proteinaceous substances.

It was assumed that initially the substrate content in the hydrolysis system was too low, and the probability of collisions between the substrate and protease was limited due to lack of substrate, therefore the progress of hydrolysis was slow. Followed with an increasing amount of

substrate, the overall hydrolysis was accelerated till the system reached its opportune saturation. As the reaction system was getting much more concentrated, excessive amount of substrate took up reaction space and reduced the availability of enzyme-to-substrate proteins, and the diffusion motions of the protease was also inhibited. Moreover, the enzymes activity was restricted. Thus, the reaction process was limited in the reaction system.

Considering a good balance in total soluble protein recovery and antioxidant activity, protein concentration of 4% was determined as the optimum substrate content for future experiments.

3.2.3. Enzyme-to-substrate ratio

Enzymes used same units as the enzyme standard, Anson Units (Au). The effects of enzyme-to-substrate ratio Au per gram of protein (Au/g) on the peptide yield and antioxidant activity of kafirin hydrolysates prepared with Neutrase was studied. Figure 2-5 showed the total protein recovery, degree of hydrolysis, and DPPH% of the kafirin hydrolysates prepared with Neutrase. The ratio was gradually elevated from 0.2 Au/g to 2.0 Au/g while keeping all other reaction conditions constant. It can be seen that, both total protein recovery rate and DH increased along with the increased enzyme-to-substrate ratio from 0.2 – 0.6 Au/g. Later after, DH began to decrease, and protein recovery kept plateau at around 90% as the enzyme amount continued to increase. The DPPH% of the resulting hydrolysates had a similar trend with DH as it reached maximum at 0.4 – 0.6 Au/g and decreased afterwards. It can be concluded that at exceptional high enzyme levels, the protein available to be hydrolyzed was the limiting factor (Zhang et al., 2012), and the additional addition of enzyme will be unnecessary for improvement of protein recovery. The experimental results showed that 0.4 Au/g was the optimum enzyme-to-

substrate ratio to produce hydrolysates with fairly high antioxidant activity. Therefore, 0.4 Au/g was used for all future experiments.

3.2.4. Hydrolysis time

Reaction time has a crucial impact on enzymatic hydrolysis process. It directly affected the degree of hydrolysis and the yield of water-soluble hydrolysates. Furthermore, the composition and protein structure of the hydrolysates were also influenced.

Kafirin was hydrolyzed with Neutrase at enzyme-to-substrate ratio 0.4 Au/g, substrate concentration 4% with varied hydrolysis time and the results were illustrated in Figure 2-6. Given an elongated hydrolysis time, both DH and total protein recovery continuously increased, which indicated that peptide bonds had been cleaved during the hydrolysis, and protein started to break down into fragments, producing more short chain peptides that are inclined to be water-soluble. After the reaction reached the steady state phase, no apparent increase in total protein recovery was observed. It was assumed that, at initial stage when both substrate concentration and the enzyme activity were high, the protein was hydrolyzed at a higher rate (Zhang et al., 2012). As the hydrolysis progressed, the rate of hydrolysis decreased subsequently due to the reduced enzyme activity and the decreased substrate concentration.

Tang and Zhuang (2014) claimed that, higher DH of hydrolysates usually associated with stronger antioxidative activities. However, a prolonged hydrolysis time or a higher DH did not necessarily produce a higher antioxidant activity in this study. As shown in Figure 2-6 (C), DPPH% of the hydrolysates gradually increased until it reached its maximum values and then decreased as hydrolysis time extended. Zheng et al. (2006) also reported that the antioxidant activity of corn gluten hydrolysate increased between hydrolysis time of 30 minutes to 120 minutes, and then decreased from 120 minutes to 150 minutes. Thus, the hydrolysis time is a key

factor in producing antioxidative peptides as specific structure and critical peptide size may be necessary to manifest a certain antioxidant activity. The longer time the substrate exposed to enzymes, the more excessive hydrolysis could possibly have occurred. Unnecessary excessive hydrolysis produced short peptides and amino acids that had lost the essential structure accounting for the antioxidative activities thus exhibited a decreased DPPH%. This was supported by both Xia et al. (2012) and Thamnarathip et al. (2016), who found that excessive hydrolysis resulted in a decreased antioxidant activity in their studies. Besides, the transformation of peptide structure could be another reason causing DPPH% decreased (Samaranayaka & Li-Chan, 2011)

Considering both total protein recovery as well as hydrolysate antioxidant activity, 17 hours of hydrolysis time was determined as an optimal reaction time for later experiments.

3.3. Ultrafiltration of kafirin Neutrase hydrolysates

Molecular weight distribution is one of the most important properties in determination of the functionality and bioactivity of hydrolysates. Kafirin hydrolysate prepared with Neutrase at optimal conditions (protein content of 4%, enzyme-to-substrate ratio of 0.4 Au/g and hydrolyzed for 17 hours) were sequentially subjected to Amicon Ultra-15 Centrifugal Filter Devices with 10 kDa and 3 kDa molecular weight cut-off membranes to categorize the distribution of hydrolysates based on molecular weight, and to study the relationship between peptide size and their antioxidant activities. As a result, permeate and retentate fractions with different molecular weight range were obtained as followed: the small-sized peptide fraction with M_w lower than 3 kDa fraction (denoted as < 3 k), the medium-sized peptide fraction with M_w between 3 kDa and 10 kDa (denoted as 3 – 10 k), and large-sized peptide fraction with M_w exceeding 10 kDa fraction (denoted as > 10 k). The proportional distribution of these three fractions were shown in

Figure 2-7. As it can be seen, smaller-sized peptide fraction (< 3 k) was the largest portion which took up about 79.77% followed with medium-sized peptide fraction (3 – 10 k) accounted for 12.58% and large-sized peptide fraction (> 10 k) accounted for 7.65%. It was speculated that, prolonged hydrolysis time provided adequate digestion of protein into short-chained peptides, which can also be perceived by a relatively higher degree of hydrolysis and total protein recovery. The released peptides might be divided into even shorter oligopeptides or free amino acids as hydrolysis proceeded. Thus, majority of hydrolysates fell into smaller-sized fraction. Since the proportional distribution of ultrafiltrated hydrolysates was not commonly reported, there were limited literatures for comparison

The three fractions from ultrafiltration along with the original hydrolysates without going through ultrafiltration (denoted as Mix) were analyzed for their total phenolic content and antioxidant activities as shown in Figure 2-8 (A-E).

The total phenolic content (TPC) was measured for sorghum flour, extracted kafirin, and kafirin hydrolysates along with its ultrafiltration fractions by using the Folin-Ciocalteu method. The results were expressed as gallic acid equivalent per gram of sample (GAE/g) as shown in Figure 2-8 (A). Among the four fractions of hydrolysates, 3 – 10 kDa fraction possessed highest TPC value (40.59 ± 0.86 mg GAE/g), followed with > 10 kDa fraction of 39.01 ± 0.68 mg GAE/g, and < 3 kDa the lowest value (30.76 ± 2.47 mg GAE/g). The TPC values of 3 – 10 kDa and > 10 kDa fractions are both significantly higher than Mix ($P < 0.05$), which indicated that process ultrafiltration concentrated the TPC in these fractions. However, the difference between 3 – 10 kDa and > 10 kDa is not significant. The TPC values of kafirin hydrolysates are all significantly higher than that of sorghum flour (2.68 ± 0.24 GAE/g) and sorghum protein kafirin (12.47 ± 2.15 mg GAE/g). This result revealed that sorghum protein kafirin contains

concentrated phenolic content within sorghum flour, and the hydrolysis process dramatically increased the total phenolic content of sorghum kafirin by releasing the peptides with phenolic amino acid residues and other free phenolic compounds. Liu et al. (2017) also found that TPC continuously increased throughout the hydrolysis process of wheat germ and reached a final increase of 147.3%. Compared to the unhydrolyzed protein, a higher quantity of free phenolic and a lower quantity of bound phenolic in the hydrolysate was also observed, which lead to the authors' conclusion that hydrolysis changed the mode of polyphenol protein interaction and converted some protein-bound polyphenols into free polyphenols. Thus, phenolic compounds could be released during enzymatic hydrolysis (Thamnarathip et al., 2016), which contributed to a higher TPC. It is important to note that, TPC may be overestimated due to the interference of other products that could react with Folin-Ciocalteu reagent such as nucleic acids (Anunciação et al., 2017).

Free radical scavenging activity of proteins or peptides is closely related to the molecular weight (Tang & Zhuang, 2014). DPPH% was measured for the kafirin ultrafiltrated hydrolysates to compare their difference in electron donating activities (Xia et al., 2012) as shown in Figure 2-8 (B). It was found that, 3 – 10 kDa and > 10 kDa fractions exhibited higher DPPH% than other hydrolysate fractions at varied substrate concentrations (1 – 10 mg/mL). Meanwhile, < 3 kDa fraction exhibited the lowest activity. This result indicated that the medium-sized hydrolysate fraction, specifically, 3 – 10 kDa fraction possessed stronger potential to act as electron donors in quenching free radicals.

The ferric iron reducing power assay (FRAP) measured the ability of an antioxidant to reduce Fe^{3+} to Fe^{2+}, which was often used as an indicator of electron-donating or hydrogen-donating abilities (Xia et al., 2012). The formation of Fe^{2+} complex was monitored using Perl's

Prussian blue at 700 nm wavelength absorbance. Thus, an increased absorbance at 700 nm indicated a higher reducing power. Many reports had revealed a positive correlation between antioxidant activities and reducing power. As shown in Figure 2-8 (C), the 3 – 10 kDa fraction exhibited the strongest reducing power ability with the highest absorbance value (0.321 ± 0.029) followed with > 10 kDa fraction (0.286 ± 0.010), and the < 3 kDa fraction had the lowest absorbance (0.201 ± 0.006) among the four peptide samples. All the four ultrafiltrated hydrolysate fractions exhibited significantly ($P < 0.05$) higher absorbance over the blank control (0.128 ± 0.010). This result could serve as the evidence that the antioxidative peptides act as electron-donors and result in reduction of Fe^{3+} to Fe^{2+}. The medium-sized peptide fraction (3 – 10 kDa) was the most active fraction in regards of the electron-donating ability. Xia et al. (2012) studied the antioxidant activity of barley glutelin hydrolysates, the authors also found that the larger molecular sized hydrolysates (> 10 kDa) exhibited strongest reducing power than 1-10 kDa and < 1 kDa fractions.

ORAC assay follows a hydrogen atom transfer mechanism where the H of the peptides neutralizes the radicals and breaks the chain reactions of thermally generated peroxyl radical from AAPH. Therefore, ORAC evaluated the hydrogen donating capacity of the antioxidant. The small-sized fraction of peptides had a significant lower ($P < 0.05$) ORAC values than the other ultrafiltrated fractions of kafirin Neutrase hydrolysates (Figure 2-8 (D)). Besides, all hydrolysate fraction had a relatively high ORAC value (> 0.4 g Trolox equiv./g) compared with other previously reported values. This data revealed the fact that the antioxidant peptides are excellent proton donors to quench radicals as counterpart of their antioxidant profile.

Transition metal ions such as Fe^{2+} and Cu^{2+} are key catalyst in generation of oxygen species that lead to oxidation of unsaturated fat and could cause physiological damages to

biomolecules and subsequent aging, cancer and diseases (Liu et al., 2017; Tang & Zhuang, 2014; Duh, Tu, & Yen, 1999). Especially, Fe^{2+} generates highly reactive and destructive hydroxyl radicals by Fenton reaction and accelerate the lipid peroxidation chain reaction (Tang & Zhuang, 2014). Thus, it is necessary to measure the chelating ability of the compound for evaluating its antioxidative activities. Figure 2-8 (E) showed the metal chelating ability of kafirin Neutrase hydrolysate fractions at different concentrations. All the hydrolysates fractions exhibited metal chelating abilities, and the ability was enhanced as the antioxidant dosage increased. 3 – 10 kDa fraction presented exceptional higher chelating capacity (24.29 ± 0.18 % at 5 mg/mL) ($P < 0.05$) than the other three fractions (11.20 – 12.68% at 5 mg/mL). This result indicated that kafirin hydrolysates prepared with Neutrase had iron binding capacity which contributed to its activity as an antioxidant. The chelation capacity may be attributed to the exposure of more acidic and basic amino acids during the process of hydrolysis as the carboxyl and amino groups in their side chains can bind Fe^{2+}, and this ability may also contribute to their hydroxyl radical scavenging effects of antioxidants due to the combined effects (Xia et al., 2012).

Some studies indicated that short-chained peptides fraction with smaller M_w are more efficient antioxidants (Jin et al., 2016; Liu et al., 2015; Wang et al., 2014; Agrawal et al., 2017; Tang & Zhuang, 2014; Alashi et al., 2014; Zhuang et al., 2013), as they are more accessible to the oxidant agents. However, other studies indicated that medium- or large-sized peptides were more active (Phongthai et al., 2018; Wang et al., 2014; Park et al., 2001). In this study, the medium-sized peptide fraction was found to be more effective. It was speculated that, the hydrolysates permeated through 3 kDa membrane yielded much small-sized peptides and free amino acids that eventually decreased the overall activity of < 3 kDa fraction. It was also explained previously that excessive hydrolysis results in breakage of essential structures

including peptide sizes and specific amino acid sequences that were responsible for the radical scavenging activities.

In addition, it was found that TPC was positively correlated to antioxidant activity measured by DPPH% (R^2 = 0.8807) and reducing power (R^2 = 0.9804), which was in accordance with previous findings of Liu et al. (2017) and Thamnarathip et al. (2016). Liu et al. (2017) determined that both TPC (R^2 = 0.972) and peptide content (R^2 = 0.937) had a strong positive correlation with and antioxidant activity of wheat germ hydrolysates measured by DPPH%. Thamnarathip et al. (2016) reported that the ABTS radical scavenging activity of rice bran protein hydrolysate was coincidental with TPC with positive correlation (coefficient R=0.7). However, others reported that there were no correlations between TPC and DPPH% (Zengin et al., 2017; Đorđević et al., 2010). Phenolic compound was not the only factor in determining the overall antioxidant activity of the hydrolysates, instead, peptides and phenolic compounds released during hydrolysis both contributed to the antioxidant activity. Throughout the hydrolysis, the mode of polyphenol-peptide interaction was changed and free phenolics were released from bound-phenolics (Liu et al., 2017). The phenolic content contributed to the radical scavenging activity via hydrogen atom donation activity of hydroxyl group in phenolic compounds (Thamnarathip et al., 2016). Meantime, amino acids with a phenolic hydroxyl group in peptide sequence may also be responsible for radical scavenging activity (Thamnarathip et al., 2016). It was proposed by Liu et al. (2017) that the antioxidant capacity was also dependent on the interaction of peptide and phenolic content at different stages of hydrolysis. At early stage of hydrolysis, the increase of peptide content might be dominant in the increase of antioxidant activity; however, as hydrolysis proceeded, the peptides were digested into free amino acids resulting in a decreased activity. The phenolic content released for the late stage were more

responsible for the activity. Therefore, the medium-sized (3 – 10 kDa) hydrolysates fraction with a concentrated phenolic content demonstrated higher antioxidant activities as revealed by DPPH%, reducing power, and metal chelating%.

In general, ultrafiltration is an effective approach to fractionate and isolate peptide fraction from hydrolysate mixture based on molecular weight distribution. The isolated peptide fraction with medium molecular range (3 – 10 kDa) exhibited stronger antioxidative activities. The antioxidative capacity of hydrolysates could be possibly utilized to reduce the oxidized intermediates in lipid peroxidation process, and therefore, act as primary and secondary antioxidants (Liu et al., 2017).

3.4. Inhibition of lipid oxidation in model systems

3.4.1. Oil-in-water emulsion system

The ability of antioxidants to inhibit lipid oxidation in food emulsions depends on a lot of factors such as reactivity and concentration of antioxidants; environmental conditions including pH, ionic strengths, and temperature; and partitioning between oil, water and interfacial phases (Zhao et al., 2012). An oil-in-water emulsion model system was utilized to mimic the chemical, physical and environmental conditions in real food products. 3 – 10 kDa fraction kafirin Neutrase hydrolysate at 50 mg and 100 mg per mL of soy oil was incorporated to prepare emulsion samples. According to the preliminary experiments results of Cheng, Xiong & Chen (2010) and Zhao et al. (2012), emulsion prepared with hydrolysates as the sole emulsifier did not reach steady state even at high concentrations, which indicated that protein hydrolysate was not an ideal emulsifier on its own. Thus, Tween-20 was incorporated as an additional stabilizer to enhance the emulsion stability. The physical properties of the emulsion samples were characterized by measuring their turbidity and stability. The degrees of lipid autoxidation and

peroxidation were determined by quantifying the concentrations of lipid peroxide (POV) and thiobarbituric acid reactive substances (TBARS) as primary and secondary lipid oxidation products, respectively (Cheng, Xiong & Chen, 2010). The results of emulsion samples incorporated with hydrolysates were compared to that of blank control during the incubation period at 37 °C until 14 days.

As shown in Figure 2-9 (A), with hydrolysate incorporated at 50 mg/mL ($A_{500} = 0.83 \pm 0.03$) and 100 mg/mL ($A_{500} = 0.80 \pm 0.03$), both emulsion sample had a lower turbidity than that of blank control ($A_{500} = 0.91 \pm 0.02$). However, the high turbidity values ($A_{500} > 0.80$) observed in all emulsion samples indicated that the soy oil was effectively dispersed. Meantime, the addition of hydrolysates slightly increased emulsion stability as determined by changes in emulsion turbidity. This was probably due to the adsorption action of hydrolysates resulted in a better dispersion of oil droplets. From this point, the addition of hydrolysate enhanced the overall emulsion stability. On the other hand, the emulsion samples with hydrolysate incorporated had a less tendency to stay stable during the extended storage time till 14 days, and with more hydrolysate added, the less stable it became. This finding was in agreement with Cheng, Xiong & Chen (2010). It was interpreted that a competitive adsorption at the interface which favored hydrolysates at high concentrations.

Most importantly, the presence of kafirin hydrolysates decreased both POV and TBARS in the entire incubation period as compared to the control. After 14 days of incubation at 37 °C, the emulsion sample with addition of 50 mg hydrolysate per mL of soy oil exhibited an average inhibition the formation of POV and TBARS by 77.14 ± 13.81% and 54.34 ± 9.78%, respectively. The emulsion sample with increased concentration of hydrolysate at 100 mg/mL

displayed a slight but not substantial enhancement of POV and TBARS inhibition, which were 76.41 ± 13.81% and 59.91 ± 13.51%, respectively.

The inhibition efficacy of kafirin hydrolysate in this study was higher than that of potato protein prepared at same concentration reported by Cheng, Xiong & Chen (2010). This result provided important evidence clarifying the antioxidative effects of kafirin hydrolysate in stabilizing the oil-in-water emulsions by reducing the formation of lipid hydroperoxide and TBARS. This activity might be associated with the free radical scavenging activities and pro-oxidative metal chelating abilities of the kafirin hydrolysates (Cheng, Xiong & Chen, 2010). Besides, in food emulsion systems due to the amphiphilicity of proteins and peptides, the hydrolysates can diffuse to the water-oil interface and act as surfactants to adsorb or loosely bind to oil droplets and form a physical barrier to hinder the access of oxidizing agents thus contribute to reduced lipid peroxidation (Samaranayaka & Li-Chan, 2011; Kong & Xiong, 2006). The protein emulsifiers can also prevent oil droplets from coalescing whilst protecting the interior of oil droplets from oxidation (Zhao et al., 2012).

3.4.2. Ground meat system

The ground meat is easily susceptible to oxidative process due to the grinding process disrupts the muscle membrane systems and therefore exposes unsaturated fatty acids and proteins to oxidative agents such as molecular oxygen, oxidative enzymes, heme compounds, and metal prooxidants (Schilling et al., 2018). The oxidation of lipid does not directly result in rancidity until fatty acids are decomposed to low M_w volatile compounds know as secondary lipid oxidation products. These products adversely affect the sensory quality of foods perceived as off-flavors and aroma. In order to evaluate the inhibiting effects of peptide antioxidants against lipid and/or fat peroxidation, 3 – 10 kDa fraction of kafirin hydrolysate prepared with Neutrase at

optimized conditions was incorporated with ground pork at 0.5 mg and 1.0 mg hydrolysate per gram of meat. The oxidative stability of meat samples was determined by quantifying their secondary oxidative products by measuring TBARS at different days during incubation at 4 °C and compared to a blank control and a positive control prepared with rosemary extract at 0.5 mg/g.

The results in Figure 2-10 clearly indicated the trend that meat peroxidation gradually proceeded along with days of incubation as reflected by the continuous increase of TBARS values. On day 0 right after the meat samples were prepared, all the meat samples showed close values of TBARS. From day 1, TBARS of meat samples with addition of hydrolysates started to become lower than that of control, and the trend of inhibition is obvious with prolonged incubation time. At the end of incubation, TBARS of meat sample was inhibited by 35.34% and 43.17% with hydrolysates incorporated at 0.5 mg/g and 1.0 mg/g, respectively. The average inhibition rate was 24.16 ± 10.14% and 30.71 ± 9.26% during the 12-day storage period. This result indicated that the addition of hydrolysates lowered amount of lipid peroxide generated, which provides important evidence showing the inhibition effect of kafirin hydrolysates against lipid peroxidation of ground pork. Rosemary extract is a well-known natural antioxidant found to exhibit inhibition on lipid oxidation at low concentrations of 100-2500 ppm (Sebranek et al, 2005). The inhibition effects of peptide antioxidants are not as strong as rosemary extract, which could be due to the impurity of hydrolysates. Thus, further fractionation to isolate critical peptide sequences responsible for the activities is needed.

3.5. Purification and identification of antioxidative peptides from kafirin 3 – 10 kDa Neutrase hydrolysates

3.5.1. Gel filtration of kafirin Neutrase 3 – 10 kDa hydrolysates

In order to further fractionate the antioxidative peptides according to its molecular weight profile, 100 mg of 3 – 10 kDa Neutrase kafirin hydrolysates prepared at optimized conditions (17 hours, 0.4 Au/g, 4%) which showed highest antioxidant activity from previous results was loaded onto a Sephadex-G25 column for gel filtration chromatography. From the elution profile, three peaks appeared, and the entire eluent were dived into four major fractions (F1 – F4) for analysis of their total phenolic content and antioxidant activities (Figure 2-12). Overall, F1 represented the fraction of peptides that had larger molelar sizes and F4 had the smallest sizes as the larger molecules were eluted first and smaller molecules are retained longer by the gel medium. Fraction 2 exhibited significant higher value of TPC as well as DPPH% than other three fractions, thus was collected for peptide sequences identification. It was notable that, F4 possessed a negative DPPH% which might be due to its relatively higher salt content as well as other prooxidant components.

3.5.2. Identification of representative peptide sequences from gel filtration

Not only peptide size, the solubility, the amino acid composition, characteristic peptides sequences, and abundance of certain amino acids may also impact the overall antioxidant activity of peptides and proteins (Samaranayaka & Li-Chan, 2011; Suetsuna & Chen, 2002). In order to characterize the peptide profile of potent antioxidant fraction, crude kafirin extracted from sorghum flour as well as isolated peptide fraction (F2) from gel filtration was subjected to RP-HPLC followed with MALDI-TOF/TOF MS analysis.

The spectrum of trypsin-digested kafirin was compared to known alpha, beta, and gamma kafirin protein patterns obtained from http://www.uniprot.org, and a 100% coverage to beta-kafirin (Figure 2-13), around 30-50% coverage to gamma-kafirin, and 0% coverage to alpha-kafirin was detected. Thus, the beta kafirin sequences were used to categorize the peptides present in hydrolysate sample. Peaks at 13.3-, 14.2-, 15.2-, and 17.2-minute represent largest area percentage from the HPLC chromatogram, which accounted for a total coverage of 26.25%, were collected and analyzed for sequences by MS. A total of 23 peptides were identified and summarized in Table 2-3.

Among the identified peptide sequences, it was found that, glutamine (Gln, Q) and alanine (Ala, A) were the top two amino acids, which were present in 18 and 16 sequences, respectively, out of the total 23 identified peptides. Tsai, Liu, Yeh, Chiu, & Yeh (2012) found that the diabetic rat fed with a diet with glutamine supplementation had a decreases oxidative stress-related gene expression and increased antioxidant capacity and antioxidant enzyme activities. Grosser et al. (2004) also identified the antioxidant action of L-alanine by stimulating the expression of the antioxidant defense proteins HO-1 and ferritin in endothelial cells. Thus, glutamine and alanine could be important constituent amino acids responsible for the antioxidant activities of hydrolysate peptides. Besides, Jin et al. (2016) determined that APLC plays a critical role in good antioxidant activity of their peptides. Coincidently, Ala (A), Pro (P), Leu (L) and Cys (C) were also in significantly high abundance in the identified sequences in this study (16 of Ala, 13 of P, 10 of L, 10 of C). Thus, they could be critically associated with the antioxidant activity of kafirin peptides.

Zhuang et al. (2013) found that all fractions of corn protein hydrolysate contained the sequence of Leu-Pro-Phe (LPF). Hence, this sequence may be related to their high antioxidant

activity. In this study, the peptide Leu-Pro was found in three peptides, and 13 of the 23 identified peptides contained Pro, while 10 peptides contain Leu in their sequences. Peptides MDMQ and VAQ were found to be the most frequently appeared sequences. VAQ tend to appear at the end terminals while MDMQ were most often found within the sequences. Methionine (Met, M), valine (Val, V), tyrosine (Tyr, Y) are generally accepted as antioxidants or important constituent amino acids in antioxidant peptides reported by massive literatures (Guo, Kouzuma, & Yonekura, 2009). The frequent presence of Met and Tyr in the identified sequences (11 of Met, 8 of Val, and 6 of Tyr) also contributed to the overall antioxidant activities.

The determination of peptide sequences and structural analysis lay a solid foundation for future artificially synthesis and amplification of antioxidant peptides.

4. Conclusions

Enzymatic hydrolysis is an effective method to produce peptides with enhanced antioxidant activity from their parent proteins. The type of enzyme is a crucial factor impacting the protein recovery, DH, TPC, and the antioxidant capacity of consequent hydrolysates. Sorghum kafirin hydrolysates prepared with Neutrase displayed a balanced total protein recovery, DH, and antioxidant activity, thus was selected for further analysis. Other critical reaction factors were optimized to be protein content of 4%, enzyme-to-substrate ratio of 0.4 Au/g, and hydrolysis time of 17 hours. Hydrolysates with medium *M*w (3 – 10 kDa) exhibited higher TPC and antioxidative activities. The positive correlation between TPC and antioxidant activities indicated the phenolic peptides and phenolic compounds released during hydrolysis might be the components responsible for higher activities. The selected fraction of hydrolysates successfully retarded oil/lipid autoxidation and peroxidation in model systems. Upon further fractionation and peptide identification, glutamine and alanine were found to be the most

abundant amino acids; MDMQ and VAQ were the most frequent sequences, which could be associated with a higher activity.

This study provided an applicable processing technique for production of peptide antioxidants from sorghum kafirin. It was suggested that this naturally-extracted antioxidant could be used as alternatives to synthetic antioxidants or as synergetic effective component in improving the oxidative stability for various foods, beverages, animal feeds, pharmaceutical, industrial, and other applications. This work also delivered positive information to the sorghum ethanol industries by utilizing by-product such as distiller's grains as a protein source to produce peptide antioxidants as a potential revenue stream.

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Table 0-1 Protein content of sorghum flour and extracted fractions.

Sample	Nitrogen (%)	Protein (%) *
Ardent Mills sorghum flour	1.493	9.333
ADM defatted sorghum flour	1.323	8.269
Kafirin (glacial acetic acid fraction)	15.570	97.313
Glutelin (Borate/NaOH buffer fraction)	10.804	67.525
Sorghum residue after protein extraction	0.098	0.612

* Used conversion factor of 6.25.

Table 0-2 Preliminary experiments of sorghum kafirin hydrolysis.

Enzyme	Origin	Enzyme Unit	pH	Temperature (°C)	Density	Ratio	Enzyme /g of protein	Time	Recovery (%)	DPPH (%) - 5 mg/mL
Neutrase	*Bacillus amyloliquefaciens*	0.8 U/g	7.0	45	1.26g/ml	0.4 Au/g	0.39682 ml	21 hours	91.02	42.57
Flavourzyme	*Aspergillus oryzae*	500 U/g	5.0-7.0	50	1.27g/ml	10 Au/g	0.015748 ml	21 hours	27.00	31.25
Alcalase	*Bacillus licheniformis*	2.4 U/g	8.3	50	1.18g/ml	0.4 Au/g	0.14124 ml	21 hours	92.00	14.09
Everlase	*Bacillus sp.*	16 U/g	8.0	50	1.27 g/ml	0.4 Au/g	19.685 ul	21 hours	36.97	39.35
Protamex	*Bacillus sp.*	1.5 U/g	7.0	50	/	0.4 Au/g	0.26667 g	21 hours	99.62	15.23
Papain - Sigma	Papaya latex	1.5-10 U/mg	6.0-7.0	50	/	180 U/g	120 mg	5 hours	74.02	40.15
Papain - EMD	Carica papaya	31850 U/mg	6.0-7.0	50	/	360 kU/g	56.514 mg	4 hours	77.89	37.52
Bromelain	Pineapple stem	1200 GDU/g	3.0-6.5	45-65	/	180 kU/g	150 mg	5 hours	71.89	49.95
Ficin	Fig tree latex	680 MCU/mg	5.0-7.5	50-55	/	60 kU/g	225.5 mg	5 hours	67.48	48.23
Trypsin	Bovine pancreas	2500 USP/mg	7.0-9.6	37	/	25 kU/g	10 mg	5 hours	14.30	41.82
Pepsin	Porcine	400 U/mg	1.5-1.6	37	/	25 kU/g	62.5 mg	5 hours	64.60	65.45

Table 0-3 Representative antioxidant peptides in kafirin Neutrase hydrolysates.

Peak	13.3 min	14.2 min	15.2 min	17.2 min
Area %	9.95%	5.77%	6.43%	4.10%
Coverage %	92.70%	40.10%	70.30%	66.70%
	QAMCGVV AMCGVVQ SASALQM PAAQALTPL LPAAQALTP LPSYCTTP SAAIPPYY CGLYQLPS YALREQT	VAQNMP VQSVVQ QPQCSP	MRMMDMQS GGGLYPCAEY VAQVAQNMPA AVAQVAQNMP TPCATSAAIPP FLYPCAEYL VQSVVQQLQ	MDMQSRCQAM MMDMQSRCQA TPLAMAVAQVAQ QQMRMMDMQ

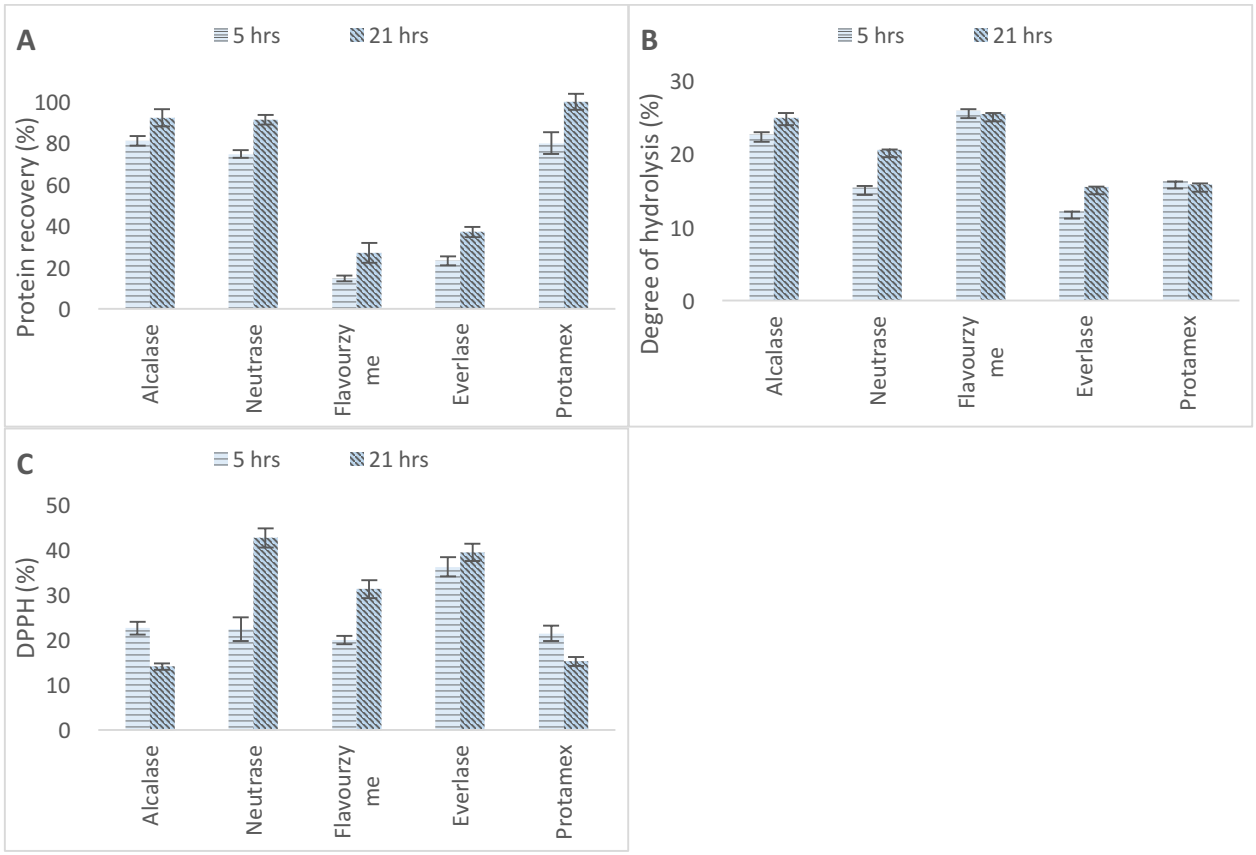


Figure 0-1 Screening of microbial origin enzymes for kafirin hydrolysis using Alcalase, Neutrase, Flavourzyme, Everlase and Protamex at protein content of 2%, enzyme-to-substrate ratio of 0.4 Au/g and hydrolyzed for 5 hours and 21 hours. A. Total protein recovery (%); B. degree of hydrolysis; C. DPPH scavenging activity (%) at 5 mg/mL.

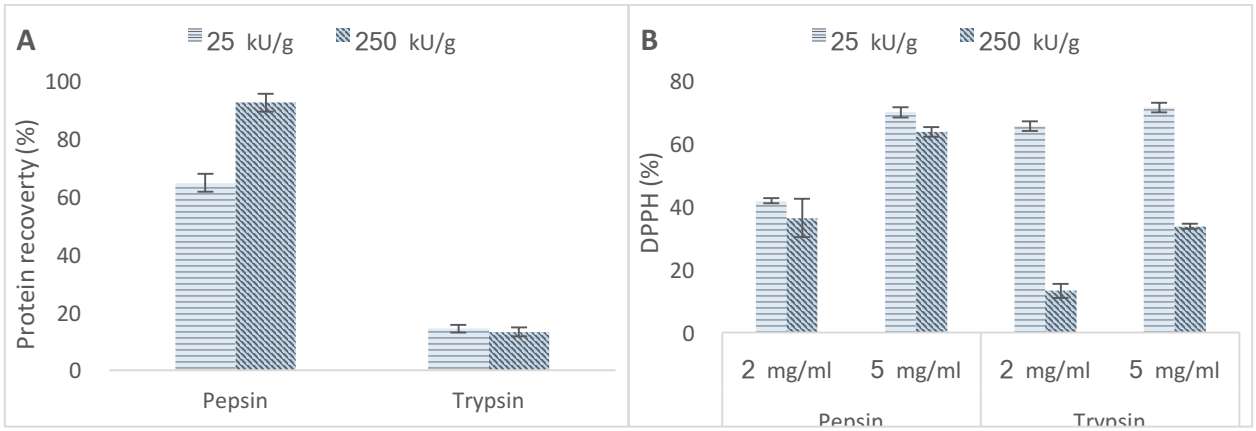


Figure 0-2 Animal origin enzyme screenings of kafirin hydrolysis using porcine Pepsin and bovine Trypsin at protein content of 4%, with enzyme-to-substrate ratios of 25 kU/g and 250kU/g and hydrolyzed for 5 hours. A. Total protein recovery (%); B. DPPH scavenging activity (%) at 5 mg/mL.

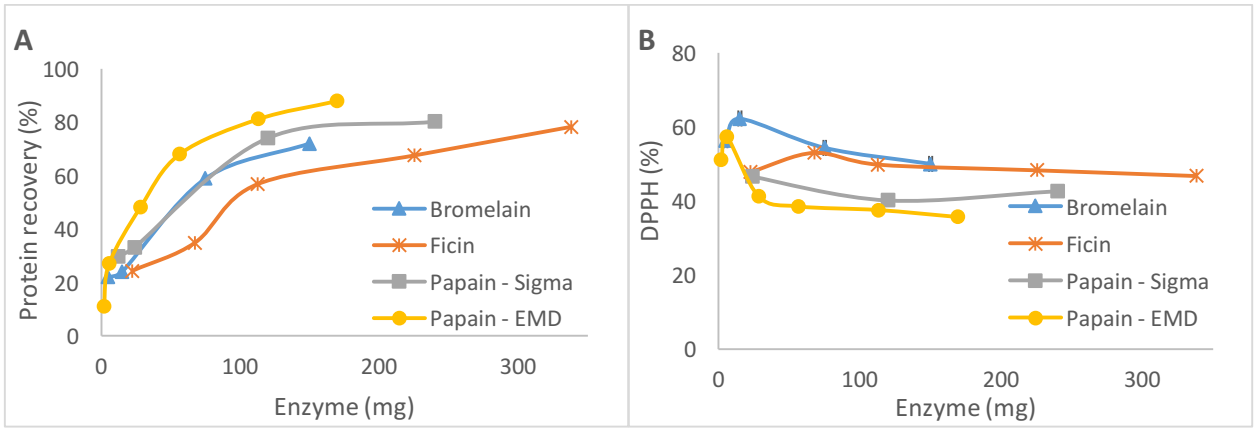


Figure 0-3 Plant origin enzyme screenings of kafirin hydrolysis using Bromelain, Ficin, Papain obtained from Sigma and Papain obtained from EMD at protein content of 4% and hydrolyzed for 5 hours. A. Total protein recovery (%); B. DPPH scavenging activity (%) at 5 mg/mL.

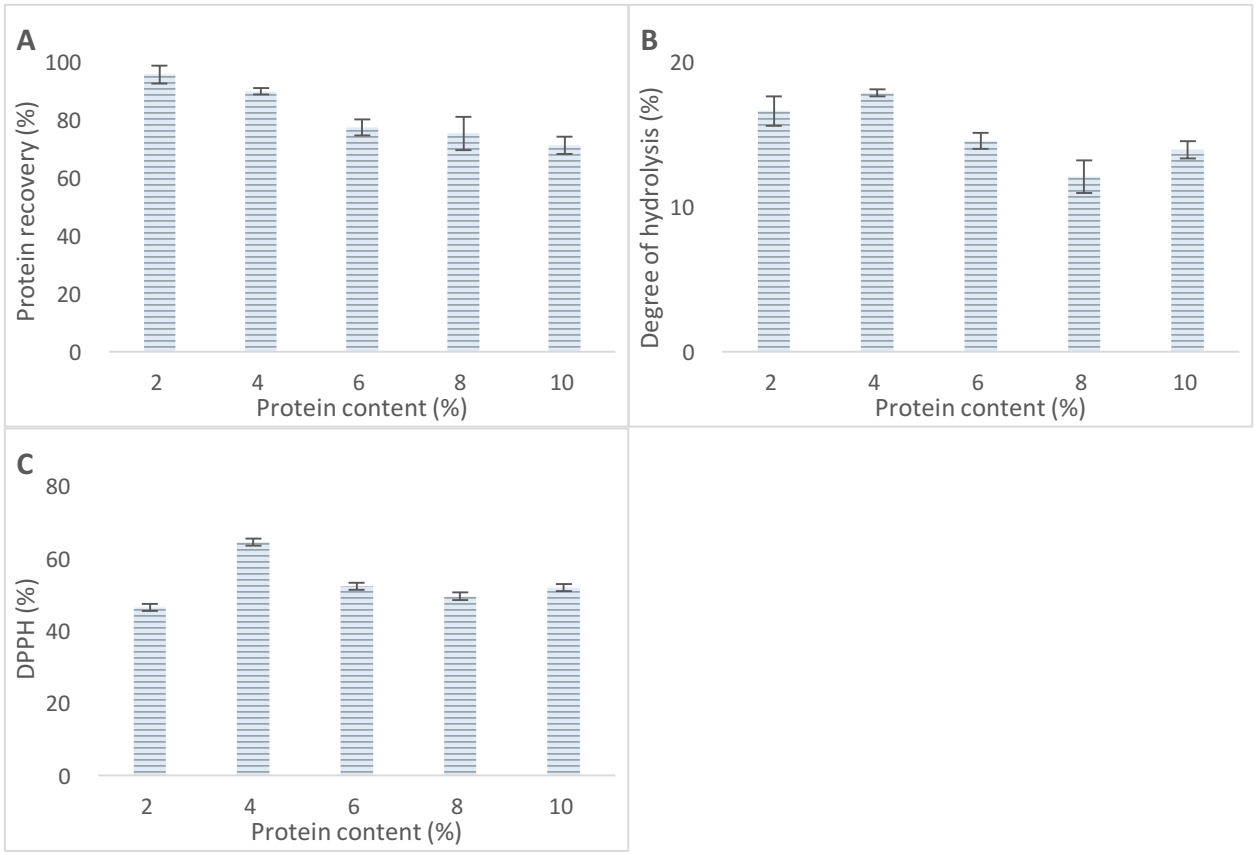


Figure 0-4 Effect of protein content on kafirin hydrolysate prepared with Neutrase at enzyme-to-substrate ratio of 0.4 Au/g, hydrolyzed for 21 hours, and substrate content at 2%, 4%, 6%, 8%, and 10%. A. Total protein recovery (%); B. degree of hydrolysis; C. DPPH scavenging activity (%) at 5 mg/mL.

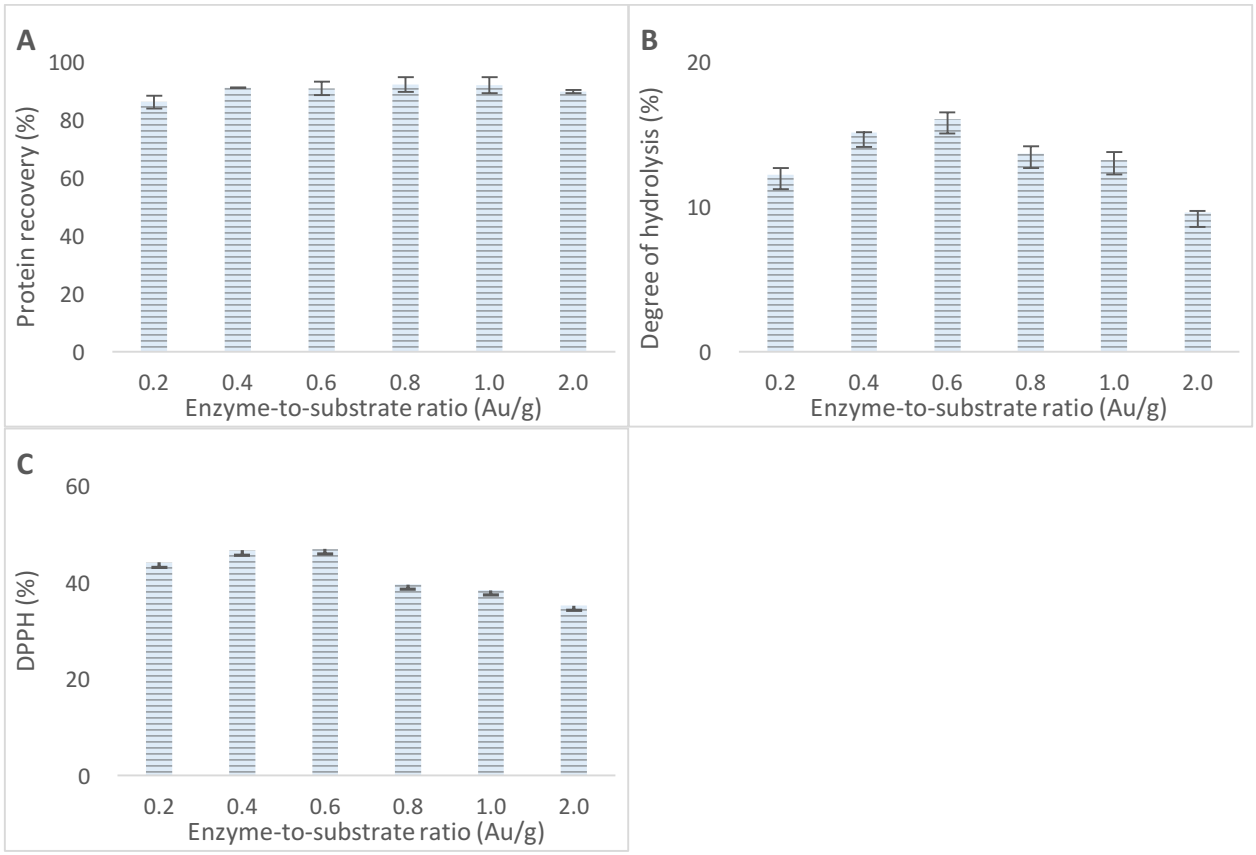


Figure 0-5 Effect of enzyme-to-substrate ratio on kafirin hydrolysate prepared with Neutrase at protein content of 4% hydrolyzed for 21 hours at enzyme-to-substrate ratios of 0.2, 0.4, 0.6, 0.8, and 1.0 Au/g. A. Total protein recovery (%); B. degree of hydrolysis; C. DPPH scavenging activity (%) at 5 mg/mL.

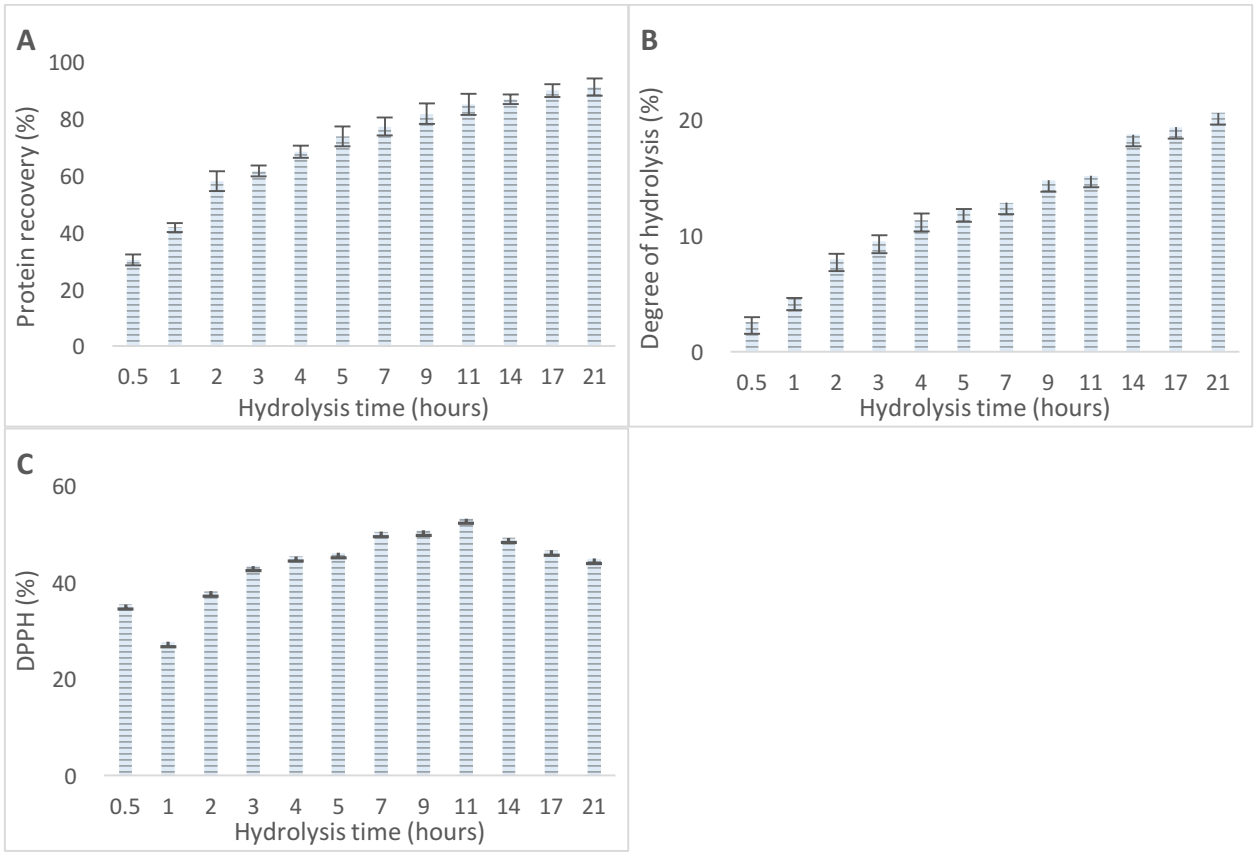


Figure 0-6 Effect of hydrolysis time on kafirin hydrolysate prepared with Neutrase at protein content of 4% and enzyme-to-substrate ratio of 0.4 Au/g with different time of hydrolysis (hours). A. Total protein recovery (%); B. degree of hydrolysis; C. DPPH scavenging activity (%) at 5 mg/mL.

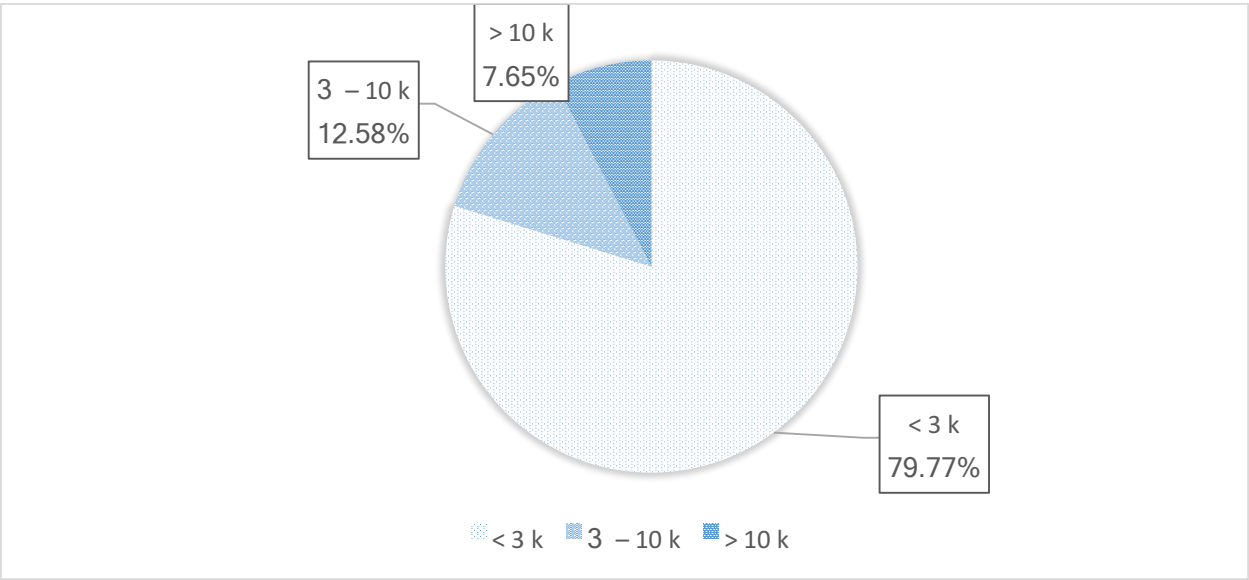


Figure 0-7 Distribution of ultrafiltrated fractions of kafirin Neutrase hydrolysate prepared at protein content of 4% enzyme-to-substrate ratio of 0.4 Au/g and hydrolyzed for 17 hours followed with membrane filtration using 10 kDa and 3 kDa membranes.

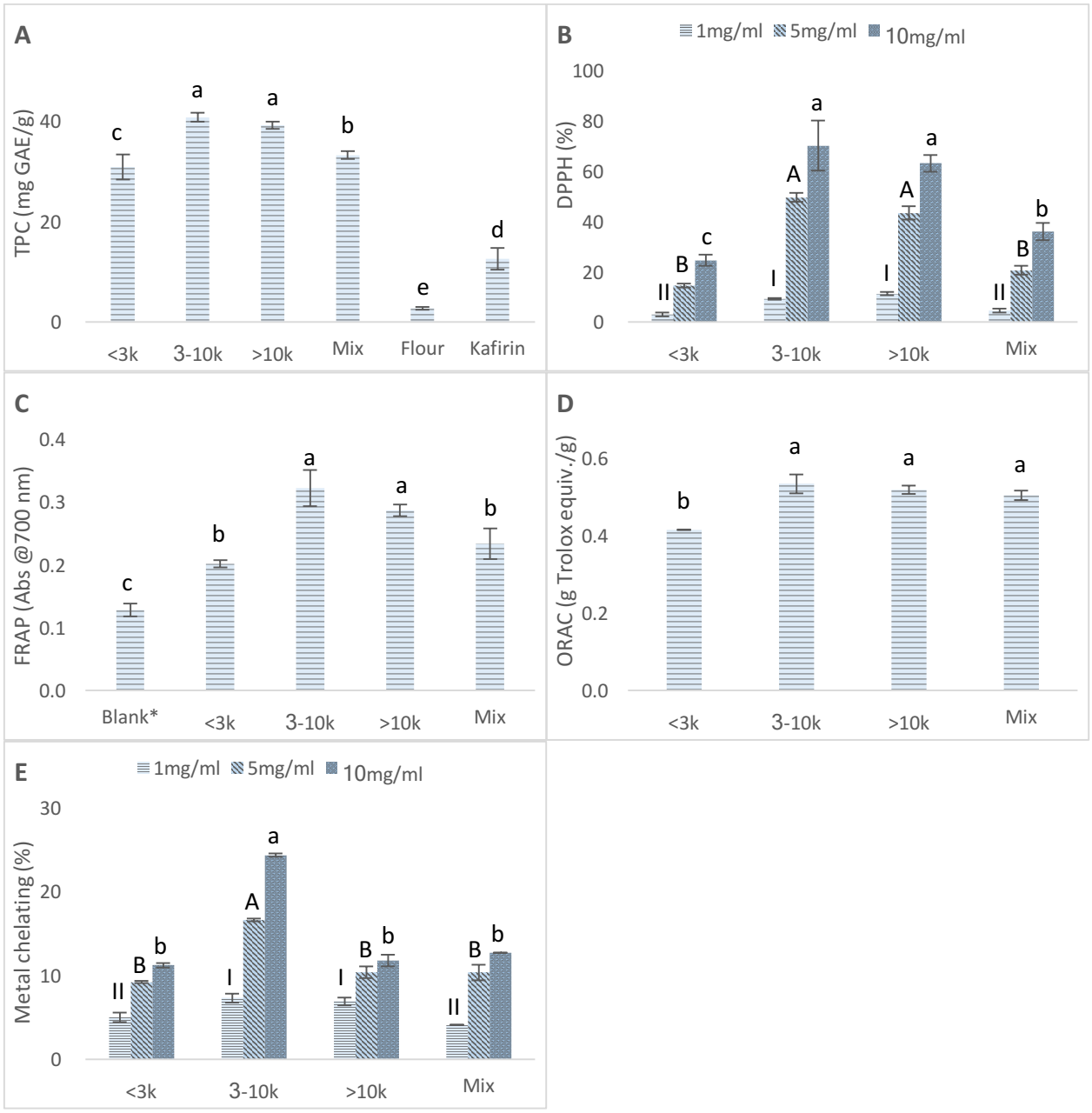


Figure 0-8 Total phenolic content and antioxidant activities of kafirin Neutrase hydrolysate ultrafiltrated fractions prepared at protein content of 4% enzyme-to-substrate ratio of 0.4 Au/g and hydrolyzed for 17 hours followed with membrane filtration using 10 kDa and 3 kDa membranes. A. Total phenolic content (mg GAE/g); B. DPPH scavenging activity (%); C. Reducing power capacity at 2.5 mg/mL (Abs at 700 nm); D. ORAC (g Trolox equiv./g); E. Metal chelating capacity (%).

* Blank represents the absorbance of reaction mixture at 700 nm using distilled water in substitute of sample.

Different lowercase letters, capital letters, and roman numerals indicated significant difference at $P < 0.05$.

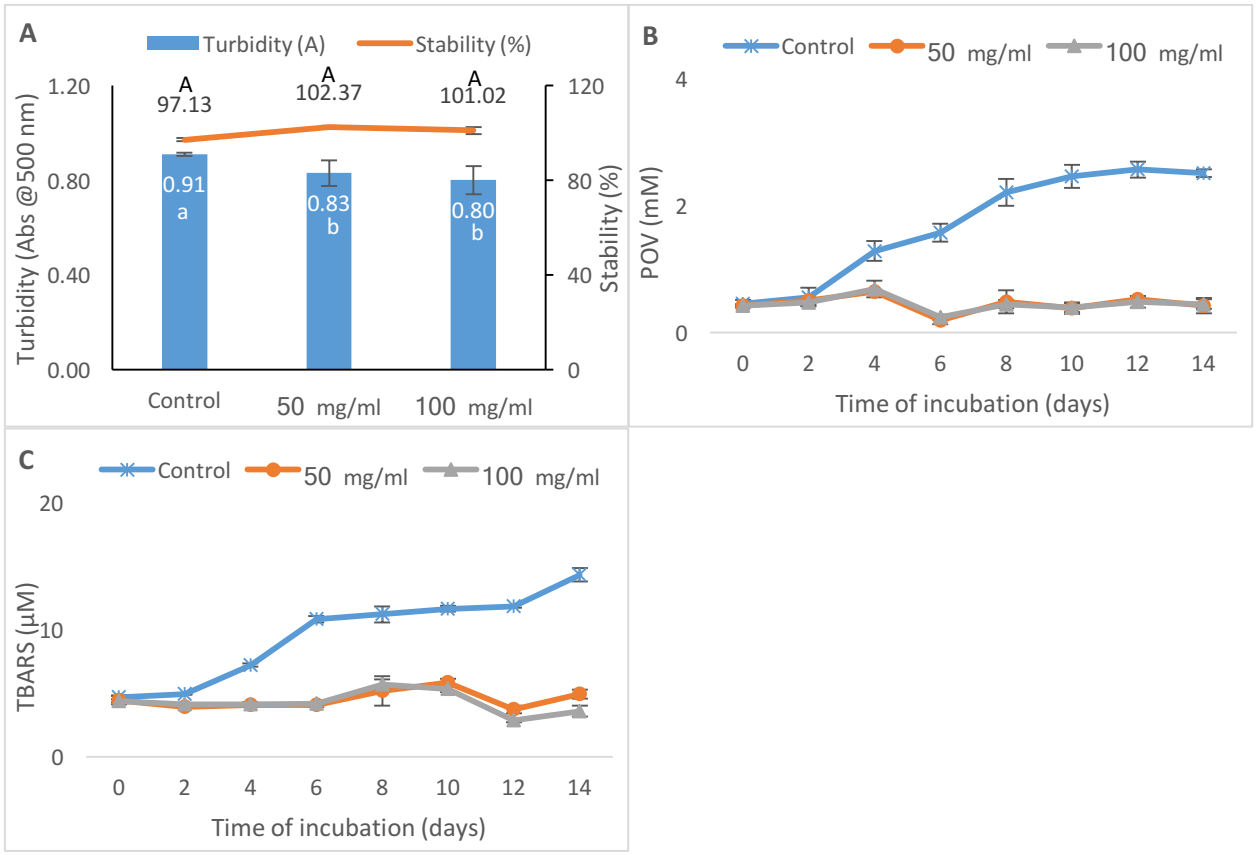


Figure 0-9 Inhibition effect of kafirin Neutrase 3 – 10 kDa hydrolysate prepared at enzyme-to-substrate ratio of 0.4 Au/g and hydrolyzed for 17 hours in an oil-in-water emulsion model system added with 50 and 100 mg/mL oil. A. Emulsion turbidity (Abs at 500 nm) and stability (%); B. POV (mM cumene hydroperoxide equivalent); C. TBARS (µM tetramethoxypropan equivalent).

Different lowercase and capital letters indicated significant difference at $P < 0.05$.

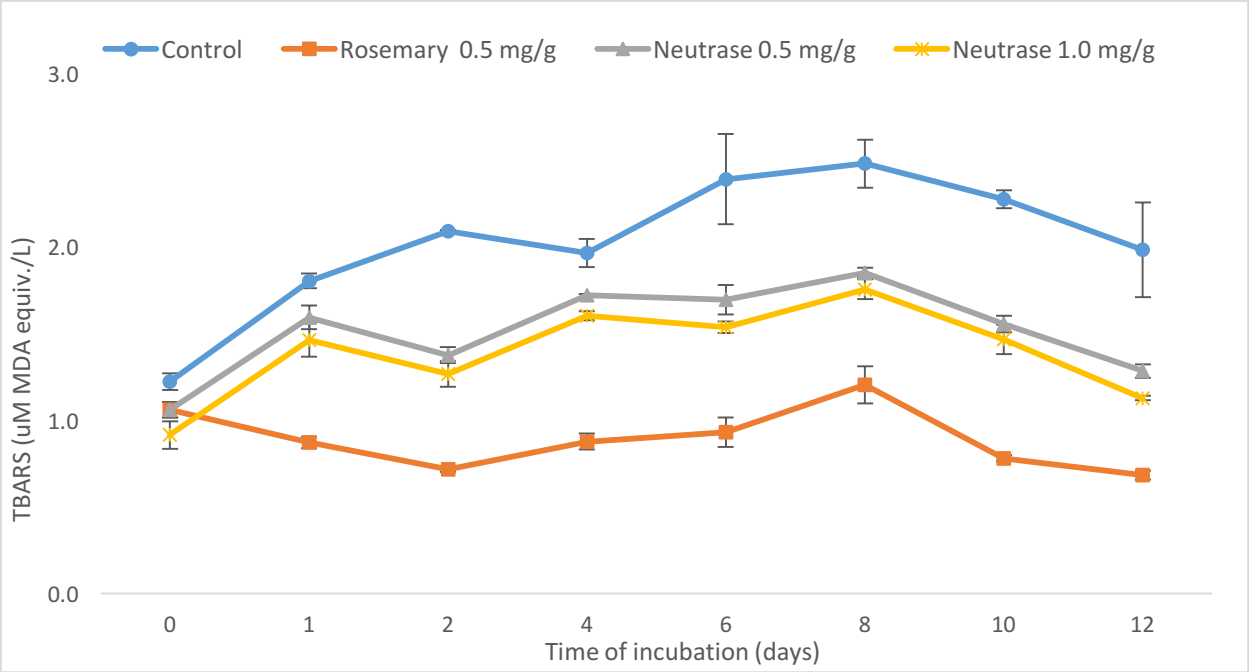


Figure 0-10 Inhibition effect shown as TBARS of kafirin Neutrase 3 – 10 kDa hydrolysate prepared at enzyme-to-substrate ratio of 0.4 Au/g and hydrolyzed for 17 hours in a ground meat system added with 0.5 and 1 Mg/g meat.

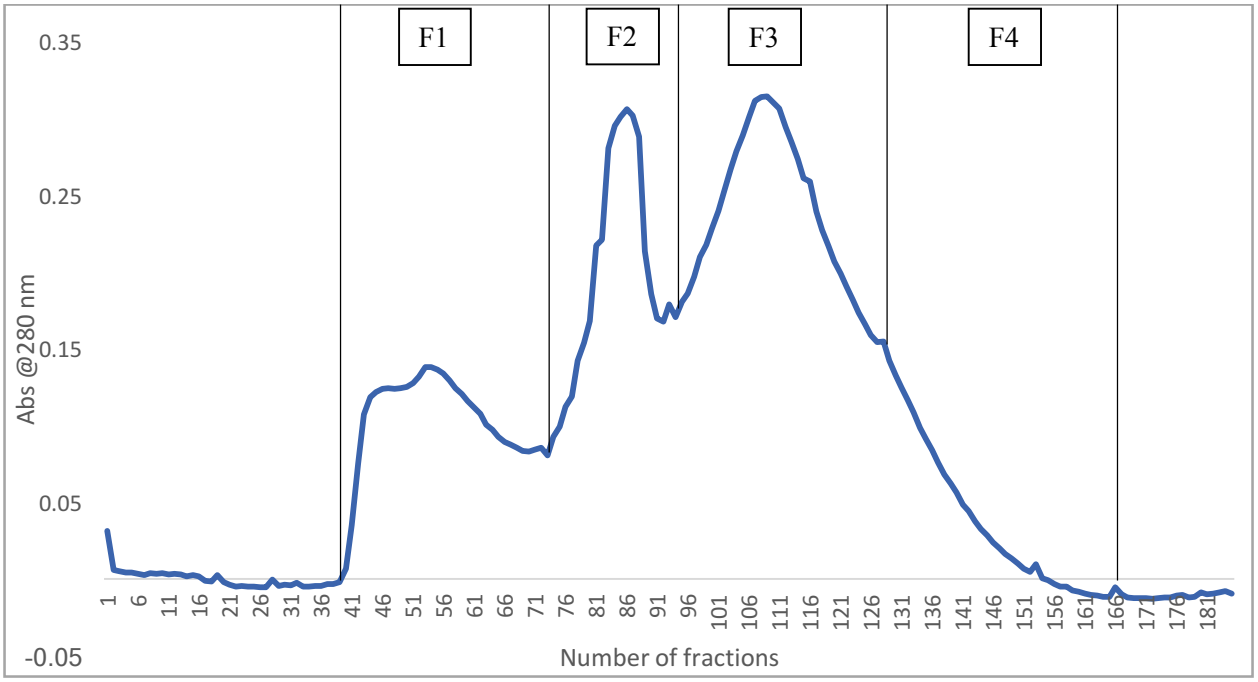


Figure 0-11 Gel filtration chromatogram of kafirin Neutrase 3 – 10 kDa hydrolysate prepared at enzyme-to-substrate ratio of 0.4 Au/g and hydrolyzed for 17 hours in a Sephadex G-25 column (26 mm × 850 mm).

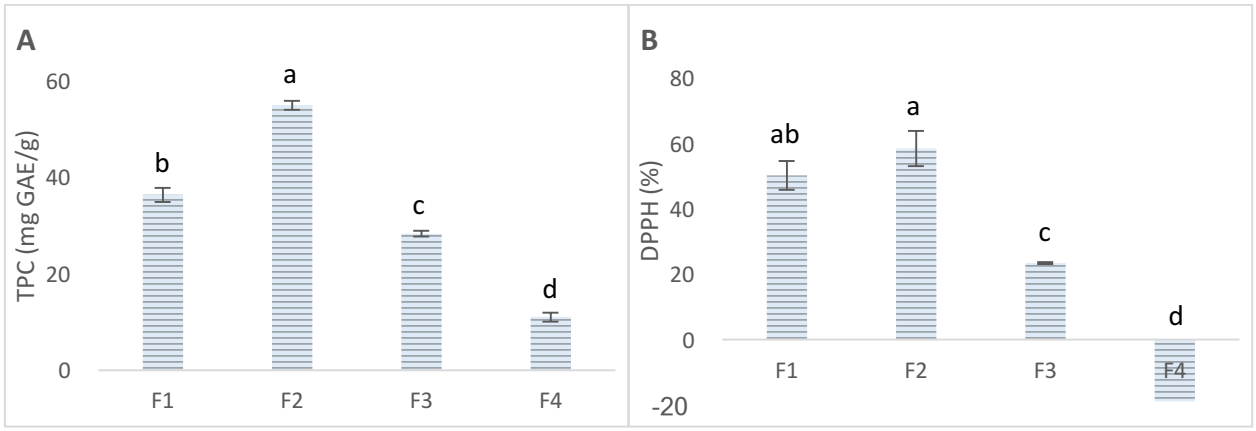
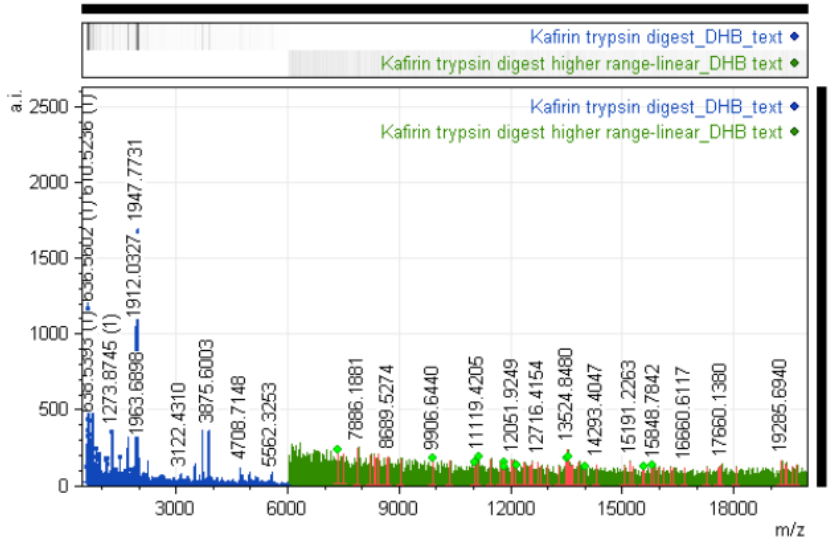


Figure 0-12 Total phenolic content and antioxidant activities of gel filtration fractions F1-F4 of kafirin Neutrase 3 – 10 kDa hydrolysate prepared at 0.4 Au/g, 4%, and hydrolyzed for 17 hours A. Total phenolic content (mg GAE/g); B. DPPH scavenging activity (%) at 4mg/mL.

Different lowercase letters indicated significant difference at $P < 0.05$.



Sequence - *Beta Kafirin*

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
	192	21619.8475	21634.8755	100.0 %	12.3 %

MKMVIVLAVC LALSAASASA LQMPGMGLQD LYGAGAIMTM MGAGGGLYPC AEYLRQPQCS PVAAPFYALR EQTMWQPNFI CQPLRQQCCQ QMRMMDMQSR CQAMCGVVQS VVQQLQMTMQ LQGVAAAASS LLYQPALVQQ WQQLLPAAQA LTPLAMAVAQ VAQNMPAMCG LYQLPSYCTT PCATSAAIPP YY

Figure 0-13 MS spectrum of crude kafirin extracted from ADM sorghum flour using glacial acetic acid method.

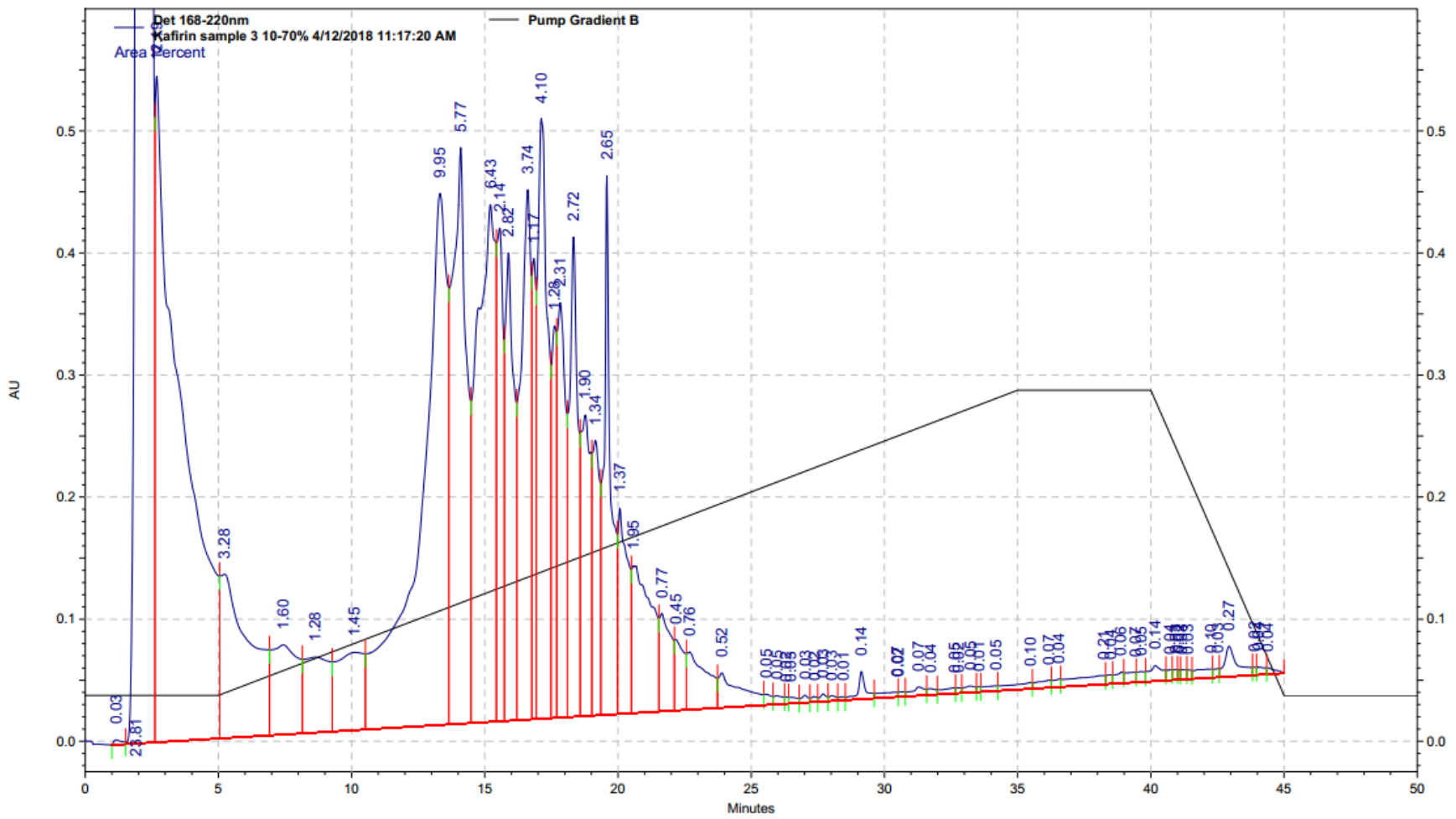
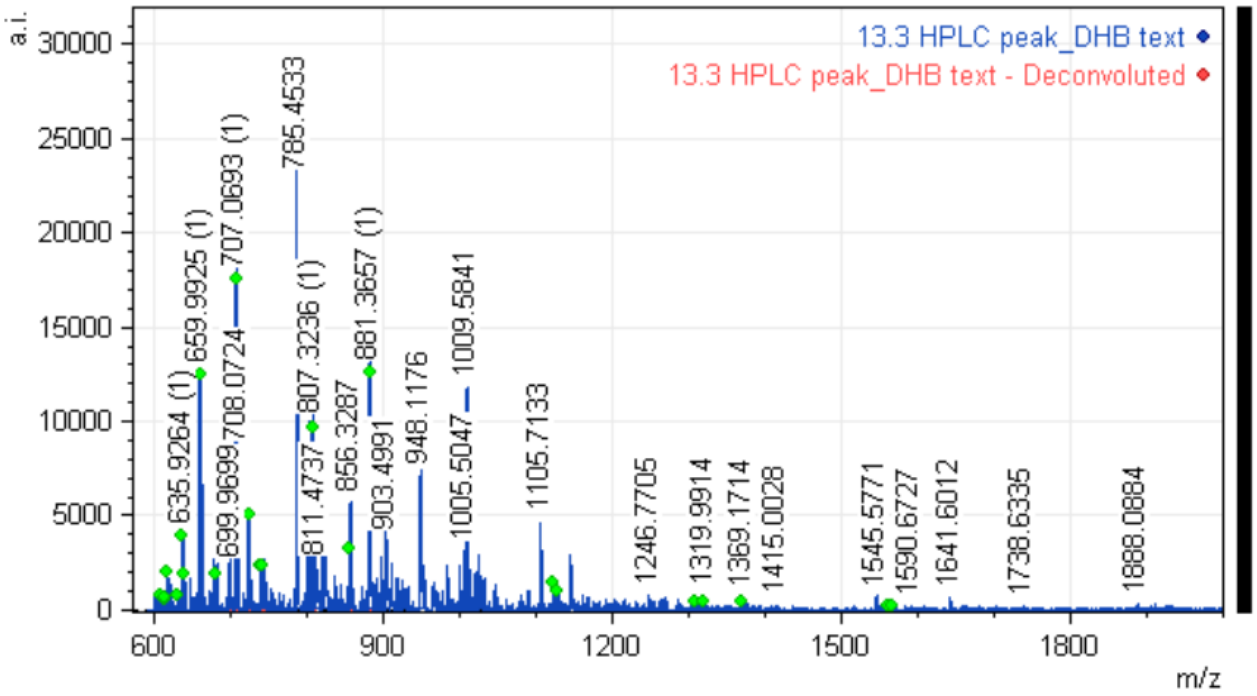


Figure 0-14 RP-HPLC chromatogram of Fraction 2 from gel filtration of 3 – 10 kDa kafirin Neutrase hydrolysates.



Sequence - *Beta Kafirin*

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
	192	20661.8889	20676.4896	92.7 %	92.8 %

MKMVIVLAVC LALSAASASA LQMPGMGLQD LYGAGALMTM MGAGGGLYPC AEYLRQPQCS PVAAPFYALR EQTMWQPNFI
CQPLRQQCCQ QMRMMDMQSR CQAMCGVVQS VVQQLQMTMQ LQGVAAAASS LLYQPALVQQ WQQLLPAAQA LTPLAMAVAQ
VAQNMPAMCG LYQLPSYCTT PCATSAAIPP YY

Figure 0-15 MS spectrum of 13.3 minute peak from RP-HPLC of 3 – 10 kDa kafirin Neutrase hydrolysates gel filtration fraction 2.

Chapter 3 - Reaction Optimization, Antioxidant Activity Characterization, and Peptides Identification of Sorghum Kafirin Hydrolysates Prepared with Alcalase

Abstract

Alcalase was a promising enzyme in producing antioxidative hydrolysates and peptides from sorghum kafirin according to preliminary experiment results. Hydrolysis of kafirin extracted from defatted white sorghum flour was conducted with combined treatment of varied hydrolysis time and enzyme-to-substrate ratios. The optimal reaction parameters were determined to be at substrate content of 4%, enzyme-to-substrate ratio at 0.4 Au/g, and hydrolysis time of 4 hours. At these conditions, hydrolysate was prepared and fractionated by ultrafiltration to categorize its molecular weight distribution, and fractions with different M_w range were evaluated for their antioxidative capacity through free radical scavenging activity, metal chelating ability, reducing power, and oxygen radical absorbance capacity. Medium sized hydrolysate (5 – 10 kDa) exhibiting promising activities through *in vitro* chemical assays was subjected to further evaluation model systems. With an incorporation at 50 mg/mL oil, the formation of primary and secondary oxidation products was successfully inhibited by the selected fraction of hydrolysates by 68.26% and 37.83%, respectively, during the 14-day incubation period. When incorporated at 1.0 mg/g meat, the lipid peroxide products were reduced by 46.92% during the 12-day storage stage. This selected hydrolysate fraction was further fractionated on a Sephadex G-25 gel filtration column and F3 demonstrating stronger activities was pooled and analyzed by RP-HPLC, and a total of 26 peptide sequences were therefore identified from major peaks of HPLC by MALDI-TOF/TOF MS. Sequences QQWQ

and QWQQ were found to be present in all isolated peaks. Hence, these sequences may be related to the antioxidant activity of kafirin hydrolysates peptides. A significant high level of hydrophobic amino acids was also found to contribute to the overall antioxidant activity of kafirin Alcalase hydrolysates by increasing the accessibility of antioxidants to hydrophobic targets. These combined results demonstrated that antioxidant peptides obtained from sorghum kafirin are capable of delaying or inhibiting the oil and fat peroxidation.

1. Introduction

Nowadays, adding values to under-utilized crops and optimize their values for various industrial applications is an increasing trend (Alashi et al., 2014). Sorghum is the third largest cereal crop in U.S. mainly used as animal feed and starch source for biofuel (Awika, 2011; Lee et al., 2011). The by-products form sorghum ethanol industries (e.g., DDGS) are a premium protein source which could be potentially used for manufacture of value-added products such as peptide antioxidants.

Driven by the heightened safety concerns over synthetic antioxidants and consumers' preference for natural ingredients, the development of novel natural antioxidants have drawn growing interests. Protein has been on the top-ten driving factor in current markets with extensive popularity. Peptide antioxidants are naturally existed (e.g., GSH) and can be produced from dietary protein hydrolysates, which exert antioxidative performances through multiple pathways such as scavenging free radicals, chelating transition metals, reducing oxidized substances, interrupting the decomposition of hydroperoxide, forming physical barriers hindering access of prooxidant to targets and so on. Peptide antioxidant is naturally-derived, efficient, generally considered as safe at high dosages, and able to serve as an energy source and amino acid and provider. Besides, due to the surface amphiphilicity, peptide antioxidants can also serve as functional ingredients with special properties (e.g., gelation, emulsifying, foaming, water and/or oil binding capacity) (Sloan, 2014). Thus, they are promising alternatives to synthetic antioxidants to be incorporated into various food products as additives in protecting lipid and/or oil from peroxidation.

Upon enzymatic hydrolysis, native globular matrix of intact proteins will be cleaved, characteristic structures (e.g., functional R groups, structural domains) attributing to the

antioxidative activities will be exposed, and specific peptide sequences will be released (Jin et al., 2016). The solubility of hydrolyzed proteins is increased, yet, the amino acid profile and antioxidant activities could remain essentially unchanged or improved in some fractions (Alashi et al., 2014). The resulting hydrolysates can be purified and isolated by various techniques to obtain peptides with enhanced activities.

The selection of enzyme is important in determining the end-use properties of hydrolysates. Alcalase is a food-grade commercial protease in liquid form prepared by submerged fermentation of a selected strain from Bacillus licheniformis, with Subtilisin Carlsberg as a major proteolytic component. It is a serine endopeptidase with broad specificity, which cleaves peptide bonds at the interior of the chain and produces peptide with varied sizes (Xia et al., 2012; Zheng et al., 2006; Kong & Xiong, 2006). Alcalase is readily soluble in water at all concentrations and has a relatively low bulk sale price, which has been widely used in food industry to hydrolyze plant and animal proteins for improvements in functional and nutritional values (Zhu et al., 2006). Alcalase, alone or as a partial step, can be used for generation of antioxidative peptides from plan and/or animal proteins such as those from wheat germ (Zhu et al., 2013), barley glutelin (Xia et al., 2012), rice bran protein (Thamnarathip et al., 2016), corn gluten (Zheng et al., 2006), zein (Kong & Xiong, 2006), green tender sorghum protein (Agrawal et al., 2017), soy protein (Park et al., 2010; Zhang et al., 2010), chickpea protein (Li et al., 2008; Kou et al., 2013), rapeseed (Pan et al., 2011), sardine by-product proteins (Bougatef et al., 2010), egg yolk (Park, Jung, Nam, Shahidi, & Kim, 2001), pollack skin (Kim et al., 2001) and so on. Xia et al. (2012) reported that barley glutelin hydrolysate prepared with Alcalase demonstrated a significantly higher antioxidant capacity including superoxide radical scavenging activity, hydroxyl radical scavenging activity and ferrous iron chelating ability than that prepared with

Flavourzyme. Thamnarathip et al. (2016) indicated that rice bran protein hydrolysate prepared with Alcalase yields a higher amount of antioxidative hydrolysates, higher degree of hydrolysis, higher protein content and shorter peptide chains compared to those of Neutrase and Flavourzyme. Pan et al. (2011) determined to use Alcalase to produce antioxidant peptides rather than using Papain, Neutrase, and Protamex due to a relatively higher degree of hydrolysis and DPPH quenching activity. The obtained bioactive peptides prepared with Alcalase are more resistant to digestive enzymes (Kim et al., 2011; Park et al., 2001). Alcalase is completely soluble in water with all concentrations and available in food grade, make it a readily used enzyme in protein dispersions in producing water soluble peptides.

According to the results of preliminary experiments, sorghum protein kafirin hydrolysates prepared with Alcalase exhibited promising antioxidative activities as well as good protein recovery yield. Thus, Alcalase was selected to hydrolyze kafirin in producing antioxidative peptides in this study. The objectives of this study were to: 1) optimize the reaction conditions of kafirin hydrolysis using Alcalase; 2) evaluate the antioxidant performances of kafirin Alcalase hydrolysates antioxidants via *in vitro* chemical assays as well as model systems; and 3) identify the structural characteristics including molecular weight distribution, amino acid composition, and characteristic peptide sequences of the antioxidative activities.

2. Materials and Methods

2.1. Materials and chemicals

The materials and chemicals used for the study in this chapter is the same as those used in Chapter 2, thus, will not be listed.

2.2. Preparation of sorghum protein hydrolysates

The procedures used in preparation of sorghum hydrolysates for this study were similar to those described in Chapter 2, which include the defatting of sorghum flour, extraction of sorghum kafirin, and enzymatic hydrolysis of kafirin, except that Alcalase protease was used.

2.3. Evaluation of hydrolysis process

The process of hydrolysis was evaluated and optimized as determined by several indicators including total protein recovery, degree of hydrolysis, and total phenolic content as previously discussed.

2.4. Fractionation and identification of antioxidative peptides

The only difference from the study in Chapter 2 was ultrafiltration using a stirred cell instead of centrifugal tubes, which will be described as followed. Other fractionation and identification procedures including gel filtration with Sephadex G-25, RF-HPLC, and MALDI-TOF/TOF MS followed the same procedure as previously described.

2.4.1. Ultrafiltration with stirred cell

The ultrafiltration was achieved using an Amicon® Stirred Cell (EMD Millipore Corporation, Billerica, MA, USA) sequentially assembled with Ultracel® Ultrafiltration Discs (EMD Millipore Corporation, Billerica, MA, USA) with molecular weight cut-off at 10 k, 5 k, 3 kDa and 1 kDa. New membranes were floated in deionized water with skin (glossy) sides adown for 24 hours with water changed at least three times to remove the pretreated glycerine residues. The membrane was then placed into the stir cell and loaded with hydrolysate solution at 5 mg/mL. The stir cell was placed on top of a magnetic stirrer at 60 rpm. Compressed nitrogen was connected to the stir cell at a maximal pressure of 60 psi to accelerate the penetration process.

2.5. Assessment of antioxidative activity

In addition to the assays and model systems described in Chapter 2, ABTS radical scavenging activity was also employed as depicted below.

2.5.1. ABTS radical scavenging assay

The ABTS radical scavenging activity of protein hydrolysates or peptides was determined according to the method of Alashi et al. (2014) with modifications. The ABTS stock solution was made by mixing equal amount of 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution and stored for 12-16 h in dark at room temperature to generate ABTS radicals. The ABTS stock solution was then diluted with deionized water to achieve an absorbance of 1.1 ± 0.02 at 734 nm on a spectrophotometer. 2.85 mL of the diluted ABTS radical solution was mixed with 0.15 mL of sample solution and incubate at room temperature in darkness for 10 minutes before reading its absorbance at 734 nm. Deionized water was used as a blank control. The ABTS radical scavenging activity was calculated using follow equation:

$$\text{ABTS\%} = \frac{A_b - A_s}{A_b} \times 100\%$$

where A_b was the absorbance of blank and A_s was the absorbance of sample.

2.6. Statistical analysis

The statistical analysis method used in this study was same as described in Chapter 2.

3. Results and Discussion

3.1. Reaction optimization of kafirin enzymatic hydrolysis

As explained in previous chapters, enzyme type, substrate, and hydrolysis conditions have a synthetic effect on the production of peptide. 4% kafirin solution was hydrolyzed with Alcalase at 0.2, 0.4, and 0.8 Au/g enzyme-to-substrate ratio with varied hydrolysis time. Degree

of hydrolysis (DH), total protein recovery, total phenolic content (TPC) and DPPH radical scavenging activities (DPPH%) were evaluated for the resulting hydrolysates (Figure 3-1).

Percentage of water-soluble hydrolysate yield was calculated as total protein recovery and used to determine the efficiency of the hydrolysis process (Alashi et al., 2014). Total protein recovery was found to increase with extended hydrolysis time. After 2 hours hydrolysis, yields for all three enzyme-to-substrate ratios are over 50%, and after 4 hours, kafirin with 0.4 Au/g enzyme reached a yield over 74%, which indicated that majority of high M_w proteins were degraded into low M_w soluble peptides and amino acids through peptide cleavage (Alashi et al., 2014). An obvious enhancement in total protein recovery was observed when increased enzyme-to-substrate ratio from 0.2 to 0.4 Au/g. However, there was no noteworthy enhanced yield when increase enzyme-to-substrate ratio to 0.8 Au/g.

Besides, DH increased consistently with prolonged hydrolysis time until reached plateau at around 4 hours. This was because at initial stage, the substrate concentration as well as the enzyme activity were relatively higher, which led a higher rate of peptide bond cleavage and protein hydrolysis (Zhang et al., 2012). As the hydrolysis progressed, the rate of hydrolysis decreased when the system tended to reach reaction equilibrium.

DPPH is a stable free radical that has an intrinsic purple color in ethanol solvent, which can be detected at 517 nm. When encounters an electron-donating substrate, DPPH radical accepts an electron and becomes a stable diamagnetic molecule hence loses the purple color as well as a reduced absorbance at 517 nm. Therefore, the ability of kafirin hydrolysate to act as electron donors in its antioxidative performances can be tested by DPPH radical scavenging activity. The DPPH% of kafirin hydrolysates after 3 hours of hydrolysis was 23.89% - 27.23% at 5 mg/mL. The abnormal high value of DPPH% at 0.5 hour might be due to the intrinsic activity

of the protease. All other hydrolysates exhibited fairly similar values of DPPH% and no obvious increase of DPPH% was observed when longer hydrolysis time was applied. This result indicated that kafirin hydrolysates are antioxidants that can act through radical scavenging activity. The antioxidant activity was not enhanced or decreased with extended hydrolysis time, which might be due to the fact that extended hydrolysis time only increased the hydrolysate yield but did not changed the hydrolysate nature.

TPC of Alcalase hydrolysates was between 31.13 and 39.47 mg GAE/g. While TPC was increased with hydrolysis time extended from 0.5 hour to 2 hours for all three enzyme-to-substrate ratios, increasing hydrolysis time from 4 hours to 11 hours did not make a huge impact on TPC. Thamnarathip et al. (2016) also reported that increasing hydrolysis time from 2 to 6 hours did not significantly changed TPC of rice bran protein hydrolyzed by Alcalase. These authors observed that large-sized peptides over 40 kDa were completely degraded after 2 hours of hydrolysis, and the large bands shown in 2-hour hydrolysate were completely digested after 4 hours of hydrolysis unveiled by SDS-PAGE patterns. Kong and Xiong (2006) indicated that, the three main components in zein protein were completely digested and only trace amount of proteins left after 5 hours hydrolysis with Alcalase. They further indicated that if the hydrolysis became too extensive (> 4 hours in their study), it could reduce the peptides' ability to act as physical barrier to prevent oxidants from reaching the lipid fraction in the liposome.

Thus, protein content of 4%, enzyme-to-substrate ratio at 0.4 Au/g, and hydrolysis for 4 hours were determined to be optimal reaction conditions with a good balance of protein recovery, antioxidant activity, and production cost for kafirin hydrolysis with Alcalase.

3.2. Ultrafiltration of kafirin Alcalase hydrolysates

Previous studies have revealed that the antioxidative activities of hydrolysate peptides is dependent on their M_w distribution (Xia et al., 2012). Kafirin Alcalase hydrolysate was prepared at previously determined optimal reaction conditions (protein content of 4%, enzyme-to-substrate ratio of 0.4 Au/g and hydrolysis time of 4 hours). It was then sequentially fractionated in an ultrafiltration stirred cell coupled with M_w cut-off membranes 10 k, 5 k, 3 k, and 1 kDa.

Figure 3-2 showed the M_w distribution profile of the five fractions. The yield of each fraction was found to decrease with increased fraction size. The hydrolysate in < 1 kDa fraction (47.98%) was significantly higher than others. 1 – 3 kDa (14.97%), 3 – 5 kDa (14.98%), and 5 – 10 kDa (16.58%) shared similar portions while > 10 kDa fraction had the lowest percentage (5.48%). Xia et al. (2012) pointed that hydrolysates obtained by hydrolysis with Alcalase contain mostly small- and medium- sized peptides due to the interior cleavage property of endo-protease. This could explain the large proportion of small-sized hydrolysate fraction. Since percentage yield of protein hydrolysates is not commonly reported, adequate comparison could not be compared. The results are similar to those reported by Alashi et al. (2014) that < 1 kDa took up the largest portion followed with 1 – 3 k, 3 – 5 k, and 5 – 10 kDa of hydrolysates obtained from canola protein hydrolyzed with Alcalase.

All fractionated fractions of hydrolysates as well as the hydrolysate mixture were evaluated for total phenolic content as shown in Figure 3-3 (A). Except for 1 – 3 kDa fraction having the lowest TPC, other hydrolysate fractions all had TPC over 39 mg GAE/g. With TPC of 45.64 ± 0.51 mg GAE/g, 5 – 10 kDa fraction had the highest value ($P < 0.05$) than other fractions.

DPPH% was measured for each of the filtrate fraction plus the mixture as shown in Figure 3-3 (B). Overall, 5 – 10 kDa (74.74 ± 2.50% at 10 mg/mL) fraction had the significantly ($P < 0.05$) higher DPPH% than other fractions, and < 3 kDa (25.70 ± 0.48% at 10 mg/mL) fraction was the lowest. The scavenging activity was linearly dose-dependent. This result revealed that the free radical scavenging activity is closely related to the M_w distribution of the substrate hydrolysate (Tang & Zhuang, 2014). Medium sized kafirin hydrolysates (5 – 10 kDa) were found to possess higher DPPH scavenging activity which might be due to the substrates in 5 – 10 kDa contain more efficient electron donors thus were able to stabilize free radicals to less reactive products (Wang et al., 2007).

ABTS radical scavenging assay (ABTS%) is widely used to test the hydrogen donating ability of the compounds in evaluation of the antioxidative activity of a substrate (Tang & Zhuang, 2014). ABTS radical showing a green color can be suppressed through radical scavenging ability of substrates. The discoloration can be monitored at decreases in absorbance at wavelength 700 nm and the inhibition percentage was used as an indicator for ABTS scavenging ability (Ragaee et al., 2006). ABST radical was scavenged by the hydrolysates in an amount-dependent manner. As shown in Figure 3-3 (C), all 6 fractions of hydrolysates displayed excellent ABTS%. The scavenging activity increased accordingly with an increased substrate concentration. There were no significant differences ($P < 0.05$) in ABTS% assay among the medium- and small-sized fractions of kafirin hydrolysates.

Reducing power assay is a typical electron-transfer method involving one redox reaction showing the presence of the compounds as a hydrogen atom donator to stabilize the free radicals (Thamnarathip et al., 2016), which is associated with the exposure of electron-dense amino acid side chain groups and with the M_w of the peptides (Thamnarathip et al., 2016). From Figure 3-3

(D), all fractions of kafirin hydrolysate resulted in significantly ($P < 0.05$) higher absorbance values ($A_{700} > 1.18$ at 10 mg/mL) over blank control ($A_{700} = 0.136 \pm 0.048$), which indicated an excellence reducing power ability of kafirin hydrolysate to reduce Fe^{3+} to Fe^{2+}. Among all six fractions, medium- (5 – 10 kDa) and large-sized (> 10 kDa) hydrolysates were found to be stronger reducers. Xia et al. (2012) also found that the larger-sized fraction (> 10 kDa) of barley glutelin hydrolysate had higher reducing power than smaller (< 1 kDa) sized fraction.

No food system can be considered free of metal ions (Duh, Tu, & Yen, 1999). Transition metals such as Fe^{2+} and Cu^{2+} are well-known catalyst of lipid peroxidation chain reactions. Thus, chelation of metal ion is an important pathway of antioxidant action. In this assay, after adding antioxidant to ferrous chloride, the un-chelated ferrous iron was determined measuring the formation of ferrous iron ferrozine complex at 562 nm. After reaction, a lower absorbance indicated a higher metal chelating ability. As shown in Figure 3-3 (E), kafirin Alcalase hydrolysates exhibited a metal chelating ability ranging from 8.36% to 22.78% at 10 mg/mL. It appeared that 5 – 10 kDa fraction had significant ($P < 0.05$) higher chelating activity than other fractions especially at low dosage. This result indicated that kafirin Alcalase hydrolysates demonstrated a remarkable iron binding capacity, which may be related to its action as peroxidation protector. The ferrous ion chelating ability may also contribute to their hydroxyl radical scavenging effects of antioxidants due to the combined effects (Xia et al., 2012).

Overall, medium sized kafirin Alcalase hydrolysate displayed higher antioxidant activities than other fractions with a good recovery. This result was agreed with other studies (Wang et al., 2014; Xia et al., 2012). Wang et al. (2007) located the M_w of wheat gluten hydrolysates with strongest activity to be 4.2 kDa by gel permeation chromatography and HPLC system.

Lastly, some researchers found a positive correlation between antioxidant activity and TPC, however, such relationship was not observed in this study.

3.3. Inhibition of lipid oxidation in model systems

3.3.1. Oil-in-water emulsion system

Oil-in-water emulsions exists in many food products such as soups, sauces, beverages, and so on (Cheng, Xiong & Chen, 2010). Oxidation of emulsions is a common problem that can cause texture alteration, development of rancid odor, and loss of nutrition profiles. Thus, the ability to improve the oxidative stability of food emulsion is an important indicator of peptides antioxidants capacity.

Kafirin Alcalase 5 – 10 kDa hydrolysate was incorporated when preparing soy oil-in-water emulsion samples stabilized with Tween-20. Changes in emulsion physical texture and structure was validated by measuring the emulsion turbidity and stability. As shown in Figure 3-4 (A), the turbidity of emulsion without hydrolysate was $A_{500} = 0.87 \pm 0.007$, and the addition of hydrolysate at 50 mg and 100 mg hydrolysate per mL of soy oil decreased the emulsion turbidity to $A_{500} = 0.77 \pm 0.053$ and $A_{500} = 0.76 \pm 0.059$, respectively. Meanwhile, the emulsion stability did not have a significant difference among the samples ($P < 0.05$).

The presence of hydrolysate displayed inhibition effect regarding the formation of both primary and secondary oxidation products, peroxide values (POV) and thiobarbituric acid reactive substances (TBARS), in a continuous phase compared to the control as illustrated in Figure 3-4 (B) & (C). During 14 days of incubation period at 37 °C, the emulsion sample with addition of 50 mg/mL displayed an inhibition rate of POV up to 76.56% (at day 8) and had an average inhibition of 68.26 ± 9.06%. Where the inhibition rate of TBARS was highest to be 51.89% (at day 8) and was averaged to be 37.83 ± 7.56%. Increased concentration of hydrolysate

at 100 mg/mL displayed an increase of inhibition effect of oxidation, where, POV inhibition rate up to 85.02% at day 8, and had an average inhibition value of 72.48 ± 9.10%, TBARS was decreased up to 54.03% at day 8, and averaged at 38.34 ± 10.50%.

This experiment results could serve as evidence indicating the antioxidative activities of fractionated kafirin hydrolysate in decreasing lipid hydroperoxide and TBARS formation, therefore, to stabilize the oil-in-water emulsions and retard emulsion oxidation. The activity of the hydrolysates could be due to multiple mechanisms, as previously discussed. Their free radical scavenging activities, chelation of transition metal ions, reducing power, and interrupting the decomposition of hydroperoxide into secondary products thus inhibit TBARS formation (Zhao et al., 2012; Kong & Xiong, 2006) all contribute to the overall antioxidant activity. In addition, Alcalase is an endo-protease which could perform both subtilisin and glutamyl activities, thus, the released peptides with Glu at C- terminal and hydrophobic patches in the sequences ultimately increased the overall hydrophobicity of hydrolysates, which assisted the adsorption of peptide molecules with oil droplets in emulsions. Proteins and peptides at oil-water interfaces could form a physical barrier to protect the interior of oil droplet, and also hinder the access of prooxidants in the aqueous phase. This concluded that kafirin hydrolysate could be used as potential antioxidants and/or functional ingredient for formulating emulsion-type foods with enhanced stability against lipid and/or oil oxidation.

3.3.2. Ground meat system

Reducing hydrogen and lipid peroxides in food products are important reactions because they are able to decompose to carbonyl molecules associated with rancid aroma and form free radicals (Elias et al., 2008). The lipid oxidation process in food products is highly dependent on the presence of pro-oxidants such as lipoxygenase, singlet oxygen molecules and transition

metals such as Fe and Cu (Zhao et al., 2012). Kafirin 5 – 10 kDa Alcalase hydrolysate fraction exhibited strongest antioxidative activities in primary experiments was inferred to be effective in inhibiting and retarding lipid oxidations through pathways as previously discussed.

Concentration of secondary oxidation product TBARS was measured for meat samples incorporated with hydrolysates and was compared to that prepared with rosemary extract and a blank control (i.e., no antioxidant addition) as shown in Figure 3-5. With hydrolysate added at 0.5 mg and 1.0 mg per gram of meat, TBARS was reduced throughout the entire 12-day incubation period compared with the blank control. At day 0, all meat samples contained similar TBARS content and from day 2, the inhibition effect of hydrolysates had begun to be apparent. At the end of the incubation, the TBARS was decreased by 35.34% and 43.17% for 0.5 mg/g and 1.0 mg/g, respectively, which indicated that the oxidation activities of lipid in ground meat were markedly inhibited by the addition of the kafirin hydrolysates. The average inhibition of TBARS during the 12-day incubation was 26.18 ± 7.06%, and 46.92 ± 15.98%. However, the inhibition performed by hydrolysates were not as efficient as rosemary extract did, which was posed an average of inhibition of 59.55 ± 6.56%. This might be due to the impurity of kafirin hydrolysates, which could contain both antioxidative and prooxidative components (Kong & Xiong, 2006). Hence, further fractionation of hydrolysates and identification of major peptide sequences responsible for the activities is needed.

3.4. Purification and identification of antioxidative peptides from kafirin Alcalase 5 – 10 kDa hydrolysates

3.4.1. Gel filtration of kafirin Alcalase 5 – 10 kDa hydrolysates

Kafirin Alcalase 5 – 10 kDa fraction exhibited relatively higher antioxidant activity from previous experiments was further fractionated on a Sephadex-G25 gel filtration column (Figure

3-6). The elution profile was divided into three fractions (F1-F3), and each fraction was collected and freeze-dried for analysis of total phenolic content and antioxidant activities (Figure 3-7).

Overall, the smaller sized fraction F3 manifested significantly ($P < 0.05$) higher values in TPC, DPPH%, ABTS%, and ORAC than the other two fractions. F3 also possessed an enhanced antioxidant activity as well as TPC than the hydrolysate mixutre without separation by gel filtration. Thus, gel filtration provided an effective tool to fractionte and isolate the most promising portions of peptides within the hydrolysate mixture.

3.4.2. Identification of representative peptide sequences from gel filtration

The antioxidant activity of protein hydrolysates was dependent upon the characteristic amino acid sequences of the peptides derived (Chen et al., 1995), which was dictated by the protease specificity (Kong & Xiong, 2006).

In order to identify the peptide profile present in the fraction of hydrolysates with potent antioxidant activity, F3 from gel filtration was further isolated and analyzed by RP-HPLC (Figure 3-8) followed by MALDI-TOF/TOF MS analysis (Figure 3-9). After digestion with trypsin, several peaks appeared and represented the major counterparts of peptides present in the sample. Peaks at 1.6-, 3.6-, 35.0-, and 36.0-minutes possessing area percentages of 30.62%, 11.03%, 2.34% and 4.15% were collected and analyzed, separately. After fitting the spectrum to protein patterns of beta kafirin protein, a total of 26 peptides sequences were identified for each peak (Table 3-1). Three major peptide fragments were detected at *m/z* 617, *m/z* 637, and *m/z* 659.

Alcalase is a serine endopeptidase with broad specificity but a preference for large, uncharged amino acid side-chain groups (Zheng et al., 2006; Kong & Xiong, 2006). It was found that, all four peaks from HPLC contained peptide sequences of QQWQ and QWQQ, which could be critical peptide sequences responsible for the antioxidant activity of kafirin Alcalase

hydrolysate. Valine (Val, V), leucine (Leu, L) and isoleucine (Ile, I) are symbolic hydrophobic amino acids, which were found to be present in almost all of the identified peptide sequences. This might be due to the cleavage nature of Alcalase that released more hydrophobic amino acids during the unfolding of kafirin protein.

The presence of these hydrophobic amino acids increased the solubility of their compositional peptides in lipid phase, thus, enhanced their accessibility to the hydrophobic lipid and/or oil targets. Besides, cysteine (Cys, C) and methionine (Met, M) are typical nucleophilic sulfur-containing amino acids that are widely accepted as antioxidant amino acids or important constituent amino acids in antioxidant peptide sequences despite some prooxidant properties under some circumstances (Samaranayaka & Li-Chan, 2011; Wang et al., 2007), which were found to be present in 10 and 5 of the identified peptides, respectively. Tryptophan (Trp, W) is an aromatic amino acid that was also reported to be an important antioxidant amino acid due to the hydrogen donating ability of the aromatic ring (Guo, Kouzuma, & Yonekura, 2009). 10 of the identified peptides were found to contain Try at terminals or in sequences.

4. Conclusions

Alcalase is an effective enzyme to produce antioxidant peptides with a relatively high protein recovery, increased total phenolic content, and enhanced antioxidative activities. The optimal reaction conditions of hydrolyzing kafirin with Alcalase was determined to be protein content of 4%, enzyme-to-substrate ratio of 0.4 Au/g, and hydrolysis time for 4 hours. By using membrane ultrafiltration, the kafirin Alcalase hydrolysate was fractionated and the antioxidant activities associated with different M_w ranges were evaluated through several different assays. Medium-sized fraction of hydrolysate (5 – 10 kDa) was found to possess the highest total phenolic content, DPPH radical scavenging activity, ABTS radical scavenging activity, reducing

power, and metal chelating capacity. In two different model systems, the selected fraction of hydrolysate unveiled excellent inhibition effect against oil and/or lipid peroxidation. The hydrolysate was further fractionated by gel filtration chromatography and smaller-sized fraction (F3) showed a significantly stronger antioxidant activity.

By using RP-HPLC followed with MALDI-TOF/TOF MS analysis, 26 representative peptides were identified for sequences, meantime, QWQQ and QQWQ were found to be present in all major peaks from HPLC. In addition, the cleavage of peptides by Alcalase yielded significant amount of hydrophobic amino acids, which accounted for its admirable antioxidative activities especially towards hydrophobic targets.

With relatively high antioxidant activity and total protein recovery, the novel kafirin hydrolysates obtained with Alcalase is attractive to be considered as alternatives to synthetic antioxidants in various food and feed products as functional ingredients to deliver multiple functionalities besides its nutritious values. The protection of oil and fats also aids the utilization of healthy but highly unstable ingredients such as unsaturated fatty acids.

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Table 0-1 Representative antioxidant peptides in kafirin Alcalase hydrolysates.

Peak	1.6 min	3.6 min	35.0 min	36.0 min
Area %	30.62%	11.03%	2.34%	4.15%
Coverage %	36.50%	82.80%	54.70%	27.10%
	KMVIV LAVCLA AVCLAL QQWQ QWQQ RQQCC MCGWQ CATSAAI	QWQQ QQWQ GVVQSV	QQWQ QWQQ GVVQSV QLQGVA VQQLQ VAQVAQ RQQCC MCGWVVQ CATSAAI DMQSR	KMVIV AVCLAL LAVCLA QQWQ QWQQ

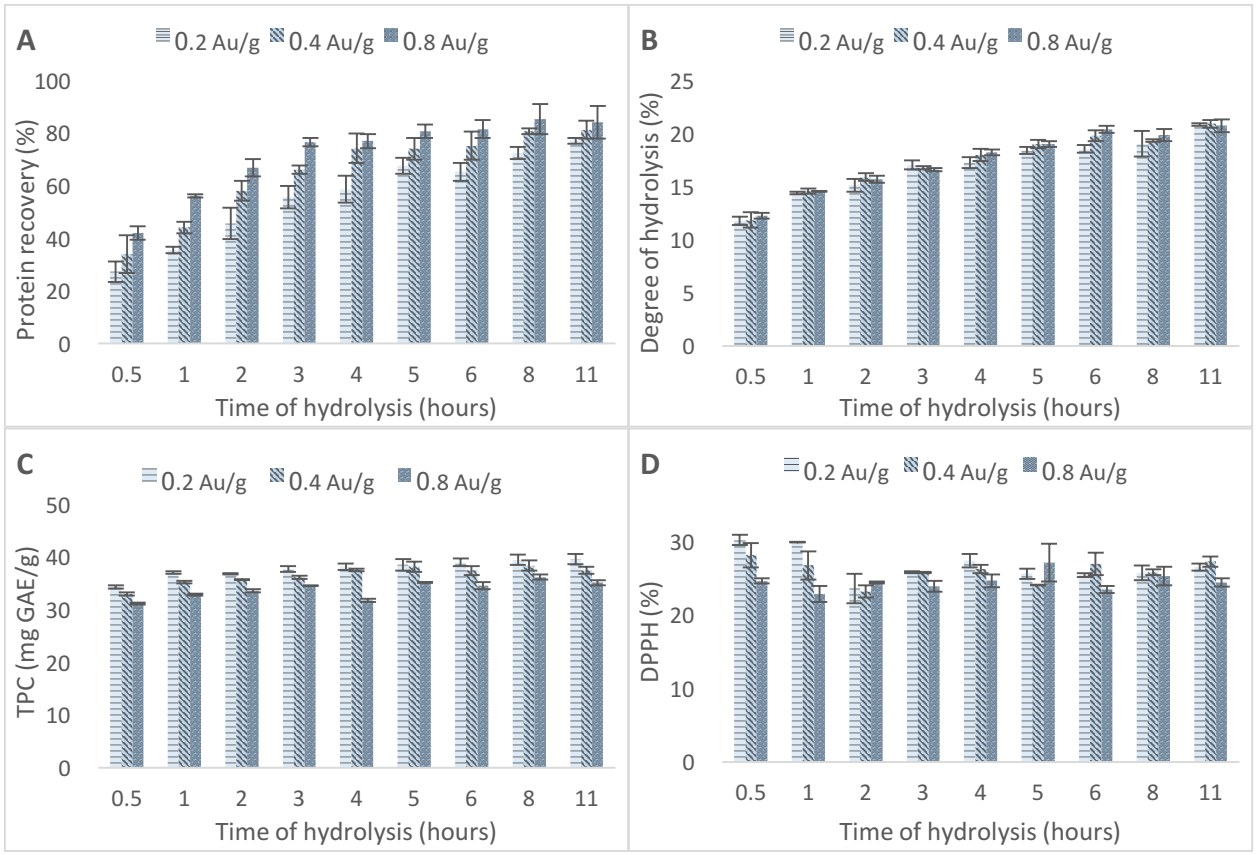


Figure 0-1 Reaction optimization and antioxidant activities of kafirin Alcalase hydrolysates prepared at combinations of different hydrolysis time and enzyme-to-substrate ratios of 0.2, 0.4, and 0.8 Au/g. A. Total protein recovery (%); B. degree of hydrolysis; C. total phenolic content (mg GAE/g); D. DPPH scavenging activity (%) at 5 mg/mL.

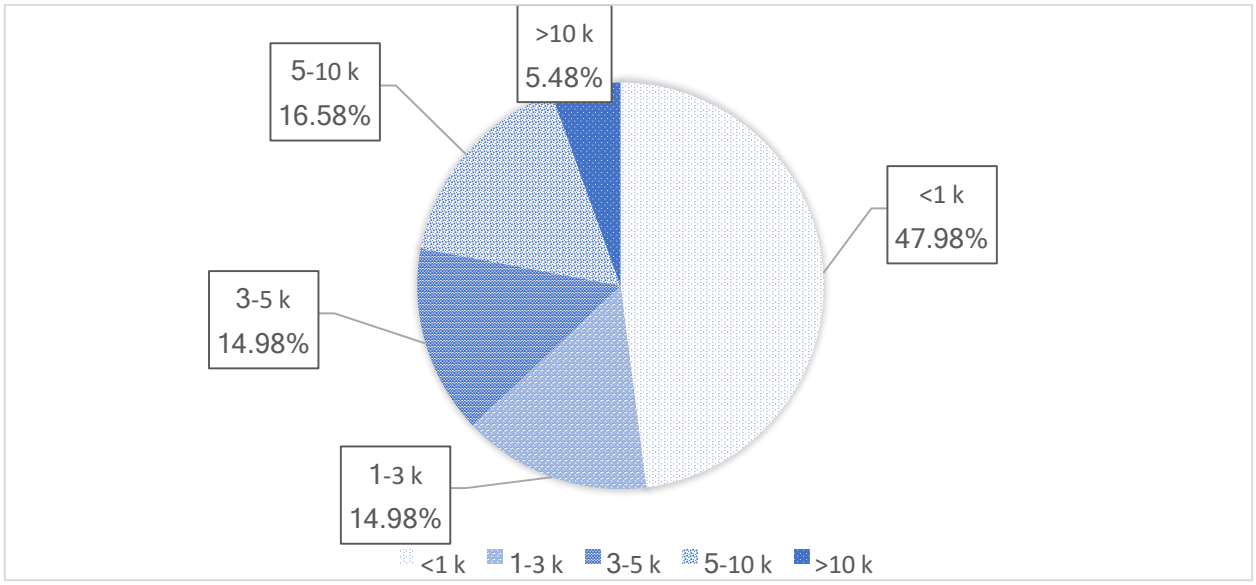


Figure 0-2 Distribution of ultrafiltrated fractions of kafirin Alcalase hydrolysate prepared at enzyme-to-substrate ratio of 0.4 Au/g and hydrolyzed for 4 hours followed with membrane filtration using 10 k, 5 k, 3 k, and 1 kDa membranes.

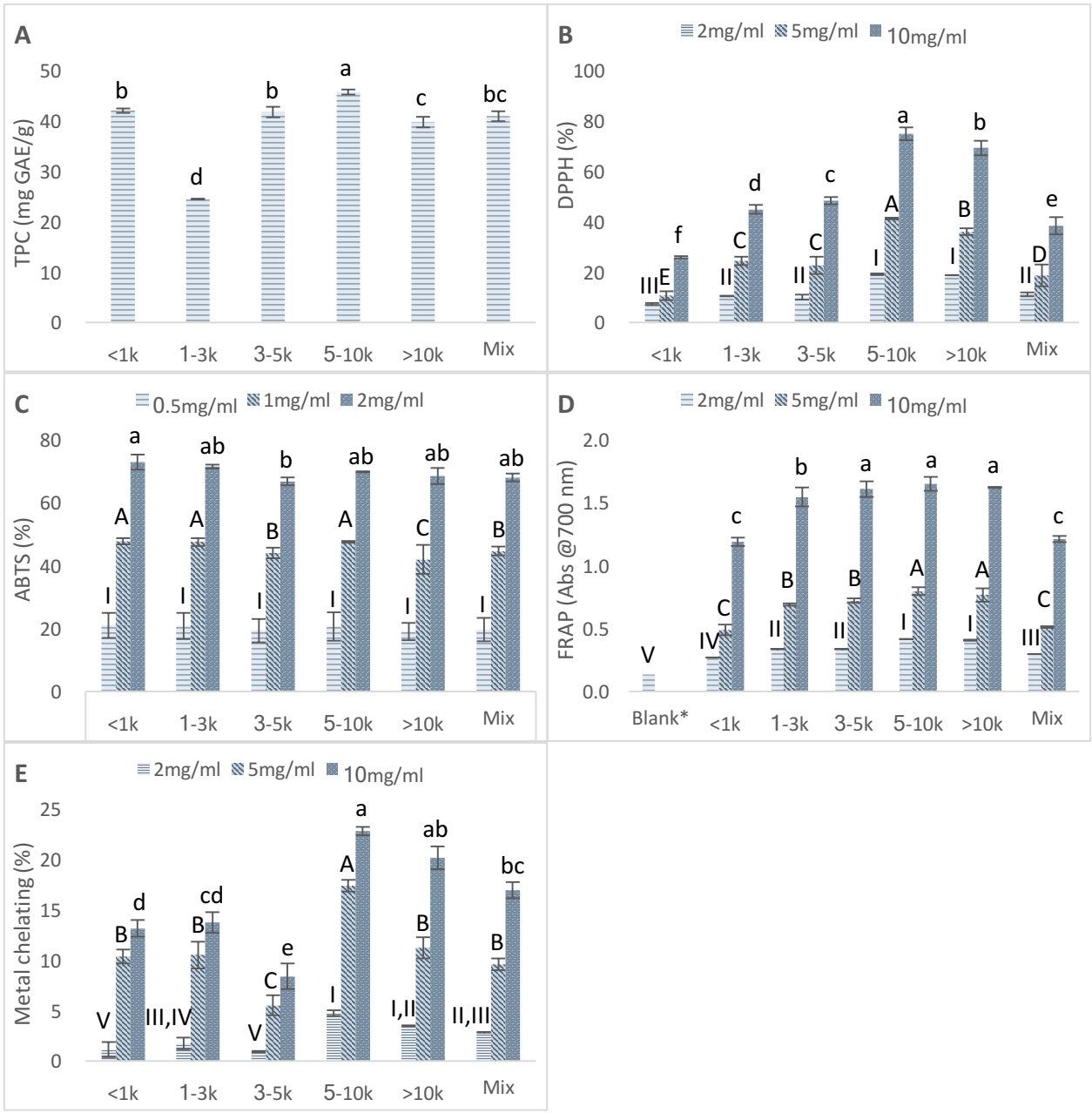


Figure 0-3 Total phenolic content and antioxidant activities of kafirin Alcalase hydrolysate ultrafiltrated fractions prepared at enzyme-to-substrate ratio of 0.4 Au/g and hydrolyzed for 4 hours followed with membrane filtration using 10 k, 5 k, 3 k, and 1 kDa membranes. A. Total phenolic content (mg GAE/g); B. DPPH scavenging activity (%); C. ABTS scavenging activity (%); D. Reducing power capacity (Abs at 700 nm); E. Metal chelating capacity (%).

* Blank represents the absorbance of reaction mixture at 700 nm using distilled water in substitute of sample.

Different lowercase letters, capital letters, and roman numerals indicated significant difference at $P < 0.05$.

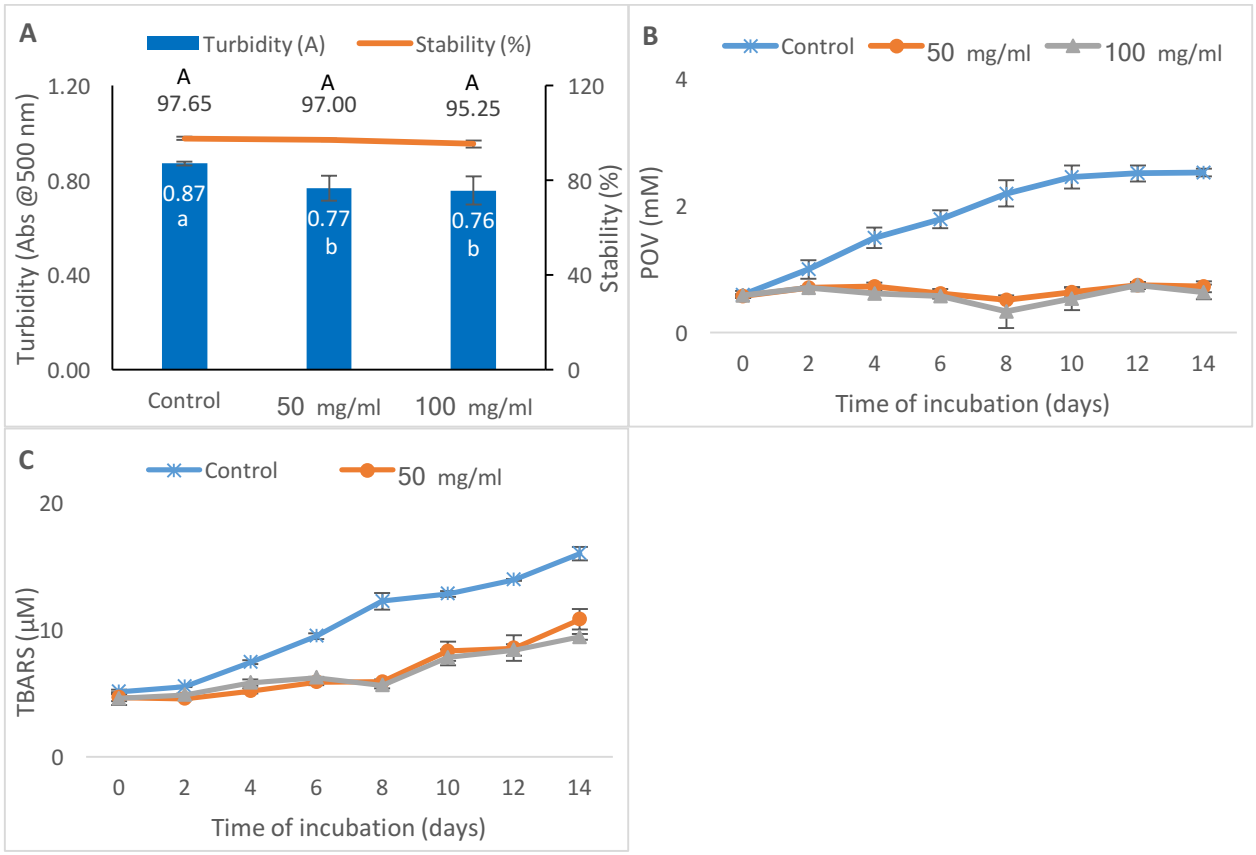


Figure 0-4 Inhibition effect of kafirin Alcalase 5 – 10 kDa hydrolysate prepared at enzyme-to-substrate ratio of 0.4 Au/g and hydrolyzed for 4 hours in an oil-in-water emulsion model system added with 50 and 100 mg/mL oil. A. Emulsion turbidity (Abs at 500 nm) and stability (%); B. POV (mM cumene hydroperoxide equivalent); C. TBARS (µM tetramethoxypropan equivalent).

Different lowercase and capital letters indicated significant difference at $P < 0.05$.

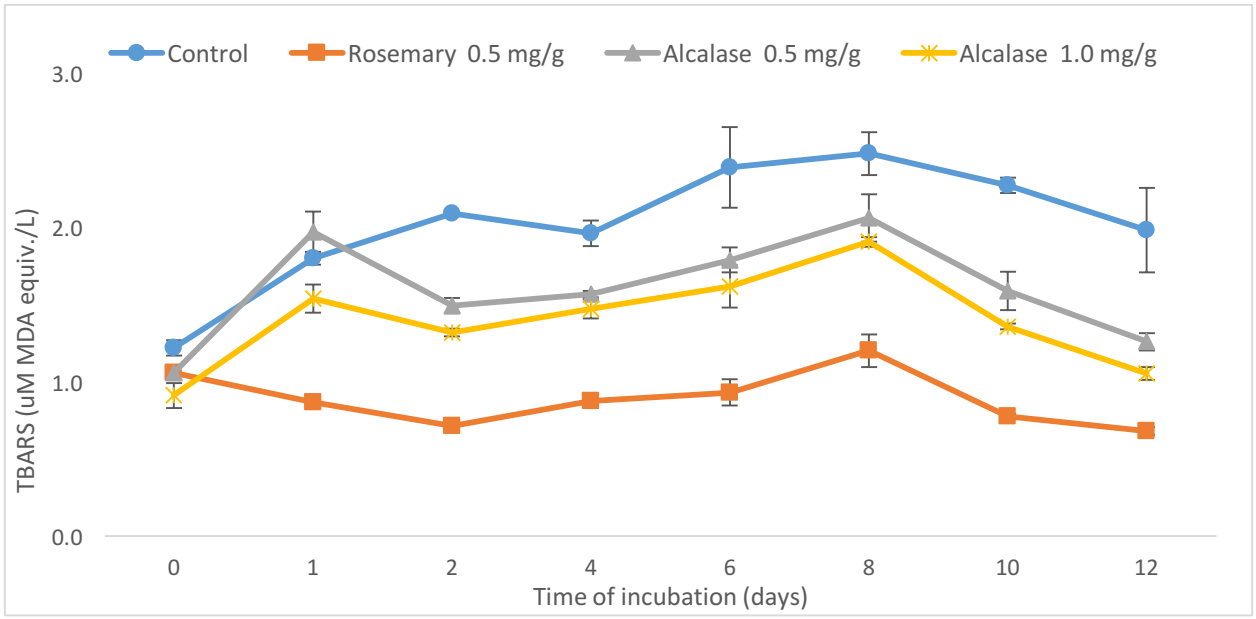


Figure 0-5 Inhibition effect shown as TBARS of kafirin Alcalase 5 – 10 kDa hydrolysate prepared at enzyme-to-substrate ratio of 0.4 Au/g and hydrolyzed for 4 hours in a ground meat model system added with 0.5 and 1 Mg/g meat.

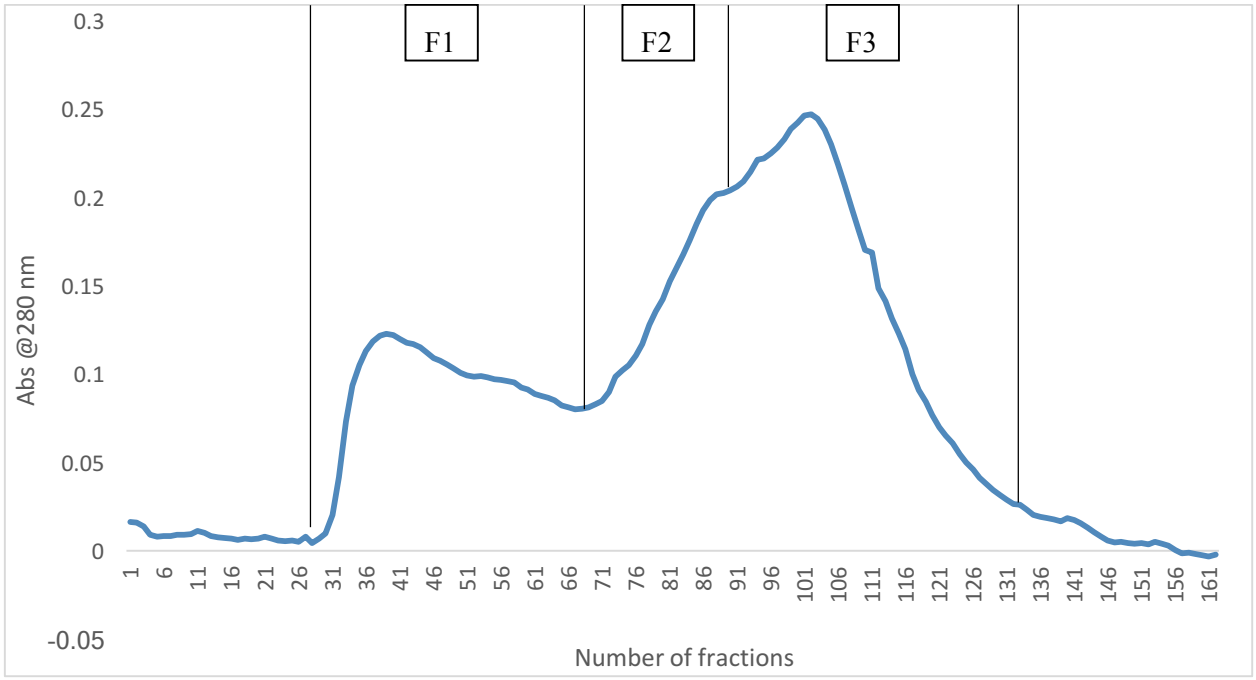


Figure 0-6 Gel filtration chromatogram of kafirin Alcalase 5 – 10 kDa hydrolysate prepared at 0.4 Au/g and hydrolyzed for 4 hours in a Sephadex G-25 column (26 mm × 850 mm).

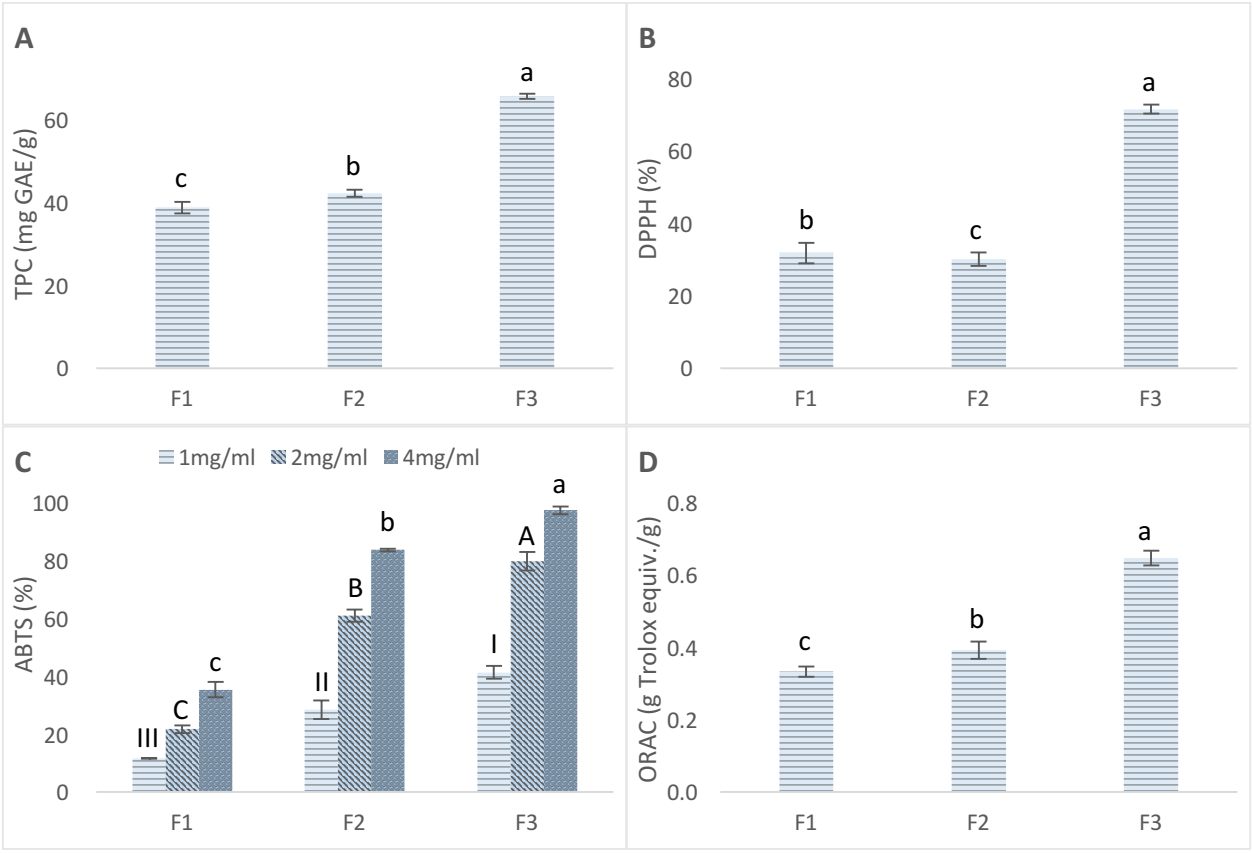


Figure 0-7 Total phenolic content and antioxidant activities of gel filtration fractions of kafirin Alcalase 5 – 10 kDa hydrolysate prepared at 0.4 Au/g and hydrolyzed for 4 hours A. Total phenolic content (mg GAE/g); B. DPPH scavenging activity (%) at 4mg/mL; C. ABTS scavenging activity (%); D. ORAC (g Trolox equiv./g).

Different lowercase letters, capital letters, and roman numerals indicated significant difference at $P < 0.05$.

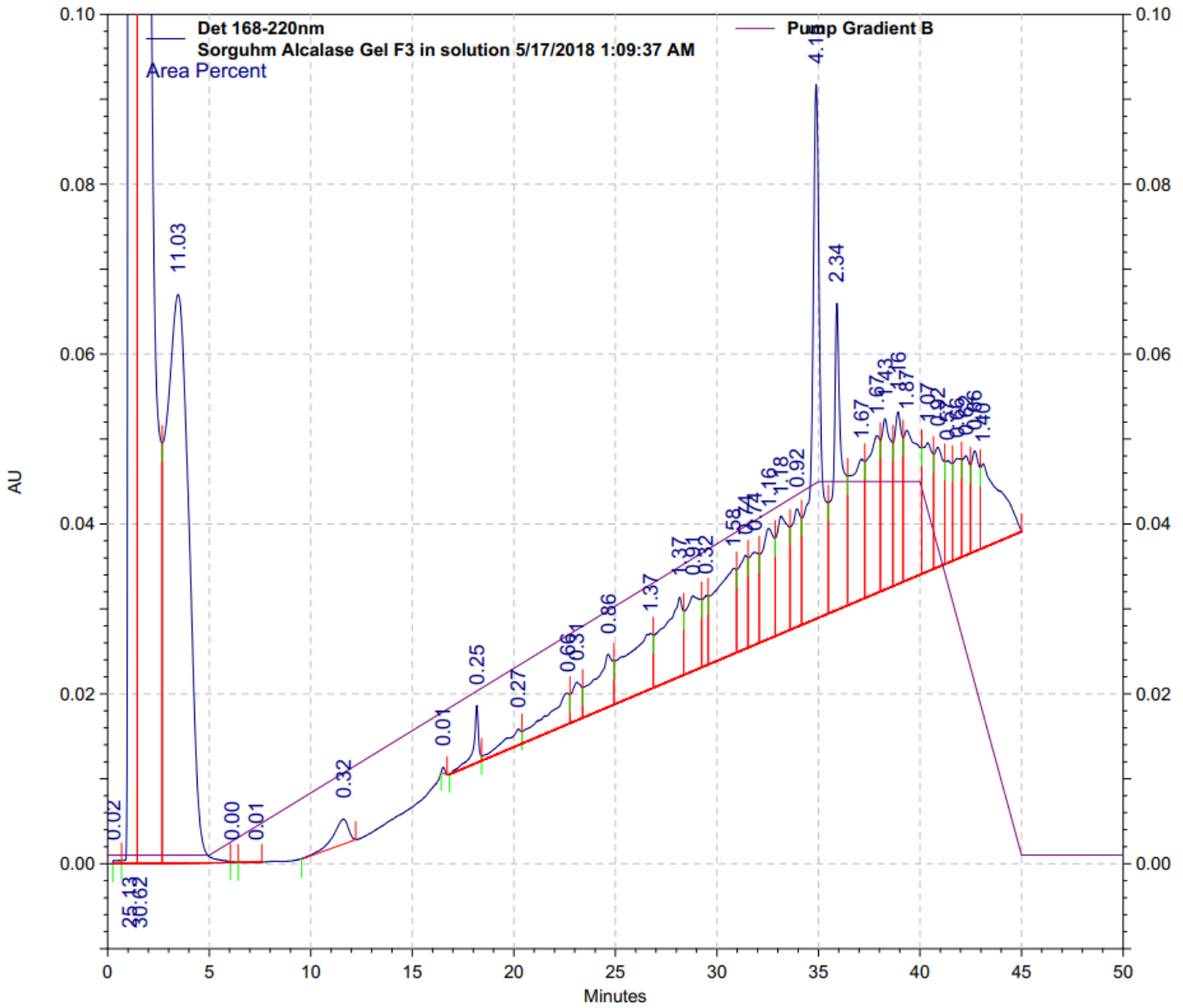
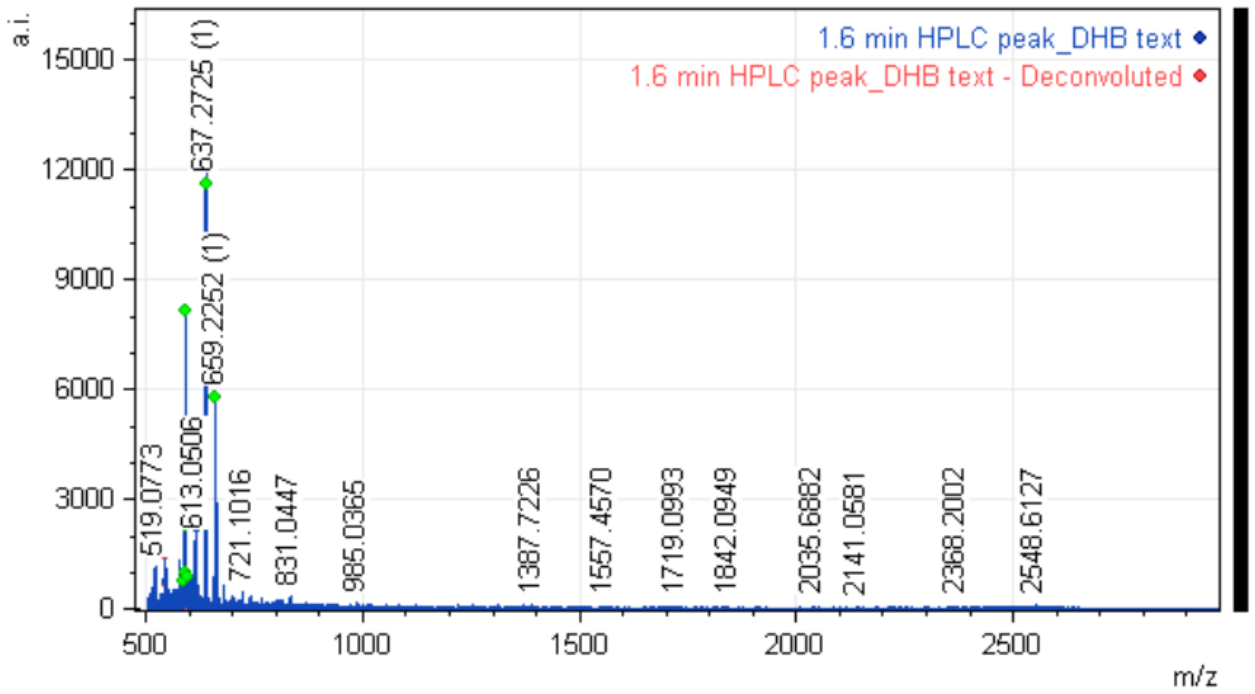


Figure 0-8 RP-HPLC chromatogram of Fraction 3 from gel filtration of 5 – 10 kDa kafirin Alcalase hydrolysates.



Sequence - *Beta Kafirin*

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
	192	20661.8889	20676.4896	36.5 %	83.8 %

MKMVIVLAVC LALSAASASA LQMPGMGLQD LYGAGALMTM MGAGGGLYPC AEYLRQPQCS PVAAPFYALR EQTMWQPNFI
CQPLRQQCCQ QMRMMDMQSR CQAMCGVVQS VVQQLQMTMQ LQGVAAAASS LLYQPALVQQ WQQLLPAAQA LTPLAMAVAQ
VAQNMPAMCG LYQLPSYCTT PCATSAAIPP YY

Figure 0-9 MS spectrum of 1.6 minute peak from RP-HPLC of 5 – 10 kDa kafirin Alcalase hydrolysates gel filtration fraction 3.

Chapter 4 - Reaction Optimization, Antioxidant Activity Characterization, and Peptides Identification of Sorghum Kafirin Hydrolysates Prepared with Papain

Abstract

Papain was used to hydrolyze sorghum kafirin in producing hydrolysates and peptides with antioxidant capacity. At combined treatment of enzyme-to-substrate ratio and hydrolysis time, optimal reaction parameters (substrate content at 4%, enzyme-to-substrate ratio at 360 kU/g, and hydrolysis time of 4 hours) were determined through assessing total protein recovery, degree of hydrolysis, total phenolic content, and DPPH radical scavenging activity. Several other assays reflecting different antioxidation mechanisms were also employed to assess the antioxidant capacity of kafirin hydrolysates. After fractionation by ultrafiltration, small-sized hydrolysate (1 – 3 kDa) had stronger antioxidant activities as well as good yield; therefore, it was subjected for assessment in oil-in-water emulsion and ground pork model system for evaluation of its lipid and oil inhibition effects. The incorporation at 100 mg/mL oil resulted in an inhibition of primary and secondary oxidation products by 43.04% and 66.91%, respectively, without hurting emulsion turbidity or stability. An average of 32.12% inhibition effect towards lipid peroxidation was also detected when incorporated at 1.0 mg/g meat. The selected fraction of hydrolysate was further fractionated by gel filtration, and the most potent fraction (F3) was analyzed by HPLC followed with MALDI-TOF/TOF MS. Peptides LRQQ, QLQGV, and WQPN were found to appear most frequently within the identified sequences, which might be related to the antioxidant activity of kafirin Papain hydrolysate. An extraordinary popularity of

glutamine among identified peptides indicated that it could be an important counterpart accounting for the antioxidative potentials.

1. Introduction

In biological systems, imbalance between oxidants and antioxidants can cause damage to biological structures such as DNA mutation, membrane phospholipids oxidation, protein damages that could eventually lead to diseases (Tang & Zhuang, 2014). In food industry, oxidation of lipids and fat in food products causes undesirable quality deterioration such as development of discoloration, off-flavor, and rancid odors, which ultimately reduces the shelf life and decreases the nutritional quality and safety of foods (Zhao et al., 2012). Antioxidants are a group of substances that can delay or inhibit the oxidation process at low concentrations, which has been commonly used in food industry as additives or functional ingredients to retard the oxidative spoilage of food products and maintain their shelf-stability and/or quality attributes. The antioxidant is a growing market that is projected to reach $3.1 billion in 2020. Synthetic antioxidants such as BHA and BHT was long being used as they are cheap and effective, but in recent years, their use has been limited or prohibited due to the potential toxicity, carcinogenic potentials, and other safety concerns over pathological conditions (Tang & Zhuang, 2014). Driven by market preference for safe and natural antioxidants, the peptide antioxidants have attracted rising interests. Some literatures have reported the excellent efficacy and multifunctionalities of antioxidants derived from cereal proteins such as those obtained from wheat, rice, and corn. These studies built the foundation of transforming sorghum protein into peptide antioxidants as potential alternatives to synthetic antioxidants. Compared to synthetic antioxidants, peptide antioxidants are considered safe even at high concentrations. They are also able to provide essential amino acid profiles, nutritional values, potential health benefits, and various functional properties such as gelling, emulsifying, foaming, water and/or oil binding capacity (Sloan, 2014).

United States is now leading in global sorghum production and distribution, whereas sorghum is mainly used for animal feed or a starch source for production of bioethanol (Lee et al., 2011). Since there is an increasing trend of transforming bioresources into value-added products, the by-products (e.g., sorghum DDGS) of sorghum bioethanol industry, which was often discarded or underutilized, could possibly be modified into novel products for various industrial applications. Producing antioxidative hydrolysates and peptides from these protein-rich by-products or co-products by enzymatic hydrolysis provides a feasible approach for this value-adding conversion.

Proteins are typically large matrix with complex tertiary structures. The antioxidant activities of proteins are limited because functional groups and structural domains associated with antioxidative activities of peptides sequences are buried inside the hydrophobic core and are inaccessible to pro-oxidants. Enzymatic hydrolysis is commonly used to prepare hydrolysates and peptides with improved functionality and enhanced bioactivities, hence, are favorable than intact proteins in a lot of industrial applications. Many studies have reported that Papain was a promising enzyme to obtain antioxidative hydrolysates and peptides from various protein origins including wheat gluten (Wang et al., 2007), corn gluten meal (Zhuang et al., 2013), zein (Kong & Xiong, 2006; Tang & Zhuang, 2014), and so on. Results of preliminary experiments also indicated that Papain was an efficient enzyme to release antioxidative peptide sequences from sorghum kafirin with a good yield of protein recovery. However, to the best of our knowledge, no report was yet found about the fractionation and characterization of antioxidative hydrolysates of sorghum protein hydrolyzed by Papain.

The objectives of this study were to: 1) optimize the reaction variables (hydrolysis time, enzyme-to-substrate ratio) of kafirin hydrolysis using Papain in production of antioxidative

hydrolysates; 2) evaluate the antioxidative profile of purified peptide antioxidants by comprehensive *in vitro* assays as well as the antioxidant performances in emulsion and meat model systems; and 3) further purify the antioxidant peptides and identify the critical sequences in the most potent fraction of hydrolysates.

2. Materials and Methods

The materials and methods used for the study in this chapter were similar to those described in Chapter 2 and Chapter 3, thus, will not be repeated.

3. Results and Discussion

3.1. Reaction optimization of kafirin enzymatic hydrolysis

Enzyme (e.g., type of enzyme, enzyme amount), substrate (e.g., type of protein, protein content), and hydrolysis conditions (e.g., hydrolysis time) altogether enforced an integrated impact on the end product of hydrolysate including its yield as well as the antioxidant activity. In order to identify the optimal reaction conditions in of kafirin hydrolysis with Papain for production of antioxidant peptides, a factorial designed experiment using combined treatment of enzyme-to-substrate ratio (90, 180, 360, and 540 kU/g) and hydrolysis times (varied from 0.5 hour to 6 hours) was conducted. The recovery rate of total soluble hydrolysates released from intact protein, degree of hydrolysis (DH), total phenolic content (TPC), and antioxidant activity measured by DPPH radical scavenging activity (DPPH%) were employed as critical indicators and the results were summarized in Figure 4-1.

In Figure 4-1 (A), with a prolonged hydrolysis time especially from 0.5 to 4 hours, a huge leap in total protein recovery was observed, despite the specific point of time to reach the reaction plateau varied for different enzyme-to-substrate ratio treatments. At lower levels of enzyme dosage (90 and 180 kU/g), the reactions were close to equilibrium at around 2 hours of

hydrolysis and the total protein recovery rates did not increase obviously afterwards with extended hydrolysis time. When elevating the enzyme-to-substrate ratio from 90 to 540 kU/g, total recovery rates greatly increased especially for the hydrolysates obtained with longer time of hydrolysis. The hydrolysis occurred at higher levels of enzyme-to-substrate ratios (360 and 540 kU/g) displayed good recovery rates even with a shorter hydrolysis time, the recovery rates were ended close to 100% given sufficient hydrolysis. Yet, a noteworthy increase did not appear when changing the enzyme-to-substrate ratio from 360 to 540 kU/g. Similarly, in Figure 4-1 (B), DH consistently increased with protracted hydrolysis time for all treatments until it reached steady at around 4 hours of hydrolysis. A remarkable increase in DH was not observed when increasing the enzyme amount from 90 to 540 kU/g. This data implied that in the initial stage of protein hydrolysis, reaction time applied to the system was the first limiting factor; meanwhile, the amount of enzyme applied to the hydrolysis system was the limiting factor in the latter stage of protein hydrolysis.

Hydrolysis of kafirin leads to the structural changes of the intact proteins including the release of phenolic peptides and phenolic compounds, which significantly contributed to the total antioxidant capacity of hydrolysates (Liu et al., 2017). The total phenolic content (TPC) is a rough estimate of the quantity of these compounds (Anunciação et al., 2017), thus, was measured for all treatments of kafirin hydrolysates as shown Figure 4-1 (C). All hydrolysates had a similar TPC value that was not impacted by the different treatment of hydrolysis time or enzyme-to-substrate ratios. The TPC was between 41.16 and 44.63 mg GAE/g, which was higher than that prepared with Alcalase or Neutrase. Thamnarathip et al. (2016) also reported that with an increased hydrolysis time from 2 to 6 hours, TPC of rice bran protein hydrolysate was not significantly changed.

DPPH% is a simple and rapid assay that tests the ability of a compound to act as electron donors, which provide straightforward information antioxidant potentials of kafirin hydrolysates. As shown in Figure 4-1 (D), kafirin Papain hydrolysates prepared with all treatments possessed DPPH scavenging capacities. The DPPH% values of hydrolysates prepared after 3 hours of hydrolysis were very much similar (35.21% - 47.88% at 5 mg/mL), and no obvious relationship between DPPH% and hydrolysis time was observed. It worth attention to note that the abnormal high values of hydrolysates prepared with less than 2 hours were detected, which was largely due to the intrinsic activity of enzyme may have conquered the activity of hydrolysates therefore was not taken into consideration. This result revealed that, the kafirin Papain hydrolysates contained substrates that were electron donors, which were able to convert free radicals into more stable products and therefore terminate the radical chain reaction (Wang et al., 2007).

As a conclusion, extending hydrolysis time after 4 hours did not result in an improved total protein recovery. The additional amount of enzyme from 360 to 540 kU/g was unnecessary. Since an extensive hydrolysis may lead to the production of free amino acids or short-chained peptides that lost the essential structures accounting for antioxidative capacity, a hydrolysis time longer than 4 hours was not suggested. Considering a good balance of protein recovery, antioxidant activity, and economic efficiency, 4% kafirin protein solution coupled with an enzyme-to-substrate ratio of 360 kU/g for 4 hours was determined to be the optimal reaction parameters for the hydrolysis of kafirin in production of antioxidant hydrolysate.

3.2. Ultrafiltration of kafirin Papain hydrolysates

It was widely reported that, the molecular mass is one of the most important determining the antioxidant activity of a peptide (Zheng et al., 2006; Liu et al., 2017). Thus, it is necessary to characterize the molecular weight distribution of the antioxidative hydrolysates and study the

antioxidant activity associated with each molecular weight range. The kafirin hydrolysate prepared with Papain at optimized conditions (protein content of 4%, enzyme-to-substrate ratio of 360 kU/g, and hydrolysis time of 4 hours) was sequentially ultrafiltrated in a stirred cell coupled with 10 k, 5 k, 3 k, and 1 kDa molecular weight cut-off membranes. Fractions with different molecular weight ranges were collected, lyophilized, and analyzed.

Figure 4-2 showed the distribution of fractions of hydrolysates with different molecular weight ranges from ultrafiltration. As it can be seen, the < 1 kDa fraction took up most of the hydrolysate (55.75%), the 5 – 10 kDa fraction was the second largest fraction (14.75%) followed with > 10 (12.10%), 1 – 3 (10.65%), and 3 – 5 kDa (6.75%) fractions. Since percentage yield of protein hydrolysates was rarely reported, there were not adequate literatures for comparison. This result was in accordance with previous study of kafirin Alcalase hydrolysates as <1 kDa was the largest portion. Alashi et al. (2014) also reported that < 1 kDa fraction took up the largest portion followed with 1 – 3 k, 3 – 5 k, and 5 – 10 kDa of hydrolysates obtained from canola protein hydrolysates.

As shown in Figure 4-3 (A), TPC was measured for the hydrolysate mixture and its ultrafiltrated fractions. It was found that, 3 – 5 kDa (43.48 ± 0.07 mg GAE/g) fraction of hydrolysate possessed highest TPC followed with 1 – 3 kDa (42.82 ± 0.12) and < 1 kDa (42.51 ± 0.22) fractions. Except for > 10 kDa fraction (25.01 ± 0.19 mg GAE/g), all the other ultrafiltrated fractions were found to have higher TPC than the hydrolysates without ultrafiltration (41.37 ± 0.20 mg GAE/g).

DPPH% assay was used to evaluate the antioxidant activity of ultrafiltrated fractions of hydrolysates regarding their ability in electron donating. In Figure 4-3 (B), all fractions of hydrolysates showed abilities to quench DPPH radicals, the scavenging effects increased along

with amplified sample concentrations, undoubtedly. Among all five ultrafiltrated fractions, medium-sized peptides, 5 – 10 kDa fraction exhibited strongest DPPH% (67.51 ± 0.33% at 5 mg/ml) followed by 3 – 5 kDa (50.68 ± 2.17% at 5 mg/ml) while the small-sized peptides < 1 kDa (33.76 ± 0.89% at 5 mg/ml) fraction had the lowest DPPH% value.

The medium-sized fraction of peptides 3 – 5 kDa also exhibited the strongest reducing power capacity reflected by the highest absorbance at 700 nm (A_{700} = 1.59 ± 0.037 at 5 mg/mL) monitored on a spectrophotometer (Figure 4-3 (D)). The increased absorbance of sample over a blank control implied the amount of ferrous iron reduced from ferric iron by the of antioxidant effects of hydrolysates. In addition, all fractions of kafirin hydrolysate possessed markedly higher absorbance values (A_{700} > 1.00 at 5 mg/mL) than the blank control (A_{700} = 0.136 ± 0.048) may be attributed to the increased availability of hydrogen ions (protons and electrons) due to peptide cleavages (Kong & Xiong, 2006).

ABTS% assay provided the evaluation of antioxidant capacity in a different aspect, which was calculated by the percentage of ABTS radicals quenched by the antioxidants. As shown in Figure 4-3 (C), small-sized peptide fractions, < 1 kDa (36.24 ± 1.82% at 2 mg/ml) and 1 – 3 kDa (37.44 ± 2.46% at 2 mg/ml) yielded a higher ABTS% than the other larger-sized peptide fractions. The small-sized peptide fractions < 1 kDa and 1 – 3 kDa also exhibited good to excellent activities in metal chelating capacity and ORAC assay as shown in shown in Figure 4-3 (E) and Figure 4-3 (F), respectively.

Smaller-sized peptides are commonly preferred for better antioxidative activity in a lot of studies as they are believed to be more accessible and easily adsorbed to the oxidative agents compared to the larger peptides (Phongthai et al., 2018; Wang et al., 2014; Zhang, Li, & Zhou, 2010; Park, Jung, Nam, Shahidi, & Kim, 2001). Based on the combined data, 1 – 3 kDa fraction

exhibiting promising antioxidant activities and acceptable yield was selected for further evaluation in model systems.

3.3. Inhibition of lipid oxidation in model systems

3.3.1. Oil-in-water emulsion system

Each *in vitro* chemical assay such as DPPH, ABTS, ORAC, reducing power, or metal chelating measured antioxidant activity represented by a single mechanism, which could not reflect the multiple mechanisms or the comprehensive outcome of peptide antioxidants (Chi et al., 2014). Thus, it is necessary to evaluate the peptides' protective action of oil/lipid oxidation in model food systems, which provides important information to processing strategies for the development of peptide antioxidants (Cheng, Xiong & Chen, 2010).

An oil-in-water emulsion model system was employed to examine the expected inhibition effect of antioxidant peptides against oil/lipid oxidation. 1 – 3 kDa fraction of kafirin Papain hydrolysates which showed relatively higher activities in chemical assays was incorporated at 50 mg and 100 mg per mL of oil to prepare emulsion samples. Tween-20 was added as a stabilizer to enhance the emulsion stability suggested by Cheng, Xiong & Chen (2010) and Zhao et al. (2012).

As shown in Figure 4-4 (A), no significant difference in emulsion turbidity and emulsion stability ($P < 0.05$) was observed for the emulsion sample incorporated with kafirin Papain hydrolysates. This indicated that the utilization of hydrolysates did not alter the texture properties of emulsions. High turbidity values ($A_{500} > 0.80$) were observed among all emulsion samples, which symbolized that the oil was effectively dispersed in the aqueous solution and fine emulsions were achieved. All emulsions exhibited good stabilities (> 94%) and stayed stable during a 14-day incubation period at 37 °C.

Besides, as shown in Figure 4-4 (B) and (C), the oxidation activities of emulsion were effectively inhibited by the addition of antioxidant peptides. The concentration peroxide values (POV) was decreased by an average of 59.27 ± 15.19% and 66.91 ± 16.16% by hydrolysates at 50 mg/mL and 100 mg/mL, respectively. The inhibition rate slowly increased with an extended incubation time, and the maximal inhibition was achieved at day 10 with 73.36% and 82.49% for 50 mg/mL and 100 mg/mL, respectively. On day 10, the inhibition rate decreased to around 61.31% and 65.50%, which reflected a loss of efficacy of antioxidant peptides. The concentration of TBARS was also reduced by antioxidant peptides. It has a highest inhibition rate of 40.39% for 50 mg/mL and at day 10, and 56.44% for 100 mg/mL at day 12. The average inhibition was 28.63 ± 10.7% and 43.04 ± 12.29% for 50 mg/mL and 100 mg/mL, respectively.

It can be concluded that kafirin hydrolysate prepared with Papain effectively act as an antioxidant in protecting the emulsion from oxidation.

3.3.2. Ground meat system

The oxidative damage to meat-based products results in problems like tissue damage, development of undesired color and odor, loss of nutrients, accumulation of toxic products, etc. The oxidative stability of meat products mainly depends on the balance of antioxidants, oxidative agents, cholesterol, and haeme pigment (Sohaib et al., 2017). Lipid peroxidation is an oxidative chain reaction where lipid molecules were oxidized to the maximum possible extent to form lipid peroxides (Alashi et al., 2014). It could be slow at the beginning but proceed rapidly once oxidative chain reaction was initiated. Lipid hydroperoxides, which are the primary products of lipid oxidation, have higher polarity than normal fatty acids, thus, they are able to disrupt the integral structure and function of the biological membranes and cause detrimental damages to the biological tissues (Sohaib et al., 2017). The application of antioxidant is considered a pragmatic

choice in controlling the lipid oxidation problem of meat and meat products as it can retard the rate of oxidation and reduce the peroxyl radicals to the hydro-peroxide before it participates the radical chain (Alashi et al., 2014). Antioxidants can further reduce protein oxidation as well as the interaction of lipid-derived carbonyls with proteins, preventing alteration of protein functionalities (Sohaib et al., 2017).

As shown in Figure 4-5, it was observed that over 12 days incubation period, the kafirin Papain hydrolysates was able to inhibit the peroxidation as revealed by the reduced concentration of TBARS compared to blank control. A mean inhibition level was calculated to be 29.26 ± 5.14% and 32.12 ± 9.03% for 0.5 mg/g and 1.0 mg/g, respectively. The inhibition rate was slowly increased in early stage of incubation and reached maximum at day 8, where the inhibition was observed to be 30.76% and 40.03% for 0.5 mg/g and 1.0 mg/g, respectively.

The inhibition of lipid oxidation has been attributed to chelation of prooxidative metal ions, quenching singlet oxygen, scavenging free radicals, and termination of free radical chain reactions (Kong & Xiong, 2006) which was accomplished through specific amino acid residue side-chain groups or specific peptide structure. The oxidation inhibitive potentials of kafirin hydrolysates revealed in this study was higher than that of soy protein hydrolysates previously reported by Zhang et al. (2010). However, the TBARS inhibition percentage was lower than that of commercial rosemary extract. More efforts need to be paid to fractionate and purify the hydrolysates to determine the active components responsible for the antioxidant activities.

3.4. Purification and identification of antioxidative peptides from kafirin Papain 1 – 3 kDa hydrolysates

3.4.1. Gel filtration of kafirin Papain 1 – 3 kDa hydrolysates

Kafirin Papain 1 – 3 kDa fraction of hydrolysate was further separated on a Sephadex-G25 gel filtration column according to its molecular weight differences. Two major peaks appeared in the elution chromatogram (Figure 4-6), and the entire elution was divided into three fractions (F1-F3).

After collecting and freeze-drying each fraction, total phenolic content and antioxidant activities of the separated fractions were analyzed. As shown in Figure 4-7, the second peak in the chromatogram (F3) exhibited higher TPC, DPPH%, ORAC, and metal chelating% than the other fractions which represented that the smaller-sized peptides possessed stronger activities in kaifrin Papain hydrolysates. The antioxidant activity as well as TPC of F3 was also significantly higher ($P < 0.05$) than that fractionated before gel filtration.

3.4.2. Identification of representative peptide sequences from gel filtration

The fraction 3 (F3) of gel-filtrated hydrolysate which showed excellent antioxidant activity in previous experiments was analyzed by RP-HPLC. Peaks at 2.6-, 5.8-, 13.6- and 15.9-minute had relatively higher area percentages from HPLC chromatogram, which were 14.9%, 6.24%, 11.67% and 9.63%, respectively. These peaks were eluted and analyzed for identification of amino acid sequence profile with MALDI-TOF/TOF MS. By fitting the MS spectrum to the beta kafirin protein patterns, 13 sequences with 100% relatively intensity were identified and summarized in Table 4-1.

LRQQ, QLQGV, and WQPN were present in both 2.6- and 5.8-minute peaks. Glutamine (Gln, Q) is an amino acid with antioxidant and immunomodulatory properties (Shabert,

Winslow, Lacey, & Wilmore, 1999). Suetsuna and Chen (2002) synthesized two sequences of two peptides LQPGQGQQG and AQIPQQ with promising antioxidant activity, where Gln was the most abundant amino acid. In this study, 11 out of 13 identified peptides contained Gln at terminals or within the sequences. Thus, the extraordinary high level of glutamine in the identified peptides might play an essential role for the antioxidant activity of kafirin hydrolysate. Bedsides, leucine (Leu, L) and valine (Val, V) are the second and third abundant amino acids among the identified peptides, which are also important hydrophobic amino acids. Along with proline (Pro, P), they increased the overall hydrophobicity of the fraction of hydrolysate, therefore caused a higher antioxidant activity especially in hydrophobic lipid and oil phase. Gu et al. (2012) reported that, the presence of Tyr at N-terminal position was responsible for the strong oxygen radical absorbance capacity of YECG (Tyr-Glu-Cys-Gly). Thus, Tyr at N-terminal position could be responsible for the antioxidant activity capacity of YLRQ in peak 13.6 minute (Gu et al., 2012). Ala at the N-terminal might be responsible for the antioxidant activities of AQVAQ and AMCGVV (Wang et al., 2007).

4. Conclusions

Kafirin hydrolysates obtained with Papain exhibited notable antioxidant activity as well as high yield. The optimal reaction parameters were determined as protein content of 4%, enzyme-to-substrate ratio 360kU/g, and hydrolysis time of 4 hours. After fractionation by ultrafiltration, peptides with lower M_w (1 – 3 kDa) exhibited favorable antioxidant activities evaluated by several different assays. Results from the assays of DPPH%, ABTS%, reducing power, and metal chelating demonstrated that the antioxidant activity of kafirin hydrolysates appeared to involve multiple modes of actions including electron/hydrogen donation, radical quenching, and metal ion chelation. In addition, in the emulsion and meat model systems, the

selected fraction of hydrolysates effectively retarded oil and/or lipid peroxidation. The inhibition of lipid oxidation has been attributed to chelation of prooxidative metal ions, quenching singlet oxygen, scavenging free radicals, termination of free radical chain reactions, and the ability to perform as shielding barriers to minimize the contact of prooxidants to susceptible oil droplets (Kong & Xiong, 2006). 13 peptide sequences were identified from HPLC analysis followed with MALDI-TOF/TOF MS of the most potent fraction from gel filtration chromatography. Glutamine and hydrophobic amino acids (Pro, Leu, and Val) were found largely present in the identified peptide sequences, which might be accountable for a high antioxidant activity.

These combined data provided important evidences showing that some kafirin peptides hydrolyzed with Papain can be used as potential antioxidants as alternatives to synthetic antioxidants to protect the susceptible ingredients within the food and feed products from peroxidation. The production of peptides from kafirin can also improve the utilization of distillers' grain and accelerate the industrial production cycle with an additional revenue stream.

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Table 4-1 Representative antioxidant peptides in kafirin Papain hydrolysates.

Peak	2.6 min	5.8 min	13.6 min	15.9 min
Area %	14.90%	6.24%	11.67%	9.63%
Coverage %	66.10%	28.60%	72.90%	29.70%
	LRQQ QLQGV WQPN	GLQDL LRQQ QLQGV WQPN	AMCGVV YLRQ TPCATS	QGVAAA AQVAQ QQLQ

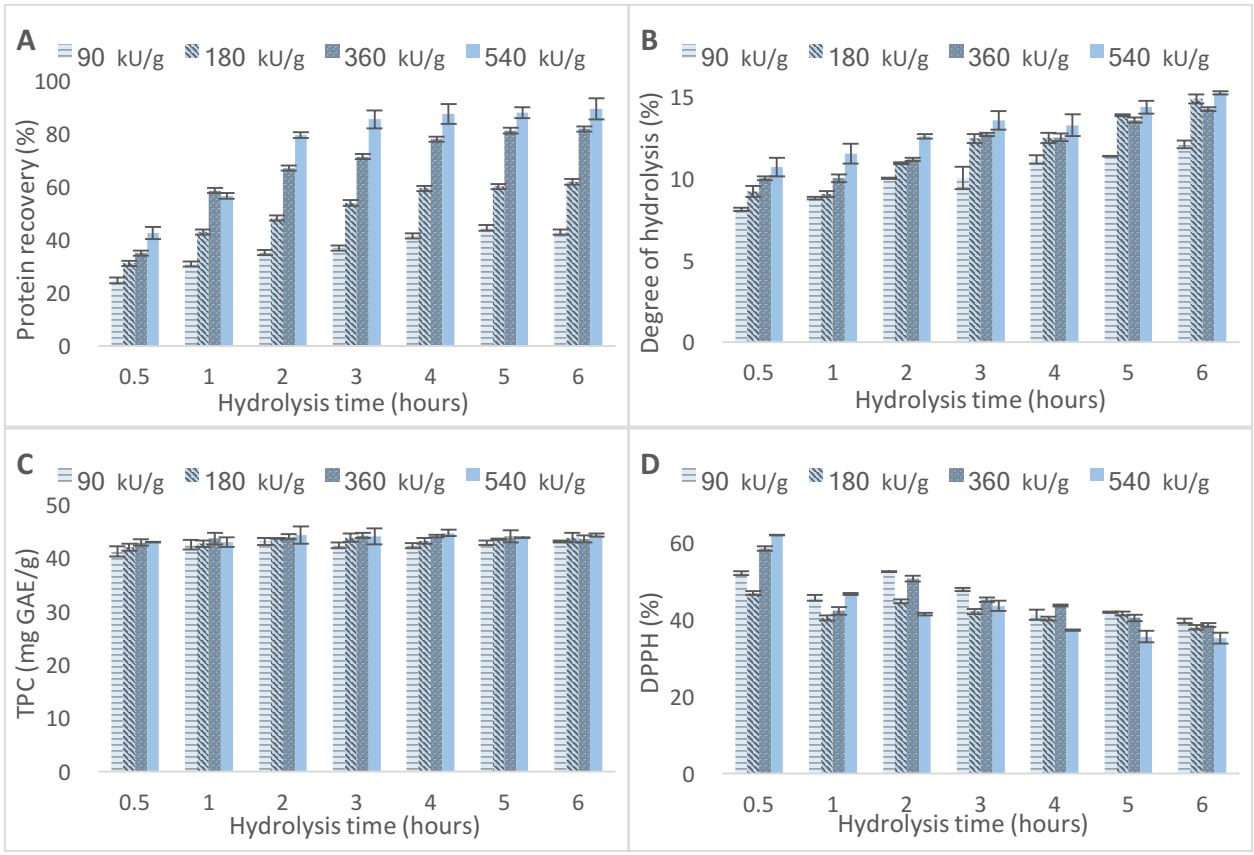


Figure 4-1 Reaction optimization and antioxidant activities of kafirin Papain hydrolysates prepared at combinations of different hydrolysis time and three enzyme-to-substrate ratios (90, 180, 360, and 540 kU/g). A. Total protein recovery (%); B. degree of hydrolysis; C. total phenolic content (mg GAE/g); D. DPPH scavenging activity (%) at 5 mg/mL.

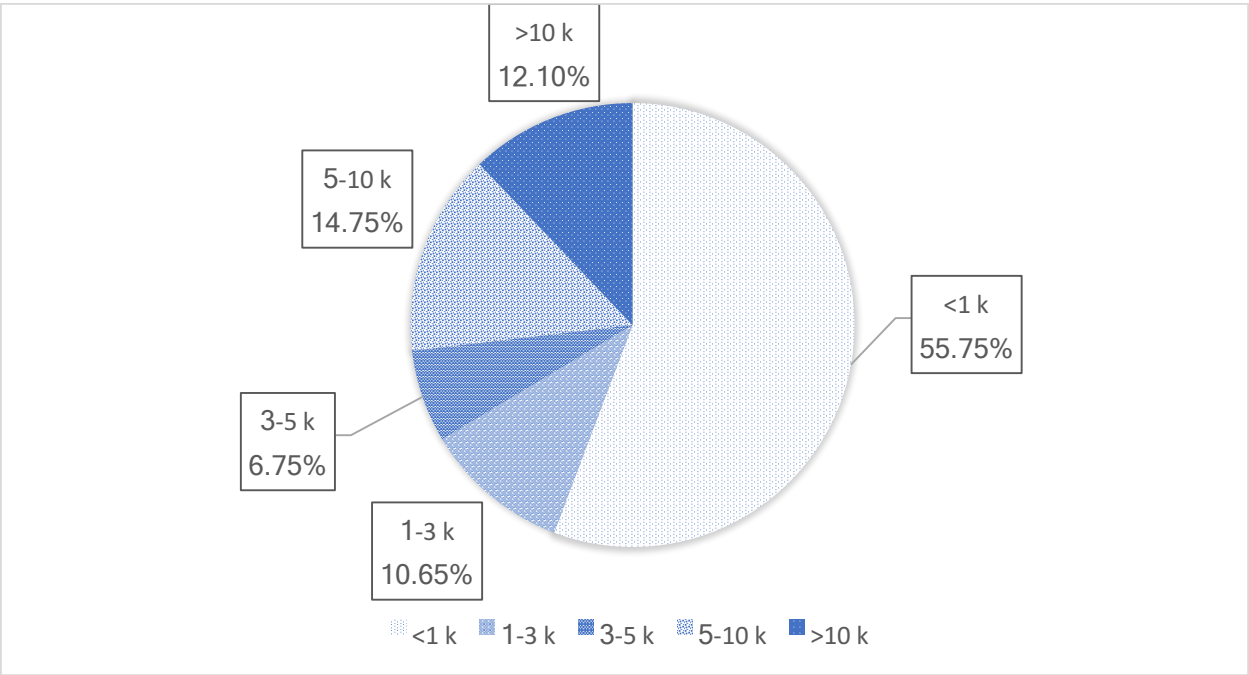


Figure 4-2 Distribution of ultrafiltrated fractions of kafirin Papain hydrolysate prepared at enzyme-to-substrate ratio of 360 kU/g and hydrolyzed for 4 hours followed with membrane filtration using 10 k, 5 k, 3 k, and 1 kDa membranes.

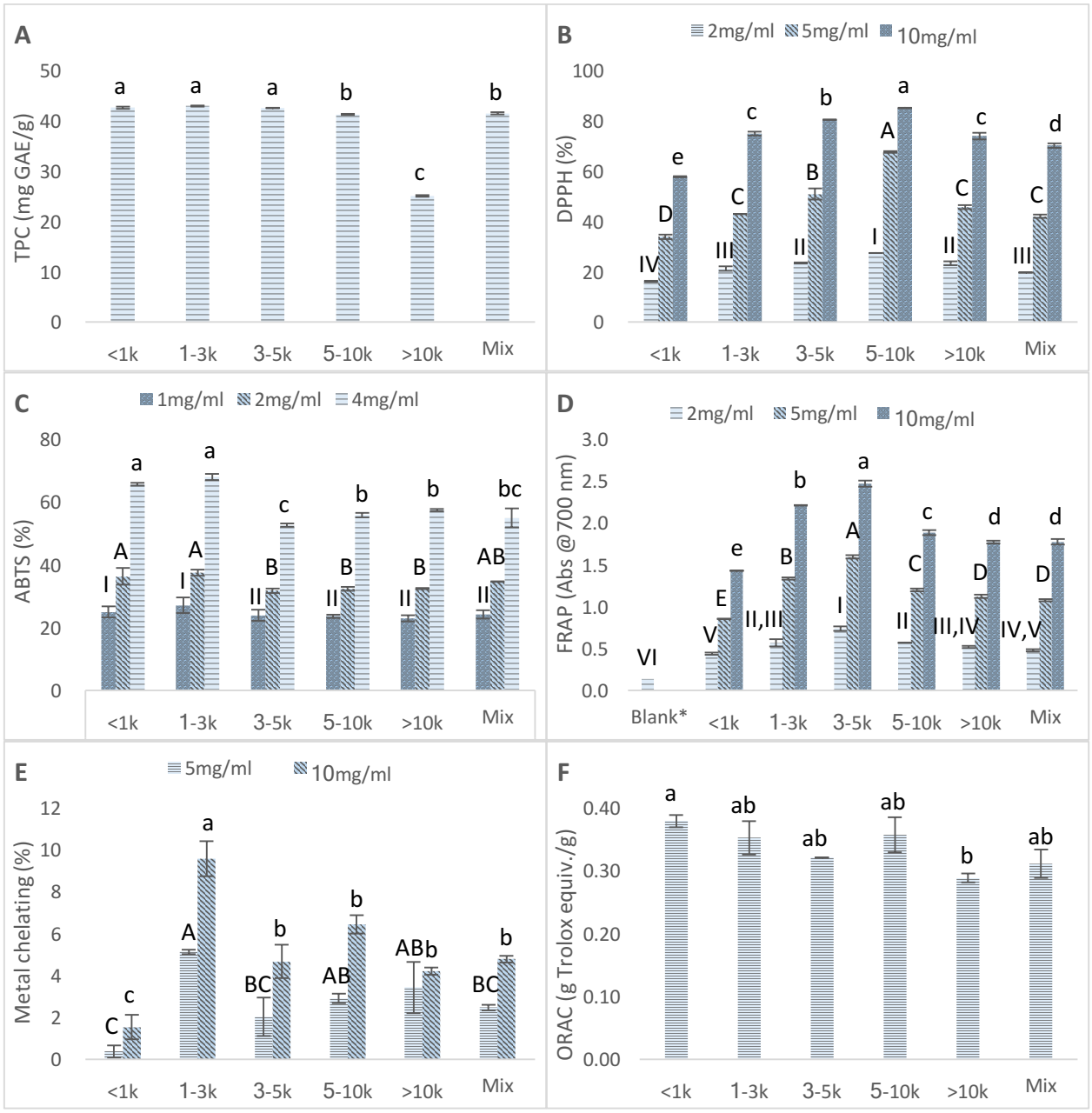


Figure 4-3 Total phenolic content and antioxidant activities of kafirin Papain hydrolysate ultrafiltrated fractions prepared at 360 kU/g and hydrolyzed for 4 hours followed with membrane filtration using 10 k, 5 k, 3 k, and 1 kDa membranes. A. Total phenolic content (mg GAE/g); B. DPPH scavenging activity (%); C. ABTS scavenging activity (%); D. Reducing power capacity (Abs at 700 nm); E. Metal chelating capacity (%); F. ORAC (g Trolox equiv./g).

* Blank represents the absorbance of reaction mixture at 700 nm using distilled water in substitute of sample.

Different lowercase letters, capital letters, and roman numerals indicated significant difference at $P < 0.05$.

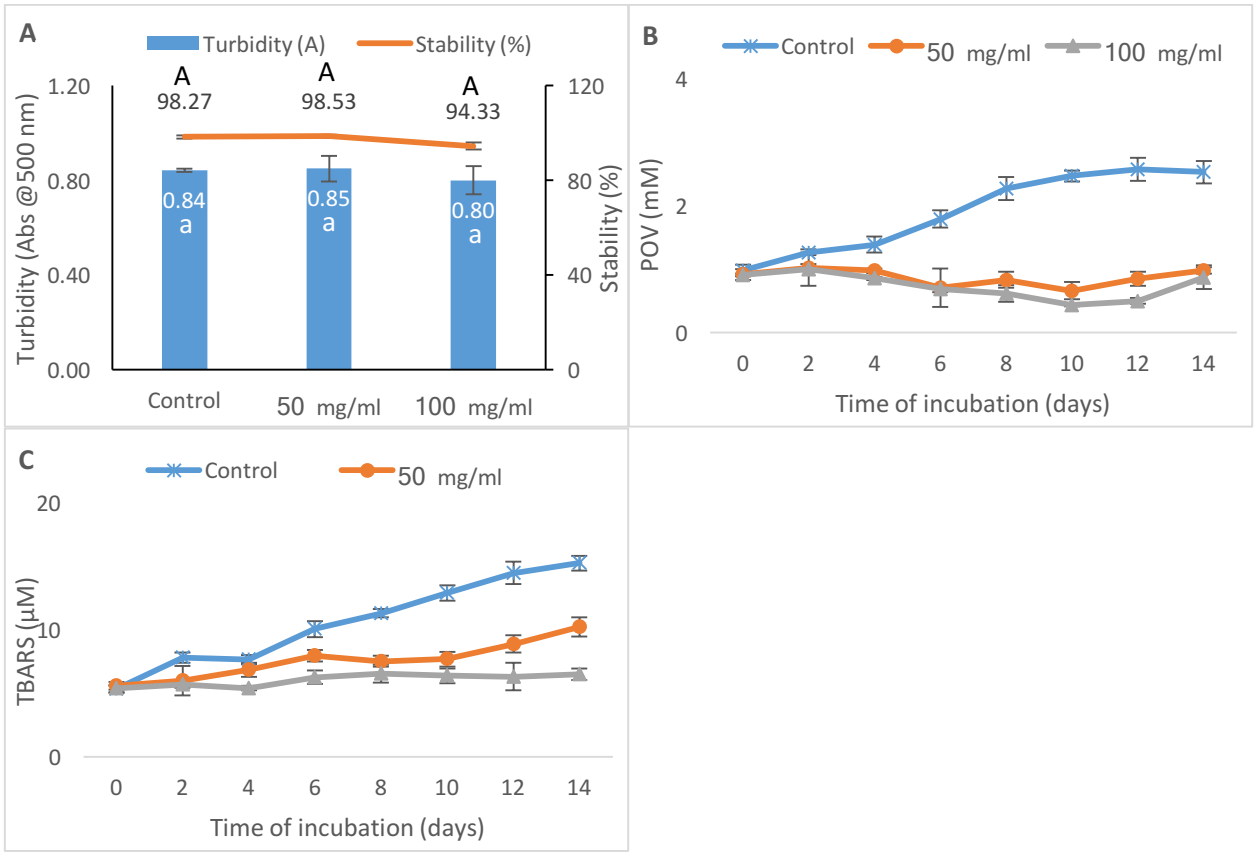


Figure 4-4 Inhibition effect of kafirin Papain 1 – 3 kDa hydrolysate prepared at 360 kU/g and hydrolyzed for 4 hours in an oil-in-water emulsion model system added with 50 and 100 mg/mL oil. A. Emulsion turbidity (Abs at 500 nm) and stability (%); B. POV (mM cumene hydroperoxide equivalent); C. TBARS (μM tetramethoxypropan equivalent).

Different lowercase and capital letters indicated significant difference at $P < 0.05$.

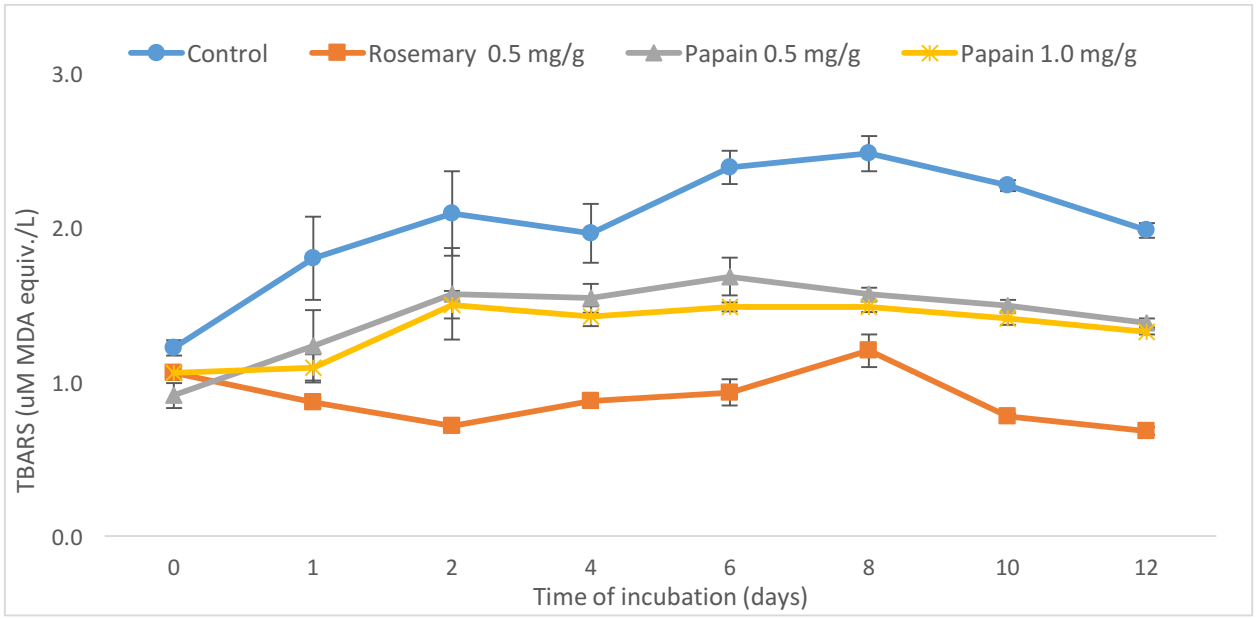


Figure 4-5 Inhibition effect shown as TBARS of kafirin Papain 1 – 3 kDa hydrolysate prepared at 360 kU/g and hydrolyzed for 4 hours in a ground meat model system added with 0.5 and 1 Mg/g meat.

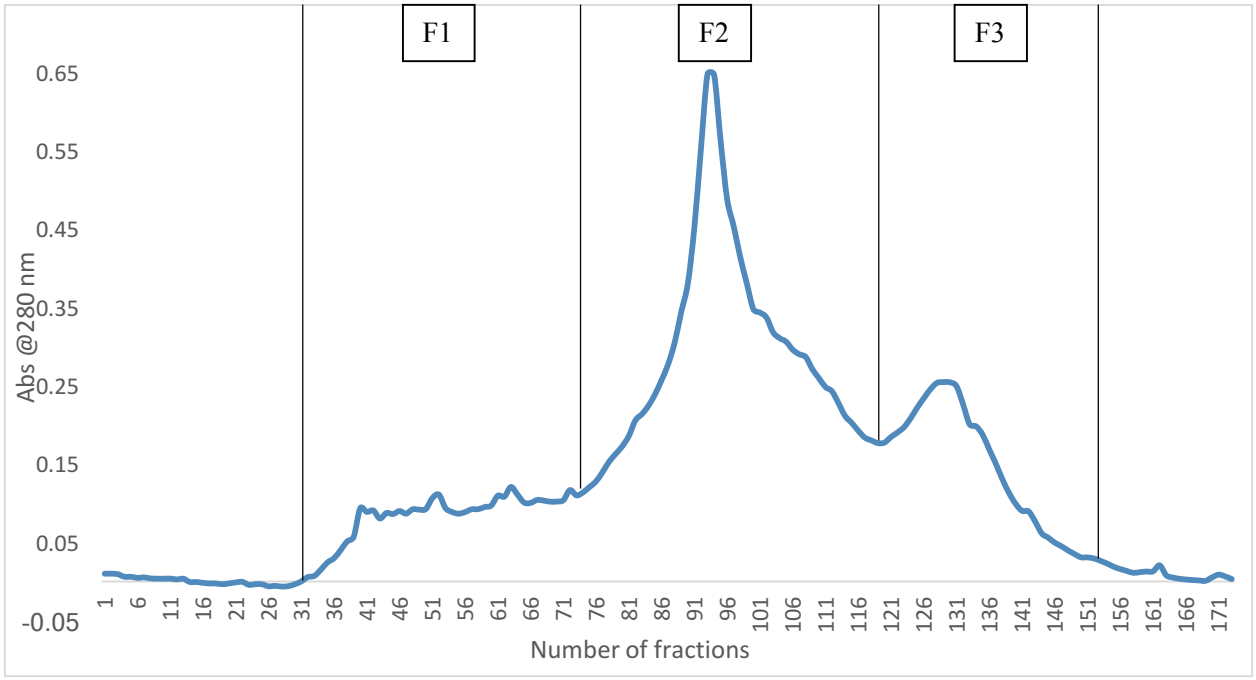


Figure 4-6 Gel filtration chromatogram of kafirin Papain 1 – 3 kDa hydrolysate prepared at enzyme-to-substrate ratio of 360 kU/g and hydrolyzed for 4 hours in a Sephadex G-25 column (26 mm × 850 mm).

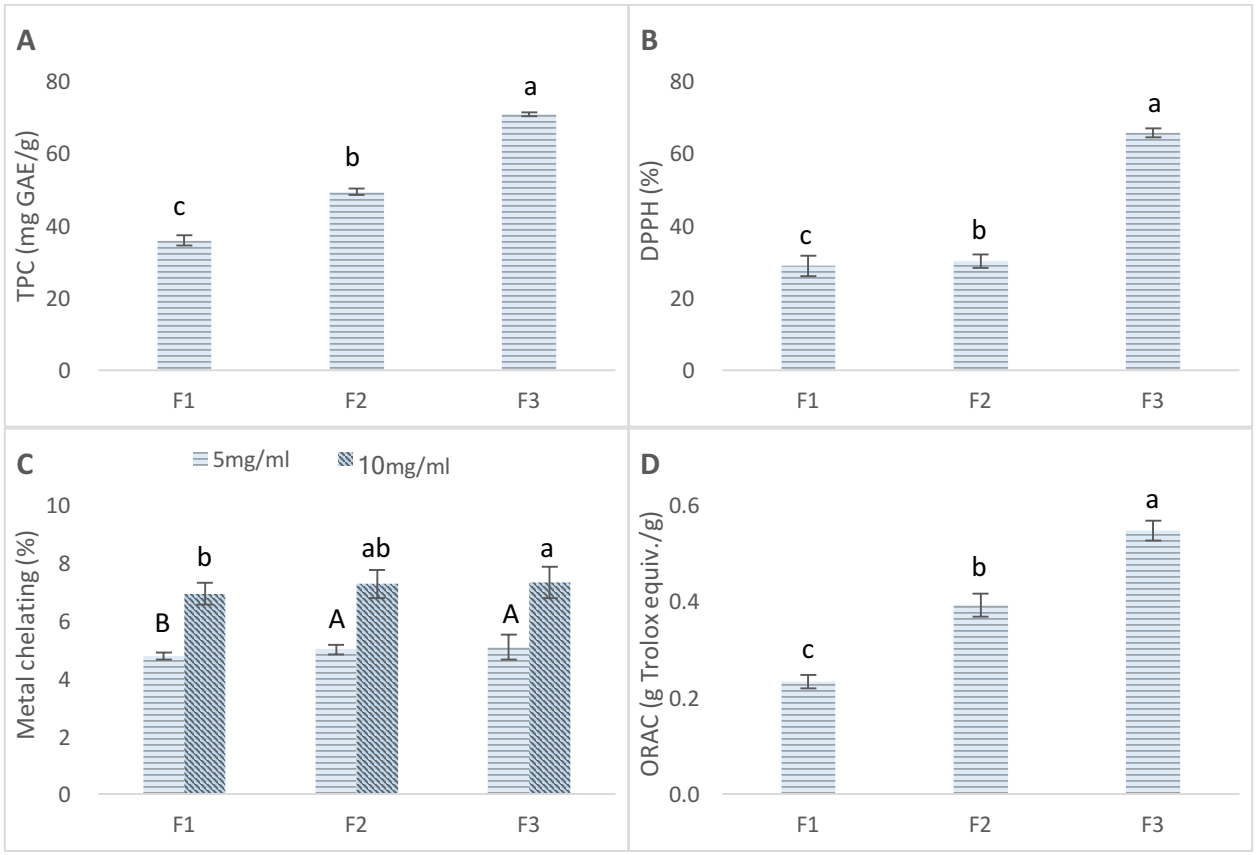


Figure 4-7 Total phenolic content and antioxidant activities of gel filtration fractions of kafirin Papain 1 – 3 kDa hydrolysate prepared at 360 kU/g and hydrolyzed for 4 hours A. Total phenolic content (mg GAE/g); B. DPPH scavenging activity (%) at 4mg/mL; C. Metal chelating activity (%); D. ORAC (g Trolox equiv./g).

Different lowercase and capital letters indicated significant difference at $P < 0.05$.

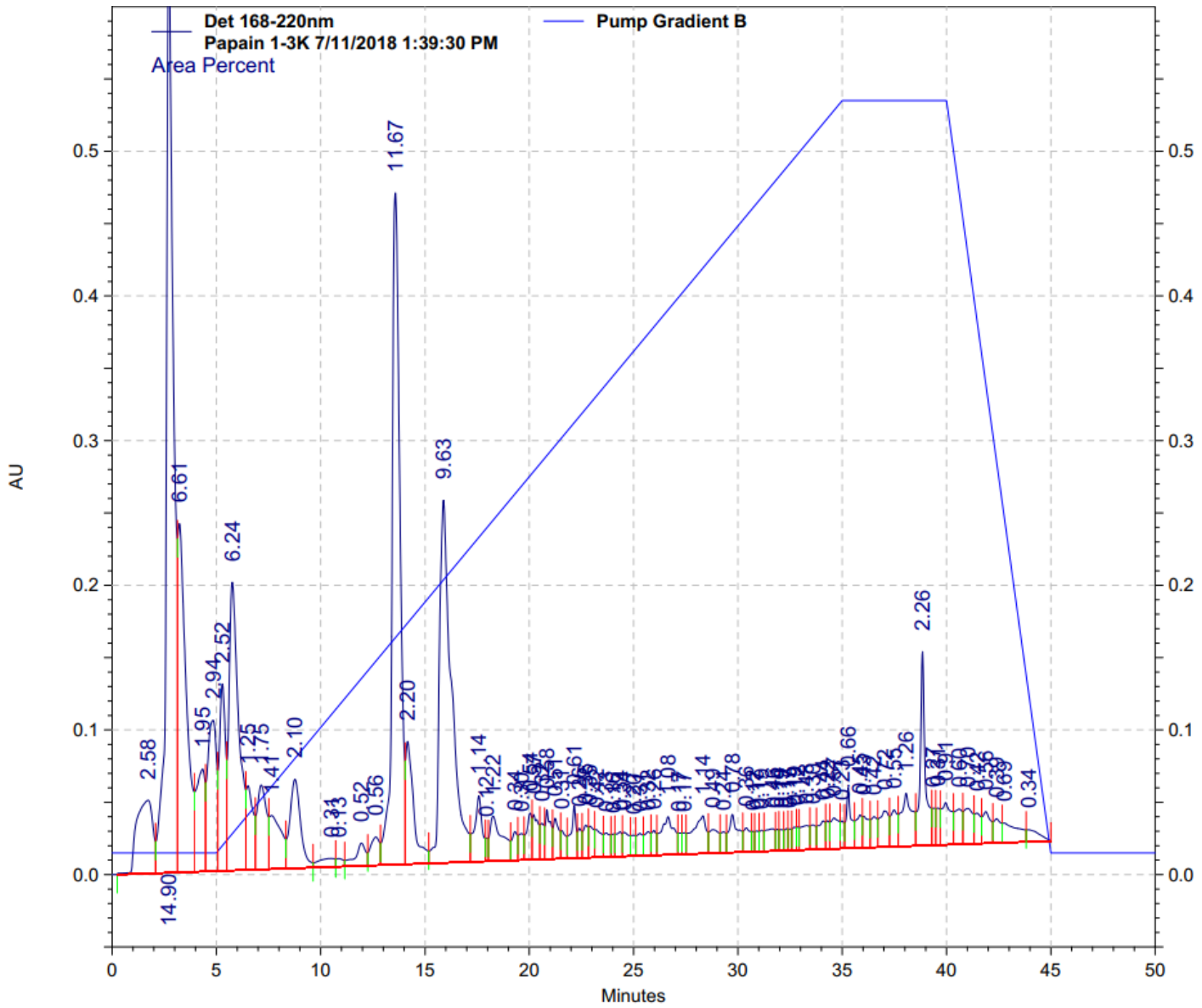
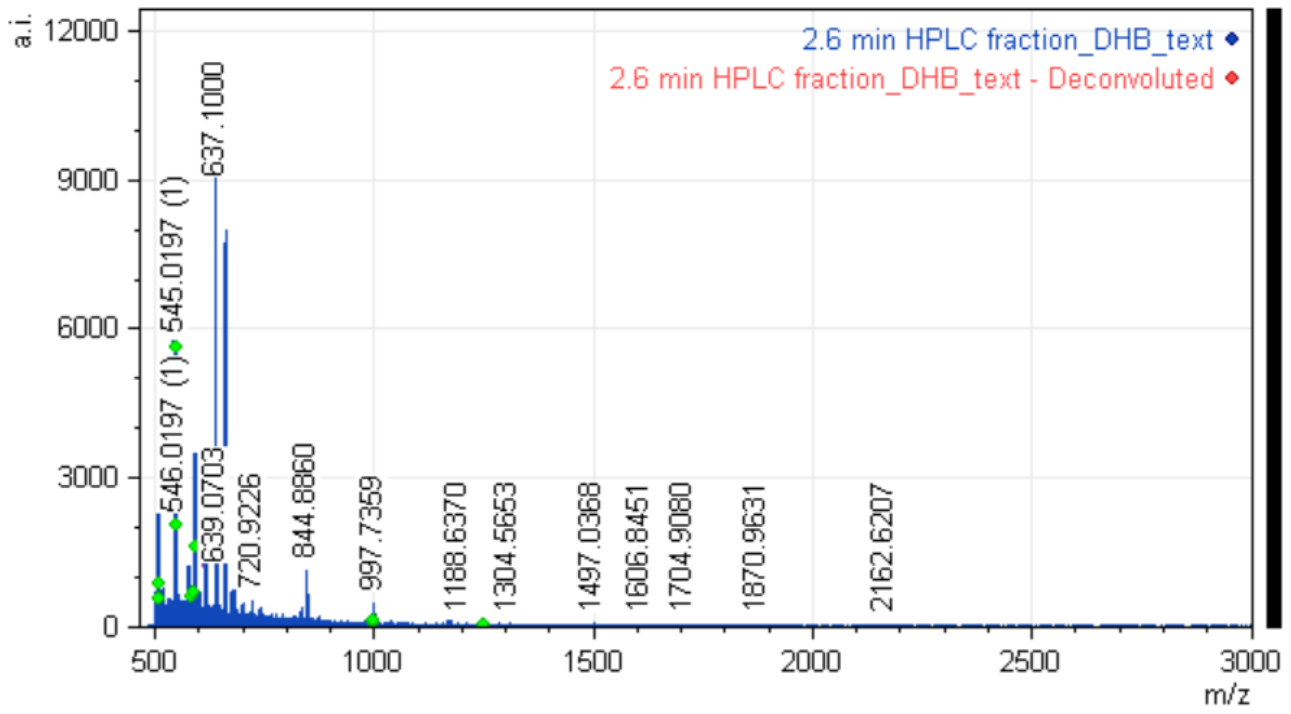


Figure 4-8 RP-HPLC chromatogram of Fraction 3 from gel filtration of 1 – 3 kDa kafirin Papain hydrolysates.



Sequence - *Beta Kafirin*

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
	192	20661.8889	20676.4896	66.1 %	86.6 %

MKMVIVLAVC LALSAASASA LQMPGMGLQD LYGAGALMTM MGAGGGLYPC AEYLRQPQCS PVAAPFYALR EQTMWQPNFI
CQPLRQQCCQ QMRMMDMQSR CQAMCGVVQS VVQQLQMTMQ LQGVAAAASS LLYQPALVQQ WQQLLPAAQA LTPLAMAVAQ
VAQNMPAMCG LYQLPSYCTT PCATSAAIPP YY

Figure 4-9 MS spectrum of 2.6 minute peak from RP-HPLC of 1 – 3 kDa kafirin Papain hydrolysates gel filtration fraction 3.

Chapter 5 - Conclusion and Future Recommendations

1. Conclusions

Numerous studies have revealed that peptides and hydrolysates with excellent antioxidant activities can be released and modified from a wide variety of dietary protein sources, especially those obtained from egg, milk, mussel proteins, nuts and pulses. However, to the best of our knowledge, studies of peptide antioxidants developed from cereal protein is limited and a systematic characterization of sorghum kafirin protein's antioxidant potential is lacked. This innovative study delivered a practical technology of producing peptide antioxidants from extracted sorghum protein. Related studies were extensively reviewed and critical processing steps (sorghum protein extraction, enzymatic hydrolysis, fractionation and purification of protein hydrolysates, identification of peptide sequences) were substantially described. The antioxidant profile of enzymatic hydrolysates from sorghum kafirin was measured by several different *in vitro* assays, the selected fractions were evaluated in model systems. The results revealed that antioxidant peptides obtained from kafirin hydrolysates possessed excellent inhibition against lipid/oil oxidation through multiple mechanism. This study provided strong evidence indicating the potential of incorporating sorghum peptide into food products as alternatives to synthetic antioxidants or as synergistic elements to protect the susceptible ingredients from oxidative spoilage (Chen et al., 2006). This work also provided a positive insight for sorghum ethanol industry for the transformation of the protein-rich by-products into value-adding products and development of an additional revenue stream.

After screening different types of enzymes, it was found that Neutrase, Alcalase and Papain were the most promising enzymes to produce kafirin hydrolysates with a desired protein recovery, degree of hydrolysis, and antioxidative activity. Thus, these three cases of hydrolysates

were selected for in-depth study described in Chapter 2 – 4. Substrate content, enzyme-to-substrate ratio, and hydrolysis time were critical reaction parameters to achieve consistent production of hydrolysates, which were optimized based on single factor method as well as factorial design for each hydrolysate. The finalized reaction parameters for kafirin hydrolysis were substrate content of 4%, enzyme-to-substrate ratio of 0.4 Au/g, and hydrolysis time of 17 hours with Neutrase; 4%, 0.4 Au/g, and 4 hours with Alcalase; 4%, 360 kU/g, and 4 hours with Papain.

Medium- and small-sized fractions of hydrolysates were found to exhibit higher activities than the large-sized or intact proteins which agreed with previously reviewed literatures. However, unnecessary extensive hydrolysis may cause breakage of critical structures and damage the ability of hydrolysate to form physical barriers that will ultimately lead to a reduced antioxidant activity. The fractionated kafirin hydrolysates displayed excellent antioxidative potentials *in vitro* revealed by multiple assays. In oil-in-water emulsion and ground meat model systems, they manifested outstanding inhibition and detention effects against lipid and/or oil autoxidation and peroxidation. Some possible acting mechanisms of peptide antioxidants include quenching or terminating free radicals, chelating prooxidative catalytic metal ions, electron or proton donating, reducing peroxide products, interrupting the decomposition of hydroperoxide, and minimizing physical contact of oxidizing agent to susceptible targets.

Representative peptides were identified for each hydrolysate. Glutamine was found to be the leading amino acid among the identified peptide sequences for all three enzymatic hydrolysates, which was also confirmed to be an antioxidative amino acid by previous studies (Tsai et al., 2012; Shabert et al., 1999). Thus, it could be an important constituent building block responsible for the antioxidant activities of kafirin hydrolysate peptides. The overall

hydrophobicity of peptides was also important for the accessibility of antioxidants to reach oil and/or lipid targets, which was mainly enhanced by exposure of hydrophobic amino acids during unfolding of proteins.

2. Recommendations and research niche

There are a wide range of research niches worth future study to commercialize the antioxidant peptides in practical applications in food and/or feed industry.

Firstly, although a flow path had been established at bench-top scale, the engineering design for scaling-up to industrial level is obliged to be carried out. Certain reaction parameters and conditions (e.g., hydrolysis time, shaking force of water-bath incubator) are projected to be modified accordingly. At industrial production level, the whole process could be assembled to a continuous phase (Samaranayaka & Li-Chan, 2011), which will save both time and cost.

Besides, due to the compositional difference of various sorghum protein-rich by-products from purified sorghum protein, specific processing steps need to be modified in practice in order to transfer this procedure to different research substrates. For instance, the removal of interferential components (e.g., lipid and fiber) before protein hydrolysis will be necessary. Moreover, various procedures such as steeping, cooking, saccharification, fermentation occurred during the production of bioethanol could have altered the structural and functional properties of the proteins, which would lead to different end-use performances of subsequent antioxidant peptides. Thus, it is necessary to conduct studies by using different subjects of protein-rich by-products such as whole stillage after distillation, wet distillers' grains, dried distillers' grains, and so forth.

Further, although the antioxidative activities of peptide antioxidants had been confirmed by prolific studies *in vitro* assays and model systems, there are inadequate *in vivo* evidences to

provide strong and convincing support their potential health-beneficial claims. The antioxidant capacity may be reduced, enhanced in the gut lumen or through receptor in the intestinal cell wall due to the *in vivo* digestion, or kept persistent inside the organisms after GI digestions (Masisi et al., 2016; Sarmadi & Ismail, 2010). Animal studies and/or human clinical trials regarding diets supplemented with antioxidant peptides could deliver important messages. Using biomarkers to track the evolutionary fate of antioxidant peptides during the GI passage is another useful tool to reveal the metabolic biotransformation of peptide antioxidants as well as their protective mechanisms to bio-tissues and macronutrients (e.g., lipids, proteins, DNA, etc.) from oxidative damages (Samaranayaka & Li-Chan, 2011).

What's more, understanding the relationships between structural characteristics (e.g., M_w, surface hydrophobicity, amino acid composition, peptides sequence, etc.) and the consequent antioxidant activities is important for establishing convincing mechanism of the peptide antioxidants. In-depth research is needed to clarify the metabolic biotransformation of the antioxidative peptides. Likewise, a noteworthy research area is to design novel peptide sequences based on the latest information of identified peptide sequences. Genomic and transcriptomic techniques as well as polymetric chain reaction (PCR) can be used to synthesize and amplify the peptide antioxidants for a large-scaled production (Masisi et al., 2016).

In addition, the use of different assays as well as variations of the same assay by different researchers makes it difficult to compare the antioxidant potentials of reported peptide sequences (Samaranayaka & Li-Chan, 2011). Official methods and standard operating procedures need to be established or standardized to quantify the antioxidative capacity of peptides or protein hydrolysates for comparison among literatures (Zulueta et al., 2009; Samaranayaka & Li-Chan, 2011; Frankel & Meyer, 2000). Development of a continues-phased evaluation method for

monitoring the protecting effect on the oxidatively unstable ingredients (e.g., unsaturated fatty acids) during the processing and storage period will be helpful for the protection of specific food products. Establishment of data base summarizing antioxidative activity of individual amino acids and oligopeptides is necessary. Statistical models that predict the relationship between structure (amino acid sequence) and activity is another approach in categorizing these information (Sila & Bougatef, 2016). This information could guide the prediction of bioactivities of peptides released from original proteins with specific enzymes.

Lastly, the changes in compositions, textures (e.g., viscosity), structures (e.g., emulsion turbidity), functionalities (e.g., gelation), and sensory features (e.g., color and flavor) caused by incorporating the peptide antioxidants into a food product shall be completely considered and studied. Sensory analysis with groups of panelists is required in assisting the formulation of food products stabilized with peptide antioxidants since bitterness and off-flavors were widely reported in food products incorporated with protein hydrolysates (Elias et al., 2008). Further studies are needed in researching the mechanism behind the flavor formation of protein hydrolysates and finding possible approaches to cover them. The storage requirements, shelf-stability, allergenicity test of the antioxidative peptides should also be assessed before introducing to market.

It will not be a rapid process until successful commercialization of the peptide antioxidants, but its endogenous advantages such as naturally-derived, nutritionally-valued, efficient, cost-effective, sustainable, multifunctional, and generally recognized as safe have drawn keen attention to this field of study.

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Sila, A., & Bougatef, A. (2016). Antioxidant peptides from marine by-products: Isolation, identification and application in food systems. A review. *Journal of Functional Foods*, *21*, 10-26.

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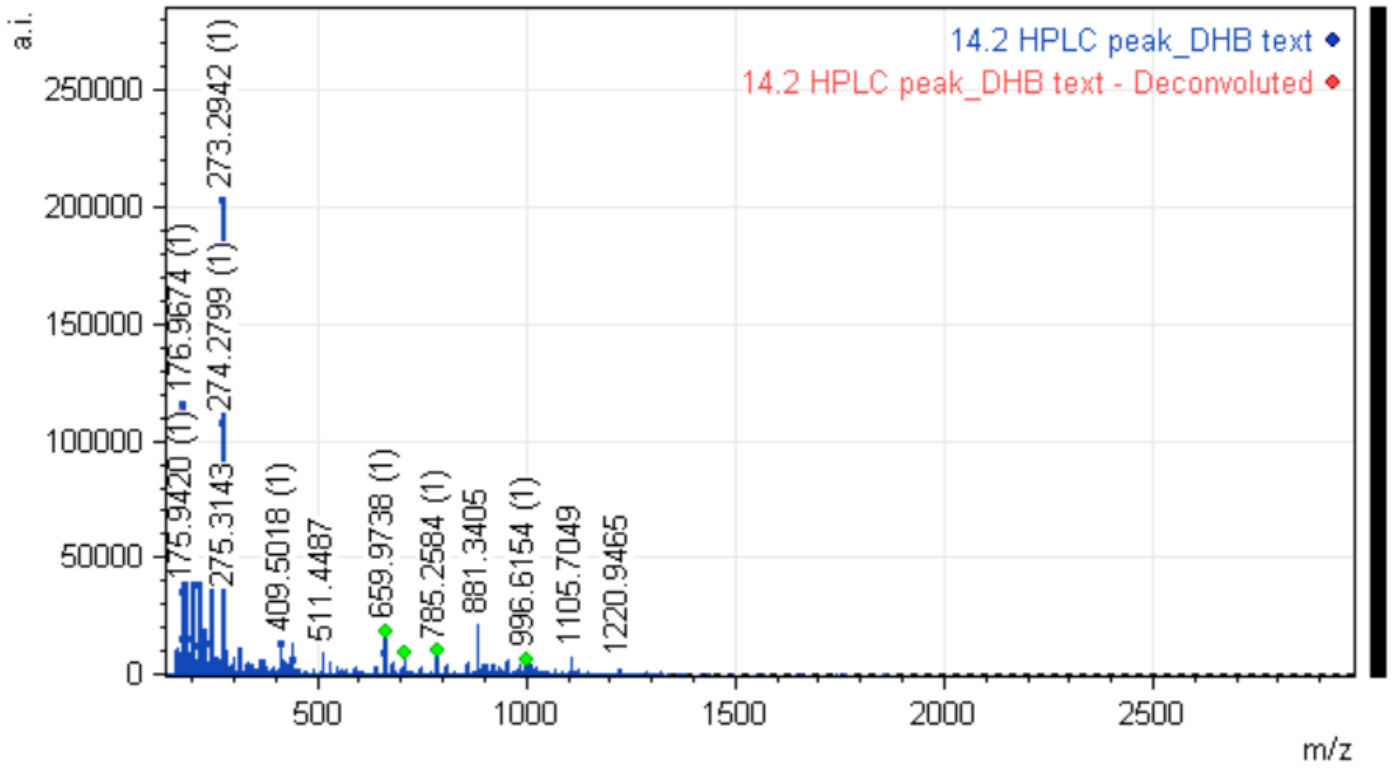
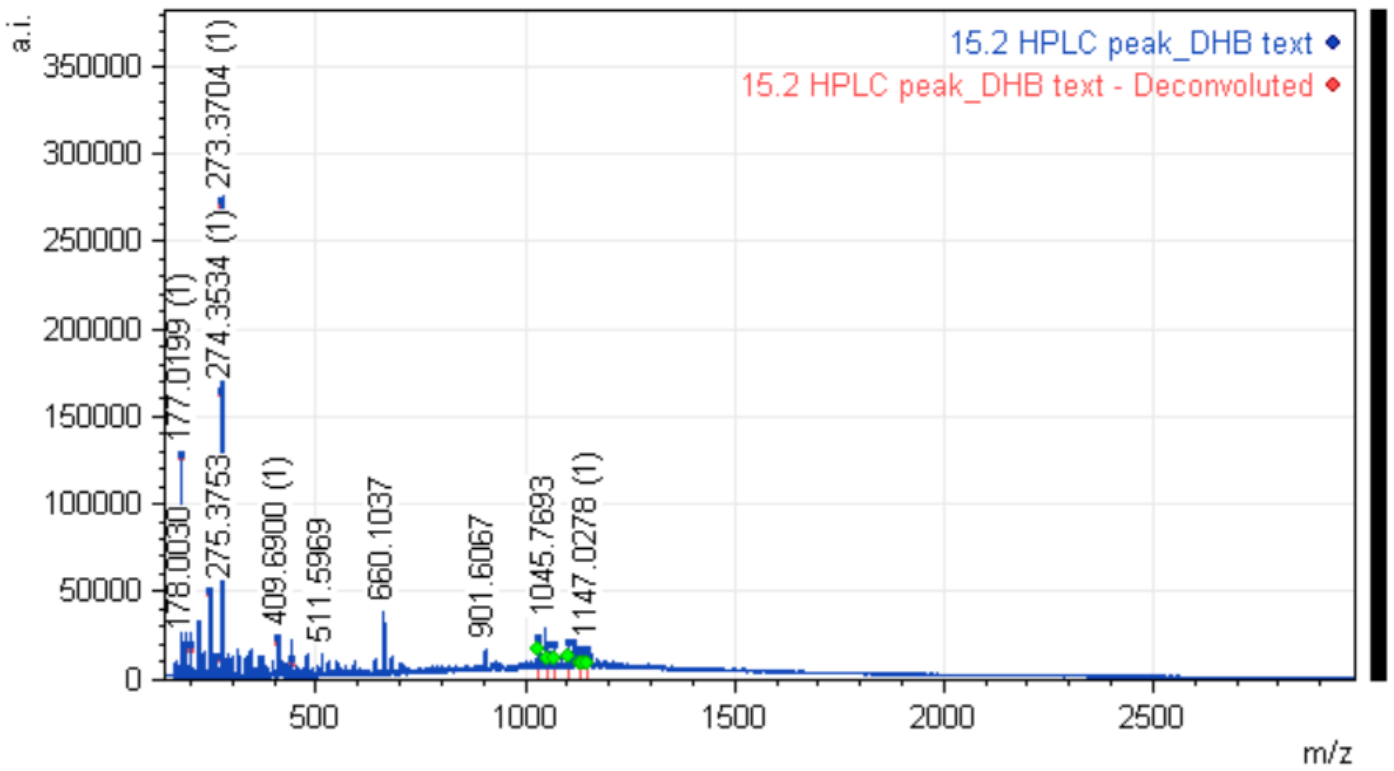
Appendix A - MS spectrum of identified peptides from HPLC peaks of 3 – 10 kDa kafirin Neutrase hydrolysates gel filtration fraction 2



Sequence - *Beta Kafirin*

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
	192	20661.8889	20676.4896	40.1 %	5.4 %

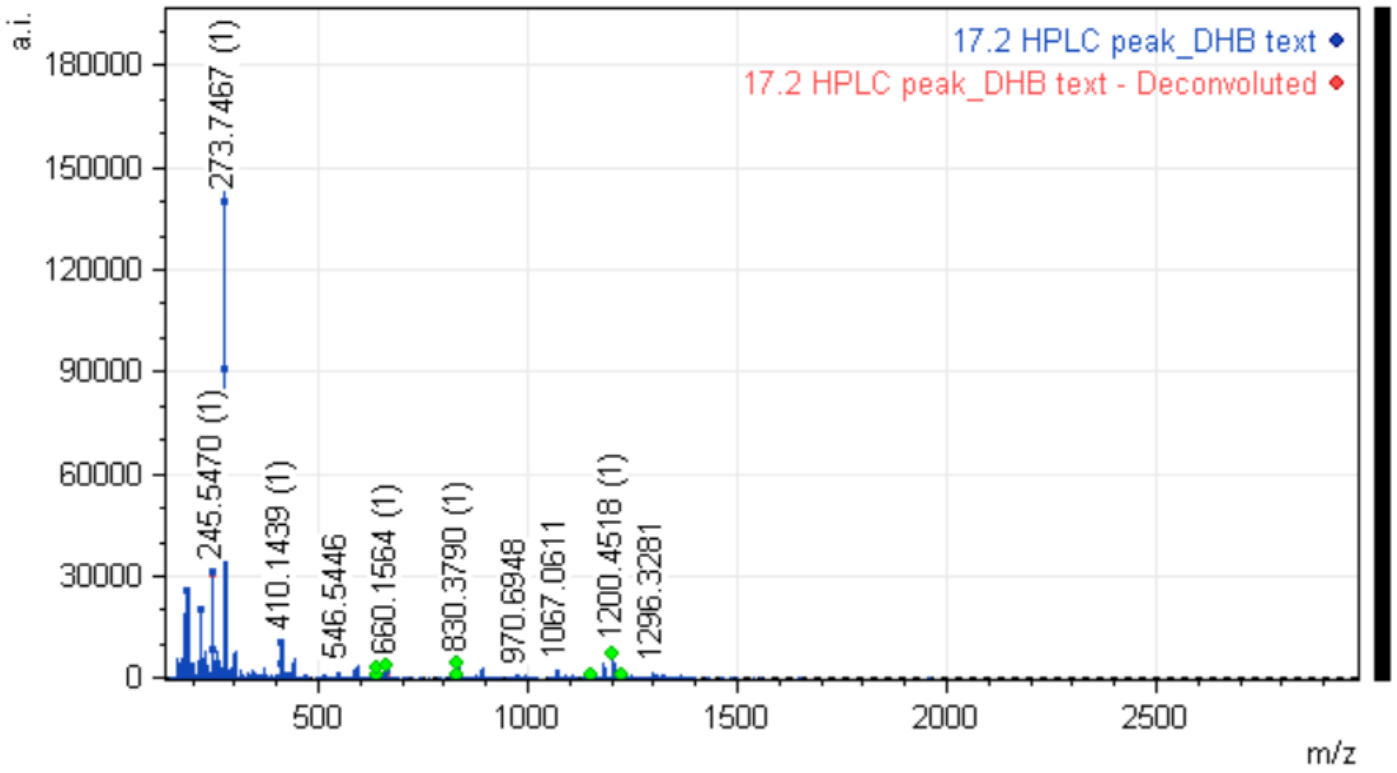
MKMVIVLAVC LALSAASASA LQMPGMGLQD LYGAGALMTM MGAGGGLYPC AEYLRQPQCS PVAAPFYALR EQTMWQPNFI
CQPLRQQCCQ QMRMMDMQSR CQAMCGVVQS VVQQLQMTMQ LQGVAAAASS LLYQPALVQQ WQQLLPAAQA LTPLAMAVAQ
VAQNMPAMCG LYQLPSYCTT PCATSAAIPP YY



Sequence - *Beta Kafirin*

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
	192	20661.8889	20676.4896	70.3 %	9.2 %

MKMVIVLAVC LALSAASASA LQMPGMGLQD LYGAGALMTM MGAGGGLYPC AEYLRQPQCS PVAAPFYALR EQTMWQPNFI
CQPLRQQCCQ QMRMMDMQSR CQAMCGVVQS VVQQLQMTMQ LQGVAAAASS LLYQPALVQQ WQQLLPAAQA LTPLAMAVAQ
VAQNMPAMCG LYQLPSYCTT PCATSAAIPP YY

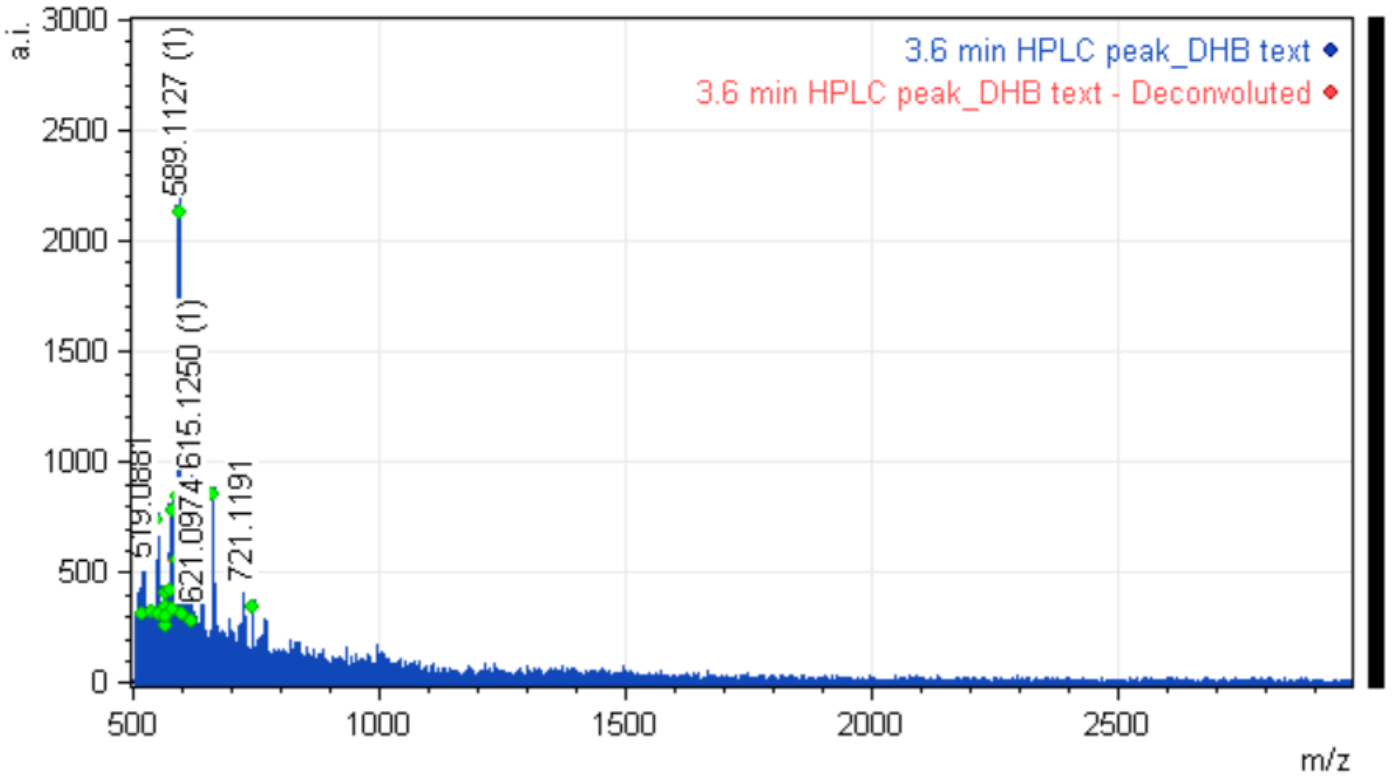


Sequence - *Beta Kafirin*

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
	192	20661.8889	20676.4896	66.7 %	6.8 %

MKMVIVLAVC LALSAASASA LQMPGMGLQD LYGAGALMTM MGAGGGLYPC AEYLRQPQCS PVAAPFYALR EQTMWQPNFI CQPLRQQCCQ QMRMMDMQSR CQAMCGVVQS VVQQLQMTMQ LQGVAAAASS LLYQPALVQQ WQQLLPAAQA LTPLAMAVAQ VAQNMPAMCG LYQLPSYCTT PCATSAAIPP YY

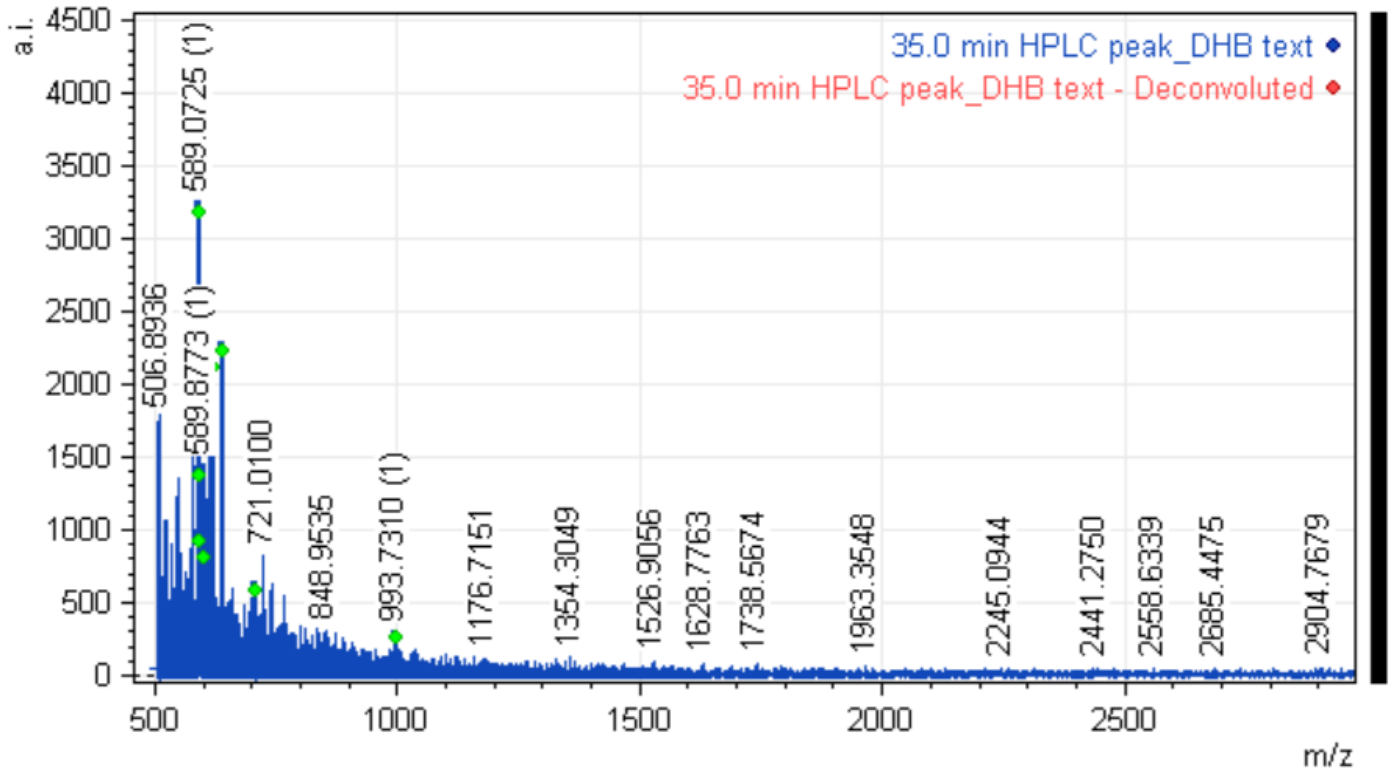
Appendix B - MS spectrum of identified peptides from HPLC peaks of 5 – 10 kDa kafirin Alcalase hydrolysates gel filtration fraction 3



Sequence - *Beta Kafirin*

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
	192	20661.8889	20676.4896	82.8 %	80.7 %

MKMVIVLAVC LALSAASASA LQMPGMGLQD LYGAGALMTM MGAGGGLYPC AEYLRQPQCS PVAAPFYALR EQTMWQPNFI
CQPLRQQCCQ QMRMMDMQSR CQAMCGVVQS VVQQLQMTMQ LQGVAAAASS LLYQPALVQQ WQQLLPAAQA LTPLAMAVAQ
VAQNMPAMCG LYQLPSYCTT PCATSAAIPP YY



Sequence - *Beta Kafirin*

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
	192	20661.8889	20676.4896	54.7 %	72.3 %

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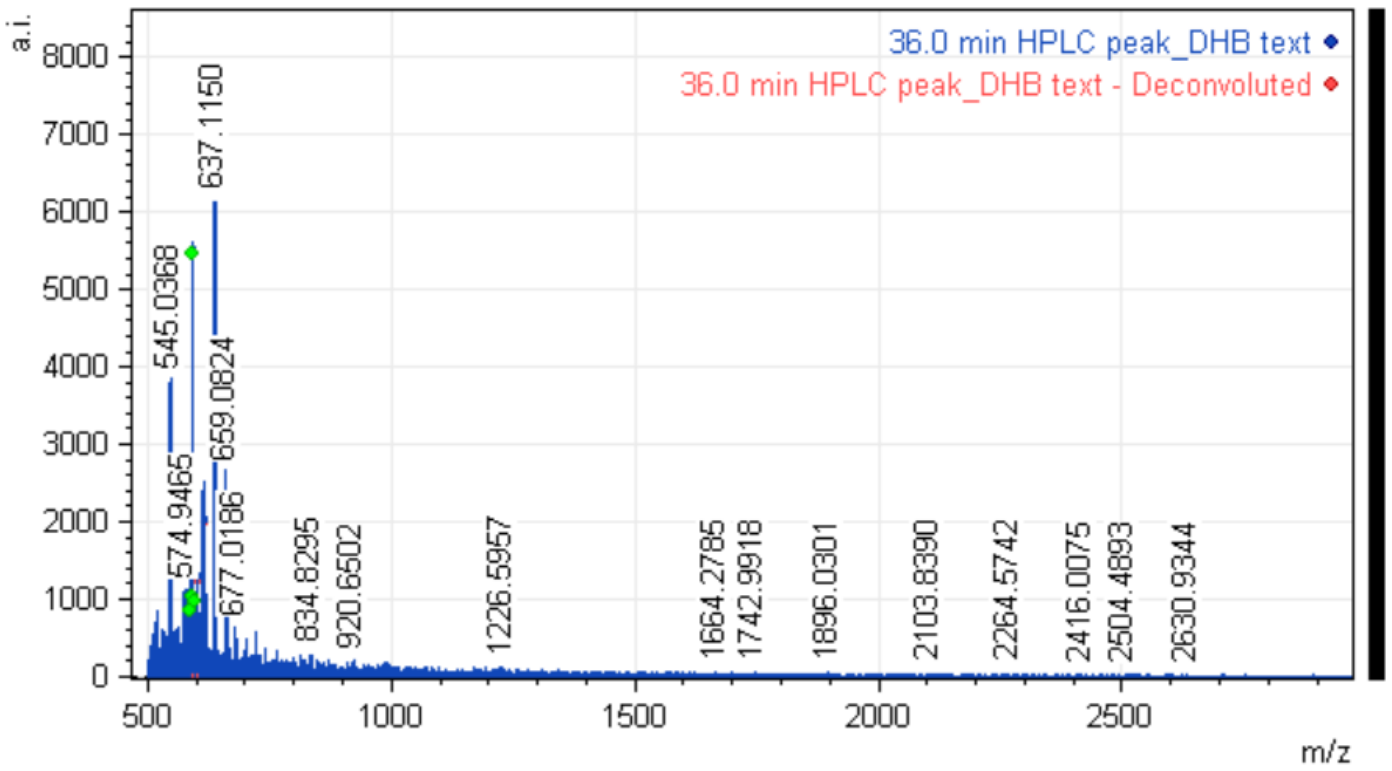
CQPLRQQCCQ QMRMMDMQSR CQAMCGVVQS VVQQLQMTMQ LQGVAAAASS LLYQPALVQQ WQQLLPAAQA LTPLAMAVAQ
VAQNMPAMCG LYQLPSYCTT PCATSAAIPP YY



Sequence - *Beta Kafirin*

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
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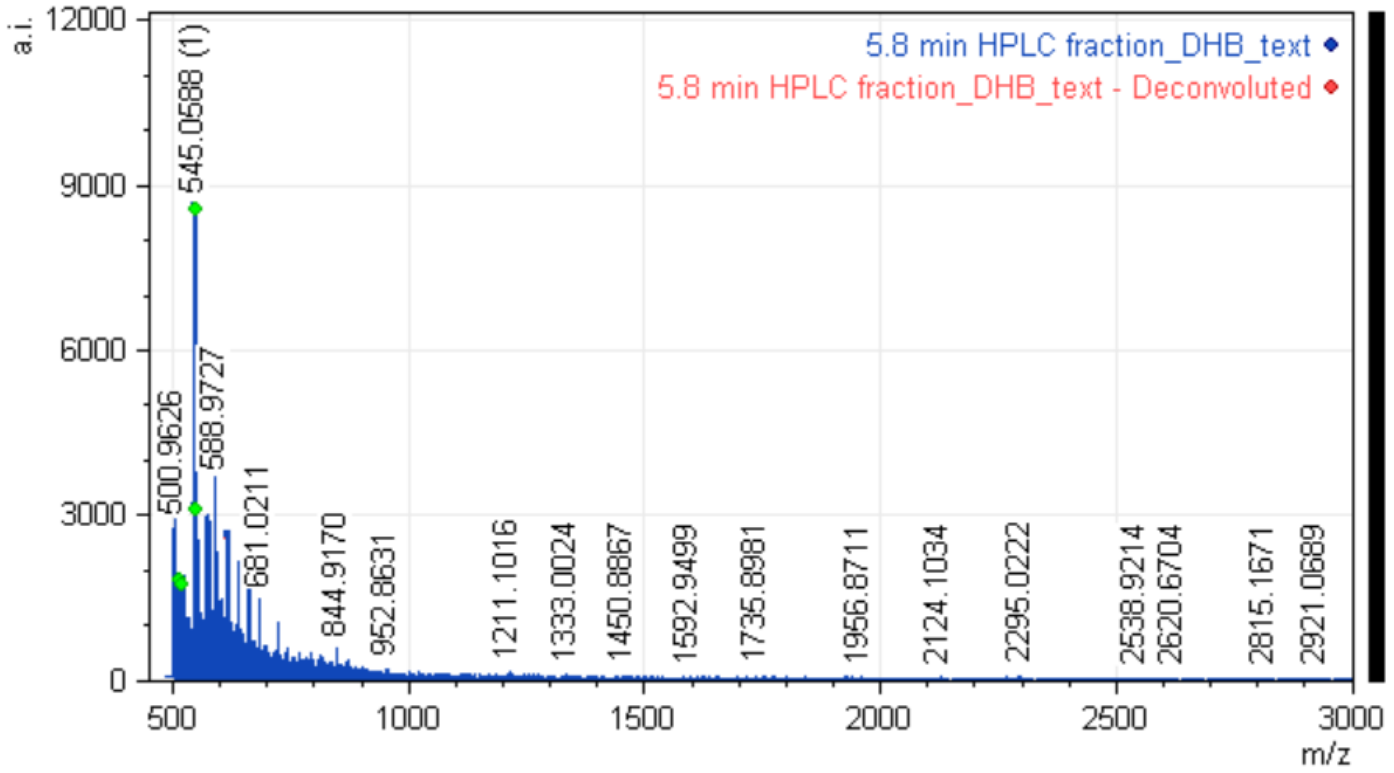
MKMVIVLAVC LALSAASASA LQMPGMGLQD LYGAGALMTM MGAGGGLYPC AEYLRQPQCS PVAAPFYALR EQTMWQPNFI
CQPLRQQCCQ QMRMMDMQSR CQAMCGVVQS VVQQLQMTMQ LQGVAAAASS LLYQPALVQQ WQQLLPAAQA LTPLAMAVAQ
VAQNMPAMCG LYQLPSYCTT PCATSAAIPP YY

Appendix C - MS spectrum of identified peptides from HPLC peaks of 1 – 3 kDa kafirin Papain hydrolysates gel filtration fraction 3



Sequence - *Beta Kafirin*

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
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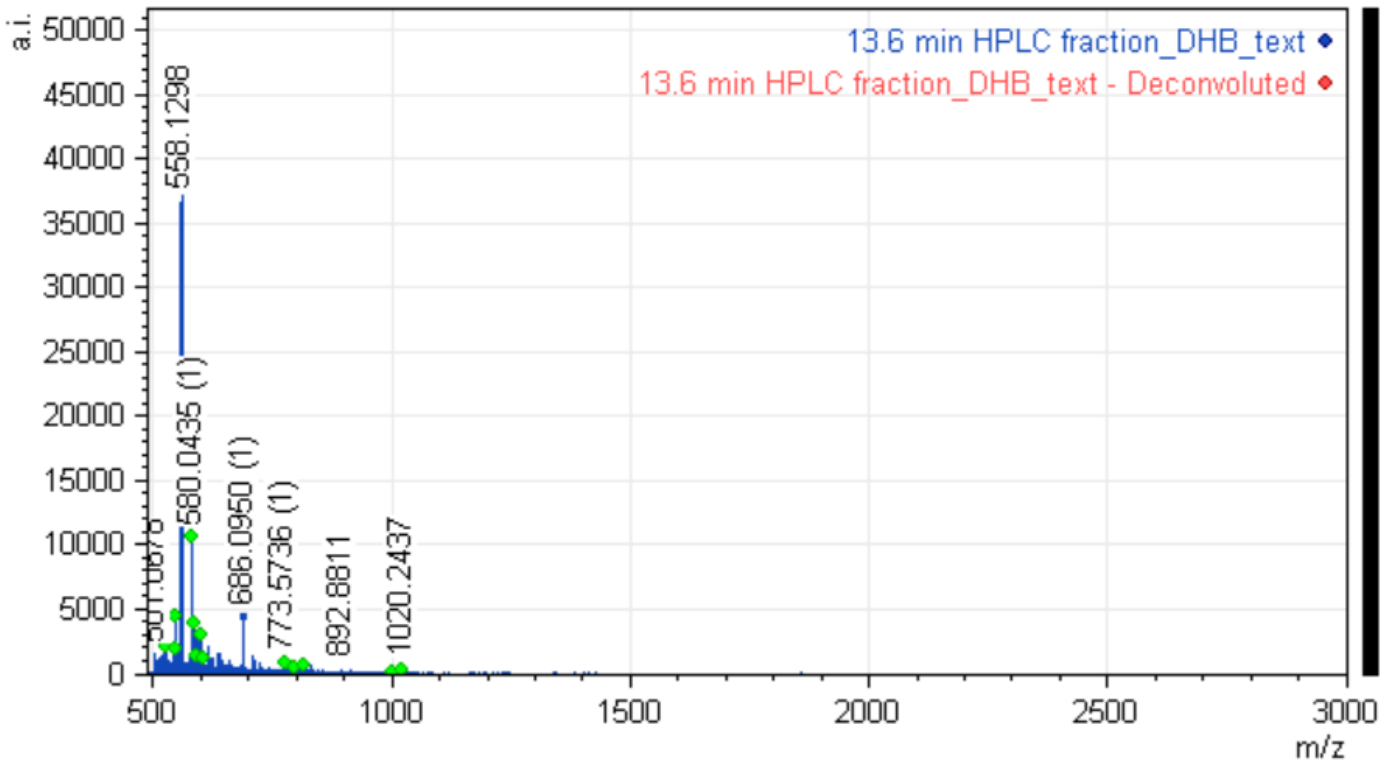
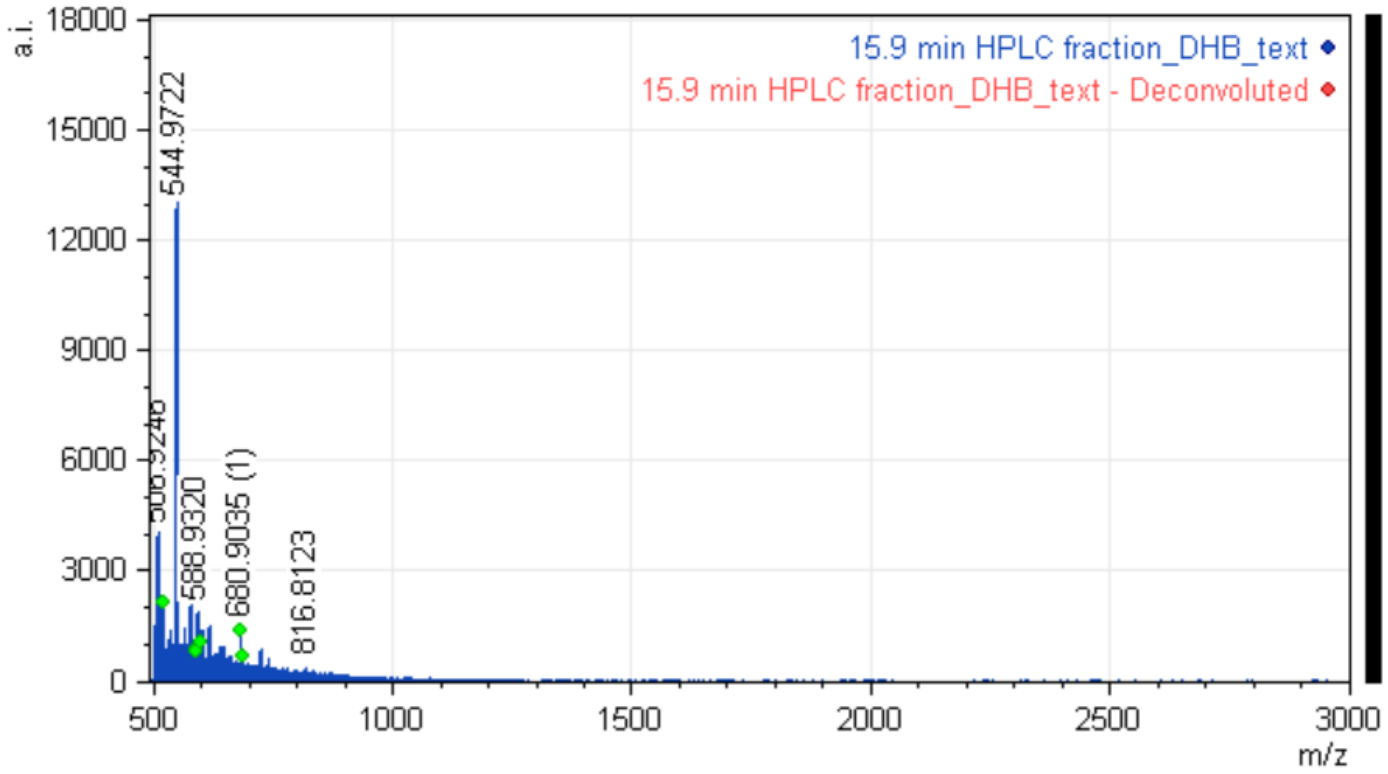
MKMVIVLAVC LALSAASASA LQMPGMGLQD LYGAGALMTM MGAGGGLYPC AEYLRQPQCS PVAAPFYALR EQTMWQPNFI
CQPLRQQCCQ QMRMMDMQSR CQAMCGVVQS VVQQLQMTMQ LQGVAAAASS LLYQPALVQQ WQQLLPAAQA LTPLAMAVAQ
VAQNMPAMCG LYQLPSYCTT PCATSAAIPP YY



Sequence - *Beta Kafirin*

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
	192	20661.8889	20676.4896	72.9 %	84.5 %

MKMVIVLAVC LALSAASASA LQMPGMGLQD LYGAGALMTM MGAGGGLYPC AEYLRQPQCS PVAAPFYALR EQTMWQPNFI
CQPLRQQCCQ QMRMMDMQSR CQAMCGVVQS VVQQLQMTMQ LQGVAAAASS LLYQPALVQQ WQQLLPAAQA LTPLAMAVAQ
VAQNMPAMCG LYQLPSYCTT PCATSAAIPP YY



Sequence - *Beta Kafirin*

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
	192	20661.8889	20676.4896	29.7 %	67.8 %

MKMVIVLAVC LALSAASASA LQMPGMGLQD LYGAGALMTM MGAGGGLYPC AEYLRQPQCS PVAAPFYALR EQTMWQPNFI
CQPLRQQCCQ QMRMMDMQSR CQAMCGVVQS VVQQLQMTMQ LQGVAAAASS LLYQPALVQQ WQQLLPAAQA LTPLAMAVAQ
VAQNMPAMCG LYQLPSYCTT PCATSAAIPP YY