Preparation of Proteins and Peptides from Heat-Stabilized Defatted Rice Bran Via Solid State Fermentation and Investigating for Skin Health

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Abstract

Heat-stabilized defatted rice bran (HDRB) is an abundant and inexpensive agro-industrial by-product of rice milling and rice oil extraction process. Furthermore, HDRB is a potential source of proteins. However, the direct protein extraction has low yield of protein due to the impact of heat stabilization and oil extraction process which made the HDRB proteins bound and interact with other components. This research is based on using solid-state fermentation (SSF), which is a natural and cost-effective approach to facilitate protein extraction from HDRB using *Bacillus subtilis* (natto) Takahashi as a microorganism.

Response surface methodology (RSM) analysis was used with Box-Behnken design (BBD) to optimize SSF conditions for extraction of the maximum water-soluble proteins and peptides (WSPP) from HDRB, and the predicted optimum conditions were 40.96% w/v water content, 6.27 Log CFU/g HDRB inoculum log, and 61.01h fermentation time. Conducting SSF under the optimized conditions facilitated extraction of 64.6 ±0.7% of total protein in HDRB as WSPP in comparison to 20.6% of protein extracted from nonfermented HDRB. Besides, the SSF increased the amount of low molecular weight of protein fractions and peptides with size of <5KDa.

The WSPP of SSF-HDRB extract was fractionated into molecular size ranges of >50, 10-50, 5-10, and <5 kDa employing ultrafiltration (UF) technique, and the fractions were evaluated for tyrosinase, the main enzyme in melanogenesis pathway, and elastase inhibition activities. The results showed that the highest in-vitro elastase and tyrosinase inhibition activities by UF peptide fraction < 5Kda and were 12.9% and 68.5%, respectively. Further separation of fraction <5 kDa using size exclusion chromatography (SEC) resulted to obtain two fractions (F5 and F4) that exhibited the highest tyrosinase inhibition activities (82.3%) and (79.1%), respectively.

The SEC fractions (F4 and F5) were characterized and purified by reverse phase HPLC using C-18 column and among the collected fractions three fractions, namely F8, F14, and F40, showed enhanced anti-tyrosinase activity of IC50 values of (870.3, 445.8, and 187.6 µg/mL) respectively, with dose dependent inhibition. Also, the fractions showed potential inhibition of melanogenesis activity without cytotoxic effect in mouse B16F10 melanoma cells, and peptide fraction (F40) had the greatest melanogenesis inhibition activity (84.2% ±1.3) and IC50 value (235.2 µg/mL) signifying the potential of F40 as a cosmeceutical agent for treatment of melanin-related skin disorder.

The most abundant peaks in the three fractions were identified by mass spectrometry and proteomic tools using rice proteomic database. Five peptides were identified having the amino acid sequence (TSFTL) and (APDLPML) from (F40), (FPLHF) and (SYVFGCF) from (F14), and (SCADGGF) from (F8).

WSPP of SSF-HDRB has potential antioxidant activities, and showed (56.0, 44.4, and 84.5 %) of DPPH free radical, superoxide radicals, and hydroxyl radicals scavenging activities, respectively.

Based on the results, bacterial SSF using the probiotic B. subtilis (natto) Takahashi is an efficient approach to enhance extraction of WSPP from HDRB with potential bioactivities and add-value to HDRB in finding applications as a functional ingredient in the cosmeceutical products that support skin health and control the oxidative stability in food products.

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CHAPTER 1

Introduction

Rice (*Oryza sativa*) is one of the most important staple foods. In the United States, the production of rough rice was 11,56 million metric tons in 2020, and Arkansas's production for 2020 was about 5.49 million metric tons (USDA, 2021) and ranks #1 in rice production in the US. Rice bran is considered the most valuable by-product of rough rice produced in abundant quantities in the Arkansas State. During the production of white rice, bran is removed from the kernel by friction. Bran accounts for about 12% of rough rice (Spaggiari et al., 2021). However, it is mostly under-utilized and used as a livestock feed supplement and as an oil extraction source in food industry which has started to attract more interest due to containing many nutritional and beneficial components including tocopherols, tocotrienols, and sterols (Yang et al., 2018). Protein is an important component in rice bran. Rice bran contains dietary fiber (10-27%), lipid (12-23%), and protein (10-20%) which composed of albumins (37%), globulins (36%), glutelins (22%), and prolamins (5%) (Fabian and Ju, 2011). Also, rice bran contains several antinutritional factors such as phytate, trypsin inhibitor, hemagglutinin-lectins**,** and oryzacystatin (Juliano, 2016a). Rice bran protein has higher protein efficiency ratio (PER) than casein (2.0 to 2.5, 2.5 respectively) (Chang et al., 2006). However, rice bran proteins from heat stabilized defatted rice bran are difficult to extract due to the interaction or tight bonding of these proteins with other components of rice bran including carbohydrate moieties, fiber, and phytic acid (Tang et al., 2002). Furthermore, to maintain the quality of rice bran oil, heat stabilization causes more interactions between proteins and carbohydrates, which in turn contributes to a decrease in protein extraction efficiency (Fabian and Ju, 2011; Tang et al., 2002). Several approaches including physical, chemical, and enzymatic methods have been followed to extract sufficient

quantities of protein and peptide hydrolysates from rice bran. Due to the intricated process and the high cost of using enzymes and chemicals in protein extraction, finding simple and cost-effective process to extract protein from rice bran has been challenging**.**

Fermentation is a widely accepted method of food processing and preservation; more recently, fermentation has dramatically been developed and included in several biotechnology sectors. Solid-state fermentation and liquid-state fermentation are the two main methods of fermentation. Based on the water content in the growth substrate, there are two main types of fermentation, solid-state fermentation (SSF) and liquid-state fermentation (LSF) or submerged fermentations (SmF) in which the microorganisms grow and develop in a liquid substrate (media) with low concentration of the biomass while in SSF, the activity of microorganisms and formation of products occur on the surface of the solid-substrate particles which performs as a supporter and a source of nutrients, with absence or near absence of free water (Rosales et al., 2018). Liquid-state fermentation is widely used and more developed in several fermentation industries, such as wine, beer, and sauce. However, solid-state fermentation has obtained prominence and played role in different fields including enzyme production, pharmaceutical, and chemicals fields due to its energy-saving, low-cost, high yield, high end-concentration of products and stability, low catabolic repression, low demand on sterility due to low water activity used, water-saving, ideal for valorization of agro-industrial by-products, and less emission release properties (Behera et al., 2019; Teigiserova et al., 2021). These properties and advantages over LSF have made SSF points toward the sustainable advancement of chemical and biological industry, hence, it has become an interested and hot spot of research area since the last decade. Furthermore, in SSF, the growth condition resembles the natural habitat of the microorganism which facilitates a better and more productive environment. Accordingly, microorganisms have

been grown on different nutritional substrates, such as agricultural wastes and food crops (soybean meal, wheat bran, sawdust, bagasse, and straw), which provides a nitrogen and carbon sources as well as the physical structure for the growth of microbes (Sadh et al., 2018; Singhania et al., 2009). Thus, SSF is a cost effect approach to yield higher number of bioactive compounds.

Recently, several studies have confirmed the role of solid-state fermentation in the value addition and nutritional improvement of many plant co-products, such as rapeseed cake (Shi et al., 2015), lupin and soya bean (Bartkiene et al., 2015), Olive Pomace (Filipe et al., 2020), Grape Pomace (Dulf et al., 2020), Oilseed Cakes (Pinela et al., 2020), Wheat Bran (Mao et al., 2020), and Oat Bran (Călinoiu et al., 2019), or to produce enzymes including proteases and alpha-amylases on corn pericarp, cellulases on maize bran, pectinases on soybean meal (Salim et al., 2017; Teigiserova et al., 2021), and chitinase on wheat bran with colloidal chitin (Waghmare et al., 2011). Rice bran has been reported to be a suitable substrate for the microbial growth and production of enzymes, including phenolic acid esterase, xylanase, and protease (Marques et al., 2018; Sumantha et al., 2006).

In addition, several strains of probiotics can produce or enrich bioactive compounds in fermented foods. *B. subtilis*, an aerobic endospore-forming bacterium commonly found in nature. There is a long history of the safe use of *B. subtilis* in fermented food production around the world, such as Maseura and Bhallae, (traditional Nepali fermented food) made from black gram, Dawadawa (traditional African flavoring product) made from African locust bean, Wari from Bengal gram, and natto from soybean (Srikaeo, 2020). In the US, several enzymes from *B. subtilis* including carbohydrase (21 CFR 184.1 148) and protease (21 CFR 184.1 150) have GRAS status for use in foods as enzymes. The confirmations are based on the use of nonpathogenic and nontoxigenic strains of *B. subtilis* (FDA, 2011). *B subtills* secrets several

extracellular enzymes including protease, xylanase, ferulic acid esterase, amylase, pullulanase, chitinase, lipase, and phytase, with higher quantities. Also, under stress conditions due to the solid-state fermentation environment including reduced water levels and non-easily available nutrients, *B. subtilis* can excrete variety of enzymes and secondary metabolites such as antimicrobial peptides, poly-gamma-glutamate (γ-PGA), and levan polysaccharide. Additionally, the presence of thiol-disulfide oxidoreductases in *B. subtilis* may be beneficial in the secretion of disulfide-bond-containing proteins. Even though, *B. subtilis (natto)* cells utilize the proteins, peptides, and amino acids in soybeans for their growth. The kinds and quantities of peptides and amino acids produced by the activities of *B. subtilis (natto)* during fermentation affect the flavor appeal of natto (Hosoi and Kiuchi, 2008). Therefore, *B. subtilis natto* might secrete more than one type of proteases through growing on heat defatted rice bran in solid state fermentation conditions, and this could be a potential approach to generate not only protein fractions and peptides which have potential in product applications as bioactive substances but also ensure the naturality, higher yield, and synergistic bioactive effects of the extracted proteins hydrolysates.

In this project, the major focus was to prepare protein isolate and protein hydrolysates from HDRB using solid state fermentation technology, and to identify and evaluate natural compounds, such as proteins and peptides in fermented HDRB which might have bioactivity to enhance skin health including anti-tyrosinase, melanogenesis inhibition, anti-elastase, and antioxidant bioactivities. Due to the biological activities of microorganisms and secreted enzymes, fermented foods and products are rich in protein fractions and peptides and secondary metabolic compounds especially phenolic compounds which are responsible from several bioactive properties protecting from disorders related to the aging, such as skin disorders (Chan et al., 2014). Bioactive peptides in general consist of 2 to 20 amino acid moieties which are an

encoded part of the primary structure of their innate proteins, and to be released, a proteolytic activity of the precursor protein is required. In fermented foods from animal and plant bases, several peptides have been isolated, described, and evaluated, and they possessed important physiological activities and functions in human (Chai et al., 2020). Results of previous studies have stated that rice proteins and peptides possessing biologically functional properties against disease progression. Although carbohydrates and phenolics have been documented as biochemical compounds that mainly contribute toward anti-disease characteristics (Liu et al., 2019).

To study the effect of bioactive peptides generated by fermentation technique, eliminating, or minimizing the interference of phenolic compounds in the fermented extracted broth is required since phenolic compounds are highly released during fermentation (Webber et al., 2014) and considered as a main source of impurities in protein extraction contributing to the dark color and undesirable taste of the extract (Xu and Diosady, 2002). polyvinylpolypyrrolidone (PVPP), a synthetic water- insoluble polymer, is used industrially to remove polyphenols from, apple juice, wine, and beer (Croitoru and Râpeanu, 2019; da Costa Lopes et al., 2016; Laborde et al., 2006). Furthermore, PVPP has been used to separate phenolic fractions from the extract especially in wine industry. The hydrophobic interaction and hydrogen bonds are responsible from the high affinity between phenolic compounds and PVPP (Cosme et al., 2019; Han et al., 2020).

Fermentation has been used to increase antioxidant capacity of protein isolate prepared from different sources including plant proteins and proteins extracted from agricultural by-products (Chai et al., 2020), such as SSF of soybean flours (Fernandez-Orozco et al., 2007), wheat germ extract (Liu et al., 2017), and corn gluten meal (Jiang et al., 2020). As a result of the

age and different environmental stresses including UV radiation exposure, the levels of antioxidant enzymes and internal, cellular, antioxidants suffer from reduction. Thus, accumulation of reactive oxygen species (ROS) becomes the main cause to initiate oxidative stress which advances skin aging characterizing by atypical pigmentation and skin wrinkles when ROS produce in the dermal fibroblasts. It is important to replenish the decrease of the antioxidants by using external compounds with antioxidant properties including protein fractions and peptides, in cosmetic formulations as topical applications. This method is considered as an effective mean for antiaging benefits by preventing of the photo-induced aging symptoms and skin aging disorders (Buranasudja et al., 2021; Masaki, 2010).

In addition to the role of antioxidant peptides in protection of the skin from the developing of skin disorders, peptides obtained from fermented products or enzymatically hydrolyzed proteins can play a potential role in treatment the main skin disorders as well, such as hyperpigmentation and skin wrinkling through inhibitory activities of their key enzymes, tyrosinase and elastase respectively.

Although, several synthetic tyrosinase inhibitors including peptides, are recognized, and used today in cosmetic products, there is increasing restrictions in the usage of these inhibitors due to their high cytotoxicity and low stability and solubility which limiting their application (Schurink et al., 2007). Furthermore, the safety of inhibitors is the primary consideration in the cosmetic products. Therefore, obtaining tyrosinase inhibitors from natural and inexpensive sources, such as agricultural by product including meals and cakes, and other natural sources is in need because of their safety and being free from undesirable side effects (Hsiao et al., 2014; Schurink et al., 2007). Although, fermented broth ingredients, such as, polyphenolic compounds and flavonoids, aglycone isoflavones, 8-Hydroxydaidzein and, 3-hydroxydaidzein, exhibited

anti-melanogenesis and anti-tyrosinase activities (Chan et al., 2014), there is no data in the literature related to evaluation of protein fractions and peptides obtained from fermentation broth of rice bran or other fermented cereal brans and grains toward inhibition of tyrosinase and melanogenesis.

To reveal the maximum potential of the bioactive peptide as melanogenesis inhibitor in cell culture or anti-tyrosinase in vitro assay, the peptide needs to be separated, isolated, and purified from other substances including protein fractions and peptides which might have potential interaction and effect on its activity. In general, studies related with characterization of bioactive peptides have applied membrane fractionation methods as an initial step at which the pool of protein hydrolysates and peptides is sequentially passed through several membranes each of them has a particular pore size so that the protein hydrolysates and peptides can be separated into several groups depending on it molecular size. In industry, food peptides have been fractionated and separated by using ultrafiltration technique, and the common range of separation is from 1 to 100 KDa (de Andrade et al., 2017). Hence, having these fractions of the protein hydrolysate and peptides with specific molecular size is potential in first-step screening for bioactivity peptides which evaluates the activity of the fractions with comparison to each other and to the innate protein source. To obtain peptides from fermented substances, fractionation usually is processed starting from higher molecular cut-off membrane (100-50 KDa) to lower MWCOs (5-1 KDa) after subjecting the fermented broth or extract to micro membrane filtration with pore size of (0.1 μm) to remove the microorganisms and their spores.

Additionally, the bioactive peptides need purification to evaluate their bioactivities more precisely and determine their accurate molecular weight and amino acid sequences. Several proteomic powerful tools are used for separation and purification of peptides from protein

hydrolysates with high resolution. The common approach in this matter is using chromatographic separation techniques to achieve the highly purified peptides. Employing size exclusion chromatography (SEC) followed by revers phase high performance liquid chromatography (RP-HPLC), with C18 columns, is the most efficient process to purify peptides. Preparative HPLC have been used to improve both the purity and yield of the peptides. The multi-processed separation, purification, and identification approach has been used widely to obtain peptides from fermented foods and grains with different bioactivities, such as Angiogenic and lipopolysaccharide neutralizing peptides from fermented soybean, natto, (Taniguchi et al., 2019), ACE-I inhibitory peptide from pea seed (Jakubczyk et al., 2013), antioxidant and ACE-I inhibitory peptide from fermented bovine colostrum (Gaspar-Pintiliescu et al., 2020), and ACE-I, antimicrobial, DPP-IV inhibitory, proliferation stimulating, and cytotoxic peptides from fermented whey proteins (Worsztynowicz et al., 2020).

Since anti-tyrosinase and melanogenesis inhibitory bioactive peptides are used as topical applied cosmetic product, evaluation the cytotoxicity of these peptides is very important as well as determining melanogenesis inhibition activity requires test the activity of the peptides in skin cell cultures. Furthermore, skin cancer, melanoma, is related with hyperpigmentation activity. Murine melanoma cells (B16) are common model used to evaluate anti-melanogenesis activity and cytotoxicity of an inhibitor.

There is growing interest in the production of pharmaceutical and cosmetic products based on proteins and peptides that possess disease-protective properties. Therefore, there is a need to evaluate the bioactivity including anti-skin ageing disorders of these non-carbohydrate components in food sources, such as fermented rice bran, we propose this research project.

This project was based on the following objectives and focused on obtaining protein hydrolysates and peptides from fermented rice bran using *Bacillus subtilis* (natto) Takahashi in solid state fermentation condition, evaluating bioactivates including anti-tyrosinase, anti-skin wrinkling, melanogenesis inhibitory in cell culture, and anti-skin cancer bioactivities, and purifying and sequencing the most effective peptides.

Objectives:

Based on the hypothesis that bacterial fermentation can add more nutritional values to the rice bran, and protein fractions obtained from fermented rice bran possess bio-functional properties against skin disorders including melanogenesis, wrinkling, and skin-cancer**,** the objectives of this proposal were as follows:

1. Optimize the growth conditions of probiotic (*Bacillus subtilis* (natto) Takahashi) in solid state fermentation (SSF) utilizing heat treated defatted rice bran (HDRB) as the medium and extract proteins and peptides.

2. Fractionate protein hydrolysates and peptide fractions and investigate bioactivities of protein hydrolysates and peptide fractions for *in vitro* anti-tyrosinase (anti-skin pigmentation), and anti- elastase (anti-skin wrinkling) activities.

3.Separate and purify peptide fractions that have the highest anti-tyrosinase activity and investigate melanogenesis inhibitory and cytotoxicity of the purified peptides in skin melanoma cell line.

4. Preform mass spectrometric analysis and sequencing of the single purified peptide that have anti-melanogenesis activity.

5. Evaluate *in vitro* antioxidants activities of the proteins and peptides extracted from fermented heat-stabilized rice bran.

CHAPTER 2

Literature Review

Rice and Rice Bran:

Rice (Oryza sativa), an important cereal crop, is one of the main food sources in human diet. Rice provides about half of the globally required dietary calories and a considerable portion of the protein consumption for people in different areas in the world. Thus, rice has been correlated with food security (Khir et al., 2019). Globally, over 100 countries are growing rice with total production of 752.8 million metric tons as rough rice in 2020 including USA (11,56 million metric tons), and Arkansas's production was about 5.49 million metric tons (USDA, 2021) ranking #1 in rice production in the US.

A freshly harvested rough rice consists of a protective husk that surrounds the rest of the rough rice (**Figure 2.1**). Rice bran represents about 8-10% of the weight of rough rice, and it consists of aleurone and sub-aleurone layers, inner pericarp, and the embryo (Pandey and Shrivastava, 2018; Zhao et al., 2018). Production of white rice requires passing the rough rice through several processing procedures which generate large quantity of different by-products such as husk, brewers, straw, and bran. During processing, the rough rice is milled to remove the husk from the rice kernel. Then, the brown rice is subjected to a whitening step at which the bran layers that surround the starchy endosperm are removed by abrasion and friction process (Juliano, 2016b).

In general, the by-products of rice milling process are underutilized as animal feed or other usages that lose or reduce the value of these by-products. The production of the by-products in rice industry will remain high since there is an expected huge demand for white rice globally for long time which reflects the economic and population growth in the world. Based on

these expectations, it is crucial to find new and efficient technologies for processing and managing the substantial yielded amounts of rice by-products and convert these valuable resources especially the rice bran to beneficial value-added products.

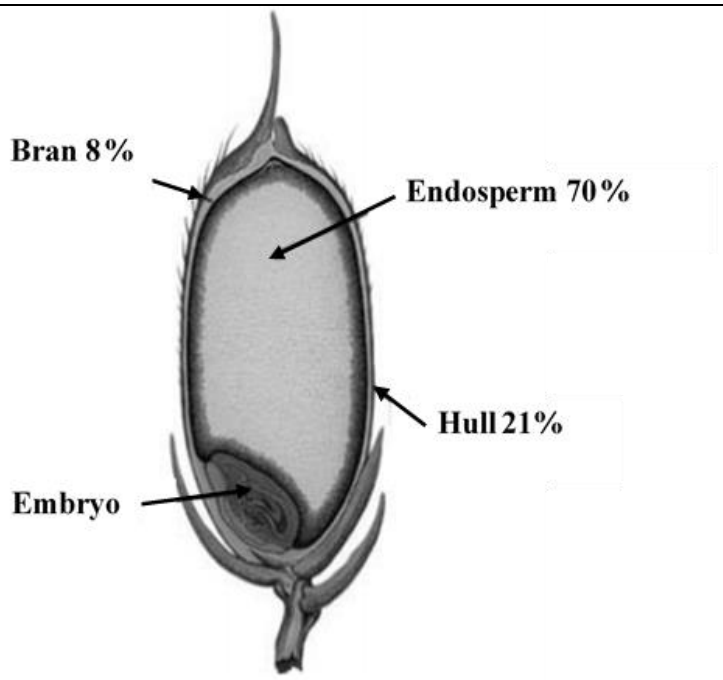

Figure 2.1. Structure and composition (%) of rice grain (modified from Kong, 2009)

Globally, the annual production of rice bran reaches about 76 million metric tons (Bodie et al.). However, over 90% of produced rice bran is underutilized as animal feed, and due to that, rice bran in US is inexpensive and the average coast is $130/ton. Three types of rice bran can be produces after the milling of rough rice. The raw full-fat bran, full-fat parboiled bran, and de-fatted bran. This classification is based on oil content of the bran (Khir and Pan, 2019).

The high oil content of rice bran and the activity of endogenous lipase have restricted the utilization of rice bran because hydrolyzing the triglycerides of the oil into free fatty acids and glycerol deteriorates the oil quality and causes the bran with bitter taste and rancid smell. Therefore, the bran and the extracted oil cannot be used for human consumption. To prevent

lipase activity in rice bran, the bran is stabilized using heat directly after milling process. This step helps to extend the shelf life of the bran and prevent lipid oxidation. After oil extraction from heat stabilized bran, the by product is called heat-stabilized defatted rice bran (HDRB) (Wang et al., 2017). In industry sector, all the rice bran is stabilized to inhibit lipase enzyme activity. Heat-treatment and chemical treatment are the two major methods for rice bran stabilization (Prakash, 1996). Heat treatment is more popular in the industry than chemical treatment. However, during the process of heating, a series of non-enzymatic browning reactions is initiated, and the rice bran proteins get bound more tightly with other components including carbohydrates and makes the protein less extractable. Furthermore, other components including fiber (12%), and phytic acid (1.7%) interact in such a way to hinder high extractability of proteins (Lv et al., 2017; Tang et al., 2002).

Rice bran (RB) is an inexpensive and important source of nutrients and phytochemicals due to the significant content of lipids, dietary fibers, protein, vitamins, and minerals (Sohail et al., 2017). Depending on RB cultivars, the main compositions of rice bran are 15-19.7% Fat, 34-62% carbohydrates, 7-11.4% total fiber, 6.6-9.9% ash, 8-14% moisture, and 11.3-14.9% protein (Juliano, 2016b). However, in defatted rice bran (DFRB), the percentage of most components including proteins increases after oil extraction due to the change in the mass percentage of the total compositions. It was reported that HDRB contains about 15% protein, 13.5% reducing sugars, 58.5% carbohydrates, 2% soluble fiber, 14.5% ash, and 8.5% moisture (Sirikul et al., 2009). In a study of (Beloshapka et al., 2016), it was reported that defatted rice bran comprises about 24.4% dietary fiber, 34.2% starch, and 17% crude protein. Also, based on the rice variety, protein content in defatted rice bran can reach 20.3 % after extraction of more than 90% of the oil from RB (Zhuang et al., 2019).

Proteins in rice bran

Proteins in rice bran which represent the main part of seed storage protein, can be grouped into four fractions including glutelin (32.5%), albumin (30.9%), globulin (24.9%) and prolamin (11.6%), according to their solubility and based on Osborne fractionation method (Chanput et al., 2009). Protein distribution also differs between the milled rice and rice bran. in milled rice, the alkali soluble glutelin represents about 65% then 20% as an alcohol-soluble prolamin while water and salt soluble protein (albumin and globulin) (15%) are the less percent fraction in milled rice proteins. In rice bran, the dominant fraction of protein is albumin and globulin (66-98%) (Juliano, 2016a). However, this percentage of each group of protein also varies based on rice cultivars. The chemical properties and characteristics of each rice bran protein group are as follows:

Glutelin is considered as a major protein in milled rice endosperm comprising about 80% of the rice endosperm protein. Also, the molecular weight of glutelin is the highest among the other protein fractions in rice (Anderson and Guraya, 2001). In general, glutelin consists of two main subunits, the basic subunits having molecular weight of 19-25 KDa and the acidic subunits with molecular size about 30-39 KDa. The two subunits in rice glutelin connect with each other by covalent interaction occurs between intermolecular disulfide bonds. The molecular weight of glutelin was observed at 10-60 kDa (Chanput et al., 2009; Xia et al., 2012). Also, it was reported that rice glutelin comprised high molecular size protein that ranges from 45 to 150 KDa (Hamada, 1997). Due to the high number of disulfide bonds in glutelin and link between its polypeptides and other hydrophobic interactions, there is difficulty in rice protein extraction. Accordingly, the water solubility of glutelin is limited (Xia et al., 2012). This protein is soluble above pH 10 and below pH 3.0 (Champagne et al., 2004).

Albumin is generally the water-soluble protein and its extraction using water combines with globulin (salt-soluble) contamination because the rice grain contains minerals that dissolve in the water during extraction (Champagne et al., 2004). Albumin in rice was analyzed using different separation techniques, such as gel filtration chromatography (Hamada, 1997; Martínez-Maqueda et al., 2013), electrophoresis (Cao et al., 2009; Chanput et al., 2009). Hamada (1997) reported that rice albumin fractions have molecular weight about 100KDa or less. Moreover, the major polypeptides bands of albumin in rice are in range of 18-20 KDa (Cao et al., 2009), while it was stated by Chanput and his co-worker (2009) that rice albumin separated by SDS-PAGE into two bands with molecular weight of 30 and 50 KDa. Albumin was extracted from rice bran and separated using SDS-PAGE. The results showed several subunits with molecular mass of 14, 17, 22, 31, 32 KDa (Wang et al., 2014).

The salt soluble protein (globulin) in rice is rich in sulfur and contains different polypeptide fractions with molecular weight ranges from 16-130 KDa based on gel filtration chromatography separation (Hamada, 1997). Thai rice protein showed globulin fractions having mass distribution at 50, 30, 15 KDa (Chanput et al., 2009). The extracted globulin from rice bran showed molecular mass distribution of 21, 36, 49, 53, 63 KDa when analyzed by SDS-PAGE (Wang et al., 2015).

Rice prolamin, alcohol-soluble protein, is extracted after albumin and globulin extraction in general (Juliano, 2016a). It's percentage in rice bran proteins ranges between 3.3-11.6% (Cao et al., 2009). The main fraction of prolamin in rice bran is 23 KDa. Furthermore, it was reported that rice prolamin fractions were monitored at 25, 15, and 10 KDa (Chanput et al., 2009) and at 16, 13, and 10 KDa bands (Cao et al., 2009). Rice protein is considered to have the highest lysine content among the cereal proteins, and this is due to the prolamin content of lysine (Juliano,

2016a). In addition to that, 13 kDa fraction of prolamin is sub-divided into Cys-rich prolamin (13a) and Cys-less prolamin (13b) (Sasou et al., 2018). Rice bran proteins have been used in different food products because of having potential nutraceutical and nutritional values and applications (Al-Doury et al., 2018; Friedman, 2013).

There is a difficulty in directly extracting protein from HDRB without treatment which is due to the interaction and tight bonding of these proteins with other components of rice bran including carbohydrate moieties, fiber, and phytic acid, through heat stabilization process (Fabian and Ju, 2011).

Extraction of Rice Bran Proteins

Rice bran contains the aleurone layers of endosperm parts which is rich in storage protein. Thus, the bran contains most of the proteins found in the rice grain. The protein content of full fat rice bran reaches 15% while the defatted bran contains about 18% (Saunders, 1990; Schramm et al., 2007). Extraction of protein from rice bran including the defatted rice bran, is believed to be valuable since there is a high demand for protein in food industry for its use in the production various food products, and rice bran is relatively an inexpensive resource for protein. However, the complex nature of rice bran complicates protein extraction. Moreover, rice bran protein has poor solubility in water due to the strong aggregation, glycosylation, and cross-linking with other components through its extensive disulfide bonds (Fabian and Ju, 2011; Gupta et al., 2008; Phongthai et al., 2017b). In addition to that, the interaction of rice bran protein with the high content of phytic acid (0.9-2.2%) affects and complicates not only the protein solubility but also protein separation from other components in the bran (Juliano, 2016a; Wang and Guo, 2021). Consequently, disruption of these kinds of aggregations and crosslinking before protein extraction from the bran is crucial to insure higher solubilization and extraction of the proteins.

Several approaches have been established to facilitate protein extraction from rice bran. The most employed method for this purpose is the alkali extraction for rice bran protein. However, this method requires high pH (9.5-11.0) and relatively has low yield of protein. It was stated that by applying heat combined with high pH value led to increase in the yield of rice bran protein (Gupta et al., 2008; Sari et al., 2015). Also, in pH range of 7.5 to 11.0, there was a linear positive correlation between pH value and the purity of the extracted protein, but this correlation became negative when pH value reached 12.0 because of solubilization of nonprotein nitrogen that occurs at higher pH values (Jiamyangyuen et al., 2005). The effect of high pH is based on the high concentration of sodium hydroxide which could initiate hydrolysis process for some hydrogen, amid, and disulfide bonds leading to reduction in aggregated protein molecular size and improving in the protein extraction activity (Phongthai et al., 2017b). By exposing protein to extreme alkali condition can cause several disadvantages, including denaturation and aggregation of proteins, changing in the functional and nutritional value and characteristics of proteins, generating toxic compounds (Ajandouz et al., 2008; Pelgrom et al., 2013; Sari et al., 2015).

Table 2.1. Alkaline extraction method for extracting rice bran protein.

Raw materials	**pH value**	**Protein recovery yield (%)**	**References**
Defatted rice bran	10.0	34.2	(Wang et al., 1999)
Defatted rice bran	2.0 -12.0	1.36- 12.20	Jiamyangyuen et al (2005)
Defatted rice bran	8.0	31.27	Silpradit et al (2010)
Defatted rice bran	9.0	37.6- 46.2	(Yeom et al., 2010)
Rice bran	9.5	32.9	(Zhang et al., 2012)
Rice bran	11.0	25	(Cheetangdee, 2014)

It was reported that at alkali environment, the toxic amino acid, lysinoalanine, can be produced from converting of protein residues, such as serine and cysteine, to dehydroalanine which transforms to lysinoalanine, and this conversion corresponded with lowered contents of both residues, cysteine and lysine (Fabian and Ju, 2011; Hou et al., 2017). (**Table 2.1**)

summarizes studies that have used alkaline extraction approach to improve extraction of rice bran protein.

Physical techniques

Physical techniques, the more economical and convenience application in industry, have been used to extract protein from rice bran. Subjecting rice bran or defatted rice bran to physical treatment including high pressure, sonication, freeze-thaw cycles, and high-speed blending, disrupts plant cell walls by shearing force or causing cell lysis due to ice crystallization that ruptures the structure of the cellular membranes (Phongthai et al., 2017b; Tang et al., 2002). Thus, these effects facilitate releasing of the proteins (Fabian and Ju, 2011). Also, the physical methods are more variable in food processing steps due to having less effect on the processed food compositions and causing minimum health concerns. Micro-fluidization and colloid milling have been used to disruption the agglomeration between protein and starch under high shearing influence and wet processing, and improve separation of protein (Xia et al., 2012). The efficiency of different physical method, high pressure, sonication, freeze-thaw, and high-speed blending, for extraction protein from heat-stabilized rice bran has been evaluated, and the protein yield ranged between 11 to 16% (Tang et al., 2002). Furthermore, rice bran protein was extracted after microwave treatment and under optimized conditions of 1K power for 1.5 min at bran to water of 0.89g:10 mL. The yield of extracted protein increased about 1.5-fold (4.37%) in comparison with non-treated rice bran (2.92%) (Phongthai et al., 2016). The summary of some related studies applying physical techniques is listed in (**Table 2.2**).

Although, using physical methods to extract protein from rice bran have many advantages, such as more economical and simpler in industrial applications, the recovered amount of protein was noticeably lower when compared to other extraction methods including

chemical extraction. Accordingly, physical processes do not have sufficient impact to disrupt the highly aggregated proteins in rice bran.

Table 2.2. Physical treatment for extraction of rice bran protein

Raw materials	Conditions	Protein yield (%)	References
Rice bran	Colloid milling + homogenizing	37.8-67.5	(Anderson and Guraya, 2001)
Defatted rice bran	Freezing thawing Sonication High-speed blending, High pressure	12 15 16 11	(Tang et al., 2002)
Defatted rice bran	Ultrasonic at 100 W	25.8-74.6	(Chittapalo and Noomhorm, 2009)
Defatted rice bran	Microwave at 800 W	67-70	(Bandyopadhyay et al., 2012)
Defatted rice bran	Microwave at 1000 W	30.7	(Phongthai et al., 2016)
Defatted rice bran	Ultrasonic- Microwave (26KHz, 300W)	59	(Zhang et al., 2019)

Enzymes in rice bran protein extraction

Utilization of enzymes is another alternative method that has been used in protein extraction from of rice bran. Proteases have been effectively used in hydrolyzing rice bran protein for better solubility and higher extractability (Silpradit et al., 2010; Tang et al., 2003). Since proteases activity hydrolyze proteins to peptides which have higher solubility in water than the innate protein, using proteases enhanced the extraction of protein from rice bran by recovering about 60-93% of rice bran proteins as large varieties of hydrolysates (Hamada, 1999).

Protein extraction can also be enhanced by using other enzymes including carbohydrases that attack the polysaccharide matrix of bran which is commonly consists of cellulose and hemicellulose (Tang et al., 2002; Wang et al., 2016). Protein was extracted from rice bran using food-grade hemicellulase or cellulase under condition of pH 4.0 and 65°C, and the extracted protein yield was 46% and 35%, respectively, which was higher than protein extraction without enzyme treatment (25.8%) (Ansharullah et al., 1997). The use of phytase and α-amylase which attack the interaction of proteins with phytate and starch respectively, have been studied in

protein extraction from rice bran. The α-1,4-linkages of starch are hydrolyzed by the activity of α-amylase enzyme, and the final product is dextrose and glucose. By using α-amylase in protein extraction from rice bran, starch-bound proteins can be liberated increasing the solubility of the protein, hence, improving the efficiency of protein extraction (Tang et al., 2002). Rice bran was subjected to α-amylase hydrolysis which effectively removed the impurities including starch from the bran, and the achieved protein yield was 13.4% under the following extraction condition, pH 6.5, 45 min, and 95 °C (Shih et al., 1999) while in the study of Tang et al. (2002), the protein yield increase to 37.3% after the treatment of rice bran with α-amylases under condition of (pH 6.5, 3.5h, and 45◦C). The summary of studies that used enzymes to facilitate protein extraction from rice bran and defatted rice bran is shown in (**Table 2.3**).

Table 2.3. Enzyme-assisted extraction of protein from rice bran and defatted rice bran

Raw materials	Conditions	Protein yield (%)	References
Defatted rice bran	Phytase & xylanase	34-74.6	(M. Wang et al., 1999)
Defatted rice bran	Amylase & Protease	66	(S. Tang et al., 2002)
Defatted rice bran	Amylase	58.4	(S. Tang et al., 2003)
Defatted rice bran	0.1% (w/w) Papain&5% (v/w) viscozyme	54-82.6	(Bandyopadhyay et al., 2012)
Rice bran	Cellulase & hemicellulase	35-46	(Shih et al., 1999)
Rice bran	0.5% w/w α-amylase & viscozyme	30-35	(Cheetangdee, 2014)

Although, using enzyme extraction method for protein extraction results in significant increase in protein yield in comparison to alkaline and physical methods, there are several disadvantages of this method including the high cost of enzymes and the difficulty in the scaling up of extraction process.

Hence, several extraction methods of rice bran protein have been developed, but they remain not efficient enough or possibly not economically feasible for commercial rice bran protein and protein hydrolysates production (Fabian and Ju, 2011). Finding a more efficient and economically viable method for extraction of rice bran protein and preparation of protein

hydrolysate is needed to be used as an ingredient in different products or as nutraceutical additive. In this proposal, we will utilize solid state fermentation as a simple and cost-effective mean to extract rice protein and protein hydrolysates.

Solid-State Fermentation

Among several methods of food preserving and producing, fermentation is the oldest and most economical approach. and it is used for food processing including meat, fish, dairy, legumes, cereal grains, fruits, and vegetables, as well as by-products of each of these categories. During fermentation process, microorganisms including fungi and bacteria, catalyze the nutrients of the substate and synthesize different metabolites under aerobic or anaerobic growth conditions, so the desired microbial metabolites and/or the microorganisms can be produced and accumulated (Chen, 2013). Fermentation can be divided into two types, solid-state fermentation (SSF) and liquid-state fermentation (LSF) or submerged fermentations (SmF), according to the water content in the growth substrate. In the SmF, the microorganisms grow and develop in a liquid substrate (media) with low concentration of the biomass while in SSF, the activity of microorganisms and formation of products occur on the surface of the solid-substrate particles (Tengerdy, 1985). Even though, submerged fermentation is widely used and more developed in several fermentation industries, such as wine, beer, and sauce, it has noticeable problems including major pollution and high consumption of energy, which are limiting the sustainable improvement of fermentation. Solid-state fermentation has obtained prominence and plays a role in different fields including enzyme production, pharmaceutical, and chemicals fields due to its energy-saving, low-cost, water-saving, and less emission release properties (Chen, 2013). These properties have made SSF toward the sustainable advancement of chemical and biological industry, hence, it has become an interested and hot spot of research area since the last decade.

The main specification of SSF is that the substrate is relatively solid because of the nearly free water absent (Lizardi-Jiménez and Hernández-Martínez, 2017). In SSF, microorganisms grow on a nutritional substrate, such as agricultural wastes and food crops (soybean meal, wheat bran, sawdust, bagasse, and straw), which provides a nitrogen and carbon sources as well as the physical structure for the growth of microbes (Sadh et al., 2018; Singhania et al., 2009).

One of the valuable and abundant agro-industrial byproducts is heat-stabilized defatted rice bran (HDRB) which is also an important natural source of many phytochemicals and bioactive phytonutrients having pharmacological importance. Polysaccharides, phenolic compounds, and proteins constitute high percentages in HDRB (Sivamaruthi et al., 2018). Accordingly, rice bran and HDRB have been used as a substrate in SSF for production of different enzymes and secondary metabolites as summarized in (**Table 2.4**).

Table 2.4. Production of enzymes and secondary metabolites by different microorganisms in solid-state fermentation using rice bran

Microorganisms	**Substrates**	**Products**	**Reference**
Bacillus subtilis DM-04	rice bran (Full fat)	alkaline protease	(Mukherjee et al., 2008)
Streptomyces albulus PD-1.	rice bran (Full fat)	ε-poly-lysine	(Xu et al., 2017)
Penicillium citrinum YS40-5	rice bran (Full fat)	β-glucosidase	(Ng et al., 2010)
Aspergillus oryzae and Rhizopus oryzae	rice bran (Full fat)	Phenolic compounds	(Abd Razak et al., 2017)
Aspergillus *spp.* (*brasiliensis*, *awamori*, and *sojae*)	rice bran (Full fat)	Phenolics, Flavonoids, and Kojic acid	(Ritthibut et al., 2021)

Rice bran has been fermented in SSF technique using *Rhizopus oryzae* CTT 7560, and the maximum protein recovery was 26.6% in 120h fermentation. Also, fermentation enhanced the extraction of phenolic compounds which resulted in increasing of antioxidant scavenging activity of the extract (87% at 96h) (Kupski et al., 2012). Other studies have been reported that

SSF mediated using *Rhizopus oligosporus* and *Monascus purpureus* and grown on rice bran leading to an increase in the total phenolic content including ferulic, caffeic, sinapic acids, syringic, and vanillic, as well as the antioxidant capacity of methanolic extraction of fermented rice bran (Abd Razak et al., 2017; Abd Razak et al., 2015; Jamaluddin et al., 2016).

Significant changes occurred in rice bran composition after SSF with *Rhizopus oryzae* for 120h at 30°C. The data showed potential increase in protein and fiber contents of rice bran, 40% and 50% respectively, while sugar content decreased about 60% (Oliveira et al., 2012). Different species of lactic acid bacteria, *Pediococcus acidilactici, Lactococcus lactis,* and *P. pentoseous,* have been used to evaluate effects of SSF on rice bran phytochemical content. After fermentation, the extract contents of α-tocopherol and γ-oryzanol were increased in comparison to non-fermented rice bran. Also, it was observed that the level of organic acids increased during SSF of RB (Abd Rashid et al., 2015). Webber and his co-workers (2014) reported that fermentation of Heat-stabilized defatted rice bran mediated with *Bacillus subtilis* subspecies *subtilis* enhanced the extraction of phenolic compounds in 96 h fermentation by releasing about 26.8 mg FAE/g sample which improved the free radical scavenging activity of the extract in contrast to the non-fermented HDRB (Webber et al.).

In another study, rice bran fermentation with the aid of *Preussia aemulans* increased the contents of protein (3%) and amino acids (20-fold) under optimized conditions of polysaccharide fermentation for 15 days (Li et al., 2016). Furthermore, HDRB from rice cultivar MR-219 was fermented with *Saccharomyces cerevisiae* (yeast), and under optimized condition (3% yeast concentration, for 17 h, at 30°C), the achieved protein yield was about (23.37%) to that of non-fermented rice bran (10.16%) (Chinma, 2014) while there was no significant change in the phytochemical content of fermented Tai black rice bran after fermentation with *Saccharomyces*

cerevisiae (Chaiyasut et al., 2017). It was stated that the change of the chemical composition of rice bran depends on the microbial strain used to mediate the fermentation process (Schmidt et al., 2014). The increase in bioactive compounds, such as phenolic compounds, that occurs after SSF was attributed to the breakdown of complex compounds including carbohydrates, lignocellulose, lipid, and proteins, as microbial activities releasing several kinds of bioactive compounds (Çabuk et al., 2018; Oliveira et al., 2012).

Among various groups of microorganisms used in SSF, the filamentous fungi are the most exploited because of their biochemical and physiological properties. Furthermore, the fungi including species from subdivision Basidiomycota and Ascomycota could grow on solid substrates and produce wide range of extracellular enzymes (Behera et al., 2019; Steudler et al., 2019) while using bacteria in general have not broadly utilized in solid state fermentation. Rice bran has been shown to be a suitable substrate for the growth of bacterial and fungal species (Geetha et al., 2015; Webber et al., 2014).

Moreover, many of the microorganisms having proteases secreting activity also can produce phenolic esterase, xylanase, and other carbohydrases when grown using SSF technique (Šelo et al., 2021; Steudler et al., 2019). Besides, the fermentation of HDRB with protease producing organisms is a potential means of integrating both enzymatic production and utilization steps. Such a process could serve as a simple, single step method for the extraction of protein and hydrolyze them to protein fractions and peptides. Therefore, the proposed research has potential to significantly increase the extraction yields by freeing bound proteins using fermentation. Rice bran fermentation might also serve as a value-added process with co-products including food grade enzymes, prebiotic oligosaccharides, or bioactive components including protein fractions and peptides.

Bioactivity of fermented rice bran

Solid state fermentation has been correlated with the improvement in the bioactivities of plant material including rice bran through increasing of bioactive compounds. Antioxidant activity effectively can be improved in rice bran by SSF technique. Defatted rice bran (DRB) was fermented using *Grifola frondose* as a starter fungus in SSF, and the water extract of fermented defatted-RB (FDRB) exhibited potential scavenging activity of hydroxyl radical at concentration of (1mg/ml). also, the FDRB extract at 2mg/ml showed 87.8% scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH), a synthetic free radical (Liu et al., 2017). In another study, *Rhizopus oryzae* mediated fermentation of FRB showed up to 50% scavenging activity against DPPH at concentration of 100µL/mL of fermented DRB extract which also had peroxidase inhibition activity (Schmidt et al., 2014). Lactic acid bacteria, *Pediococcus. acidilactici*, was inoculated in DRB, and the phenolic compound extraction of fermented DRB showed 82.6% DPPH radical scavenging activity while scavenging activity of the extracts that obtained from DRB fermented with *Lactobacillus lactis* and *P. pentoseous* were about 77.2% and 71.5% respectively (Abd Rashid et al., 2015).

In addition to antioxidant activities, fermented rice bran showed other bioactivities including anti melanogenesis activity which was attributed to the effect of phenolic compounds. Fermentation of RB with different fungus species, *A. oryzae*, *R. oryzae,* and *R. oligosporus*, improved the total phytochemical content and enzyme inhibitory activities, such as tyrosinase and elastase enzymes. Inhibition of tyrosinase and elastase prevents skin hyperpigmentation and skin wrinkling respectively. The results showed that 56% and 60% of tyrosinase and elastase activities respectively, were inhibited by *A. oryzae* fermented RB extract (Abd Razak et al., 2016; Abd Razak et al., 2017).

Furthermore, rice bran fermentation was mediated with the combination culture of *Lactobacillus rhamnosus* and *Saccharomyces cerevisiae,* and the fermented RB extract inhibited synthesis of melanin in melanoma cells, B16F1. Also, Human fibroblast cells irradiated with ultraviolet-B (UVB) showed an increase in synthesis of collagen activity and reduction in IL-1α production in the cells after exposure the cells to fermented RB extract (Seo et al., 2010). Although, SSF is an advantageous method for bioactive peptide production, most of the studies related with the solid-state fermentation of rice bran and the bioactivities have evaluated the bioactivities of phytochemicals, such as phenolic compounds.

Protein and Peptides from Fermented Foods

Fermented foods and products are a potential source for bioactive peptides which has been an interested subject of research because of the beneficial health properties that can be provided by theses peptides. Bioactive peptides in general consist of 2 to 20 amino acid moieties which are an encoded part of the primary structure of their innate proteins, and to be released, a proteolytic activity of the precursor protein is required. In fermented foods from animal and plant bases, several peptides have been isolated, described, and evaluated, and they possessed important physiological activities and functions in human (Chai et al., 2020).

The two main ways by which the release of peptides may occur during fermentation are the microbial proteolytic activity which is the dominated system and the endogenous proteolytic enzymes. Different body systems, such as cardiovascular, endocrine digestive, and immune systems, get impacted by the bioactive peptides after they consumed or applied and absorbed, and their activities depend on the peptide composition of amino acid, the sequence of peptide, and the peptide size (Martinez-Villaluenga et al., 2017). Many biological activities including antioxidant, antimicrobial, antihypertensive, anticancer, opioid, and immunomodulatory

activities, are represented in fermented foods and substances, and several of these activities are attributed to the existence of peptides (María Hebert et al., 2010). Thus, the process of protein fermentation from different sources using a variety of microbial species and strains is considered as a useful and novel method to produce bioactive peptides with valuable health impacts through hydrolyzing of food protein. Production of bioactive peptide using fermentation is cost effective in comparison to the use of pure enzymes, and the produced peptides do not need further analysis for purification. Cereals and their by-products including rice bran are high protein content substances (10-15%), so they are a potential candidate for growing of microorganisms as fermentable substrates. Hence, bioactive peptides can be generated due to cereal protein hydrolysis by microbial secreted protease activity during fermentation (Chai et al., 2020). Many bioactive peptides have been isolated from various solid-state fermented such as food sources, wheat, oat, quinoa, rapeseed, corn gluten meal, and red bean (Ayyash et al., 2019; Ding et al., 2019; Jiang et al., 2020; Xiao et al., 2018).

Rice bran is an excellent plant source for highly nutritious proteins, rice bran proteins, are very attractive for using in food and nutraceutical products as potential ingredients due to being rich in amino acids especially the essentials, efficiently digested, hypoallergenic proteins (Juliano, 2016a). Furthermore, many remarkable bioactive peptides have been generated from rice bran proteins by the enzymatic hydrolysis method using different proteases for this purpose (Liu et al., 2019). The most stated properties and reported bioactivities of the peptides obtained from rice bran protein hydrolysates are antihypertensive (Uraipong and Zhao, 2016; Wang et al., 2017), anti-diabetic (Boonloh et al., 2015; Hatanaka et al., 2012; Uraipong and Zhao, 2016), anticancer (Kannan et al., 2009; Kannan et al., 2010; Li et al., 2014), Antimicrobial (Taniguchi et al., 2019), and antioxidant activities. The main method used in the literature for generating

bioactive peptides from rice bran protein is the direct enzymatic hydrolysis of alkaline extracted protein isolate.

Bioactive protein fractions and peptides derived from rice bran

Defatted rice protein has been digested with chymotrypsin, alcalase, papain, flavorase, and neutrase, to generate peptides with antioxidant activities and the neutrase produced protein hydrolysates had the highest antioxidant activities against DPPH (85.8%), hydroxyl radicals (82.9%) and superoxide radicals (75.7%) at fraction concentration of 2mg/mL. Antioxidant peptides were identified by analyzing with MALDI-TOF/TOF mass spectrometry and had the sequence and mass of (FRDEHKK, 959.5 Da) and (KHDRGDEF, 1002.5 Da) (Zhang et al., 2010).

In another study, rice bran protein hydrolysate obtained by optimal digestion with proteases and pepsin, exhibited high antioxidant activity, and the peptide activity was positively correlated with the increase in the degree of hydrolysis. The molecular masses of the isolated peptides were 1373.6, 1038.09 and 907.53 Da (Adebiyi et al., 2009). The antioxidant activity of trypsin hydrolyzed rice bran protein fractions has studied, and the highest reducing activity was caused by the albumin fraction of the protein followed by the other fractions, globulin, prolamin, and glutelin, respectively (Chanput et al., 2009). Furthermore, different proteases, Protamex: Flavourzyme: Papain, were mixed in the ratio of (1:3:2) and used to hydrolyze rice residue protein. An isolated peptide (NFHPQ, MW: 641.7 Da) strongly scavenged the radicals ABTS and DPPH with (IC_{50} =0.11 and 0.14 mg/mL, respectively (Yan et al., 2015).

Also, rice bran albumin has been extracted from defatted rice bran which had been treated with 70% ethanol for removing of the phenolic compounds. Then the water extracted albumin was subjected to acid precipitation at pH 4.1 before hydrolyzing the rice bran albumin

using different commercial proteases including Alcalase, Protamax, Flavourzyme, and Neutrase. The albumin hydrolysates were fractionated and purified using ultra-filtration membranes with MW cut-off 3 and 10KDa and ion-exchange chromatography, respectively before the fractions were evaluated for antioxidant activities including ABTS and DPPH radical scavenging activities. Alcalase hydrolysate of rice bran albumin exhibited the highest antioxidant activities among other used proteases, and the scavenging activity of ABTS and DPPH radicals was 76.7% and 49.4%, respectively (Uraipong and Zhao, 2016).

Trypsin was also used to prepare protein hydrolysate from rice bran protein, and an antioxidant peptide was isolated from fraction F2-a which was more purified using gel filtration and RP- HPLC. The highest antioxidant activities of the peptide against DPPH free radicals and reducing power were achieved at IC_{50} 0.15 mg/mL and 0.05 mg/mL, respectively. The peptide has sequenced using Q-TOF ESI mass spectroscopy, and it consists of three amino acids with sequence (YSK, MW 395.0 Da) (Wang et al., 2017).

Heat-stabilized defatted rice bran has been used to prepare protein hydrolysate and peptides by hydrolyzing alkaline extracted HDRB protein isolate with Alcalase. Then the protean hydrolysate was used to prepare the GI-resistant peptides by using sequential simulated gastro-intestinal enzyme digestion. The final prepared protein hydrolysate was fractionated with ultra-filtration membrane into different size of protein and peptide fractions, >50, 10–50, 5–10, and <5 kDa. The HDRB protein fraction with molecular size of <5 kDa showed potential growth inhibitory and cell cytotoxicity of breast (HTB-26) and human colon (HCT-116) cancer cell lines at concentration of 0.5mg/mL, and the highest cytotoxicity activity of the fraction was with an IC_{50} of 0.75 mg/mL, so the <5 KDa fraction of rice bran protein hydrolysates have anti-cancer activities (Kannan et al., 2009). In another study conducted by the same group, a bioactive

peptide was purified from <5 kDa fraction of rice bran protein, and the pure peptide showed significant anticancer activity. The peptide concentration of 0.6-0.7mg/mL caused growth inhibition of 84%,84%, and 80% on colon (Caco-2, HCT- 116), breast (MCF-7, MDA-MB-231), and liver (HepG-2) cancer cell lines, respectively. The mass and the amino acid sequence of the bioactive peptide was revealed by MALDI-TOF-MS mass spectrophotometry analysis and de-novo peptide sequencing. The result stated that the peptide is a pentapeptide having the mass and amino acid sequence of 685.4 Da and (EQRPR) respectively (Kannan et al., 2010; Li et al., 2014).

In addition to that, rice bran albumin has been extracted from rice bran by distilled water and hydrolyzed using chymotrypsin and trypsin to prepare albumin hydrolysates which further fractionated and separated using size exclusion and reverse phase HPLC which resulted to purify the peptide (LQPSHY) with melanogenesis inhibition activity in rodent melanoma cell line (Ochiai et al., 2016a). It was stated that hydrolyzing rice bran proteins (RBP) using subtilisin which is an alkaline serine protease produced by *Bacillus sp.* including *B. subtilis* varieties, produced the highest amount of peptides that have low molecular weight and exhibited higher bioactivities in comparison to the RBP hydrolyzed using pepsin, papain, and cysteine endopeptidase (Zhang et al., 2012).

Bacillus subtilis (natto)

Bacillus subtilis, a Gram-positive, spore-forming, and an aerobic bacterium, has been used in the industry sectors to produce enzymes (Contesini et al., 2018; Su et al., 2020). Due to the excellent ability of secretion of numerous enzymes including proteases (Neutral proteinase, subtilisin serine protease, and metalloprotease) and various carbohydrases (α-Amylase, β-Glucanase, cellulase, and Xylanase), that assist the degradation of different types of substrates,

B. subtilis strains can survive in a variety of environments. The fermentation cycle of *B. subtilis* is around 48 h which is shorter than that of *Saccharomyces cerevisiae*, around 180h (Sahin et al., 2020; Webber et al., 2014). The *B. subtilis* showed extracellular protease productivity about 45% higher in SSF performed on soy cake in comparison to submerged fermentation (Soares et al., 2005). Furthermore, *B. subtills* and other species have also been reported to possess several abilities, such as antimicrobial (Shobharani et al., 2015), pathogen exclusion (Ripert et al., 2016), and food fermentation (Terlabie et al., 2006) abilities.

One of the important and widely used *B. subtilis* variant in food production is *Bacillus subtilis* (natto) which is a potential probiotic bacterium used as a starter in the production of the traditional Japanese fermented soybeans "natto" (Kamada et al., 2014; Nishito et al., 2010), and this variant of *B. subtills* is generally recognized as safe (GRAS) (Lampe and English, 2016; Su et al., 2020). *Bacillus subtilis* (natto) has been reported to produce high amounts of menaquinones-7 (MK-7), a subtype of vitamin K (Chatake et al., 2018), nattokinase or subtilisin NAT which is an extracellular alkaline protease (Ju et al., 2019; Lampe and English, 2016), and poly- γ -glutamic acid (γ-PGA), a biodegradable, and nontoxic biopolymer (Luo et al., 2016).

Bacillus subtilis WX-17 has been used to enhance nutritional values of Okara, a soybean industry byproduct, through solid-state fermentation. The fermentation improved the nutritional content of okara. The total amino acid and total fatty acids contents increased from 3.04 to 5.41 and 153.04 to 166.78, respectively after fermentation in contrast of unfermented samples. Moreover, the extract of fermented okara showed 6.4 times higher antioxidant content evaluated by DPPH free radical scavenging activity then before fermentation (Mok et al., 2019). In another study, Tomato processing waste mostly composing of seeds has been fermented with *B. subtilis*, and the protein hydrolysates of the fermented tomato waste fractionated and isolated. Several

bioactive peptides have been identified as ACE-I inhibitors and antioxidant peptides. Peptide with sequence (DGVVYY) had highest ACE-I effect having IC_{50} value of 2 µM, and the highest antioxidant activity exhibited by the peptide GQVPP which scavenged 97% of DPPH free radicals at 0.4mM (Moayedi et al., 2017).

Accordingly, *B. subtilis var.* natto, as probiotic agent, might play a potential role in enhancing protein extraction and producing bioactive peptides during SSF of HDRB due to its enzymatic excretion activity especially proteases and the remarkable amino acid sequence of HDRB proteins.

Fractionation and separation of protein hydrolysates and peptides

Many fermented substances and food sources contain abundant amount of proteins, peptides, and amino acids. Since these kinds of resources have important bioactivities, fractionation, isolation, and purification of interested proteins and peptides due to their functional role are valuable and required process (Hettiarachchy et al., 2011). Although, characterization of a peptide is a challenging task due to several peptides could related and have similar physicochemical properties, using advanced separation and identification techniques would considerably benefit in discovering novel bioactive peptides (Piovesana et al., 2018). Several separation and purification systems and techniques are widely available, and using these techniques depends on the purpose and the application that the final molecular or product has been rendered applicable.

After the extraction process of generated protein hydrolysates and peptides from fermented substance or food, the characterization step can be initiated by subjecting the extract to filtration process to fractionate the proteins, polypeptides, and peptides into several groups

based on the general molecular size which easily can be more fractionated and identified (Martínez-Maqueda et al., 2013).

Membrane separation system / Ultrafiltration

The complex pool of enzymatic hydrolysate of protein fractions and peptides requires a separation step of these fractions and peptides before performing evaluation of any activity. Furthermore, to maintain the properties and physicochemical characteristics of peptide and protein which depends on their structure and sequence, mild separation methods with high selectivity should apply, and membrane separation technologies have been ensured to be suitable for these kinds of process (Ambrosi et al., 2014; Sridhar et al., 2021).

In purification process, membrane systems included nanofiltration, ultrafiltration, and microfiltration have been used in laboratory or industrial scales (Boukil et al., 2018; Suwal et al., 2015).

Ultrafiltration is the commonly used technique in bioactive peptide fractionation and separation. Also, other macromolecules including proteins and polysaccharides, can be removed as well based on the molecular size of these molecules. In general, the principle of this technique is based on the retention and diffusion of peptides depending on the membrane pore size (diameter) and the hydrodynamic volume of peptides and proteins. This process is run by a pressure gradient provided by a pump (Bazinet and Firdaous, 2009). Thus, the separation of peptides occurs with certain molecular weight cut-off and in environmentally friendly and economical way which easily can be scaled up (Dullius et al., 2018; Sridhar et al., 2021). Cellulose derivatives and polysulfone are the main materials that the membranes are made of and offering a broad range of molecular size cut offs (1-500 KDa) (Boukil et al., 2018). As an example of membrane filtration method, ultrafiltration process was used to fractionate protein

hydrolysate from germinated soybeans and recover bioactive peptides. The protein hydrolysates were subjected to sequential filtration using ultrafiltration membranes with MWCO 5 and 10 KDa to obtain three fractions of peptides, larger than 10 kDa, between 5 kDa and 10 kDa, and less than 5 kDa fractions, (González-Montoya et al., 2018).

In another study, corn proteins, zein and gluten, were enzymatically hydrolyzed using commercial alcalase, and the protein hydrolysates were fractionated by passing through regenerated cellulose ultrafiltration membranes to recover and separate corn peptide fraction rich in hydrophobic amino acids and with specific molecular weight. Three low molecular weight cut-off membranes, 5,3,1 KDa, have been used to get peptide fractions with MW of (> 5 KDa, 5-3 KDa, 3-1KDa, and <1KDa), and the purities of peptides in the fractions were 92.07%, 93.76%, and 91.72% in the fractions <1 KDa, 5-3 KDa, and <5KDa, respectively. Furthermore, fraction <1 KDa showed the highest activity to activate hepatic alcohol dehydrogenase (HAD) in comparison to the other fractions and protein hydrolysates (Yu et al., 2013).

It has been reported that increase in the bioactivity is related with fractionation (Bazinet and Firdaous, 2009). Wang and his co-workers suggested that ultrafiltration membrane fractionation improved the antioxidant activity of a peptide fraction, and the activity is associated with peptides' molecular weight. In this study, enzymatic hydrolysis of corn protein was followed with the fractionation of protein hydrolysates through ultrafiltration membranes (MWCO 50, 20, 10, and 6 KDa), and the smallest peptide fraction (<6KDa) exhibited the highest scavenging activity toward free radicals (Wang et al., 2014). The comparative characteristics of membrane filtration types are listed in (**Table 2.5**) (Pouliot et al., 2006). A scheme of fractionation cascades and recovery of peptides and proteins using Ultrafiltration is presented in (**Figure 2.2**).

Table 2.5. Comparative characteristics of membrane filtration types

Specification	Reverse Osmosis (RO)	Nanofiltration (NF)	Ultrafiltration (UF)	Microfiltration (MF)
Pore size	< 0.1 nm	0.1-1 nm	1-500 nm	> 0.1 μm
Separation products	Monovalent ions (K, Cl)	Amino acids, salts, solutes	Proteins/peptides (1-300Kda)	Particles, globules (>01μm)
Sieving mechanism	Size	Size	Size	Size
Operating pressure	30-50 bars	15-30 bars	1-10 bars	0.1-2 bars

Adapted from Pouliot et al, 2006.

Characterization and purification of bioactive peptides

Analytical and preparative liquid chromatography techniques are the most used methods in the separations of bioactive peptides produced from proteins of fermented substances, enzymatically hydrolyzed protein, and foods. Chromatographic methods can separate peptides with high purity and quality (Dullius et al., 2018), and the separation of peptides is based on the interaction between the peptide and the mobile and stationary phases (Coşkun, 2016).

The commonly used techniques for purifying proteins and peptides from a pool of complex compounds are size exclusion chromatography, Ion-exchange chromatography, affinity chromatography, and hydrophobic interaction chromatography (Coşkun, 2016). The above-mentioned methods are the initial separation steps for isolating peptides of interest from the fraction that shows the bioactivity. Then, by using reverse phase High Pressure Liquid Chromatography (RP-HPLC) technique, the required peptide can efficiently be purified (Kannan et al., 2010). Besides, other methods might be used for structural characterization of peptides and proteins after chromatographic purification, such as SDS-electrophoresis, amino acid analysis, and mass spectrometry.

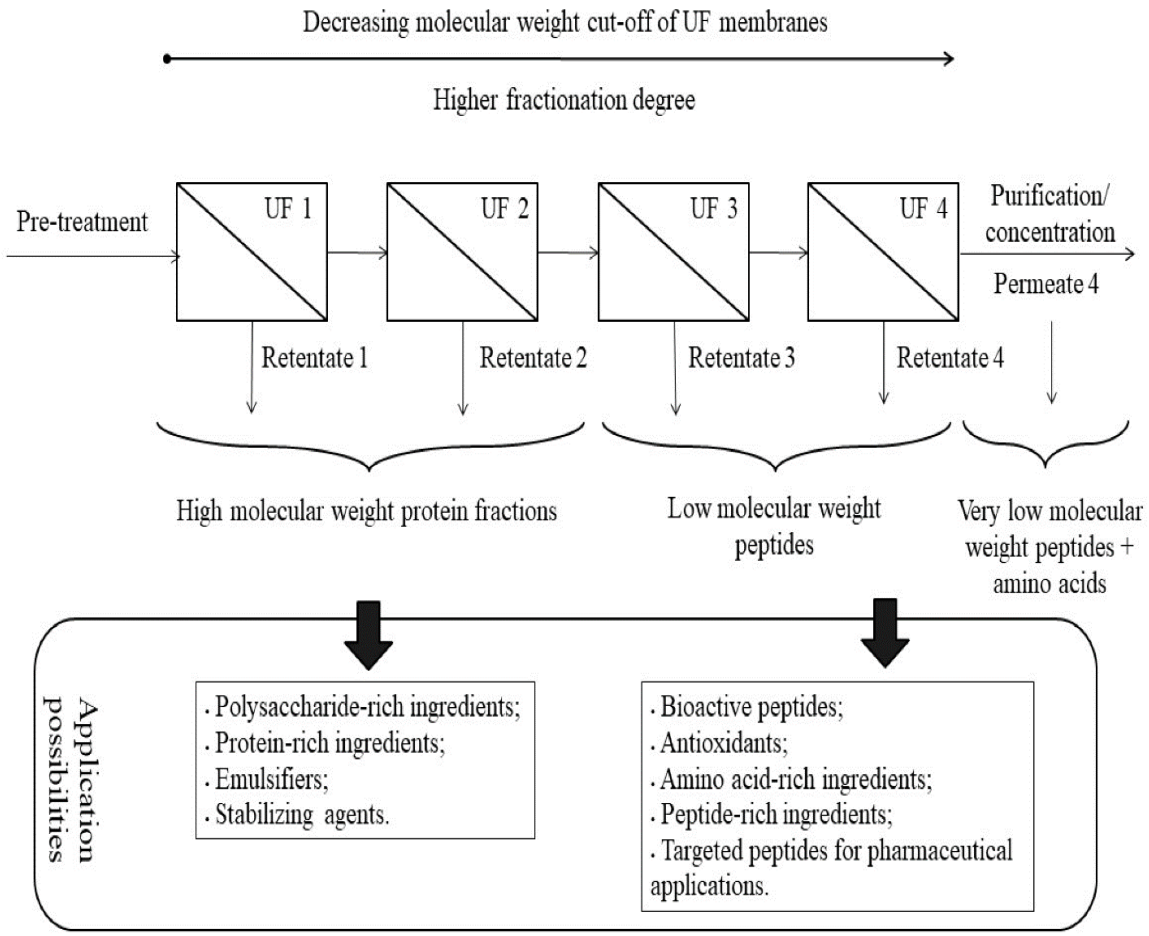

Figure 2.2. Fractionation cascades and recovery of peptides and proteins using Ultrafiltration (UF) membranes with molecular weight cut-off 500 – 1 KDa (Adapted from (Vollet Marson et al., 2021).

Ion-exchange chromatography

Molecules and peptides separation are based on their surface charge which is opposite to that of the anionic and cationic resin exchangers as stationary phase of the column. Two types of interactions, the induced electrostatic and the intrinsic charge, between the molecule and stationary phase control the retention of the peptide or molecules in the column. Isoelectric point (IP) of the peptides and proteins play an important role in the peptide retention on the matrix. Ion exchange chromatography has been used to effectively purify several peptides and proteins.

Peptides having antihypertensive activity have been isolated from peas (Jakubczyk et al., 2013) and lentil globulin protein (Jakubczyk and Baraniak, 2014). Ion-exchange column packed with DEAE cellulose resin has been used to fractionate the globulin protein hydrolysates of peas and lentil. Gel filtration SephadexG-10 column was used for more separation process of peptides.

Size exclusion chromatography (SEC)

Size exclusion chromatographic is also called a gel filtration technique and has fractionation capacity of molecules with molecular size greater than 1 KDa based on the specific pore size of the gel matrix. This separation technique is regularly used for separation of proteins and bioactive peptides based on the molecular size range from protein hydrolysates. Gel filtration technique is characterized with simplicity, great separation, and precise elution time (Mora et al., 2014). Several protein hydrolysates of different protein sources have been fractionated using size exclusion chromatography toward getting purified peptides, such as hydrolysates of canola meal (Wu et al., 2009), rapeseed (Yu et al., 2013), and corn gluten meal (Tang and Zhuang, 2014). Sephadex G-15 gel matrix has been used to purify bioactive peptides from protein hydrolysates fraction of high oleic acid soybean protein (Rayaprolu et al., 2017). In general, many studies have concluded that fractions with small size peptides (<3kDa) exhibit higher biological activities than the fractions contain large size of peptides.

Purification of bioactive peptides by RP-HPLC

Reverse phase high performance liquid chromatography (RP-HPLC) is the most applied chromatographic technique to identify and purify bioactive peptides. Purification by this method depends on the hydrophobicity of peptides by which the peptides differentially interact with the column material. A C_{18} reverse phase column is used to separate peptides generated from cleaving high molecular weight proteins by enzymatic digestion or chemical treatment.

The stationary phase of RP-HPLC columns is non-polar matrix, so the peptides or the analytes are retained due to the interactions with the stationary phase. The elution of retained peptides occurs when the polarity of the mobile phase becomes sufficient to release the peptide from the stationary phase. Smaller peptides are preferred to be separated using column packed with particles having pore sizes (60-100A), while peptides with MW greater than (4 KDa) are analyzed using silica particles with pore diameter of (300A). Using other types of columns including C4 and C8, is useful and related with the physicochemical characteristics of peptides (Chen et al., 2013).

Characterization and proteomic tools

Several advanced tools aid to simplify characterization protocols and make them more reliable and faster because these tools can precisely and quickly identify and detect the structural components of pure and complex mixtures. Also, the combination between chromatographic separations and spectrometers in tandem MS dramatically enhanced the capabilities to separate molecules and determine the mass at the same time. Accordingly, these features decreased analysis time and increased the sensitivity. The most popular technique available for characterization studies especially with proteins is the mass spectrometer including the accessory coupling instruments.

Mass spectrometry (MS)

The best and crucial method or tool for the protein and peptides characterization and identification is mass spectrometry which improved greatly the field of peptide and protein sequence identification and discovering their profiles. The principle of MS consists of two steps. First, the chemical compound is ionized to produce charged fragments or molecules. Then, the ratio of mass-to-charge of these molecules is measured. The development in the ionization

technology assisted in the vaporization/ionization step to generate ions from temperature-sensitive and not volatile molecules. Furthermore, other techniques in mass spectrometry, such as matrix assisted laser desorption/ionization (MALDI-TOF) and Electrospray ionization (ESI), have played a very useful role in peptide sequence identification. The compatibility between ESI/MS (electrospray ionization mass spectrometry) and tandem mass spectrometry and liquid chromatography (LC) has increased the sensitivity of the analysis and the ability to analyze complex mixtures. The direct interface between ESI and LC has added the capability of transforming of samples having similar electric density in aqueous solution into gaseous phase ions by applying a very high voltage through a thin capillary tube to the sample. Then ions carrying one or more charges are produced, this step added more accuracy in the measurement of MW of peptides (Jakubczyk and Baraniak, 2014; Nefedov et al., 2011).

Another technique in proteomics is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) which is important due to its high sensitivity, very accurate mass detection, rapid analysis for the sample, and analyzing heterogeneous samples and with wide range of masses, such as proteolytic digests. By connecting MALDI with TOF/MS, the method provides the results of mass/charge ratio (*m*/z) mapping after measuring the ion's flight time toward the detector. (Nefedov et al., 2011; Zhang et al., 2010). In addition, tandem mass spectrometry (MS-MS) is very useful in the production of structural information related to a compound. The sample ions are fragmented inside the MS before identifying the produced fragment ions. The structural information of the intact molecule is regenerated by combining the information obtained from the fragmentation. Further, by tandem mass spectrometry, it is possible to identify and detect specific compounds that exist in complex mixtures based on their characteristic and specific fragmentation patterns (MacMahon et al., 2012).

After protonating the molecules, they fragment alongside of the peptide backbone. The three types of bonds (NH-CH**,** CH-CO, and CO-NH) are fragmented along the amino acid backbone. CO-NH bonds are the common sites of cleavage. After breaking the bond, two species emerge, the charged and the neutral segment, and the mass spectrometer can monitor only the charged part. Thus, the suggestion of a specific amino acid on the sequence depends on the difference in the mass between two adjacent ions (y ions or b ions). The identification of peptide or protein is achieved by using the sequence data obtained from the analysis by tandem MS/MS and this sequencing is called *de novo* (Cotte-Rodriguez et al., 2011).

Furthermore, antioxidative peptides have been purified and identified from rice protein hydrolysates. The protein hydrolysates were fractionated using ultrafiltration membranes (MWCO 10 and 3 KDa) followed by further separation into four fractions using size exclusion chromatography with Sephadex G15 column. The fractions with the highest antioxidant activity were subjected to FPLC separation on a reversed phase column (SOURCE 15 RPC). Nano-UPLC/MS/MS occupied with C8 column which was coupled to electronic spray ion quadrupole time of flight analysis system (ESI-Q-TOF) has been used to identify the amino acid sequence of peptides that exhibited the highest antioxidant activities. These identified peptides were: Thr-Ser-Gln-Leu-Leu-Ser-Asp-Gln, Asn-Phe-His-Pro-Gln, Arg-Pro-Asn-Tyr-Thr-Asp-Ala, and Thr-Arg-Thr-Gly-Asp-Pro-Phe-Phe (Yan et al., 2015).

Skin and Skin disorders

Skin is an important organ in the human body covering all body surface and playing a role in the protection of the human body by becoming as a barrier between the external environment and the body. It protects the body from infection, microorganisms, mechanical trauma, and UV radiation. Also, skin regulates temperature, synthesis of vitamin D, and

sensation of pain, pressure, and cold / heat (Duval et al., 2014). The main structure of skin has been divided into three tissue layers: epidermis, dermis, and subcutaneous. The outer layer represents the epidermis which is subdivided into five sublayers: stratum basal, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum, from bottom to the top. This layer of skin contains different specialized cell types, such as Langerhans cells, Merkel cells, melanocytes, and keratinocytes which is the dominant cell type (95%). The stratum basal sublayer generates and proliferates the Keratinocytes that gradually move toward the surface layer. Melanocytes is one of the important cell types in epidermis because it secrets melanin pigment which protects the skin from ultraviolet (UV) radiation. Melanocytes are located also in stratum basal layer. The cells that are responsible for the immune system in the skin are Langerhans cells while Merkel cells represent the sensory receptor cells (**Figure 2.3**).

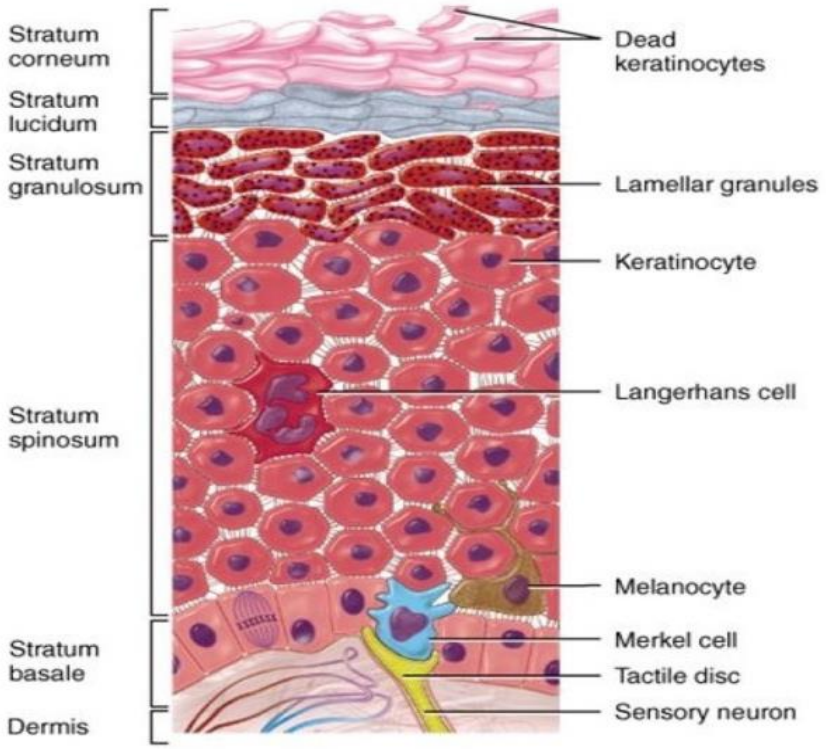

Figure 2.3: Structure of skin epidermis (Adapted from Human Anatomy Library)

The second layer of the skin is dermis which consists of two sublayers: the papillary region (superficial area), and reticular region which is thicker and deeper area. The main structure of dermis comprises from elastin and collagen fibers, in addition to Fibroblast cells. Other cellular components, such as sweat and sebaceous glands, hair follicles, and blood and lymph vessels, are in dermis. The last layer of skin is the subcutaneous tissue or hypodermis which is mainly a loose connective tissue and fat cells in addition of the blood vessels and nerves.

Skin Pigmentation

Melanocyte and its characteristics

Melanocytes are found not only on the skin, the basal layer of the epidermis, but also other body parts, such as the uvea of the eyes, nervous system, heart, and inner ear, in epidermis, several keratinocytes (about 30-40) connect with an associated melanocyte to form the "epidermal melanin unit" which is responsible from production and distribution of melanin in the skin. The ratio between melanocytes and keratinocytes in the epidermis is about 1:10, and with ageing, melanocytes become larger and have more dendritic while the tyrosinase activity starts decreasing (Hedley et al., 2002). Melanin is not only produced in the melanocytes in the skin, but there are other cells that have the capability to synthesize melanin, such as pigmented epithelium of retina, iris and ciliary body of the eye, adipocytes, and in neurons (Tsuchiyama et al., 2013).

Melanogenesis: Pathway and Inhibition

Melanogenesis and related enzymes

Human skin and hair color are caused by the pigment melanin which is derived from epidermis of the skin. Melanin produced through an activity process called melanogenesis (Solano et al., 2006). The structure of melanin as heterogeneous polymer helps to absorb

ultraviolet (UV) radiation before transforming the absorbed energy to heat by processing internal conversions and keeping the harm of UV at minimum level (Park et al., 2009; Slominski et al., 2015). Series of chemical and enzymatic reactions are involved in the melanogenesis pathway (**Figure 2.4**). The main enzymes that catalyze this pathway's reactions and transform tyrosine to melanin are: tyrosinase, dopachrome tautomerase (DCT or TRP-2), and tyrosine-related protein-1 (TRP-1).

Melanogenesis takes place in melanosomes which are the cytoplasmic organelles in melanocyte. This process as shown in (Figure 4) starts with a reaction catalyzed by tyrosinase, the rate limiting enzyme, when tyrosine as an amino acid is hydroxylated by tyrosine to form L-3,4-dihydroxyphenylalanine (DOPA) which goes through rapid oxidation by tyrosinase to form DOPA-quinone which in turn becomes a substrate for the synthesis of eumelanin or pheomelanin (Hida et al., 2020). In pheomelanin formation, presence of cysteine and glutathione leads DOPA-quinone to form cysteinyl-DOPA which is oxidized and has sequential polymerization to form pheomelanin, the yellow-red melanin type. However, when the cysteine and glutathione are nonexistent, DOPA-quinone is cyclized to DOPA-chrome through undergoing amino group addition reaction to generate cyclodopa (leukodopachrome). Then, leukodopachrome transfers to 5,6-dihydroxyindole (DHI), and DHI-melanin, the dark-brown-black melanin, is produced from the oxidation of DHI. Tyrosinase related protein-2 (TRP-2) catalyzes this process. DOPA-chrome also converted to DHI-2-carboxylic acid (DHICA), and this rection is catalyzed by DOPA-chrome tautomerase (DCT). The DHICA forms lighter brown melanin through oxidation with TYRP1. These dihydroxy-indole compounds, DHI and DHICA, copolymerize to form dark brown/black eumelanin. Eumelanin or melanin pigments is responsible for developing the diversity in skin color. Furthermore, eumelanin plays an essential role in photoprotection and has

higher resistance to degradation in comparison to pheomelanin (Hida et al., 2020; Tsuchiyama et al., 2013).

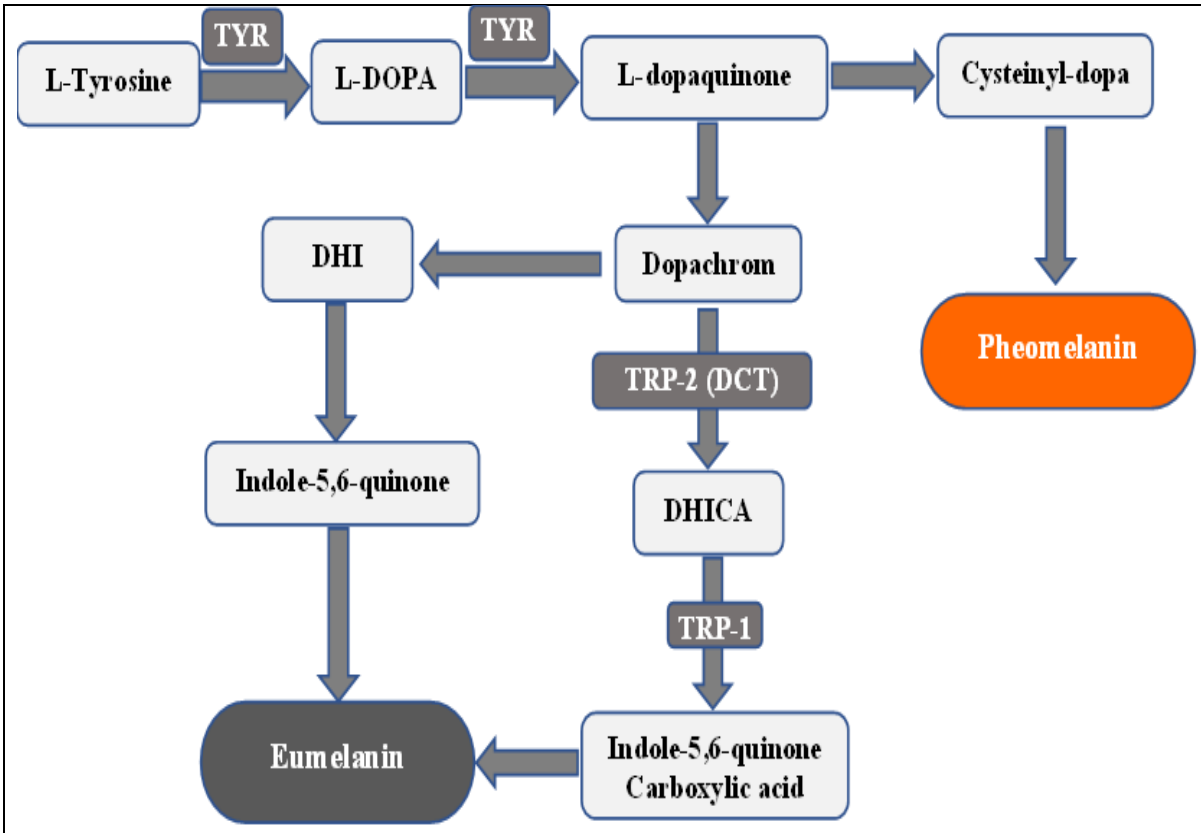

Figure 2.4. Pathway of melanin synthesis in melanocytes. TYR: Tyrosinase, TRP-1 & 2: Tyrosinase related protein-1 & 2, DHI: 5,6-dihydroxyindole, DHICA: DHI-2-carboxylic acid.

Inhibition of Melanogenesis:

The frequent exposure of human skin to environmental agents that cause DNA damage especially ultraviolet (UV) radiation (Mancebo and Wang, 2014) needs various endogenous systems of protection (Natarajan et al., 2014). Skin pigmentation on epidermis by melanin deposition to the upper layers are induced by UV radiation to form a physical barrier which scatters, absorbs, and limits the penetration of UV radiation. Even though, melanin pigmentation is crucial for human skin health (Park et al., 2009), the excess in production and accumulation of

melanin can cause many skin diseases and dermatological disorders including melanoderma and freckles, solar lentigo and age spots (Lynde et al., 2006).

Therefore, melanogenesis inhibitors have gained extensive interesting and demand in research and cosmetic markets. Many well-known commercial whitening agents, such as hydroquinone (HQ), arbutin, kojic acid, azelaic acid, niacinamide, and magnesium ascorbyl-phosphate, are used widely in cosmetic products (Kamakshi, 2012; Zhu and Gao, 2008). The main target of the whitening agents is the key enzyme of melanogenesis pathway, tyrosinase.

Tyrosinase (EC 1.14.18.1) is a monophenol oxygenase and found across a huge range of bacterial and fungal species and animals (Solomon et al., 2001). There is a great interest to tyrosinase in life science research, drug discovery, and food industry and cosmetics industry because the important role that tyrosinase plays in the biosynthesis of melanin. The enzyme is a membrane-bound and copper-containing glycoprotein. Tyrosinase is in the melanosome's cell membrane and consists of three domains: a cytoplasmic domain, a short transmembrane domain, and an inner domain where the catalytic center is located (Ando et al., 2007). A great attention is focused on the screening and development of tyrosinase inhibitors related with melanoma and hyperpigmentation disorders. Consequently, tyrosinase inhibition is anticipated to provide valuable therapeutic strategies for the cure of different skin disorders.

Melanogenesis inhibition by peptides

Many tyrosinase inhibitors are recognized today and used in different food and cosmetic products. However, there is increasing restrictions in the usage of these inhibitors due to their high cytotoxicity and low stability for water and oxygen, limiting the application of these kinds of tyrosinase inhibitors (Schurink et al., 2007). In cosmetic and food products, the safety of inhibitors is the primary consideration. Therefore, it has been very important to obtain tyrosinase

inhibitors from agricultural by product including meals and cakes, and other natural sources since they are safe and almost free from undesirable side effects. Protein hydrolysates and peptides extracted from natural sources have generated interest in the inhibition of tyrosinase. Several peptides with tyrosinase inhibition activity have been recognized, and these peptides have been reported to be safe and without inherent toxicity (Girelli et al., 2004; Hsiao et al., 2014; Schurink et al., 2007).

In studies where peptide sequences have been applied for TRY inhibition using computational approach an octapeptide (RANSRADC) P3, and another decapeptide P4 (YRSRKYSSWY) were identified with tyrosinase inhibitory effect. They showed mushroom tyrosinase inhibition with IC_{50} value of 123 and 40 µM, respectively in contrast with hydroquinone (680 µM). Furthermore, cytotoxicity effect of the oligopeptides on melanocyte at concentration of (100 µM) was not detected (Abu Ubeid et al., 2009).

A designed tripeptide (CRY) showed effective tyrosinase inhibition with IC50 value of (6.16 µM) which was stronger than arbutin, a positive control, having IC50 value of (1008.7 µM) (Hsiao et al., 2014). Hsiao et al. also reported other tripeptides (RCY, FFY, and RWY) exhibited anti-tyrosinase activity, but they have higher IC50 (>200 µM). The higher activities that CRY and RCY showed are due to the existence of the cysteine residue having thiol group coordinating with copper (Cu) ions in tyrosinase's active site (Hsiao et al., 2014).

In a study, three anti-tyrosinase oligopeptides have been synthetically designed and reported to be safe and not toxic. The peptides were numbered 35, 36, and 37 with amino acid sequences of (RRWRRYY), (RRRYRYYR), and (RRYWYWRR), respectively, and their inhibitory activity using L-DOPA as substrate showed dose dependent response. In comparison to hydroquinone (HQ), all the peptides were more effective as inhibitors of tyrosinase in human

skin cells. Peptide 35, 36, and 37 inhibited the activity of human tyrosinase by 30, 12, and 26% respectively, at peptide concentration of (200 μM) while only 5% inhibition was observed by HQ (Ubeid et al., 2012). A strong inhibitor of mushroom tyrosinase, TH10 (MRSRERSSWY), has been synthesized after it was identified from the genomic translation search of rice DNA database. The IC50 value of the peptide TH10 was 102 μM. This peptide is homologous to peptide P4 (YRSRKYSSWY) that was designed in previous study and had IC50 = 123 μM with Tyrosine as a substrate of tyrosinase (Abu Ubeid et al., 2009; Ochiai et al., 2016a). Both peptides contain tyrosine (Y) in their sequence on C-terminal, and peptide P4 contains two more tyrosine residues, in the middle and N-terminal of the peptide. Simulation analysis including sequence-shuffled variants of peptide P4 and docking simulation of peptide TH10 variants showed that the inhibitory activity of the peptide P4 does not related with the tyrosine residues at the center and N-terminal position of the peptide sequence, while the C-terminal tyrosine residue on both peptides plays very important role in the inhibition activity of tyrosinase through coordinating with copper ions of the tyrosinase enzyme and acting as a substrate analogue at the active site of tyrosinase (Ochiai et al., 2016a). The majority of peptides with tyrosinase inhibitory activity contains tyrosine residue at C-terminus location of the peptide (Ochiai et al., 2016b).

However, there are limited studies related to natural bioactive peptides with tyrosinase inhibition activity. In a study, rice bran protein was enzymatically digested using chymotrypsin and trypsin simultaneously to generate peptides. Then, six bioactive peptides designated CT-1-6 having anti-tyrosinase activity have been isolated from rice bran protein hydrolysates. Amino acid sequencing of the purified peptides revealed that three of the six peptides had tyrosine residue at the C-terminus side of the peptides (CT-1: HGGEGGRPY, CT-2: LQPSHY, and -3: HPTSEVY), and they showed substantial tyrosinase inhibition activity in monophenolase,

tyrosine substrate, reaction but not diphenolase, L-DOPA substrate, reaction. Additionally, Peptide CT-2 exhibited important inhibition of melanogenesis in melanoma cells (>50% at 500 μM) without any observed cytotoxicity toward the cells (Ochiai et al., 2016b). According to the data, Ochiai and his coworkers (2016b) stated that rice bran protein hydrolysates are a good source of bioactive peptides with tyrosinase inhibitory activity.

Recently, rice bran protein fractions, albumin, globulin, glutelin, and prolamin were extracted from rice bran and hydrolyzed using papain. Albumin hydrolysates demonstrated higher tyrosinase inhibitory activity in comparison to other fractions. Furthermore, after fractionation of rice bran albumin hydrolysates using reverse phase high performance liquid chromatography (RP-HPLC) into 11 fractions, and the fraction's eluting time negatively correlated with in the tyrosinase inhibitory activity. Hence, fraction 1 exhibited the highest tyrosinase inhibition activity with IC50 value of (1.31 mg/mL), and it contained 13 peptides with MW ranged from 1.3 to 4.8 KDa and the peptide with amino acid sequence (SSEYYGGEGSSSEQGYYGEG) had the most reported residues and features which were related with tyrosinase inhibition and chelating Cu ions activities (Kubglomsong et al., 2018).

Although, fermented broth ingredients, such as, polyphenolic compounds and flavonoids, aglycone isoflavones, 8-Hydroxydaidzein and, 3-hydroxydaidzein, exhibited anti-melanogenesis and anti-tyrosinase activities (Chan et al., 2014), there is no data in the literature related to evaluation of protein fractions and peptides obtained from fermentation broth of rice bran or other fermented cereal brans and grains toward inhibition of tyrosinase and melanogenesis.

Melanogenesis and skin-cancer (Melanoma)

The most dangerous type of skin cancer is melanoma which progresses from melanocytes and occurs especially in the body areas exposed to direct sunlight. Prolonged exposure to the UV

radiation leads to melanocytes' DNA damages which is the major reason of developing melanoma. Malignancy in general is related with deficit in many specific cellular functions. However, in melanoma, the expression of melanogenic enzymes including tyrosinase, is increased which triggers higher rates of melanogenesis and melanin production, and this up regulation in melanogenesis pathway is linked with malfunctioning of melanosomes (Borovanský et al., 1991). Although, the accumulation of the melanin in the malignant cells are considered as an essential factor in determining of the efficiency of therapy, melanin building up in malignant melanoma cells can cause different negative effects. Initially, presence of melanin can cause reduction in the efficacy of chemotherapy by its binding ability to various cancer medicines, such as cytostatic drugs. In addition, the accumulated melanin can result in hypoxia status and in turn decrease the effectiveness of radiotherapy (Riley, 2003). Accordingly, inhibition of melanogenesis activity in malignant melanoma cells might support and increase the efficiency of cancerous cell therapies. Furthermore, melanoma cells have been used as a model for evaluation of melanogenesis inhibitory activity of several compound. The cytotoxicity of the inhibitors also can be tested at the same time by monitoring the cell proliferation and viability of the cells after the treatment (Couto et al., 2019). In this study, B16F10 mouse melanoma cells were used to evaluate the melanogenesis inhibitory activity, the cytotoxicity, and cell proliferation effects of the purified peptides from water-soluble protein fractions and peptides extract of SSF-HDRB.

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CHAPTER 3

Optimization of the growth conditions of *Bacillus subtilis* (natto) Takahashi in solid state fermentation (SSF) utilizing heat treated defatted rice bran (HDRB) as the medium and extraction of proteins and peptides.

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Abstract

Heat-stabilized defatted rice bran (HDRB) is an abundant, underutilized, and inexpensive agro-industrial by-product of rice milling and rice oil extraction process in Arkansas. *Bacillus subtilis* (natto) Takahashi is a probiotic that is commonly used to prepare the traditional fermented soybean food, natto, and recently it has been introduced into other pharmaceutical and industrial applications. The current study was aimed to evaluate HDRB as growth media for *B. subtilis* (natto) Takahashi in solid-state fermentation (SSF). Followed with the extraction of protein and protein hydrolysates from heat-stabilized defatted rice bran using *B. subtilis* (natto) Takahashi in solid-state fermentation. The composition of HDRB was 11.2 ±0.4% moisture, 16.4 ±0.5% total protein, 2.1 ±0.3% crude lipid, 55.4 ±1.1% total carbohydrates, 33.2 ±0.9% total dietary fiber, and 14.9 ±0.6% ash. In SSF of HDRB, the population of *B. subtilis* (natto) Takahashi increased from an initial inoculated size of 6.1 ±0.2 Log CFU/g HDRB to 10.1 ±0.4 Log CFU/g HDRB within 36h incubation at 35% initial water content and 37°C. The maximum protease activity that achieved was 93.6 ±1.0 U/g HDRB.

The Response Surface Methodology (RSM) was used to optimize the effect of three parameters, *Bacillus subtilis* (natto) Takahashi inoculum log (5-7 Log CFU/g HDRB), initial water content (25-45 % w/v), and fermentation time (24-72h), as independent variables on the extraction of the maximum water-soluble proteins and peptides (WSPP) from heat stabilized

defatted rice bran (HDRB). The optimum conditions were achieved by fitting the second-order polynomial equation. The result showed that all the factors, initial water content, inoculum log, and fermentation time, were critical in extracting WSPP from HDRB. The predicted optimum conditions for highest amount of WSPP extracted were 40.96% w/v water content, 6.27 Log CFU/g HDRB inoculum log, and 61.01h fermentation time. Under these optimized conditions of solid-state fermentation (SSF), 64.6 ±0.7% of total protein in HDRB was extracted as WSPP which was closer to the predicted value of 63.3%. While the WSPP extracted from nonfermented HDRB was 20.6%. The majority of the WSPP from SSF-HDRB had MW <5KDa.

This study showed that bacterial SSF using the probiotic *B. subtilis* (natto) Takahashi is an efficient method to add value to HDRB as inexpensive growth media to produce nutritional and beneficial chemical compounds including protein fractions and peptides. Furthermore, the extracted proteins and hydrolysates from SSF-HDRB and can find application as an ingredient in suitable products.

Introduction

Rice (*Oryza sativa*) is one of the main food sources in human diet. Globally, over 100 countries are growing rice including USA which produced 11,56 million metric tons in 2020, and Arkansas's production for 2020 was about 5.49 million metric tons (USDA, 2021) and ranks #1 in rice production in the US. The rice bran, a co-product derived from dry milling of rough rice, mostly contain the germ and outer layers, and it comprises approximately 10% of the rough rice. Rice bran is mostly underutilized and used as a livestock feed supplement and as a source of natural healthy oil in food industry. To keep the quality of the oil, rice bran is heat stabilized to inactivate the hydrolytic enzymes. The defatted rice bran is termed heat-stabilized defatted rice bran (HDRB). Defatted rice bran is a rich source of protein, fiber, vitamins, minerals, and

nutraceutical compounds (Devi and Devi, 2021). It was reported that HDRB contains about 15% protein, 13.5% reducing sugars, 58.5% carbohydrates, 2% soluble fiber, 14.5% ash, and 8.5% moisture (Sirikul et al., 2009). It was reported that HDRB has been used successfully as a growth substrate in both types of fermentation, submerged and solid-state, to grow different microorganisms for enhancing the nutritional qualities of HDRB, producing enzymes, or liberating phytochemical compounds (Geetha et al., 2015; Webber et al., 2014). Specifically, solid-state fermentation has been adopted using various starter cultures, such as *Saccharomyces boulardii*, (Ryan et al., 2011), *Aspergillus sp.* or *Rhizopus sp.* (filamentous fungi), (Ranjan et al., 2019; Ritthibut et al., 2021) to develop antioxidant or preservative compounds, or to enhance bioactive properties. Lactic acid bacteria (LAB), in the other hand, is the most investigated bacteria in rice bran fermentation to enhance the viability of LAB, extraction of phytochemical, or producing lactic acid or other useful compounds (Le et al., 2019; Wang et al., 2015).

In general, the growth of fungi in SSF requires longer incubation time, between 5-7 days in comparison to bacterial growth that can develop successfully in 48 -72 hours (Matkawala et al., 2021). Although, HDRB, as a culture substrate, can support growth of fungi without additional nutrients, the growth of LAB on HDRB is difficult and requires addition of complex nutrients, such as fatty acids, vitamins, amino acids, and nucleic acids (Mienda et al., 2011; Pandey, 2003). *Bacillus spp.* strains have been grown under solid-state fermentation conditions on different agricultural byproducts including rice bran to produce proteases, phytase, or xylanase (Su et al., 2020).

Bacillus subtilis, a Gram-positive, spore-forming, and an aerobic bacterium, has been used in the industry sectors to produce enzymes (Contesini et al., 2018; Su et al., 2020). Due to the excellent ability of secretion of numerous enzymes that assist the degradation of different

types of substrates, *B. subtilis* strains can survive in a variety of environments. The fermentation cycle of *B. subtilis* is around 48 h which is shorter than that of *Saccharomyces cerevisiae*, around 180h (Sahin et al., 2020; Webber et al., 2014). One of the important and widely used *B. subtilis* variant in food production is *Bacillus subtilis* (natto) which is a potential probiotic bacterium used as a starter in the production of the traditional Japanese fermented soybeans "natto" (Kamada et al., 2014; Nishito et al., 2010), and this variant of *B. subtilis* is generally recognized as safe (GRAS) (Su et al., 2020).

Heat-Stabilized defatted rice bran effectively was used as an alternative growth media for *S. cerevisiae* and supported *Lactobacillus acidophilus* growth (Webber et al., 2014; Webber et al., 2014), however, HDRB has not been evaluated as a growth media of *B. subtilis* (natto) for secretion of proteases and enhancing the extraction of protein and peptides from HDRB without utilizing any additional supplements to support the growth of *B. subtilis* (natto).

The high cost of using pure enzymes and chemicals in enzymatic extraction of protein is a main drawback of this technique. While extraction of protein in severe alkaline conditions leads to damage of cysteine and lysine, causing racemization of amino acids, and reduced protein quality and digestibility (Schwass and Finley, 1984) in addition to overuse of chemicals. Hence, finding a simple and cost-effective process to extract protein from HDRB is the focus in our study.

The *B. subtilis* showed extracellular protease productivity about 45% higher in SSF performed on soy cake in comparison to submerged fermentation (Soares et al., 2005). Rice bran successfully has been used as a substrate to grow *Saccharomyces spp*. (Hettiarachchy, 2015), and it supported the production of proteolytic enzymes in SSF condition (Rukmi and Purwantisari, 2020).

Solid-state fermentation (SSF) using *B. subtilis* (natto) Takahashi can be an efficient method to extract proteins from HDRB because this microorganism is able to produce variety of enzymes that can degrade cell wall components and proteins and liberate proteins and peptides in a single economically viable process. Therefore, the objectives of the present chapter were to determine the percentage of the macronutrient's composition of HDRB including lipid, total carbohydrates, crude protein, ash, and moisture content. Investigate the growth of *B. subtilis* (natto) Takahashi on HDRB without supplementing the culture media with additional nutrients via solid-state fermentation at different fermentation times, initial inoculum levels, and evaluate proteases production by Azocasein method (Ginther 1979). Finally, demonstrate microbial assisted extraction of protein and protein hydrolysates from heat-stabilized defatted rice bran using *B. subtilis* (natto) Takahashi in solid-state fermentation.

Materials and Methods

Materials

Heat-stabilized defatted rice bran (HDRB) from Arkansas rice varieties was supplied by Riceland Foods, Inc. (Stuttgart, AR, U.S.A.) that was subjected to heat stabilization at (~120 ℃) through steam expander prior to the oil extraction. *B. subtilis* (natto) Takahashi was purchased as a natto starter from Yuzo Takahashi laboratory Co., (Yamagata, Japan). Nutrient agar and nutrient broth medias for preparing *B. subtilis* (natto) Takahashi were purchased from Difco Laboratories (Detroit, MI, USA). Polyvinylpyrrolidone and azocasein were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other analytical-grade chemicals and supplies were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Sigma Chemical Co. (St. Louis, MO, USA).

Methods

Preparation of HDRB

Heat-stabilized defatted rice bran was ground using an IKA M20 universal mill (IKA Works Inc. Wilmington, NC, USA) and passed through 80 mesh (177um) sieve to obtain uniform size particles and increase the surface area of HDRB for bacterial action.

Preparation of *B. subtilis* (natto) Takahashi culture

Spore suspension of *B. subtilis* (natto) Takahashi was cultured first on nutrient agar (gelatin peptone, beef extract, and agar) to select a single cell colony from which the stock culture was prepared in nutrient broth (gelatin peptone, beef extract) and cryopreserved with 25% glycerol as final concentration in -80 °C.

To prepare an active culture, the *B. subtilis* (natto) Takahashi was inoculated into nutrient broth (10 mL) after removing from frozen storage and thawing it in water bath at 37°C. Then, the cultured media was incubated at 37°C in a shaking incubator (Lab Line Inc., Fullerton, CA) at 200 rpm for 24 h. The final inoculum was standardized by inoculating 100 µL of 24h growth culture into 100 mL of nutrient broth before incubation for 10h at the same growth condition mentioned above. Then, the culture was centrifuged (3,500xg for 15 min) to harvest *B. subtilis* (natto) Takahashi cells as a pellet. Finally, to remove the residual growth culture, the bacterial pellet was washed twice with 0.85% saline and harvested before suspending in saline to be used as inoculum in fermentation process.

Determination of proximal composition of HDRB

Moisture content

To determine the moisture content of HDRB, oven-drying method (AACC Method 44-15.02) (AACC, 2000) was used. HDRB sample (2 g, in triplicate) in aluminum pan was dried at

105 °C for 4 hr. Then the samples were transferred to a desiccator for cooling and weighed. The same steps were repeated till the samples reached a constant weight. The moisture percentage was calculated as the ratio between the change of sample weight (moisture lost) after and before drying and the initial sample weight using the following equation:

$$\text{Moisture Content (\%)} = \frac{W2 - W3}{W2 - W1} * 100$$

where, *W1*: weight of container; *W2*: weight of container with sample (before drying); and *W3*: weight of container with sample (after drying).

Total protein content

The total protein content of HDRB flour was determined by the Kjeldahl method (AACC method 46-12.01) (AACC, 2000). In digestion tubes, the HDRB flour samples, in triplicate, were weighed (0.5g) and 5 mL of concentrated sulfuric acid and a Kjeldahl tablet were added before digestion for 1.0 h at 420 °C. Then the digested samples in duplicate were distilled using micro Kjeldahl distillation system (RapidStill-I, Labconco, Kansas, MO, USA) before they were titrated with 0.025N HCl. The total protein content as a percentage was calculated using 5.95 as a nitrogen conversion factor. The final percentage value is stated as a mean of three determinations. The following formula was used to calculate the total protein content:

$$\text{Total Protein Content (\%)} = \left(\frac{HCl\ Vol.\ (L) * 0.025N * 14.007}{\text{Sample Weight (g)}} * 100\right) * 5.95$$

Crude oil content

The crude oil content in HDRB meal was determined using Soxhlet extractor method (AACC method 30-25.01) (AACC, 2000). Into an extraction thimble, 5.0 g of HDRB flour was added, and the thimble was placed in Soxhlet extraction system. Petroleum ether (250) mL was added into the system's flask. Then the solvent was heated to reflux for 20 cycles through the

sample before it was dried overnight, and the oil content percentage was calculated from the ratio between the change of sample weight (oil removed) before and after the process and the initial sample weight. The final value is stated as a mean of three determinations.

Ash content

The ash content of HDRB flour was determined based on AACC method 08-03 (AACC, 2000). Into crucible, 2.0 g of HDRB flour in triplicate was weighed. Then the samples were heated using an electrical ashing furnace at 600 °C for about 2h. Before weighing the crucibles, all the crucibles were transferred and cooled in a desiccator. The ash percent was calculated as a difference between the sample weighs before and after ashing process

Total dietary fiber content

The percent content the total dietary fiber in HDRB flour were determined using an enzymatic- gravimetric method based on AOAC method 985.29 (AOAC, 2016) and utilizing the specific kit, TDF-100 kit, from Sigma Chemicals Inc. (St. Louis, MO, USA). In brief, HDRB flour (1 g) in triplicate were sequentially digested by heat stable α-amylase (100 °C, 20min, pH 6.0), protease (60 °C, 30min, pH 7.5), and amyloglucosidase (60 °C, 30min, pH 4.5), with agitation. After that, 4 volumes of ethanol (95%) were added to the mixtures for precipitation of the soluble dietary fiber and filtration. The precipitates were dried, weighed, and corrected for ash and protein contents. The final calculation was carried out by following this equation:

$$\text{Total Dietary Fiber (TDF) (\%)} = \frac{R - p - A - B}{m} * 100$$

Where: ***m*** is sample weight, ***R*** is residue weight from *m*, ***p*** is protein weight from *R*, ***A*** is ash weight from *R*, and ***B*** is blank. Values are expressed as a mean of three determinations on a dry weight basis.

Total Carbohydrates

The total amount of carbohydrates was calculated by difference applying the following formula:

$$\text{Total Carbohydrates (\%)} = 100 - (moisture + fat + protein + ash)$$

Determination of water activity (a_w) of HDRB

The water activity and moisture content of HDRB were conducted after autoclaving to determine the initial water content of HDRB suitable for conducting the fermentation, since *B. subtilis* requires a minimum water activity of 0.91 to grow (Fontana and Anthony, 2020). Ten grams of HDRB (80 mesh, 10% moisture content) in each of the twelve (250 mL) flasks were mixed with varying amounts of dH_2O, from 1.0 to 11.0 mL at 1 mL increments, and the first flask was left without adding dH_2O. All the flasks containing HDRB were autoclaved at 121 °C for 60 min. After cooling, water activities and moisture contents were determined using water activity meter (Aqua-Lab Dew point water activity meter, model 4TE, WA, USA) and oven-drying method (AACC Method 44-15.02), respectively.

Solid-State Fermentations at different inoculum levels

Heat stabilized defatted rice bran was examined as a culture medium to inoculate different levels of *B. subtilis* (natto) Takahashi. Twenty grams of prepared HDRB flour were placed in in separate 250 mL flasks and moisturized with dH_2O and mixed to get the initial water contents of 35 % w/v where the water activity is about 0.91. Then all the samples were sterilized by autoclaving for 60 min at 121°C and cooled to room temperature. The samples were inoculated with different concentrations of activated *B. subtilis* (natto) Takahashi cells (10^3, 10^4, 10^6, 10^8 CFU/g HDRB), incubated at 37°C for 120 h. All the flasks were manually shaken (30 rounds) every 6h to enhance the gas exchange and delivering of oxygen for better growth of

microorganisms. The growth of *B. subtilis* (natto) Takahashi on HDRB in solid-state fermentation was investigated.

To investigate the growth of the microorganism, aliquots of 1g from the cultures were withdrawn at (0, 6, 12, 24, 36, 48, 72, 96, 120 h) under the biosafety hood. These samples were suspended with 5 ml of sterilized 0.85% NaCl solution and serially diluted before the counting of *B. subtilis* (natto) Takahashi cells were performed by plating on nutrient agar plates. The plates were incubated at 37 °C for 24-36h, and the colonies were counted and calculated as (log CFU/g HDRB), and the results were expressed as a mean of triplicate samples.

0020Crude Enzyme production

To evaluate crude protease production, solid-state fermentation of HDRB (50g) in triplicates was carried out as mentioned above and under the following conditions: initial moisture content (35% w/v), *B. subtilis* (natto) Takahashi inoculation level (10^6 CFU/g HDRB), and incubation temperature (37 °C) for 120 h. Samples (2g) were taken at (0, 6, 12, 24, 36, 48, 72, 96, 120 h) to monitor the protease activity. The samples were suspended in distilled water (10 mL/g fermented HDRB). Then the slurries were stirred for 15 min at room temperature before removing the solids by centrifuging at 4,500xg for 10 min at 4 °C. The supernatants were used to determine the enzyme activity.

Protease Activity Assay

The total protease activity was determined using azocasein assay according to the method of (Coêlho et al., 2016) with slight modifications. In this assay, azocasein was used as a substrate which liberates free dye into the supernatant due to protease activity and casein degradation, and the liberated dye can be quantitatively analyzed. In the final reaction mixture, 0.5 mL of 0.5% azocasein solution (in 0.05 M Tris-HCl buffer, pH 7.4, 1.0 mM CaCl2) and 0.5 mL of the

fermented HDRB extract solution were incubated for 30min at 25°C. Then 0.5 mL of 10% v/v trichloroacetic acid (TCA) was added to terminate the reaction, and the mixture was incubated for 30 min at ambient temperature before centrifugation at 10,000 ×g for 20 min at 4°C. The supernatant was neutralized by mixing 1:1 ratio of the supernatant and 0.5N NaOH. The absorbance of the neutralized supernatant was measured at 440nm. In blank assay, the extract was substituted by the buffer. The results were expressed as the mean of triplicate independent experiments. One unit (U) of protease activity was defined as the amount of enzyme that produces a 0.01 increase in the absorbance within minute under the assay conditions.

Experimental design for optimizing fermentation

To optimize the conditions of solid-state fermentation, the following three factors, time (h, X_1), inoculum concentration (Log CFU/g HDRB, X_2), and initial moisture content (% w/v, X_3) were used as independent variables for a Box-Behnken design (BBD) in response surface methodology (RSM) analysis to consider the influence of these parameters on the extracted percent of water-soluble proteins and peptides (WSPP) as a dependent variable (Y). The three variables were evaluated at 3 levels (-1, 0, +1) and 3 replicates at the center points (Table 3.2). A total of 15 experiments in triplicate were conducted (Table 3.2) and the outcomes were applied to fit the following quadratic polynomial (second order) model:

$$Y = a_0 + \sum_{i=1}^{3} a_i X_i + \sum_{i=1}^{3} a_{ii} X_{ii}^2 + \sum_{i \neq j=1}^{3} a_{ij} X_i X_j$$

where Y is the response variable, a_0 is a constant, a_i, a_{ii}, and a_{ij} are linear, quadratic, and interactive coefficients, respectively, and Xi and Xj are levels of the independent variables. The optimum value for each factor toward the highest extracted percent of WSPP from SSF-HDRB

were obtained based on solution of the regression equation and analysis of response surface contour plots.

The central points of independent factors were chosen based on the preliminary experiments and taking the following information in the consideration. In general, *B. subtilis* grows in minimum water activity (a_w 0.91), and its optimum growth temperature is (25-37°C) (Warth, 1978). Based on the data obtained from above mentioned experiments (Solid-State Fermentations at different inoculum levels and Determination of water activity (a_w) of HDRB), the lower and higher points of the experimental factors were set in Box-Behnken experimental design (BBD) to be as follow: 24-72h, 5-7 Log CFU/g HDRB, and 25-45 % w/v for incubation time, inoculation log, and initial water content, respectively.

Optimizing Fermentation Conditions of *B. subtilis* (natto) Takahashi for RSM

Ground and sieved HDRB (10g) in separate 250 mL flasks (15 flasks) were moisturized with dH_2O and mixed to get the initial water contents of 25%, 35%, or 45 % w/v. After autoclaving for 60 min at 121°C to kill microorganism's spore and cooling to room temperature, the samples were inoculated with 1 mL of *B. subtilis* (natto) Takahashi to get 10^5, 10^6, or 10^7 CFU/g HDRB. Then, the samples were incubated at 37°C for 24, 48, or 72h. The maximum value of incubation time (72h) in RSM design was selected because after 72h fermentation, the activity of proteases secreted by *B. subtilis* (natto) Takahashi grown on HDRB decreased (Figure 3.3). All the flasks were manually shaken (30 rounds) every 6h to enhance the gas exchange and delivering of oxygen for better growth of microorganisms.

Fermentation under Optimized Conditions

The final fermentation was performed under the following conditions obtained from RSM analysis: incubation time 61h, inoculation log $3x10^6$ CFU/g HDRB, and initial water

content 41 % w/v. The optimized fermentation was set up in 1L flask by moisturizing 250g of HDRB with DI water to get 41 % w/v of water content and sterilized by autoclaving (60 min at 121°C). After cooling to room temperature, the HDRB samples were inoculated with *B. subtilis* (natto) Takahashi in sterilized 0.85% NaCl to get $3x10^6$ CFU/g HDRB. For control sample, the 250g of HDRB was inoculated with sterilized 0.85% NaCl solution without *B. subtilis* (natto) Takahashi. All the samples were incubated at 37°C for 61h and manually shaken (30 rounds) every 6h. The same conditions were applied with the control samples (HDRB). All fermentations were done in triplicate.

Protein extraction from fermented HDRB

The fermented HDRB (SSF-HDRB) samples were suspended with 1:10 sterile DI water and stirred for 30 min before centrifuging at 3,500 for 20 min at 4 °C (Wu et al., 2014). The supernatant was subjected to filtration through 0.2 µm microfiltration membrane column (model: CFP-2-E-4A, polysulfone membrane, GE Healthcare) to remove the remaining *B. subtilis'* cells and spores.

Removal of phenolic compounds from the SSF-HDRB extract

To minimize the interference of phenolic compounds (PC) in the SSF-HDRB extract with the bioactivities of protein fractions and peptides, the WSPP from SSF-HDRB and HDRB were treated with PVPP by using the method of (Bridi et al., 2014) with minor modifications. Polyvinylpolypyrrolidone (PVPP) was added to the filtered SSF-HDRB extract in ratio of 1:10 and stirred for 1h at ambient temperature. Then, the mixture was centrifuged at 10,000×g for 20 min at 4 °C to separate PVPP from the SSF-HDRB extract. This process was repeated twice to remove phenolic compounds. Total phenolic and protein contents before and after the treatment were determined by Folin-Ciocalteu method and Bicinchoninic acid method, respectively. The

final supernatant containing WSPP from SSF-HDRB and HDRB was freeze-dried and stored at -20 °C for further experiments.

Determination of protein content

The protein content of the supernatants was quantified using the Pierce Rapid Gold BCA Protein Assay Kit (A53225, Thermo Fisher Scientific) based on Bicinchoninic acid (BCA) method. Bovine serum albumin was used to establish the standard curve.

The protein percent extracted from HDRB and SSF-HDRB was calculated from the following equation:

$$\text{Water} - \text{soluble protein and peptides (WSPP) extracted (\%)} = \frac{\text{Protein content in supernatent (g)}}{\text{\%Protein of HDRB X Total weight of HDRB}}\ X\ 100$$

Determination of total phenolic content of SSF-HDRB extract.

The total phenolic content in the supernatant of the SSF-HDRB and HDRB samples were determined by the Folin–Ciocalteu method as described by (Ainsworth and Gillespie, 2007) with minor modifications. The diluted extract solution (0.2 mL) was reacted with 0.8 mL of 0.2N Folin–Ciocalteu reagent for 5 min. Then, 2 mL of 0.7 M sodium carbonate solution was added to the solution and incubated in the dark for 2h at ambient temperature. The absorbance was determined at 765 nm using a Shimadzu UV-1601 spectrometer (Shimadzu Inc., Kyoto, Japan). Since the ferulic acid is the main phenolic in the rice bran, the results were expressed as mg Ferulic acid equivalents (FAE) per 100 g HDRB on dry basis using Ferulic acid standard curve.

Determination of molecular size distribution

The distribution of molecular mass of proteins and peptide fragments in WSPP from SSF-HDRB and HDRB was done using size exclusion chromatography on a Superdex 75 10/300 GL 10x300 mm column (GE Healthcare, NJ, USA) using a HPLC system LC-8A (Shimadzu,

USA) based on the method of (Moayedi et al., 2017). An aliquot of 100 µL from each WSPP sample solution (5 mg/mL) was injected into the column after filtering through 0.22 µm syringe microfilter. The mobile phase of separation was PBS (0.05 M phosphate buffer, 0.15 M NaCl pH 6.8) The proteins and peptides were eluted at a flow rate of 0.5 mL/min and monitored by ultraviolet detection at 215 and 280 nm. The following gel filtration molecular weight standard proteins were used to calibrate the column and prepare a calibration curve from the elution volume of the standards: Conalbumin (Mr= 75 KDa), Carbonic Anhydrase (Mr= 29 KDa), Ribonuclease A (Mr= 13.7 KDa), and Aprotinin (Mr= 6.5 KDa).

Statistical analysis

All experiments were performed in triplicate determinations. The results are presented as means ±SD. For statistical analysis of data including Box-Behnken design and RSM analysis, JMP®, a statistical program, (Ver.15.1. SAS Institute Inc., Cary, NC, USA) was used with a statistical significance between the samples at level of $p < 0.05$. One-way analysis of variance (ANOVA) was performed for comparison of means with statistical significance at level $p < 0.05$.

Results and Discussion

Composition analysis

The results showed (Table 3.1) that the HDRB was composed of 11.2±0.4% moisture, 2.1 ±0.3% crude lipid, 55.4 ±1.1% total carbohydrates, 33.2 ±0.9% total dietary fiber, 16.4 ±0.5% total protein, and 14.9 ±0.6% ash. These values are within the range reported in the literature (15-22% lipids, 34.1-52.3% carbohydrates, 7-11.4% fiber, 6.6-9.9% ash, 8-12% moisture, and 10-16% protein (Faria et al., 2012; Juliano, 2016b). These pecentages of the RB composition increase after removing the oil. The carbohydrates including fibers and starch, proteins, and other nutrients could support the growth of *B. subtilis* (natto) Takahashi by

providing potential sources of carbon, nitrogen, and other nutrients. In addition to that, the nitrogen content of HDRB, about 2.8%, supplies enough nitrogen source in the growth media. In general, the minimum concentration of nitrogen source required for growth of *Bacillus spp.* is about 10-20 mM (Hu et al., 1999).

Moisture content and water activity of HDRB

The typical moisture level of growth medium in SSF varies depending on the microorganism, and for fungus growth, it ranges from 20% to 70% while bacterial growth requires about 70% (Krishna, 2005). In SSF, the importance of water availability in the substrate can be related with water activity (a_w). Water activity is one of the critical factors for the growth of different microorganisms especially bacteria which requires higher a_w than fungi (Tapia et al., 2020). Since *B. subtilis* in general requires a minimum water activity of 0.91 to grow (Fontana and Anthony, 2020), the initial water content of HDRB providing a_w of 0.91and suitable for conducting solid-state fermentation was determined. The results showed that HDRB with initial moisture content of about 35% w/v provides a water activity of 0.91 (Figure 3.1) hence this percentage was chosen to be used in SSF in this study. Pandey and others (1994) reported that in SSF, there is a strong correlation between the water activity of wheat bran as a growth substrate and the growth of *Aspergilus niger* and production of glucoamylase (Pandey et al., 1994). The growth of Aspergillus oryzae and the production of neutral protease on a substrate of rice bran and rice hulls were facilitated at initial water content of 35-40 % (Battaglino et al., 1991).

Solid-State Fermentations at different inoculum levels of *B. subtilis* (natto) Takahashi

Inoculation using various levels of *B. subtilis* (natto) Takahashi was to determine the inoculum range of *B. subtilis* (natto) Takahashi that can survive and grow on HDRB as growth media in SSF. The data in (Figure 3.2) demonstrated that HDRB was a suitable substrate for the

growth of *B. subtilis* (natto) Takahashi inoculated at 10^4 -10^8 CFU/g HDRB (high level) in SSF under the experiment conditions of 35% initial moisture content and 37 °C. The maximum cell populations of the high-level inoculums (10^8 and 10^6 CFU/g HDRB) were $4x10^{11}$ and $3x10^{10}$ CFU/g HDRB respectively, in 24h of incubation while the inoculum size of 10^4 CFU/g HDRB required about 48h to reach the ultimate cell population ($2.5x10^{10}$ CFU/g HDRB). The results also showed that inoculating HDRB with 10^8 CFU/g HDRB of *B. subtilis* (natto) Takahashi had very short fermentation cycle (<48h) in comparison to that of inoculum size of 10^4 and 10^6 CFU/g HDRB, which had about 120h and 72h fermentation cycle respectively.

The quick decline in the growth of *B. subtilis* (natto) Takahashi in HDRB inoculated at 10^8 CFU/g HDRB could be attributed to the deficiency of available nutrients required to support the large biomass of cells and accumulating metabolic compounds that raise the cellular toxicity levels (Sandhya et al., 2005). Thus, the short stationary phase is not preferable in fermentation, because the bacterial production and activity and the activity of the secreted enzymes may not be enough to achieve the highest yields of final products, such as degradation of HDRB to enhance the extraction of protein and peptides from HDRB in this study. Soares and his co-workers (2005) reported that when protease production by *B. subtilis* in SSF using soy cake as a culture media was studied in different inoculum concentrations, the highest levels of protease were obtained at inoculum range of 0.7-2.0 mg/g substrate while at higher level inoculum (2.5 mg/g substrate) the protease production level was decreased to about 45% from that of 2 mg/g substrate. This decline in the protease production level may attribute to the collapse of nutrients in the fermentation media or due to decomposition of enzymes resulted by interactions and interfering with compounds generated in the fermented substrate. However, the protease activity peak for 0.7 mg/g inoculum concentration was reached after 114 h fermentation, and it took only

62.5h of fermentation for inoculum size of 2 mg/g substrate (Soares et al., 2005). Furthermore, the results showed that more time was required for fermenting HDRB when inoculated with 10^4 CFU/g HDRB of *B. subtilis* (natto) Takahashi to reach the stationary phase because of the slow growth rate and the low initial inoculum level.

In addition to that, the data of the low-size inoculation (10^3 CFU/g HDRB) demonstrated that at this inoculated level, the growth of *B. subtilis* (natto) Takahashi could not develop under the experiment condition, 35% initial moisture content and 37 °C. This could be due to reduction in water content that lost during fermentation. According to the results mentioned above, 10^6 CFU/g HDRB as inoculum size of *B. subtilis* (natto) Takahashi is the most suitable to be used in this study and as central value in the experiment design of the optimization.

Screening of protease activity

Production of protease by *B. subtilis* (natto) Takahashi in SSF using HDRB as a growth substrate was determined under the following fermentation conditions: 35% initial water content, 10^6 CFU/g HDRB inoculation size, and incubation at 37 °C for 120 h. The kinetic profiles of *B. subtilis* (natto) Takahashi growth and protease production are shown in (Figure 3.3). Based on the data, the profile of protease production appeared to be correlated with bacterial growth. Also, the incubation time is directly associated with the production of protease up to 72h. The amount of protease production increased with increase in the exponential phase, and in 24h fermentation, protease activity was 54.3 ±1.2 U/g HDRB. Before the beginning of stationary phase and when the population of *B. subtilis* (natto) Takahashi was 10.1 ±0.4 Log CFU/g HDRB, the protease production reached the maximum activity (93.6 ±1.0 U/g HDRB) at 36h fermentation and started declining after 72h fermentation reaching the minimum activity (31.2 ±2.7 U/g HDRB) at 120h of fermentation. The protease inactivation after 72h fermentation may be attributed to the

diminishing of nutrients supplied by HDRB to *B. subtilis* (natto) Takahashi, and it can be either because of cleavage by another protease in the media or self-breakdown (Romero et al., 1998). It was stated that in several *Bacillus species* including *B. subtillis*, the maximum production of proteases occurs in the post exponential phase or in the stationary phase of the growth cycle (Contesini et al., 2018; Matkawala et al., 2021). Rice bran is the best substrate in solid-state fermentation for production of proteases by a Bacillus sp. (Jayaramaiah, 2010). the maximum protease production by B. subtilis in SSF has been reported using wheat bran at incubation time of 24 h (Sahin et al., 2020).

Optimization of SSF conditions for higher protein extracted percent

Response surface model and Box-Behnken Design (BBD)

Based on the single-factorial experiments, the range of values for the three independent factors, incubation time (X_1), inoculum log (X_2), and initial water content (X_3), were chosen. Furthermore, the optimal values of the three independent factors in solid-state fermentation to extract highest amount of protein from HDRB were determined by BBD. The pattern of the BBD design and the related experimental results are shown in (Table 3.2). The experimental WSPP percent extracted from SSF-HDRB varied from 19.6% ±0.3 to 59.0% ±1.4 (Table 3.2). The optimum conditions of SSF were fermentation time of 61.01 h, inoculum log of 6.27 Log CFU/g HDRB, and initial water content of 40.96 w/v % (Figure 3.4), and the maximum predicted value of the WSPP percent extracted from SSF-HDRB was 63.4% under these optimum conditions. The experimental data of the BBD was used to run the quadratic regression model, and the value of WSPP percent extracted was predicted from the second-order polynomial Equation (3.1):

$$Y= 56.93 + 8.11X_1 + 3.47X_2 + 12.59X_3 + 1.48X_1X_2 + 1.68X_1X_3 + 1.758X_2X_3 - 8.77X_1X_1 - 9.89X_2X_2 -11.73X_3X_3 \quad \textbf{(Equation 3.1)}$$

where Y is WSPP percent extracted, and X_1, X_2, and X_3 are the independent variables for fermentation time, inoculum log, and initial water content, respectively.

Statistically, an ANOVA test (F-test) was done to fit the response function to experimental results. The model has low probability value (P >F=0.0001), and the F-value was 79.3106 (Table 3.3), so there was very low chance (0.01%) for the occurrence of the model due to noise. This demonstrated that the regression model was highly significant at a confidence level of 99% (P <0.01).

To verify the suitability of the model, the coefficient of determination (R^2) was taken in the consideration. The value of R^2 (0.9533) indicated that the model can explain 95.3% of the response variation which might be attributed to the independent factors (Table 3.3). Furthermore, the high value of adjusted R^2 (0. 9412) implies that the independent variables contributed to the improvement of the model. The model has good predictive quality (R^2 0. 9175) allowing the prediction of a new set of data. All these analyses evidenced a good fit of the model.

The data from the analysis of variance (ANOVA) of WSPP percent extracted from SSF-HDRB (Table 3.3) showed that the linear and quadratic relationship of fermentation time (X_1, X_1^2), inoculation log (X_2, X_2^2), and initial water content (X_3, X_3^3) had significant effect (P<0.0001) on the WSPP percent extracted from SSF-HDRB. The interactions between the independent variables, X_1X_2 (P= 0.1503), X_1X_3 (P=0.1056), and X_2X_3 (P=0.0894) did not have significant effect at 95% of confidence level inferring that there is no interaction effect between the examined factors.

The 3D response surface plots (Figure 3.5 A-C) were created based on the polynomial equation (Equation 3.1) to show the interactions between the 3 independent factors on extracted WSPP at intermediate levels. The percentage of extracted WSPP increased over the time as the

fermentation approached 61h on linear response (Figure 3.5 A). Also, there was a significant increase in the extracted WSPP from SSF-HDRB ($P < 0.0001$) linked with the increasing of the initial water content and inoculation log (Figure 3.5 B and C), respectively. However, the interactions between time and inoculation log, time and initial water content, and inoculation Log and initial water content did not have significant effect on WSPP percent extracted from SSF-HDRB in the selected range of the factors' values. The convex shape of the response surface plots supported the reasonable setting of the variables' range (Wong et al., 2017).

Verification of the Optimum Conditions for HDRB fermentation

The capability of the model for estimating the highest percent of extracted WSPP from HDRB was tested by conducting SSF under the following predicted optimum conditions: the initial water content was 41.0%, fermentation time was 61.0 h, and the inoculation log was 6.3 Log CFU/g HDRB. Validation tests (in triplicate) were performed under the above-mentioned conditions, and the actual percent of extracted WSPP from SSF-HDRB was 64.6 ±0.7 % (Table 3.4) confirming the predicted value (63.4%) (Figure 3.4) and acceptability of the RSM model. As shown in (Table 3.4), the WSPP percent extracted from SSF-HDRB (64.6 ±0.7) was significantly higher ($p\text{-}value \leq 0.05$) than that of non-fermented HDRB (20.6 ±0.3). The increase in protein content of WSPP from SSF-HDRB in comparison to that from HDRB can be due to the activity of *B. subtilis natto* during fermentation. Many species of *Bacillus* including *B. subtilis* can excrete several enzymes, such as proteases, carbohydrases, and phytases (Su et al., 2020), that hydrolyze and degrade proteins, complex carbohydrates, and other plant materials. Rice bran has been reported to support the growth of *B. subtilis* (Bettiol et al., 2005) and excretion of proteases (Sumantha et al., 2006). This biodegradation helps to release bound proteins and provides more access for proteases to hydrolyze peptide bonds and break downing

the proteins during the fermentation. Hence, protein hydrolysis facilitated by protease activity and a synergistic action among proteases and other secreted enzymes including carbohydrase could have contributed to the higher protein percent extracted from SSF-HDRB. Non-defatted rice bran fermented with *Rhizopus oryzae CCT 7560* in solid-state fermentation for 120 h with 49% protein was reported by (Kupski et al., 2012). In another study, the combination of different lactic acid bacteria (*Lactobacillus acidophilus*, *Streptococcus thermophilus*, and *Bifidobacterium lactis*) was used to study the effect of fermentation on pea protein extraction. The results showed that due to the fermentation, the protein content and yield of albumin rich fraction were increased about 20% and 30%, respectively (Emkani et al., 2021). In our study, SSF of HDRB with *B. subtilis* (natto) Takahashi led to extract the highest amount of soluble protein in comparison to the studies mentioned above.

Most published studies related to fermentation of rice bran or HDRB focused on the extraction of phenolic compounds and their antioxidant activities. Fermentation of rice bran using fungal strains requires longer fermentation cycle, about 5 days, in comparison to bacterial fermentation which needs additional supplements to be added with rice bran. In this study, HDRB was successfully fermented via *B. subtilis* (natto) Takahashi without introducing any additional substances to support the growth of *B. subtilis* (natto) Takahashi. Furthermore, this fermentation facilitated extracting 64% of HDRB proteins as water soluble fractions and peptides predominantly with molecular size less than 5KDa.

Removing of Phenolic Compounds

To minimize the interference of phenolic compounds (PC) in the SSF-HDRB extract with the bioactivities of protein fractions and peptides including anti-tyrosinase, anti-elastase, and antioxidant activities, the WSPP from SSF-HDRB and HDRB were treated with PVPP. The

results (Table 3.5) showed an 87% decrease in the total PC from 15.9 ±0.4 to 2.4 ±0.3 (FAE mg/g HDRB) after the treatment with PVPP. The results also showed that the protein content in the WSPP from SSF-HDRB was not changed significantly ($P>0.05$) with PVPP treatment as shown in (Table 3.5). Fermentation assisted in the removal of phenolic compounds in rice bran by releasing PC in contrast to non-fermented HDRB because of the enzymic activity of *B. subtilis* that produces phenolic esterase and xylanase as reported by (Webber et al., 2014), and these enzymes play a role to free up the bound phenolics from the cell wall. A significant decrease in PC after PVPP treatment of SSF-HDRB extract can be due to the high affinity of PC by PVPP via hydrophobic and hydrogen bonds and PVPP has been used to separate phenolic fractions from the extract especially in wine industry (Cosme et al., 2019; Han et al., 2020). The effect and interaction of the phenolic compounds on the antioxidant activities of WSPP from SSF-HDRB and non-fermented HDRB were studied and evaluated in chapter 6.

Size distribution profiles of WSPP from SSF-HDRB and HDRB

The protein and peptide profiles of WSPP from SSF-HDRB and non-fermented HDRB were analyzed using size exclusion HPLC. In this technique, using a wide range of standard proteins with different molecular weights (MW) can help in the observation of the distribution of proteins and peptides in the WSPP samples (Figure 3.6).

The chromatogram of the size exclusion HPLC (Figure 3.6) showed four distinct regions (F1, F2, F3 and F4), and based on the column calibration, these regions correspond to the following molecular weights ranges: >75, 75–20.0 kDa, 20.0-5.1 and below 5.1 kDa, respectively. The chromatogram showed HDRB storage protein, albumin and other water-soluble proteins, in region F1 till F3 with main peaks corresponding to MW of about 46, 32, and 14 KDa. Furthermore, two small peaks appear in region F4 which could represent amino acids,

peptides, and other small MW impurities. While the profile of WSPP from SSF-HDRB illustrated a significant change in the protein and peptides distribution after SSF**,** the majority of HDRB albumin fraction was extensively decreased leaving behind small peaks in the regions F1 to F3, but several peaks appeared in area F4 (MW less than 5 KDa) with remarkable increase in the amount of short protein fractions, peptides, and amino acids. The changes in the pattern of MW distribution between WSPP from HDRB and SSF-HDRB could be due to bacterial proteases hydrolysis during SSF by cleaving the peptide bonds of high MW proteins and converting them into low MW of proteins and peptides. Several studies have been reported obtaining similar alterations in the proteins during fermentation. Fermentation of cocoa bean (Hue et al., 2016) and defatted Adlay (*Coix lachryma-jobi* L.) (Xu et al., 2021) led to degradation of high MW proteins and increasing in the content of peptides and amino acid.

Conclusion

Bacillus subtilis (natto) Takahashi is an important probiotic bacteria used in preparing fermented food. In this study, *B. subtilis* (natto) Takahashi was successfully grown in HDRB under SSF conditions.

The chemical compositions of HDRB were 11.2 ±0.4% moisture, 16.4 ±0.5% total protein, 2.1 ±0.3% crude lipid, 55.4 ±1.1% total carbohydrates, 33.2 ±0.9% total dietary fiber, and 14.9 ±0.6% ash. The complex composition of HDRB is an excellent source of carbon and nitrogen and supported the growth of *B. subtilis* (natto) Takahashi that produced enough proteases and enhanced the extraction of water-soluble protein fractions and peptides from HDRB. Therefore, SSF of HDRB with *B. subtilis* (natto) Takahashi is effective in the extraction of proteins, in the production of low molecular weight peptides and phenolic compounds. The SSF with *B. subtilis* (natto) Takahashi technology can be an effective processing method to add

value to HDRB as the fermented HDRB extract has the potential as an ingredient for functional and nutraceutical products

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Tables and Figures

Table 3.1. Proximate composition (%w/w on dry basis) of Heat-Stabilized Defatted Rice Bran

Moisture	Total Protein	Total Carbohydrates	Dietary Fiber	Crude Lipid	Ash
11.2 ±0.4	16.4 ±0.5	55.4 ±1.1	33.2 ±0.9	2.1 ±0.3	14.9 ±0.6

The data represents mean ± standard deviation of three determinations

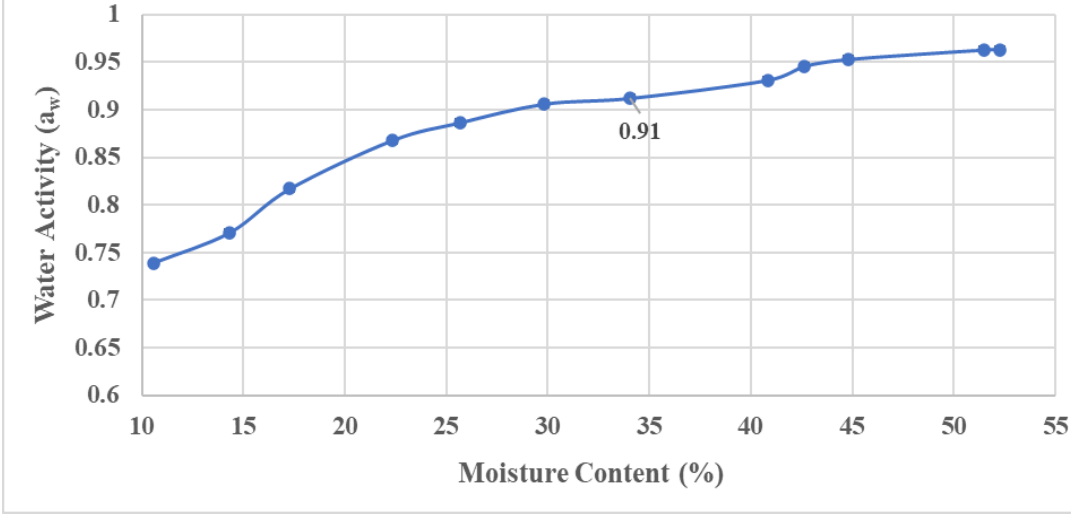

Figure 3.1. The relationship between moisture content and water activity (aw) in Heat-Stabilized Defatted Rice Bran (HDRB) 80 mesh flour after 60 min autoclaving at 121°C. The values represent mean ±SD with significant difference at $p \leq 0.05$.

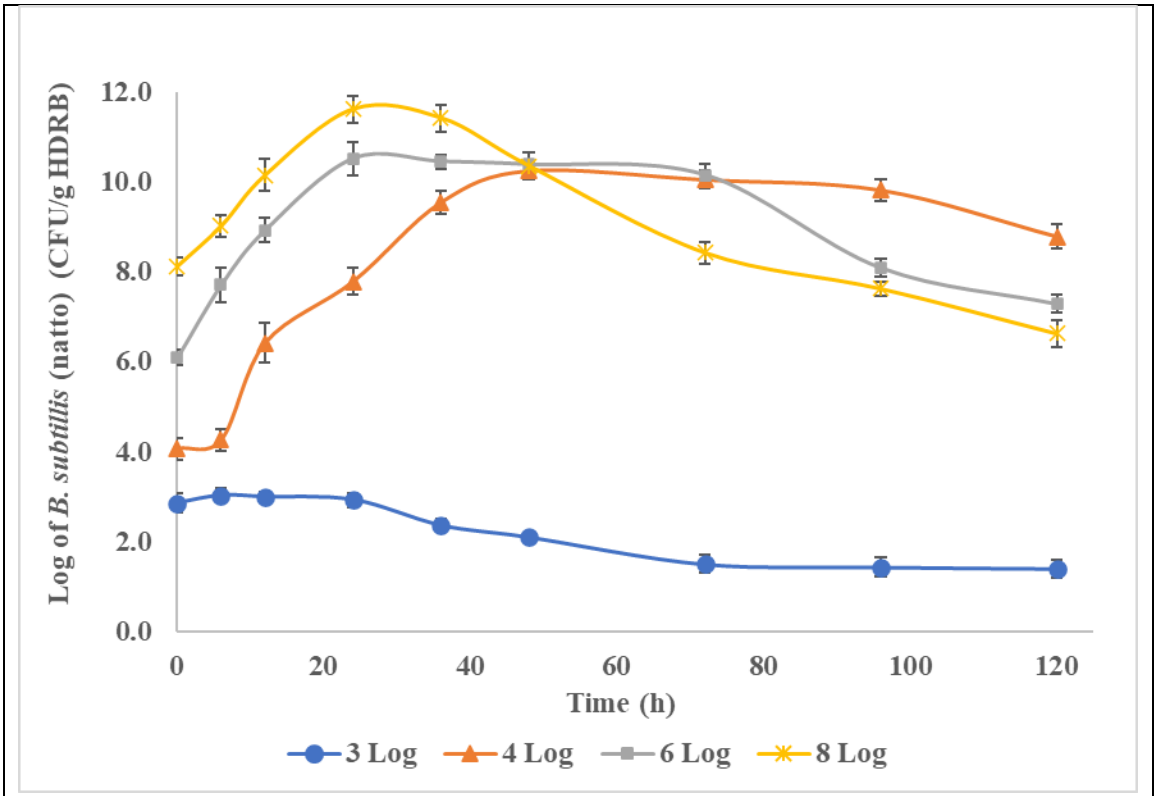

Figure 3.2. Growth of *B. subtilis* (natto) Takahashi in solid-state fermentation of heat-stabilized defatted rice bran at different initial inoculation levels. 3Log: 10^3 CFU/g HDRB, 4Log: 10^4 CFU/g HDRB, 6Log: 10^6 CFU/g HDRB, 8Log: 10^8 CFU/g HDRB of *B. subtilis* (natto). The values represent mean ±SD with significant difference at $p \leq 0.05$.

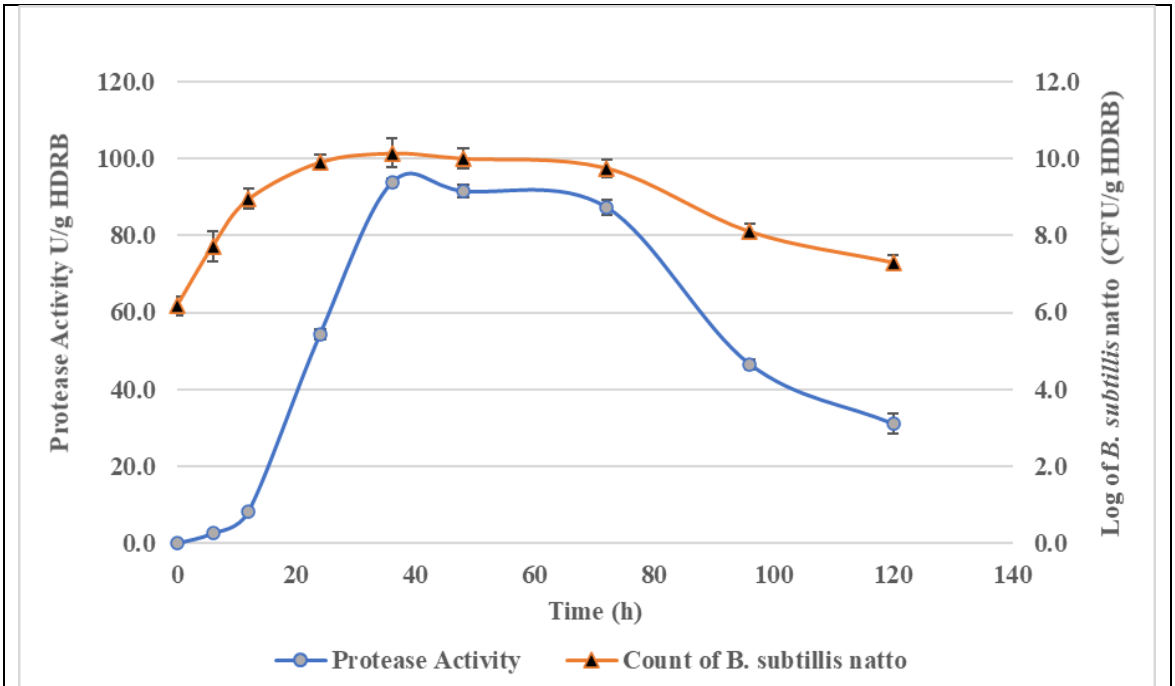

Figure 3.3. Profile of protease production and cell count in solid-state fermented heat-stabilized defatted rice bran inoculated with 106 CFU/g HDRB of B. subtilis (natto) Takahashi and incubated for different periods of time at 37°C and 35% initial moisture content. The values represent mean ±SD with significant difference at $p \leq 0.05$

Table 3.2. The Box-Behnken design for optimizing solid-state fermentation conditions with water-soluble proteins and peptides (percent) extracted.

	Coded Levels of Variable			**Experimental Values**			**Water-Soluble Proteins and Peptides Percent Extracted (%)****	
Run	X_1	X_2	X_3	Time (h)	Inoculum Log (CFU/g HDRB)	Water Content (w/v %)	Actual Response Value (Y)*	Predicted Response Value (Y')*
1	1	1	0	72	7	35	55.1 ± 0.7	51.3
2	1	0	-1	72	6	25	27.5 ±0.8	30.3
3	1	-1	0	72	5	35	45.5 ±0.7	41.4
4	-1	1	0	24	7	35	28.0 ±1.1	32.1
5	1	0	1	72	6	45	53.7 ±0.3	58.8
6	0	-1	-1	48	5	25	19.6 ±0.3	21.0
7	-1	0	-1	24	6	25	22.5 ±0.4	17.4
8	-1	-1	0	24	5	35	24.4 ±1.0	28.2
9	0	1	-1	48	7	25	23.4 ±0.4	24.4
10	0	-1	1	48	5	45	43.6 ±0.9	42.7
11	-1	0	1	24	6	45	42.0 ±1.1	39.2
12	0	1	1	48	7	45	54.5 ±1.2	53.1
13	0	0	0	48	6	35	57.7 ±0.5	56.9
14	0	0	0	48	6	35	57.1 ±0.9	56.9
15	0	0	0	48	6	35	59.0 ±1.4	56.9

* To conserve space, the data represents the mean of triplicate experiments while the experimental design was based on two replicates with 45 runs.

** Total protein content in HDRB: 16.4%±0.3 by Kjeldahl method.

Table 3.3. Analysis of variance for the experimental data of the Box-Behnken Design (BBD)

Source	Nparm[a]	Sum of Squares	DF[b]	Mean Square	F Ratio	Prob > F
Model		8777.209		75.25	79.0332	<.0001
X_1 - Time (h)	1	1577.882		1577.88	127.8704	<.0001
X_2 - Inoculation Log (CFU/gHDRB)	1	289.12		289.12	23.4301	<.0001
X_3 - Initial Water Content (w/v %)	1	3802.684		3802.68	308.1669	<.0001
X_1*X2	1	26.403		26.40	2.1397	0.1525
X_1*X3	1	34.003		34.00	2.7556	0.1058
X_2*X3	1	37.101		37.10	3.0066	0.0917
X_1^2	1	852.12		852.12	69.0552	<.0001
X_2^2	1	1084.736		1084.74	87.9062	<.0001
X_3^2	1	1523.889		1523.89	123.4949	<.0001
Error		431.889	35	12.340		
Total		9209.098	44			
R-Squared		0.9531				
Adj R-Squared		0.9410				
Predicted R-Squared		0.9175				

a: Nparm, number of parameters; b: DF, degrees of freedom.

Table 3.4. Percentage and amounts (g/100g) of water-soluble proteins and peptides (WSPP) extracted from heat-stabilized defatted rice bran using solid-state fermentation

Sample Type	Proteins Percent in the Supernatant and Residue (%)			Protein Extracted from original HDRB (g/100g HDRB)*		
	HDRB	SSF-HDRB	*p-value*	HDRB	SSF-HDRB	*p-value*
Supernatant 1 (Water-Soluble)	20.6 ±0.3	64.6 ±0.7	0.007	3.384 ±0.05	10.594 ±0.1	0.00009
Residue 1	79.8 ±0.8	36.3 ±1.0	0.002	12.994 ±0.1	5.789 ±0.16	0.00001

Sample type: supernatant and residue of solid-state fermentation (SSF). HDRB: heat-stabilized defatted rice bran. SSF-HDRB: Solid State Fermented HDRB. *p-value* ≤ 0.05 represents significant difference.

* Total Protein Content in HDRB: 16.4%.

Table 3.5. Total phenolic compounds (TPC) and protein content of solid-state fermented and non-fermented HDRB extract before (S1) and after (SPVPP) Polyvinylpolypyrrol-idone (PVPP) treatment.

Sample Type	Total Phenolic Compounds (mg FAE/g HDRB)*		Protein Content (mg protein /g HDRB)*	
	S1	S_{PVPP}	S1	S_{PVPP}
SSF-HDRB	15.9 ±0.4^{a}	2.4 ±0.3^{b}	106.1±1.2^{e}	103 ±1.3^{e}
HDRB	3.7 ±0.1^{c}	0.74 ±0.1^{d}	33.9 ±0.3^{f}	32.1 ±0.4^{f}

The values represent mean ±SD. Different letters denote significant difference at $p \leq 0.05$
HDRB: heat-stabilized defatted rice bran. SSF-HDRB: Solid State Fermented HDRB. FAE: Ferulic acid equivalent.

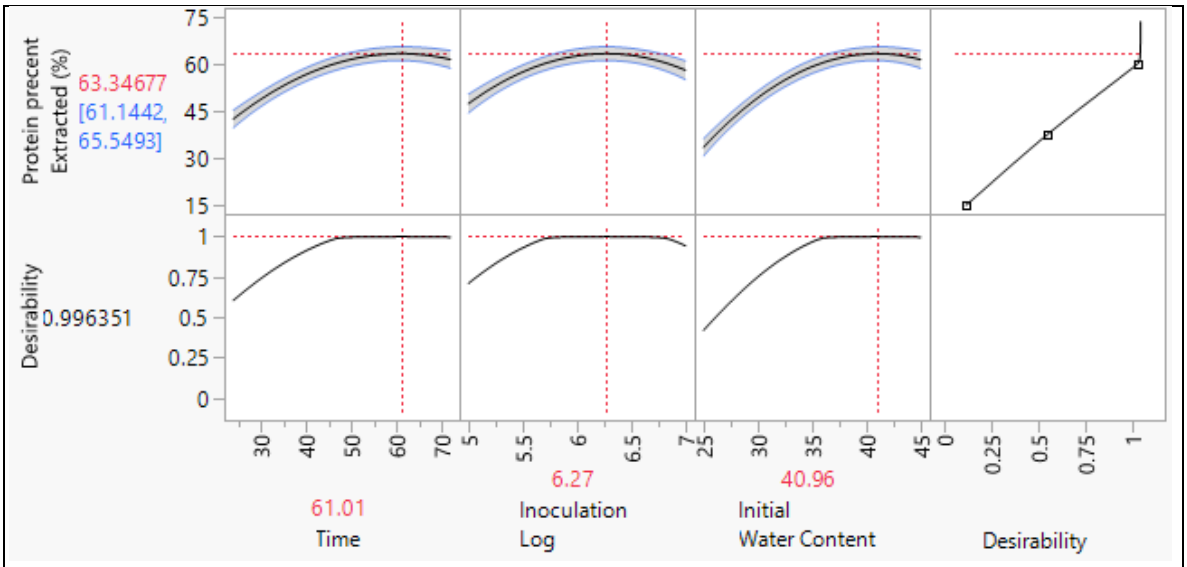

Figure 3.4. The prediction profiler showing the optimized conditions at the maximum desirability of the water-soluble protein and peptides percent extracted from the solid-state fermented heat-stabilized defatted rice bran. Generated using the software, JMP Pro. 15.2.1

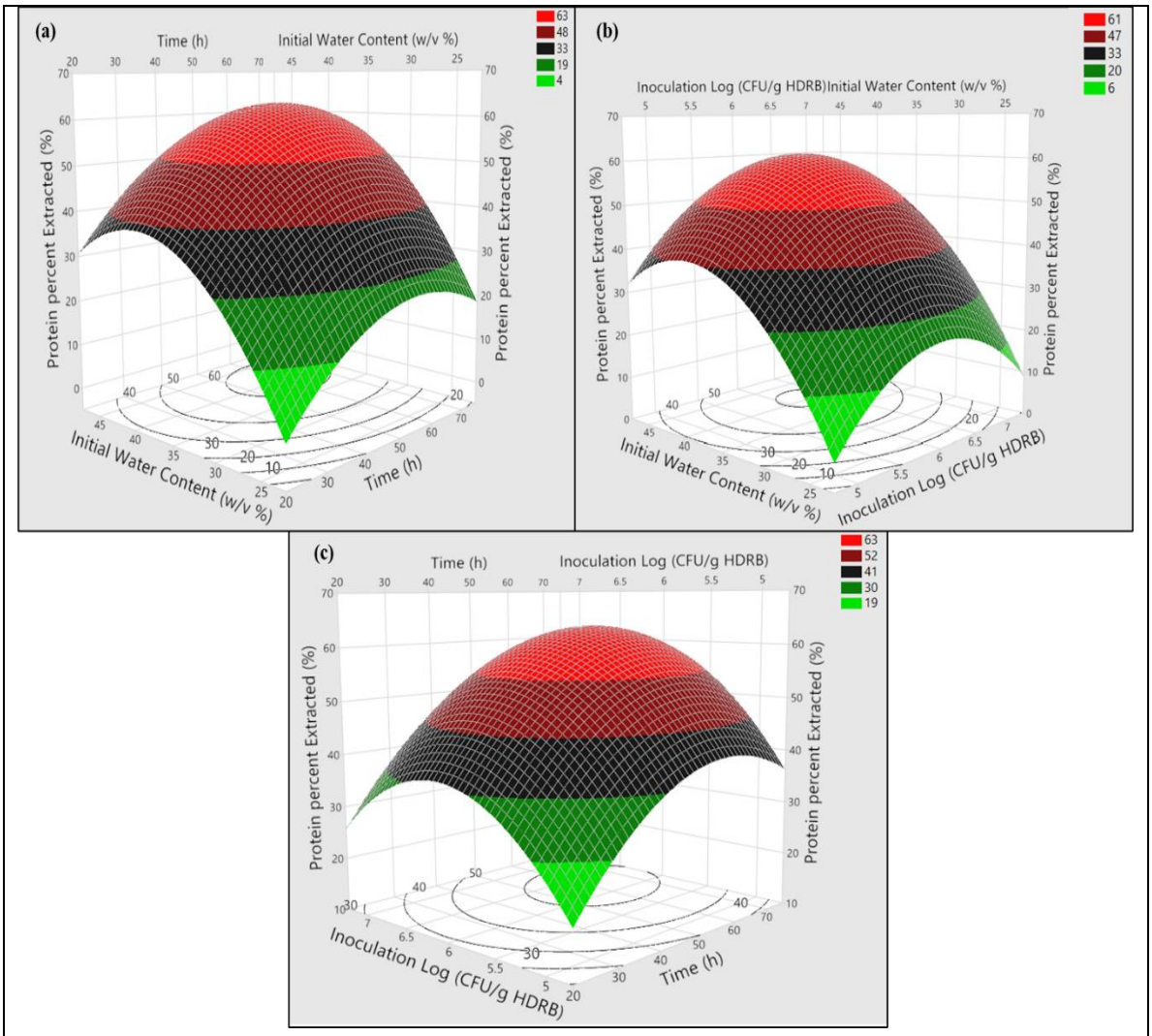

Figure 3.5 Response surface plots, WSPP percent extracted from SSF-HDRB fitted from the experimental results of the Box-Behnken design (BBD) represent the interactions between the independent variables.; **A**: Interaction between the water content and fermentation time; **B**: Interaction between water content and the inoculum concentration; **C**: Interaction between the inoculum concentration and fermentation time

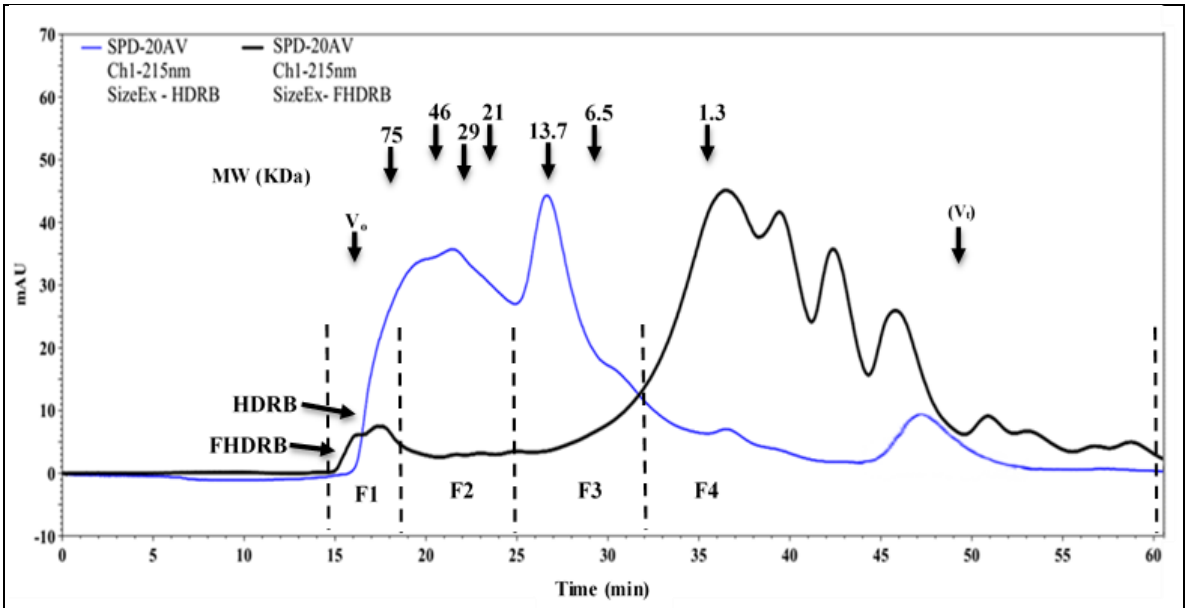

Figure 3.6 Size exclusion-HPLC chromatograms of water-soluble proteins and peptides from heat-stabilized defatted rice bran (HDRB) and fermented HDRB (FHDRB) V_o: void volume, V_t: total volume of column, MW: molecular weight

CHAPTER 4

Fractionation of the Water-Soluble Proteins and Peptides Extracted from Solid-State Fermented Heat-Stabilized Defatted Rice Bran and Evaluation the Tyrosinase and Elastase inhibitory activities of the Fractions

Abstract

The objective of this study was to evaluate the impact of solid-state fermentation (SSF) on the cosmeceutical bioactivities including skin anti-pigmentation and anti-wrinkling, of heat-stabilized defatted rice bran (HDRB) protein hydrolysates and peptides using a bacterial probiotic, *Bacillus subtilis* (natto) Takahashi. Water-soluble protein hydrolysates and peptides (WSPP) extracted from fermented HDRB showed an enhancement in the inhibition of elastase and tyrosinase activity in comparison to that of unfermented HDRB extract. Fractionation of WSPP using ultrafiltration (UF) and size exclusion chromatography (SEC) demonstrated that small peptide fractions inhibited aging related enzymes including elastase and tyrosinase. Based on the results, the highest *in-vitro* elastase and tyrosinase inhibition activity was shown by UF peptide fraction < 5Kda and were 12.9% and 68.5%, respectively. The SEC separation of the peptide fraction < 5KDa generated 8 fractions, and among them, the fractions (F5 and F4) exhibited the highest tyrosinase inhibition activities (82.3%) and (79.1%), respectively. Elastase inhibitory activity in general was very low, and the activity of SEC fractions did not have significant ($P > 0.05$) inhibition effect in comparison to that of un-separated fraction (<5KDa). The small size peptides produced by SSF are potent in inhibiting elastase and tyrosinase and enhancing skin health through preventing skin wrinkling and pigmentation. Based on the results, the SSF can be a cost-effective and an efficient approach to enhance the bioactivities of HDRB

proteins and add-value to HDRB in finding applications as a functional ingredient in the cosmeceutical products that support skin health.

Introduction

Several factors involve in the aging of human skin. These factors can be classified into extrinsic factors which are caused generally by UV radiation effects triggering photoaging, and intrinsic factors including oxidative metabolism processes that lead to developing of natural skin aging characteristics due to changes in the skin structure, such as elasticity (skin wrinkling) (Farage et al., 2008). The activation of many enzymes in the skin tissue due to the consequence of these factors is responsible for skin aging, and one of the potentially impacting enzymes is elastase. Elastase is a protease that belongs to the chymotrypsin family, and it is one of the important extracellular matrix proteins. Elastin is the primary substrate of elastase that can also cleave other proteins in the extracellular matrix, such as collagen and fibronectin. Hence, overproduction of elastase plays an important role in the alteration of the three-dimensional structure in dermal elastic fibers and leading to the decrease in the elasticity of the skin which is highly correlated with the development of the skin-wrinkles especially in UV-exposed skin (Tsukahara et al., 2006).

In addition, pigmentation is another process that is activated due to skin aging causing several skin disorders. Skin pigmentation occurs because of activation of melanogenesis process in melanocytes of skin epidermis, and the key factor of this pathway that biosynthesize melanin is tyrosinase (Kammeyer and Luiten, 2015). Tyrosinase catalyzes the first two reactions in melanin biosynthesis pathway, the hydroxylation of tyrosine to dopa (3,4-dihydroxy-L-phenylalanine) and oxidation of dopa to dopaquinone, and after a series of nonenzymatic reactions, dopaquinone is converted to eumelanin which represent dark brown/black melanin

pigments (d'Ischia et al., 2015). Therefore, the inhibition of tyrosinase is a potential target in the prevention of melanin accumulation and of interest in the fields of cosmetics, medicine, and food. Consequently, inhibition of elastase and tyrosinase can delay skin aging and support the skin against aging disorders. The inhibitors of these enzymes are widely incorporated as a main component in cosmetic products to prevent or reduce skin aging avoiding pigmentation and losing the elasticity of the skin. Although, in cosmetic products, the generally used tyrosinase inhibitors, such as hydroquinone, arbutin, and kojic acid, are therapeutically effective, they are recognized as carcinogenic and genotoxic compounds (Nohynek et al., 2004; Ochiai et al., 2016a; Westerhof and Kooyers, 2005). Thus, inhibitors with stable and safe characteristics and harmless on human health are strongly desired. Besides, natural cosmetic substances and agents are more preferred to be used by consumers instead of synthetic alternatives, so natural sources have been a target and investigated for obtaining elastase and tyrosinase inhibitors.

Bioactive peptides are protein fractions which are released from innate proteins after proteolytic hydrolysis and having biological effects (Bhandari et al., 2020), due to hydrolytic cleavage of native proteins, the active moieties of amino acids are exposed, and new formed structures are produced by hydrolysis. The bioactive peptides have high efficiency and specificity in supporting health as well as they have high tissue affinity (Daliri et al., 2017), hence the search and application of bioactive peptides have increased exponentially. Rice bran contains several bioactive components including proteins which are widely investigated as a natural and beneficial source of alternative ingredients in nutraceutical and food product industries. Several studies have reported different bioactive peptides, such as antihypertensive (Uraipong and Zhao, 2016; Wang et al., 2017), anti-diabetic (Boonloh et al., 2015; Hatanaka et al., 2012; Uraipong and Zhao, 2016), anticancer (Kannan et al., 2009; Kannan et al., 2010; Li et

al., 2014), Antimicrobial (Taniguchi et al., 2019), and antioxidant activities. These peptides have been generated enzymatically from rice bran protein hydrolyzed using different proteases. Microbial fermentation could enhance the bioactivity and bioactive peptides of rice bran (Ryan et al., 2011; Schmidt et al., 2014). Thus, the process of protein fermentation from different sources using several varieties of microbial species and strains is considered as a useful and novel method to produce bioactive peptides with valuable health impacts through hydrolyzing of food protein. Production of bioactive peptides using fermentation is coast effective in comparison to use of pure enzymes, and the produced peptides may not need further analysis for purification. Although, fermented broth ingredients, such as, polyphenolic compounds and flavonoids, aglycone isoflavones, 8-Hydroxydaidzein and, 3-hydroxydaidzein, exhibited anti-melanogenesis and anti-tyrosinase activities (Chan et al., 2014), there is no data in the literature related to evaluation of protein fractions and peptides obtained from fermentation broth of rice bran toward inhibition of elastase and tyrosinase. Therefore, in this study, the objectives were to prepare the water-soluble protein fractions and peptides (WSPP) of solid-state fermented heat-stabilized defatted rice bran (SSF-HDRB), fractionate WSPP of SSF-HDRB into different fractions (>50, 50-10, 5-10, and <5 kDa) using ultrafiltration membrane, and investigate the elastase and tyrosinase-inhibitory activities of the fractions. Then, the fraction with the highest bioactivity was separated by size exclusion-High performance liquid chromatography (SEC), and the bioactivities of the collected fractions were determined.

Material and Methods

Materials.

Heat-stabilized defatted rice bran (HDRB) was supplied by Riceland Foods, Inc. (Stuttgart, Ark., U.S.A.) as a rice bran meal that was subjected to heat stabilization at (~120 °C)

through steam expander prior to the oil extraction. *B. subtilis* (natto) Takahashi was purchased as a natto starter from Yuzo Takahashi laboratory Co., (Yamagata, Japan). Nutrient agar and nutrient broth medias for preparing *B. subtilis* (natto) Takahashi were purchased from Difco Laboratories (Detroit, MI, USA). L-3,4-dihydroxyphenylalanine (L-DOPA), N-Suc-(Ala)3-p-nitroanilide (SANA), Tyrosinase from mushroom (EC 1.14.18.1), and elastase from porcine pancreas (EC 3.4.21.36) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrafiltration system (Romicon) used for fractionation (Koch membrane systems, USA).

Methods

Preparation of protein hydrolysates from SSF-HDRB

The extract of water-soluble protein fractions and peptides (WSPP) was prepared from HDRB using *B. subtilis* (natto) Takahashi under optimized conditions of solid-state fermentation as described in previous chapter (chapter 3).

Determination of protein content

The protein content of the fractions was quantified using the Pierce Rapid Gold BCA Protein Assay Kit (A53225, Thermo Fisher Scientific) based on Bicinchoninic acid (BCA) method. Bovine serum albumin was used to establish the standard curve.

Fractionation of WSPP of SSF-HDRB by ultrafiltration

The Fractionation process was done using an ultrafiltration system equipped with a Romicon hollow-fiber ultrafiltration cartridge (1" diameter, polysulfone membrane) from (Koch membrane systems, Wilmington, MA, USA). The WSPP of SSF-HDRB samples were subjected sequentially through ultrafiltration membrane columns. Three columns with different molecular weight cut off (MWCO) membranes (50 KDa, 10 KDa, 5 KDa) were used for the fractionation of the sample. The retentate from each filtration step was subjected to the diafiltration (DF)

process with two volumes of dH2O. Then, the permeates from ultrafiltration (UF) and diafiltration steps were pooled and passed through columns with MWCO 10KDa and 5KDa, respectively. The permeates from MWCO 5KDa membrane column and the retentates from each MWCO membrane column were freeze-dried. The molecular weight ranges of the final obtained protein and peptide fractions were >50 KDa, 50-10 KDa, 10-5 KDa, and <5KDa fraction. The fractions were stored at (4°C) and used for evaluation of anti-tyrosinase, and anti-elastase activities of the WSPP of SSF-HDRB fractions.

Elastase inhibitory activity of Fractions

Elastase inhibitory activity of WSPP of SSF-HDRB, HDRB, and SSF-HDRB fractions was determined spectrophotometrically following the method described by (Abd Razak et al., 2017) with minor modifications. In this method, the activity of porcine pancreatic elastase was determined through monitoring the release of *p*-nitroaniline from the substrate, *N*-Succ-Ala-Ala-Ala-*p*-nitroanilide (SANA), for 20 min at 25°C and measuring the absorbance of the reaction mixture at 410nm using a microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA). The reaction mixture was first prepared by mixing 100 µL of 1mg/mL sample (without normalizing peptides content), 100 µL of 0.1 M Tris buffer (pH 8.0), and 10 µL of elastase (0.5U/mL in Tris buffer). After preincubation of the mixture for 10min at 25°C, 50 µL of 10mM SANA was added, and the mixture was incubated for 20 min at 25°C. Then, the absorbance of the mixture was measured at 410nm. Phenylmethylsulfonyl fluoride (PMSF) was used as a positive control at concentration of (0.5mM). The inhibition of elastase was calculated using the following equation:

$$\text{Elastase inhibition activity (\%)} = \left[\frac{Abs.control - Abs.sample}{Abs.control}\right] * 100$$

Abs. control and *Abs. sample* were the absorbance at 410 without and with the sample.

Tyrosinase inhibitory activity of Fractions

Tyrosinase inhibitory activity of WSPP of SSF-HDRB, HDRB, and SSF-HDRB fractions was determined spectrophotometrically following the method described by (Abd Razak et al., 2017) with minor modifications. The activity of tyrosinase was determined through measuring the formation rate of dopachrome using L-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate. To each well of 96- well microplate, 100 µL of 1 mg/mL sample (without normalizing peptides content), 50 µL of 50 mM phosphate buffer pH6.8, and 50 µL of mushroom tyrosinase (10units) were added. After incubation of the mixture for 10 min at 25°C, 50 µL of 10mM L-DOPA was added, and further incubated for 20min at 25°C. The absorbance of the reaction mixture was measured at 470nm using a spectrophotometric microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Kojic acid was used as a positive control at concentration of (3mM). The following equation was used to calculate the tyrosinase inhibition activity:

$$\text{Tyrosinase inhibition activity (\%)} = \left[\frac{Abs.control - Abs.sample}{Abs.control}\right] * 100$$

Abs. control and *Abs. sample* were the absorbance at 470 without and with the sample.

Separation of the highest bioactivity <5KDa ultra-membrane filtered fraction

To determine the molecular weight distribution of the protein hydrolysates and peptides having the highest bioactivity (<5 KDa) among the fractions of ultra-membrane filtration process, size exclusion chromatography (SEC) was used with preparative HPLC system LC-8A (Shimadzu, USA) on a Superdex peptide 10/300 GL column (10x300 mm, 13um) from (GE Healthcare, NJ, USA) based on the method of (Moayedi et al., 2017). An aliquot of 100 µL from sample solution were injected into the column after filtering through 0.22 µm syringe microfilter. The mobile phase of separation was PBS (0.05 M phosphate buffer, 0.15 M NaCl, pH 6.8) The

proteins and peptides were eluted at a flow rate of 0.5 mL/min and monitored by ultraviolet detection at 215 and 280 nm. At different retention times 8 fractions (F1-F8) were collected, pooled, and lyophilized. All the fractions were subjected to dialysis against dH_2O for desalting process using a SpectraPro Tube-A-Lyzer dialysis dynamic device (MWCO 100-500Da, 23x2.2 cm L.D.) from (Spectrum medical industries. Inc., LA, US). The fractions were assayed for elastase and tyrosinase inhibition activities following the same method described earlier.

Statistical analysis:

The experimental data was statistically analyzed using JMP®, a statistical program, (Ver.15.1. SAS Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) was performed for comparison of means with statistical significance at level $p < 0.05$. All the experiments were performed in triplicate determinations. The results are presented as means ±SD.

Results and Discussion

Fractionation by ultrafiltration membrane

Three columns with ultrafiltration (UF) membranes having molecular weight cut-off of 50 KDa, 10 KDa and 5 KDa were used for the fractionation the WSPP of SSF-HDRB and HDRB. The final permeates and the retentates from each column were collected. The four specific peptide fractions of sizes <5kDa, 5-10kDa,10-50kDa, and >50KDa for fermented HDRB extract were collected to be tested for the inhibitory activity of aging-related enzymes, elastase and tyrosinase.

Elastase inhibition activity of ultrafiltration fractions

Figure 4.1 represents the *in vitro* elastase inhibition activity of WSPP of SSF-HDRB ultrafiltration fractions, SSF-HDRB, and HDRB. The results showed that optimized SSF using

Bacillus subtilis (natto) Takahashi enhanced elastase inhibition activity of WSPP of SSF-HDRB in comparison of that from unfermented HDRB. Furthermore, after UF fractionation, the highest inhibitory activity against elastase was shown by the peptides fraction <5KDa (12.8% ±1.1) at (1mg/mL) which was higher about 2.4 and 16 folds than the activity of unfractionated WSPP of SSF-HDEB (5.2%±0.6) and HDRB (0.8%±0.1) at the same concentration, respectively. However, the inhibition activity of the UF fractions in general was vastly lower than that of the positive control, phenylmethylsulphonyl fluoride (PMSF) (72.5% ±2.1 at 0.5 mM). The peptides fraction of size 10-50KDa had the second highest activity of (6.7% ±0.5) toward inhibition of elastase, and the activity was not significantly deferent ($P < 0.05$) than that of unfractionated WSPP of SSF-HDRB. Among the UF fractions, peptides fraction 5-10KDa had the lowest elastase inhibition activity (4.1% ±0.2) with nonsignificant change ($P < 0.05$) from the unfractionated fermented HDRB extract. Fraction with peptides of size >50KDa did not show any inhibition activity. From the result, it can be implied that fermentation and fractionation enhanced the anti-elastase activity of WSPP extracted from HDRB, however the elastase inhibition activity for the fraction <5KDa is weak and the IC_{50} value is high about (4.9 mg/ml). Elastase is the most important enzyme responsible for formation of the skin wrinkling due to degradation of elastin protein in skin dermis. The increase in the elastase inhibitory activity of fermented and fractionated WSPP of SSF-HDRB might be due to the short peptides generated by proteolytic activities of proteases secreted during fermentation by *B. subtilis* (natto) Takahashi, and UF facilitated the concentration of these small size peptides and protein fractions in the peptide pool of sizes <5KDa. The low elastase inhibition activity was also reported in the study of (Hong et al., 2019). In this study, the porcine collagen has been hydrolyzed using Alcalase, and the protein hydrolysates and UF fraction of size <3KDa did not show elastase inhibitor

activity. However, peptides fraction of sizes <3KDa obtained from hydrolysates of Alcalase-hydrolyzed sorghum protein exposed highest elastase inhibition activity (27.2%) at 300µg/mL after inducing of elastase production on human skin cell cultures by irradiation with ultraviolet B *in vitro* (Montalvo-González et al., 2019). In recent studies, chia seed protein hydrolysates with molecular weight <3KDa and fermented salty-shrimp water-soluble protein fractions extract have shown elastase inhibition activity with IC_{50}, the needed concentration of inhibitor to inhibit 50 % of the enzyme under the assay conditions, of (0.43 mg/mL) and (182.8 ±12.38 µg/ mL), respectively (Aguilar-Toalá and Liceaga, 2020; Anh et al., 2020). Since the low elastase inhibition activity of WSPP of SSF-HDRB fraction <5KDa was observed, more separation for this fraction using SEC might enhance the bioactivity of peptide fractions against elastase.

Tyrosinase inhibition activity of ultrafiltration fractions

The tyrosinase inhibition activity of WSPP of HDRB, and UF fractionated and unfractionated SSF-HDRB are shown in (Figure 4.2). The results showed that after solid-state fermentation, the anti-tyrosinase activity of WSPP of HDRB was increased significantly at ($P <$ 0.05) from (10.5% ±0.7) to (43.3% ±2.1) and enhanced more after UF. The peptides fraction <5KDa exhibited the highest tyrosinase inhibitory activity (68.6% ±3.2) at (1mg/mL) among all the UF fractions, and it was represented about (70%) of the kojic acid, a positive control, inhibition activity (99.3% ±0.7) at (3mM). Furthermore, the fraction with peptides of sizes 5-10KDa had (23.9% ±1.7) anti-tyrosinase activity. There was no significant change at ($P < 0.05$) between the inhibition activity of fraction 10-50KDa (8.4% ±1.1) and nonfermented HDRB extract while the fraction of peptides with sizes >50KDa did not have any inhibition activity toward tyrosinase. The highest tyrosinase inhibition activity by the fraction <5KDa with a half-maximal inhibitory concentration (IC_{50}) of 0.9 mg protein/mL was linked to the amount of the

small molecular weight of the peptides as shown in (Figure 4.3) that represents the fractionation of <5KDa ultrafiltrate fraction by size exclusion chromatography (SEC) using a Superdex Peptide 10/300 GL gel filtration column having separation range of 7000-800Da. Potential tyrosinase inhibitors have been derived from protein hydrolysates prepared enzymatically from different sources especially by using Alcalase as a protease. Peptide fraction <3KDa obtained from Alcalase hydrolysis of porcine collagen shoed 30.2% anti-tyrosinase activity at concentration of (5mg/mL) (Hong et al., 2019). In another study, peptide fractions have been derived from rice bran albumin showed potential anti-tyrosinase activity, and the IC_{50} value of the fraction activity was 1.31 mg/mL (Kubglomsong et al., 2018) which is higher relatively than that (0.9 mg/mL) obtained in this study. Furthermore, peptide fraction of sizes 3-10KDa obtained from UF of squid (*Todarodes pacifcus*) skin collagen hydrolysates presented 39.65% inhibition of tyrosinase activity at (1 mg/mL) (Nakchum and Kim, 2016). While it was reported that rice protein extracted from rice flour and hydrolyzed with hydrolytic protease (not mentioned) inhibited 50% of tyrosinase at protein hydrolysates concentration of 107.6 mg/mL (Chen et al., 2021).

Consequently, peptides fraction <5KDa having highest inhibition activity toward elastase and tyrosinase was chosen for additional separation using SEC which might determine the different elastase and tyrosinase inhibition levels and peptide fractions.

Bioactivity of SEC fractions

To distinguish the peptides related with the inhibition of elastase and tyrosinase, the peptides fraction of size <5kDa was further separated using size exclusion chromatography (SEC). Based on the eluted peaks, eight fractions numbered F1-F8 were obtained (Figure 4.3).

Each fraction was collected and further evaluated for their abilities to inhibit elastase and tyrosinase activities.

Although, all the eluted fractions collected from SEC as shown in (Figure 4.4) exhibited anti-elastase activity except fraction F7 and F8, fraction F5 had the highest elastase inhibitory activity (13.1%±0.9) at (1 mg/mL) which was not significantly ($P < 0.05$) different from the activity of unfractionated UF peptide fraction <5KDa (12.8%±1.1). The rest of the fractions had anti-elastase activity ranged between (2.1%±0.3) for F1 and (9.7%±1.0) for F4. Furthermore, the inhibition bioactivity of all fractions was significantly ($P < 0.05$) lower than that of positive control, PMSF, (75.2%±1.5, at 0.5mM).

However, tyrosinase inhibition activity (Figure 4.4) was noted in all SEC fractions with the highest activity of F5 (82.3%±2.2) followed by F4 (79.1%±1.5). The anti-tyrosinase activity of these two fractions was significantly ($P < 0.05$) higher than UF peptides fraction of sizes <5KDa (68.4%±2.9), and represented about 83% of positive control, kojic acid, inhibition activity (98.5%±1.1 at 3mM). Fractions, F2, F6, F3, F8, and F7 showed (42.4% ±2.4), (31.4%±1.1), (27.6%±1.5), (19.5% ±1.6), and (15.2% ±0.6) of tyrosinase inhibition activity, respectively, but their activities were significantly ($P < 0.05$) lower than that of unseparated UF fraction <5KDa. In SEC separation, later eluted fractions contain smaller sizes peptides. Hence, the fractions (F4 and F5) showed highest anti-tyrosinase activity (Figure 4.4). The smaller size peptides produced by SSF are potent in inhibiting tyrosinase and enhancing skin lighting.

The anti-tyrosinase activity of the peptide fractions obtained from WSPP of SSF-HDRB may attributed to the amino acid profile of these peptides. It was reported that the peptides with potential tyrosinase inhibition activity contain uncharged, polar residues including cysteine and serine (Schurink et al., 2007). given that, the glutelin and albumin fractions of rice bran proteins

have been stated to contain higher amount of uncharged, polar amino acids and sulfur-containing amino acids (Wang et al., 2014), respectively, this could be associated with the tyrosinase-inhibition activity of WSPP of SSF-HDRB peptide fractions which are generated from degradation of the glutelin and albumin fractions during SSF due to secreted proteases by *B. subtilis* (natto).

The SEC fractions from WSPP of HDRB, F5 and F4, which exhibited higher tyrosinase activity, will be further investigated and purified to obtain single peptide using reverse phase HPLC.

Conclusions

In conclusion, water-soluble protein hydrolysates and peptides prepared by solid-state fermentation of HDRB using *B. subtilis* (natto) Takahashi under optimized conditions showed greater elastase and tyrosinase inhibition activity than non-fermented HDRB. Fractionation of WSPP of SSF-HDRB using Ultrafiltration and SEC separated peptide fractions with remarkable inhibition of tyrosinase activity but not elastase activity. The most active separated fraction of WSPP of SSF-HDRB exhibited tyrosinase-inhibition close to the effect of kojic acid, the Known tyrosinase inhibitor used in cosmetic products. Thus, heat-stabilized defatted rice bran has a potential for serving as a functional material in the production of nutraceutical and cosmeceuticals products produced by using probiotics, *B. subtilis* (natto), in SSF that increased the cosmeceutical activities of HDRB extract toward inhibition of tyrosinase.

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Tables and Figures

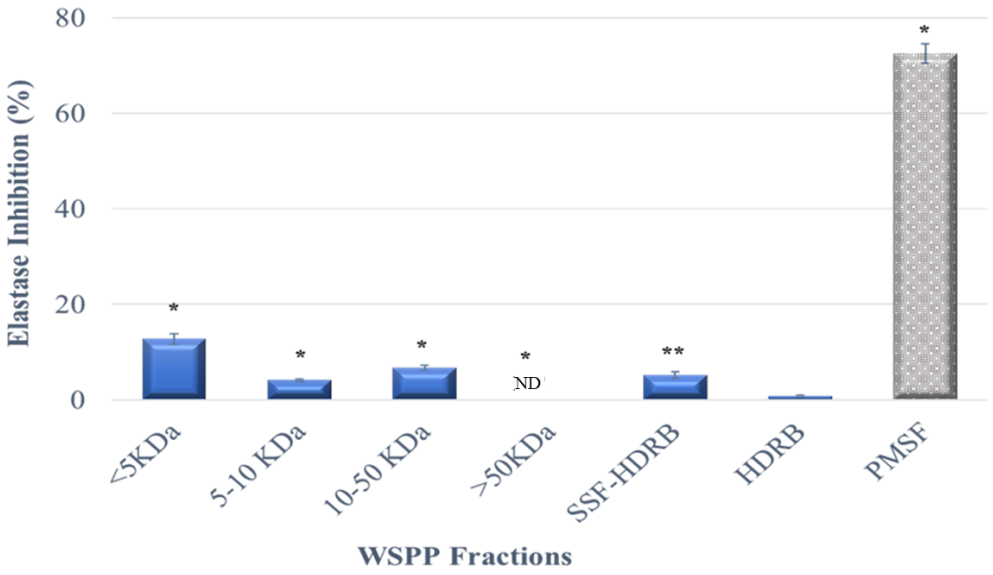

Figure 4.1 The elastase inhibition activity of water-soluble protein and peptides (WSPP) of SSF-HDRB and HDRB, and WSPP ultrafiltration membrane fractions of SSF-HDRB (1mg/mL, without normalizing peptides content). SSF-HDRB: Solid-state fermented HDRB. HDRB: Unfermented HDRB. PMSF: Phenylmethylsulfonyl fluoride (0.5 mM), positive control. ND: Not detected. Bars represent means ± standard deviation. (*) and (**) indicate the significant change at ($p<0.05$) compared to SSF-HDRB and HDRB, respectively.

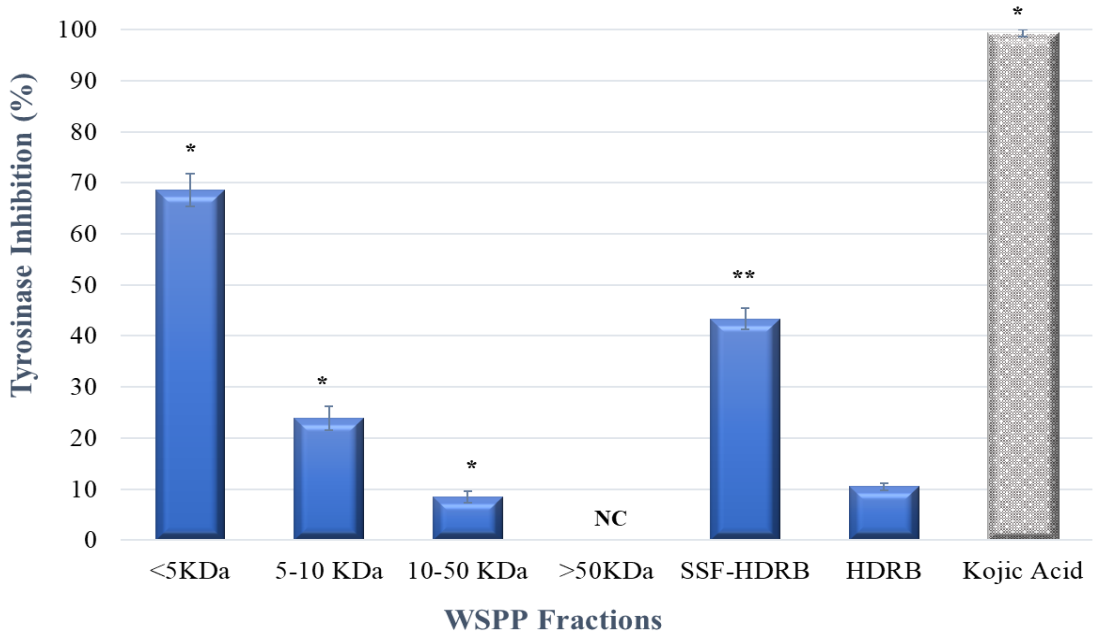

Figure 4.2 The tyrosinase inhibition activity of water-soluble protein and peptides (WSPP) of SSF-HDRB and HDRB, and WSPP ultrafiltration membrane fractions of SSF-HDRB (1mg/mL, without normalizing peptides content). SSF-HDRB: Solid-state fermented HDRB. HDRB: Unfermented HDRB. Kojic acid: Positive control (3mM). ND: Not detected. Bars represent means ± standard deviation. (*) and (**) indicate the significant change at ($p<0.05$) compared to SSF-HDRB and HDRB, respectively.

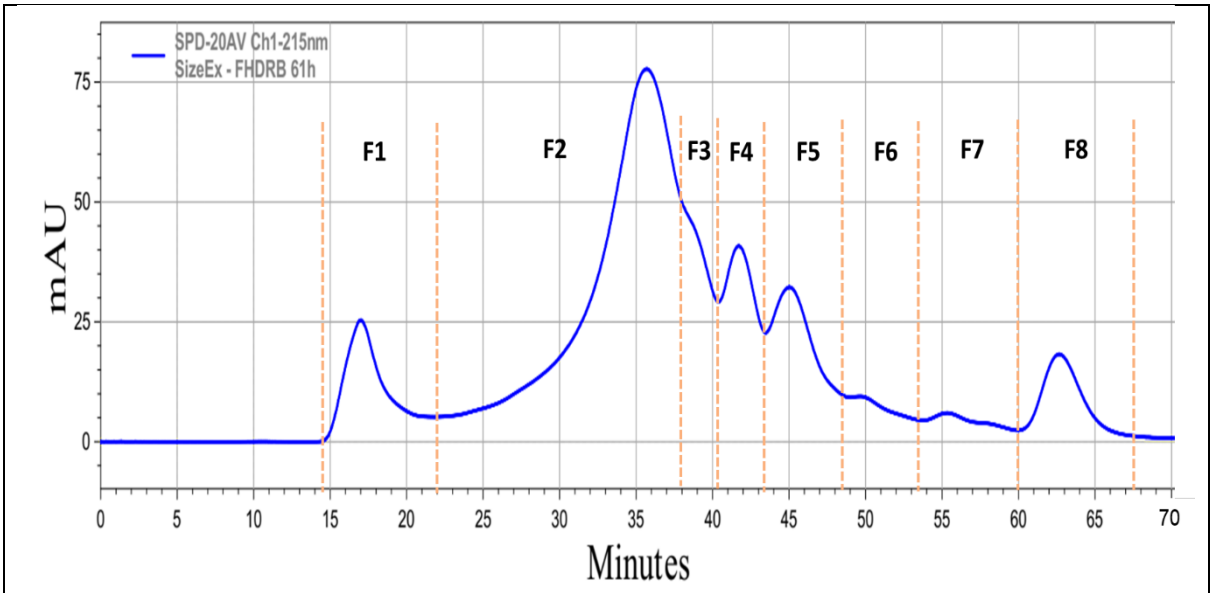

Figure 4.3 Separation of <5KDa water-soluble protein and peptides (WSPP) fraction of SSF-HDRB on a Superdex Peptide 10/300 GL gel filtration column, elution chromatogram. F1-F8 represent the collected fractions.

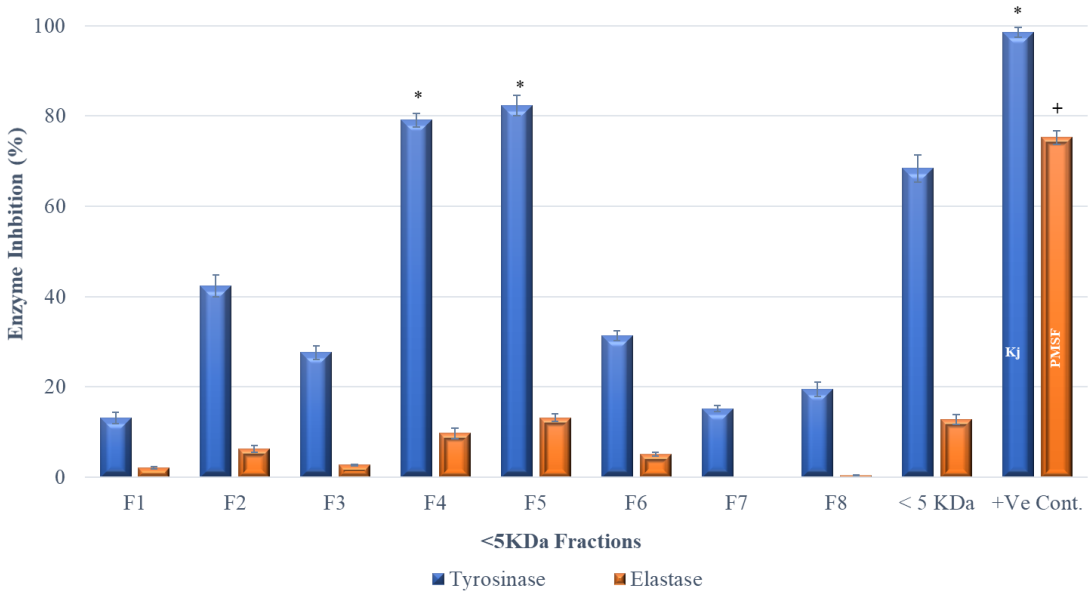

Figure 4.4 The tyrosinase and elastase inhibition activities of <5KDa size exclusion chromatography fractions (F1-F8) (1mg/mL, without normalizing peptides content) of WSPP from SSF-HDRB. +Ve Cont.: Positive control, Kj: Kojic acid (3mM), PMSF: Phenylmethylsulfonyl fluoride (0.5 mM). Bars represent means ± standard deviation. (*) and (+) represent significant increase at (P<0.05) in the tyrosinase and elastase inhibition activities, respectively compared to <5KDa.

CHAPTER 5

Purification and characterization of peptides from the highest anti-tyrosinase peptide fraction, evaluation of melanogenesis inhibition activity, cytotoxicity, and amino acid sequencing of the peptides

Abstract

Peptides with sequences of specific amino acids are considered to act as modulators in biological systems. Peptides under investigation requires characterization which include separation, purification, and identification of the peptide basic components. This will facilitate bioactive peptides to be introduced into functional and nutraceutical products that can play potential roles in decreasing different malfunctions in human body.

The data from previous study indicated that peptides fraction of size <1KDa separated from water-soluble protein fractions and peptides (WSPP) of solid-state fermented heat-stabilized defatted rice bran (SSF-HDRB) using size exclusion chromatography (SEC) demonstrated highest tyrosinase inhibition activity *in vitro*. Further characterization of the peptide fraction (<1KDa) was conducted to investigate pure peptide(s) with enhanced or similar tyrosinase inhibition properties.

Purification by reverse phase HPLC using peptide-specific column was performed to obtain pure peptide(s) from the 1kDa fraction, and 42 peptide fractions were collected and their bioactivity toward tyrosinase inhibition was evaluated. It was observed that three fractions, namely F8, F14, and F40, showed enhanced anti-tyrosinase activity of IC_{50} values of (870.3, 445.8, and 187.6 µg/mL) respectively with dose dependent inhibition. Furthermore, the three fractions showed potential inhibition of melanogenesis activity in mouse B16F10 melanoma cells without cytotoxic effect, and peptide fraction (F40) had the greatest melanogenesis

inhibition activity (84.2% ±1.3) and IC_{50} value (235.2 µg/mL) signifying the potential of F40 as a cosmeceutical agent for treatment of melanin-related skin disorder.

The analysis of the peptide fraction (F40) by MALDI-TOF-TOF-MS and MS/MS search of rice proteomic database revealed presence of two peptides having molecular masses of (567.290 and 755.388) with amino acid sequences of (TSFTL) and (APDLPML), respectively. The other peptide fractions (F8 and F14) were analyzed and identified as well.

The data of the present study suggest that WSPP of SSF-HDRB is a good source of peptides with tyrosinase inhibition activity and that solid-state fermentation of HDRB with *Bacillus subtilis* (natto) Takahashi efficiently generates these kinds of peptides which have potential for application in the cosmetic industries as a natural tyrosinase inhibitor.

Introduction

Peptides with sequences of specific amino acids are considered to act as modulators in biological systems. Food proteins are potent source for bioactive peptides and several peptides have been isolated and fully characterized by preforming a systematic approach including various steps and methods. Three general steps are involved in the characterization process: separation, purification, and identification of the structural components of the investigated peptides. The separation of bioactive peptides which have been generated after breaking down the innate proteins generally via direct enzymatic hydrolysis or indirect process (fermentation) provides an opportunity to create functional and nutraceutical products that could have beneficial and positive effects (Chai et al., 2020). The number of residues of bioactive peptides ranges between 3 to 16 amino acids, and several food-derived bioactive peptides including cosmeceutical peptides, have been isolated from the hydrolysates of different food proteins. The bioactive peptides incorporated in cosmetic products have effects on the intracellular and

extracellular matrix and cellular components of the skin, act as structural peptides, or modulators for neurotransmitter functions (Lima and Pedriali Moraes, 2018). Several amino acid residues can contribute to the bioactivity properties of the peptides, such as hydrophobic residues (Tyr, Val, or Leu) at peptides' C- and N-terminus, uncharged polar residues (Ser or Thr), and in sequences containing residues (Glu, Asp, His, and Pro) (Hariri et al., 2021). Using bioactive peptides as cosmeceuticals agents have several advantages include lacking immunogenicity, simple regulatory requirements for using in premarket, and their selectivity and involvement in many physiological functions of the skin (Pai et al., 2017).

The purity of these peptides is a crucial criterion. Therefore, purification of peptides needs preparative chromatography used in two steps. The initial purification step of peptides starts by separating the peptides of interest out from a pool of peptides that showed the bioactivity in the whole fraction. In this step, chromatographical separation techniques based on size exclusion, hydrophobic interaction, ion exchange, or affinity interactions are generally utilized. Then an efficient purification step is performed using reverse phase HPLC (RP-HPLC) to purify the peptide of interest (Coşkun, 2016; Hettiarachchy, 2013; Kannan et al., 2010). Papain hydrolyzed rice bran Albumin hydrolysates demonstrated higher tyrosinase inhibitory activity and fractionated using reverse phase high performance liquid chromatography (RP-HPLC) into 11 fractions. Fraction 1 exhibited the highest tyrosinase inhibition activity with IC50 value of (1.31 mg/mL), and it contained 13 peptides with MW ranged from 1.3 to 4.8 KDa. The peptide with amino acid sequence (SSEYYGGEGSSSEQGYYGEG) had the most reported residues and features which were related with tyrosinase inhibition and chelating Cu ions activities (Kubglomsong et al., 2018). Further characterization is required to identify the structural constituent of amino acid residues within the bioactive peptide. To achieve that,

proteomic tools can be used, and mass spectrometry (MS) have been employed to characterize various food peptides. Mass spectrometry is a multipurpose tool for determining the accurate molecular weight of the pure peptide and providing its structural information. Mass spectrometry in conjunction with database searching properties plays a significant role in the peptides characterization. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) which is important due to its high sensitivity, very accurate mass detection, rapid analysis for the sample, and analyzing heterogeneous samples and with wide range of masses, such as proteolytic digests. By using MALDI with TOF/MS, the method provides the results of mass/charge ratio (*m/z*) mapping after measuring the ion's flight time toward the detector (Nefedov et al., 2011; Zhang et al., 2010). In addition, tandem mass spectrometry (MS-MS) is very useful in the production of structural information related to a compound. The sample ions are fragmented inside the MS before identifying the produced fragment ions. The structural information of the intact molecule is regenerated by combining the information obtained from the fragmentation after comparing and searching. Further, by tandem mass spectrometry, it is possible to identify and detect specific compounds that exist in complex mixtures based on their characteristic and specific fragmentation patterns (MacMahon et al., 2012).

The objectives of this chapter were 1. Using RP-HPLC for purifying the peptides of the size exclusion chromatographic fractions (F4 and F5) which was separated from water-soluble protein fractions and peptides (WSPP) of solid-state fermented HDRB (SSF-HDRB) and showed higher tyrosinase inhibition activity, 2. Evaluating the bioactivity of RP-HPLC peptide fractions for anti-tyrosinase activity toward melanogenesis inhibition, 3. conducting cytotoxicity study on mouse melanoma cell line (B10F16), and 4. characterizing and identifying the amino acid sequence of these peptides by MALDI-TOF-TOF-MS.

Materials and Methods

Materials

Preparative HPLC system LC-8A and fraction collector FRC-10A from Shimadzu (MD, USA). HP 1050 series analytical HPLC system was from Agilent Technologies (Santa Clara, CA). Bruker Reflex III (Bruker Daltonics GMBH, Bremen, Germany) and Bruker Ultraflex II time-of-flight mass spectrometers at the Statewide mass spectrometry facility, University of Arkansas. Reverse-phase C-18 peptide column, analytical grade, and preparative reverse-phase C-18 peptide column (Supelco DiscoveryBio Wide-pore C-18 Prep ID 22 x L 250 mm part # 34955) were purchased from Sigma Aldrich (St. Louis, MO). L-3,4-dihydroxyphenylalanine (L-DOPA), and Tyrosinase from mushroom (EC 1.14.18.1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse melanoma cell line B16F10 (ATCC CRL-6475) and Dulbecco's modified Eagles's medium (DMEM) were purchased from ATCC (Virginia, USA). Fetal bovine serum was purchased from Atlanta Biologicals (Altlanta, GA, USA). MTT kit was purchased from Promega (USA). All other chemicals purchased were of HPLC grade (Sigma, MO, USA).

Methods

Preparation of protein hydrolysates from SSF-HDRB

The extract of water-soluble protein fractions and peptides (WSPP) was prepared from HDRB using *B. subtilis* (natto) Takahashi under optimized conditions of solid-state fermentation as described in previous chapter (chapter 3).

Determination of protein content

The protein content of the fractions was quantified using the Pierce Rapid Gold BCA Protein Assay Kit (A53225, Thermo Fisher Scientific) based on Bicinchoninic acid (BCA) method. Bovine serum albumin was used to establish the standard curve.

Purification of SEC eluates showing highest anti-tyrosinase activity by RP-HPLC

The combined fractions (F4 and F5) of WSPP from SSF-HDRB having the highest tyrosinase inhibition activity and obtained by separation using size exclusion chromatography (SEC) in the previous chapter (Chapter 4) was subjected to purification of peptides using revers phase high performance liquid chromatography (RP-HPLC). A preparative C18 column (Supelco DiscoveryBio Wide-pore C-18 Prep ID 22 x L 250 mm part # 34955) from Sigma Aldrich (St. Louis, MO) connected to a preparative HPLC system LC-8A (Shimadzu, USA) was used to separate peptides from SEC combined fractions (F4 and F5) and the absorbance of the eluate compounds was monitored at 215nm. The gradient run of mobile phase from solvent B (0.1% TFA in deionized water) to solvent A (0.1% TFA in Acetonitrile (ACN)) was varied as follow: solvent B from 100% to 70% in 45 min, and from 70% to 0% in 15 min at 6ml/min flow rate. The eluted peptides were collected every 1 min using a fraction collector FRC-10A (Shimadzu, USA). The collected peaks were tested for anti-tyrosinase activity, and the peaks that showed over 50% inhibition activity toward tyrosinase were evaluated for anti-melanogenesis activity and fully characterized and sequenced using mass spectrometry.

Tyrosinase inhibitory activity of RP-HPLC fractions

Tyrosinase inhibitory activity of WSPP of SSF-HDRB, HDRB, and SSF-HDRB fractions was determined spectrophotometrically following the method described by (Abd Razak et al., 2017) with minor modifications. The tyrosinase activity was determined by measuring the formation rate of dopachrome using L-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate. To each well of 96- well microplate, 100 µL of 1 mg/mL peptide sample, 50 µL of 50 mM phosphate buffer pH 6.8, and 50 µL of mushroom tyrosinase (10units) were added. After incubation of the mixture for 10 min at 25°C, 50 µL of 10mM L-DOPA was added, and further

incubated for 20min at 25°C. The absorbance of the reaction mixture was measured at 470nm using a spectrophotometric microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Kojic acid was used as a positive control at concentration of (3mM). The following equation was used to calculate the tyrosinase inhibition activity:

$$\text{Tyrosinase inhibition activity } (\%) = \left[\frac{Abs\ C - Abs\ S}{Abs\ C}\right] * 100$$

Where *AbsC* and *AbsS* were the absorbance at 470 without (control) and with the sample.

Anti-melanogenesis activity in cell culture

To evaluate the inhibitory activity of isolated peptides (F8, F14, and F40) toward melanogenesis in cell culture for reduction in melanin production, the mouse melanoma cell line B16F10 (ATCC CRL-6475) was used following the method of (Ochiai et al., 2016b) with minor modifications. The culture of B16F10 melanoma cells were prepared in high glucose Dulbecco's Modified Eagle's Medium (DMEM) which was supplemented with fetal bovine serum (FBS) at final concentration of (10% v/v) and incubated in a humidified atmosphere containing 5% CO2 at 37°C. After the cell culture reached the confluent stage (70-80%), the cells were detached from culturing dish by using 0.25% trypsin and 0.5 mM of ethylenediamine tetra acetic acid (EDTA) solution. Then, B16F10 melanoma cells were used at density of $1x10^5$ cells/well in 6-well culture plate and allowed to attach by incubating for 24 h in the growth media. After replacing the growth medium with 3 mL of fresh medium, 100 µl of varying concentrations (125, 250, 500, 750, 1000 µg/mL) from each purified tyrosinase inhibitory peptide (F8, F14, F40) was added into a well, and incubated under the same conditions mentioned above for 72 h. For positive and negative controls, kojic acid and saline were used, respectively. At the end of the incubation time, the adherent cells were washed with cold and sterilized phosphate buffer,

dissociated by treating with 0.75 mL of 0.25% trypsin/0.5 mM EDTA solution and pelleted by centrifugating (500xg, 5min) and used for melanin content determination.

Determination of melanin contents

The content of melanin of the cells after treatment with purified tyrosinase inhibitory peptides was determined following the method of (Arung et al., 2007) with minor modifications. The cell pellets recovered from cell cultures in previous section were lysed using NaOH solution (0.5mL, 1N) for 1.5 h at 60 °C. After centrifuging of lysates at 3500xg for 5 min, the melanin content in the supernatants was determined spectrophotometrically using a micro-plate reader (Bio-Tek, USA) at the absorbance of 420 nm. The results of treated samples and positive control were expressed in percent to the control culture (without sample). Also, the IC50 value, which represent the amount of peptides required to inhibit 50% of melanin production activity in the cell, of purified peptides (F8, F14, and F40) was calculated from the concentration values.

Cytotoxicity Assay

The cytotoxicity of the peptides purified from WSPP of SSF-HDRB was determined by quantifying the viability of B16F10 melanoma cells using [3-(4,5- dimethyl thiazole-2-yl)]-2,5-diphenyl tetrazolium bromide (MTT) dye-based assay. The viable cells reduce MTT reagent to formazan crystals due to the cellular metabolic activity of NAD(P)H-dependent oxidoreductase enzymes. The amount of formazan crystals formed reflects the number of the live cells since the dead cells unable to reduce MTT. The B16F10 melanoma cells were cultured in the complete DMEM medium in 24-well plate and at a density of $1x10^5$ cells/well. After 24 h of incubation at 37 °C and 5% CO_2, fresh complete medium (1 mL) containing different concentrations (125, 250, 500, 750, 1000, and 1500 µl/mL) of each identified peptide was added in the wells after removing the old culture medium from each well. The cell cultures were incubated for 72 h

under the same conditions. Then, the medium was exchanged with fresh complete medium, and 50 µl of MTT reagent (5mg/mL, in PBS) was added into each well. The plates were further incubated for 4 h at 37 °C and 5% CO_2 before removing the media and adding (1 mL) of dimethyl sulfoxide (DMSO) to each well for dissolving the formazan crystals. The absorbance was measured at 570 nm using microplate reader, Epoch, (BioTek, Winooski, VT). Kojic acid was used as positive control, and PBS was used instead of the sample in negative control wells. The percentage of viable cells was calculated as follow:

$$\text{The cell viability } (\%) = \frac{(Abs.S - Abs.b)}{Abs.C - Abs.b} * 100$$

Where *Abs. b*, *Abs. C* and *Abs. S* are the absorbance of blank, Control and the sample, respectively.

Mass spectrometry characterization of pure peptide

For preliminary intact mass determination, 1.0 µl of each HPLC purified peptide was mixed with 1.0 µl saturated alpha-Cyano-4-hydroxycinnamic acid (HCCA) and spotted on Bruker MTP 384 ground stainless steel MALDI target. MALDI-TOF-TOF (time of flight) mass spectra were acquired on a Bruker Reflex III (Bruker; Billerica, MA, USA) time-of-flight mass spectrometers operated in the positive-ion reflectron mode.

Protein/peptide database searching

Tandem mass spectra were extracted from data file. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK). Mascot was searched the National Centre for Biotechnical Information (NCBI) database (selected for *Oryza Sativa*, unknown version, 135619 entries) assuming the digestion enzyme was Chymotrypsin. Mascot was searched with a fragment ion mass tolerance of 0.5 Da.

Statistical analysis:

The experimental data was statistically analyzed using JMP®, a statistical program, (Ver.15.1. SAS Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) was performed for comparison of means with statistical significance at $p < 0.05$. All experiments were performed in triplicate determinations. The results are presented as means ±SD.

Results and Discussion

Isolation of Tyrosinase Inhibitory Peptides.

The separation of WSPP from SFF-HDRB using size exclusion chromatography (SEC) done in the previous chapter revealed two fractions (F4 and F5) which showed higher activities for inhibition of tyrosinase, so further purification of these fractions after combining them was conducted using RP-HPLC with C18 column to obtain more purified peptide fractions. In (Figure 5.1) which represents RP-HPLC chromatogram of eluted peptides from the combined fractions (F4 and F5) of SEC separation, several fractions of eluted peptides were separated, collected, and designated as (F1-F42). After evaluation of the anti-tyrosinase activity of all the collected peptide fractions, three fractions (F8, F14, and F40) showed significant tyrosinase inhibition activity (over 50%) at (1mg/mL) (Figure 5.2). The isolated peptide fraction (F40) showed the highest anti-tyrosinase activity (81.7% ±3.6) followed by (F14) and (F8) with activities of (71.8% ±3.3) and (55.5% ±2.4), respectively. The inhibition activity of the positive control, kojic acid, was (96.2% ±1.6). Tyrosinase inhibition activity was not detected in 23 of eluted fractions, while the rest of the peptide fractions showed lower than 30% anti-tyrosinase activities. The isolated peptides (F8, F14, and F40) had different IC_{50} inhibitory response (870.3, 445.8, and 187.6 µg/mL) which obtained by logarithmic equation generated using different concentrations of the peptides (0.5-1.5 mg/mL) under the same experiment conditions (Figure

5.3). The three isolated peptides (F8, F14, and F40) were chosen to be tested on melanoma cell culture for melanogenesis inhibition activity and their cellular cytotoxicity.

Melanogenesis inhibitory activity and melanin contents of Isolated Peptides

Melanoma cells have been used as a model for evaluation of melanogenesis inhibitory activity of several compounds including peptides. Assay of melanin content in mouse B16F10 melanoma cells was used to determine the effects of peptides F40, F14, and F8 on melanogenesis activity. All the peptides showed dose response inhibition of melanogenesis activity by reducing melanin production in B16F10 melanoma cells, and the highest reduction was observed by peptide (F40) (84.2% ±1.3) at (1000 µg/mL) while the other peptides (F14 and F8) showed (64.1 % ±3.1 and 57.9 %±2.7), respectively with IC_{50} values of (235.2, 591.6, and 565.8 µg/mL) of (F40, F14, and F8), respectively. In comparison with the inhibition activity of kojic acid as a positive control, peptide (F40) was as effective as the kojic acid (Figure 5.4). Although, peptides F14 and F8 were less effective on melanin production than kojic acid, they showed >50% reduction in the melanin content in B16F10 melanoma cells at concentration of (1mg/mL). The results show that the peptides (F14 and F40) bear strong anti-tyrosinase activity better than the protein and peptide fraction with size of (<5kDa) from which these peptides were separated and purified. It is convincing to notice that purification process of a peptide from the pool of peptides of <5kDa obtained from WSPP of SSF-HDRB resulted in identifying the tyrosinase inhibitory component within HDRB. The short length and amino acid type and sequence are the more related to the high activity of peptides toward inhibition of tyrosinase (Bhandari et al., 2020).

Furthermore, during fermentation, innate proteins of HDRB are hydrolyzed due to the proteolytic activity of bacterial proteases producing short peptides with C-terminals having different amino acids including large side chains (F, Y, W) and uncharged residues (L, I, V, P)

(Lampe and English, 2016; Matkawala et al., 2021). An oligopeptide (SSEYYGGEGSSSEQGYYGEG) was purified using reversed-phase liquid chromatography from enzymatically hydrolyzed rice bran albumin and showed tyrosinase inhibition effect with IC_{50} value of 1.31 mg/ml (Kubglomsong et al., 2018). However, oligopeptides are not preferable as bioactive peptide in cosmetics products due to the hurdles of skin penetration and their vulnerability to be hydrolyzed by cellular proteases which can lead to losing the bioactivity (Ochiai et al., 2016b). While the identified peptides in our study are short peptides (4-7 residues), and they observed significant melanogenesis inhibition activities in cell culture environment. This means that the peptides have not impacted with cellular protease enzymes. Fermentation of HDRB and subsequent purification of WSPP extract improve the chemical and bioactivity properties of HDRB proteins and peptides.

Cytotoxicity of Isolated Peptides

The cytotoxicity of the inhibitors was tested by monitoring the cell proliferation and viability of B16F10 melanoma cells after treatment with isolated peptides. None of the isolated peptides in this study showed cytotoxic effect in mouse B16F10 melanoma cells as demonstrated in (Figure 5.5). However, kojic acid at concentration of (1mg/mL) significantly ($p<0.05$) decreased cell viability (15%) in comparison to the control. Overall, the results demonstrate that the three peptides (F40, F14, F8) isolated from WSPP of SSF-HDRB can be safely used as an anti-skin pigmentation agent in skin care products due to their bioactivity to inhibit tyrosinase and melanogenesis in melanoma cell culture without causing any cytotoxicity.

Identification and sequencing of Isolated Peptides

The isolated peptides (F8, F14, and F40) were analyzed using matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-TOF-MS) to identify the

mass (molecular weight) for each isolated peptide. The spectrums of MALDI-TOF-TOF-MS as shown in (Figure 5.6) revealed that the abundant peak in the peptide fraction (F8) had the mass (mass + H^+) of (656.2), while the spectrum of each one of the peptide fractions (F14 and F40) showed two abundant peaks with masses of (660.2 and 822.3) and (568.3 and 756.6), respectively. Then, in the MS/MS analysis and database searching process, the amino acid sequences of these peptides were identified (Table 5.1). Five peptides were identified from the three PR-HPLC isolated peptide fractions based on the most abundant MS peaks in each fraction that have been searched, compared, and scored as follows: In the MS/MS analysis, the molecular weight (mass) of each peptide experimentally acquired during MALDI-TOF-TOF analysis step was compared to theoretical masses of peptides which have been generated from *in silico* digestion process of *Oryza sativa* proteins or nucleic acid sequences that were included in a UniProt database (https://www.uniprot.org/). After the comparison and selection of the peptides with right masses, calculation of fragment ions for candidate peptides was performed (Figure 5.7). By matching between the experimental spectrum and the MS/MS fragmentation ion, a score of probability and similarity was generated. The best matching peptides were selected based on the highest score obtained after the candidate peptides were scored against the experimental spectrum. Since determining the experimental mass is crucial for identifying the peptide from the database, using MS instrument and technique such as the configuration of time-of-flight is important. From RP-HPLC fraction (F8), one peptide with predicted sequence (SCADGGF) was identified. The corresponding sequences of the abundant MS peaks in peptide fractions (F14 and F40) were (FPLHF and SYVFGCF) and (TSFTL and APDLPML), respectively. All the identified peptides were short peptides having short sequences, and this signifies the activity of proteases excreted by *Bacillus subtilis* (natto) Takahashi during solid-state fermentation.

Furthermore, since subtilisin NAT, an extracellular serine protease, is the main protease secreted by *B. subtilis (natto)* (Ju et al., 2019) and cleaves at C-terminal of aromatic and large nonpolar residues (F, Y, W, L, M), the identified peptides carry phenylalanine (F) or leucine (L). Peptide F8 (SCADGGF) is a part of two unreviewed proteins (genes: H0801D08.5 and H0402C08.15). Peptides identified from F14, (FPLHF) and (SYVFGCF), are a partial five and seven-amino acids sequences of ribosomal protein L5 (gene: rpl5) and another unreviewed protein (gene: H0716A07.10), respectively. Furthermore, the peptides (TSFTL) and (APDLPML) from fraction (F40) are fractional sequences of two uncharacterized proteins (gene: H0613H07.7) and (gene: OSIGBa0148A10.11), respectively.

It was reported that effective tyrosinase inhibitory peptides contain polar and /or uncharged amino acids, such as serine (Schurink et al., 2007). Silk sericin hydrolysate prepared by enzymatic hydrolyzation showed tyrosinase inhibition activity (IC_{50} 10mg/mL) which was related to the high content of serine and threonine (30%) and the proportion of short peptides lower than 1000 Da (65%) (Wu et al., 2008). Likewise, peptides having tyrosinase inhibitory activity showed additional interactions to the enzyme's active site when hydrophobic amino acids present at the peptide's beginning and end parts (Jung et al., 2012). Accordingly, the identified peptides from RP-HPLC fractions of our study (F8, F14 and F40) possess these criteria as shown in their amino acid sequences in (Table 5.1).

In another study, the presence of two proline residues combined with tyrosine and serine in the peptide sequence increased the peptide's effectiveness toward inhibition of tyrosinase activity (Kawagishi et al., 1993). It was reported that the most potent natural peptide having tyrosinase inhibition activity is a cyclopeptide (Gly-Gly-Tyr-Leu-Pro-Pro-Leu-Ser) with IC_{50} value of (50µM) (Hariri et al., 2021; Morita et al., 1994). The amino acid content in the peptide

(APDLPML) identified from the peptide fraction (F40) in this study resembles the amino acid sequence of the cyclopeptide mentioned above. Furthermore, the isolated peptide (F40) demonstrated the highest tyrosinase and melanogenesis inhibition activities with the lowest IC_{50} values (Figures 5.2-5.4) among the three RP-HPLC isolated peptides.

The anti-tyrosinase and anti-melanogenesis activities observed for the identified peptides (SCADGGF, FPLHF, SYVFGCF, and TSFTL) containing serine, histidine, and threonine residues may be due to their copper ion binding capacity as tyrosinase activity depends on the copper ions located in the active site. It was reported that serine binds to copper ions through the oxygen atom of its carboxylate group and nitrogen atom of the amino groups (Mandal et al., 2015), and threonine and serine residues can chelate copper ion by hydroxyl groups of their side chains (Wu et al., 2008).

In addition, the presence of leucine, valine, and alanine (hydrophobic amino acids) were associated with the tyrosinase inhibitory effect of peptides (Karkouch, 2017). Consequently, the existence of specific amino acids (Tyr, Ser, Thr, Val, Ala, Leu, Phe, Trp, and Cys) in the structure of peptides seems to be associated with the significant activity of the peptides toward inhibition of tyrosinase. This may be related with the preferred interactions between these residues and the enzyme active site. For instance, π-π stacking interactions of the aromatic moiety in (Trp, Tyr, and Phe), hydrophobic interactions by aliphatic moiety of (Val, Ala, and Leu), and formation of hydrogen bonding through hydroxyl groups of (Tyr, Ser, and Thr) are the more proposed interaction between amino acids of inhibitor peptides and tyrosinase. Also, the position of the above-mentioned residues in the peptide sequence may play a role in the inhibition activity of the peptides (Hariri et al., 2021).

Conclusion

Heat-stabilized defatted rice bran is an important source of bioactive peptides including tyrosinase and melanogenesis inhibitory peptides which is released from innate proteins after solid-state fermentation using *B. subtilis* (natto) Takahashi, and this process efficiently produced tyrosinase inhibitory peptides. Purification of these inhibitory peptides using RP-HPLC and structural identification by MALDI-TOF-TOF-MS technique revealed five short peptides from three peptide fractions (F8, F14, and F40) with IC_{50} values of (565.8, 591.6, and 235.2 µg/mL) respectively. Furthermore, none of the isolated peptides showed cytotoxic effect in mouse B16F10 melanoma cells, so they can be used safely in cosmetic products.

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Tables and Figures

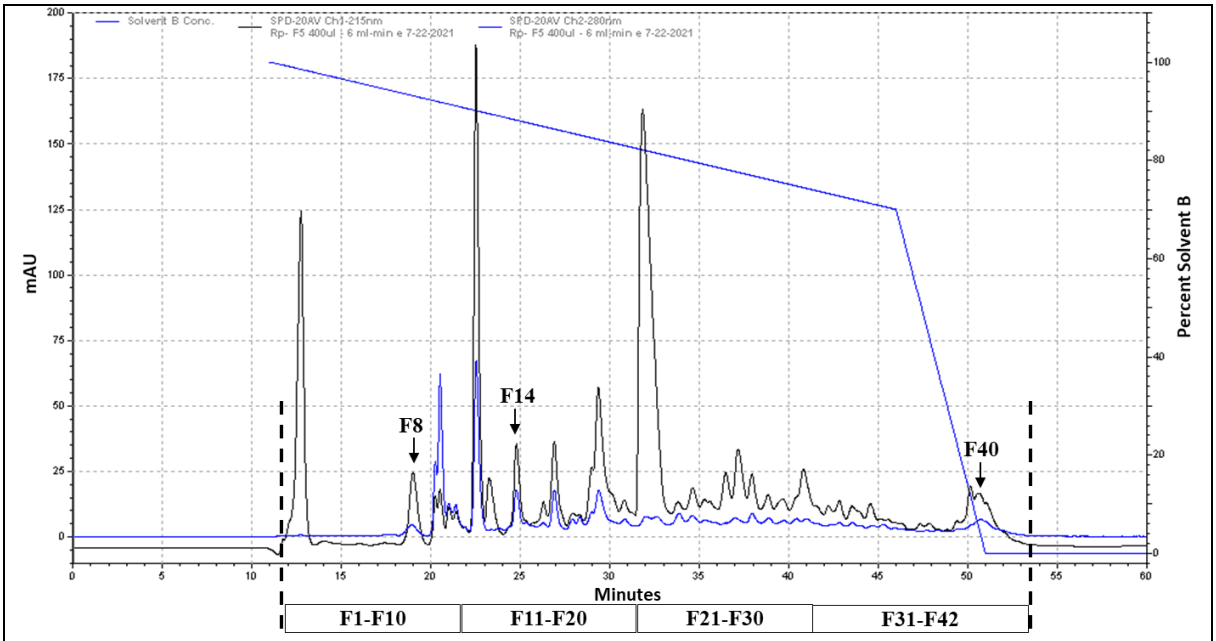

Figure 5.1. Chromatogram of eluted peptides of WSPP from SSF-HDRB (SEC fraction) separated by RP-HPLC using a DiscoveryBio wide-pore C18 column. F1-F42 denotes the collected fractions at (1 min base) and shown below the chromatogram. The black and blue peaks represent the absorbance at 214nm and 280nm, respectively. The indicated peaks (F8, F14, and F40) are peptide fractions having tyrosinase inhibitory activity above 50%.

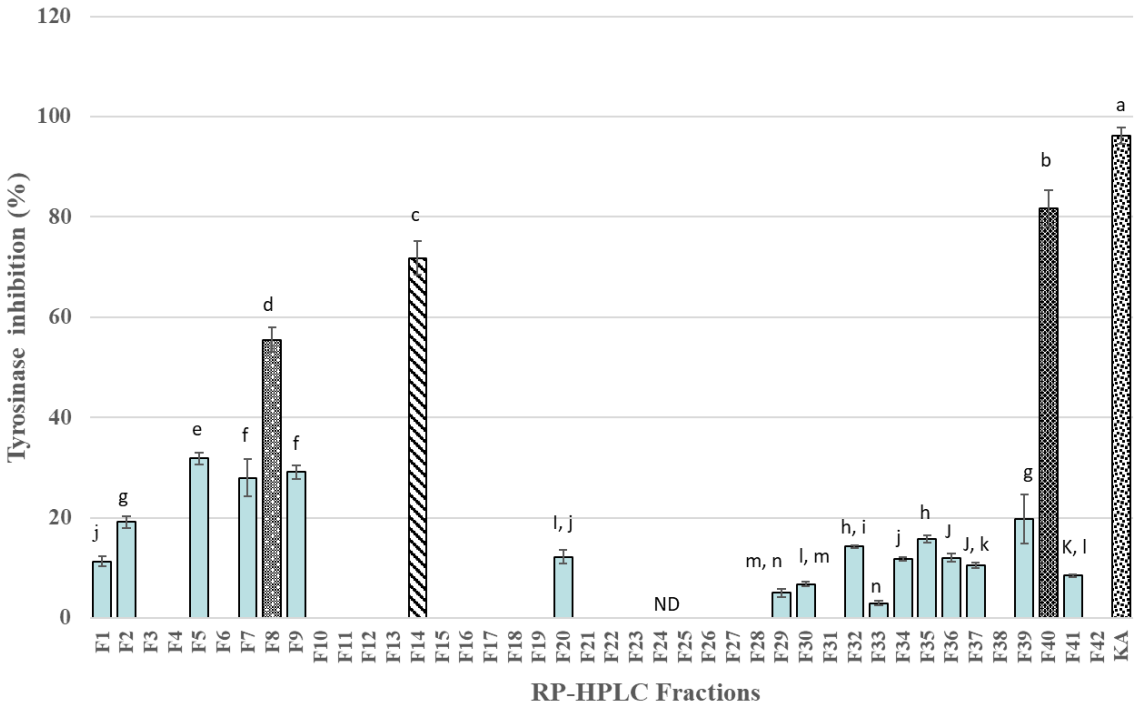

Figure 5.2. Tyrosinase inhibition activity of RP-HPLC separated peptide fractions at (1mg/ml). KA: Kojic acid (positive control). Data represents means of three independent experiments ± SD (standard deviation). Bars bearing same letter indicate non-significant change at ($p<0.05$)

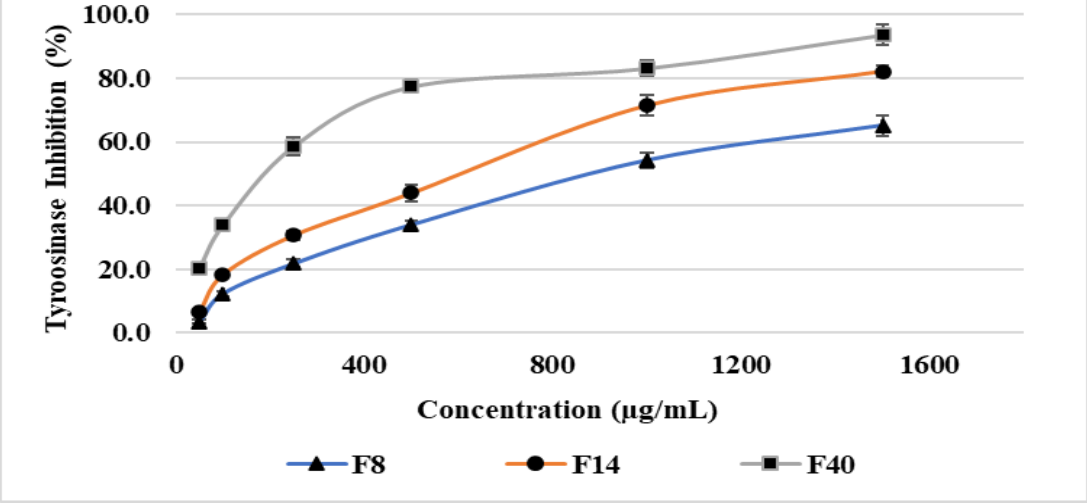

Figure 5.3. Profiles of dose−response effects of RP-HPLC isolated peptides (F8, F14, and F40) on the inhibition of mushroom tyrosinase activity. Data represents means of three independent experiments ± SD with significant difference at p-value ≤ 0.05

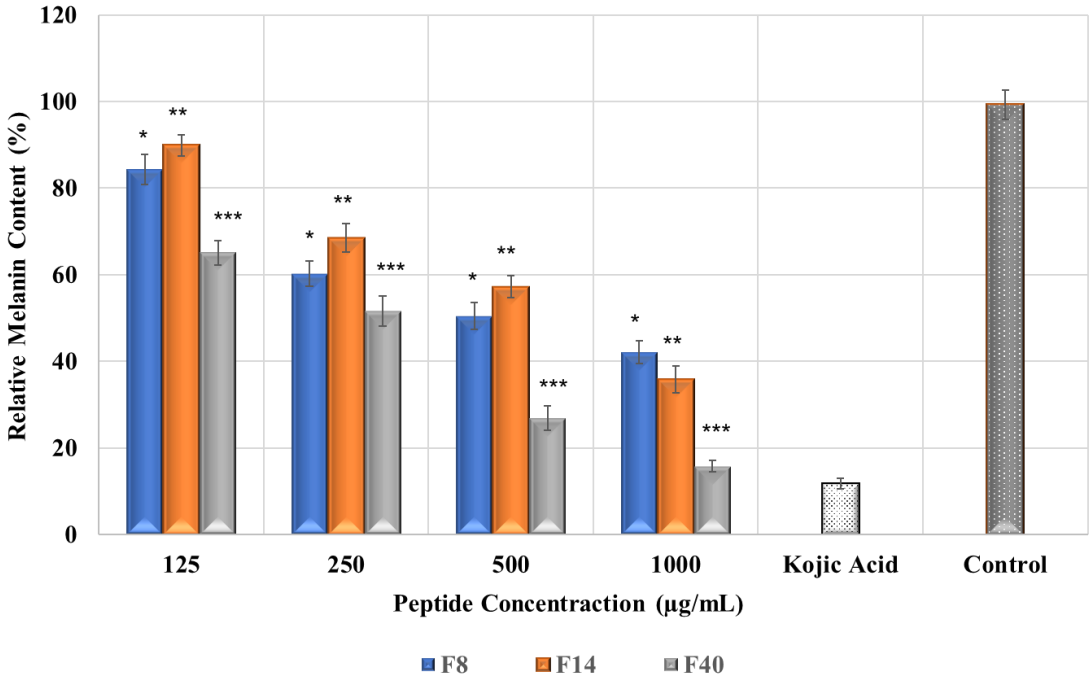

Figure 5.4. Effect of isolated peptides (F8, F14, and F40) on melanin contents in mouse melanoma cells (B16F10). Kojic acid: positive control. Data represents means of three independent experiments ± SD (standard deviation). The mean comparison is between the same sample (F8, F14, or F40) only at different concentrations and (*), (**), and (***) indicate the significant change in the melanin contents at ($p<0.05$) compared to the control.

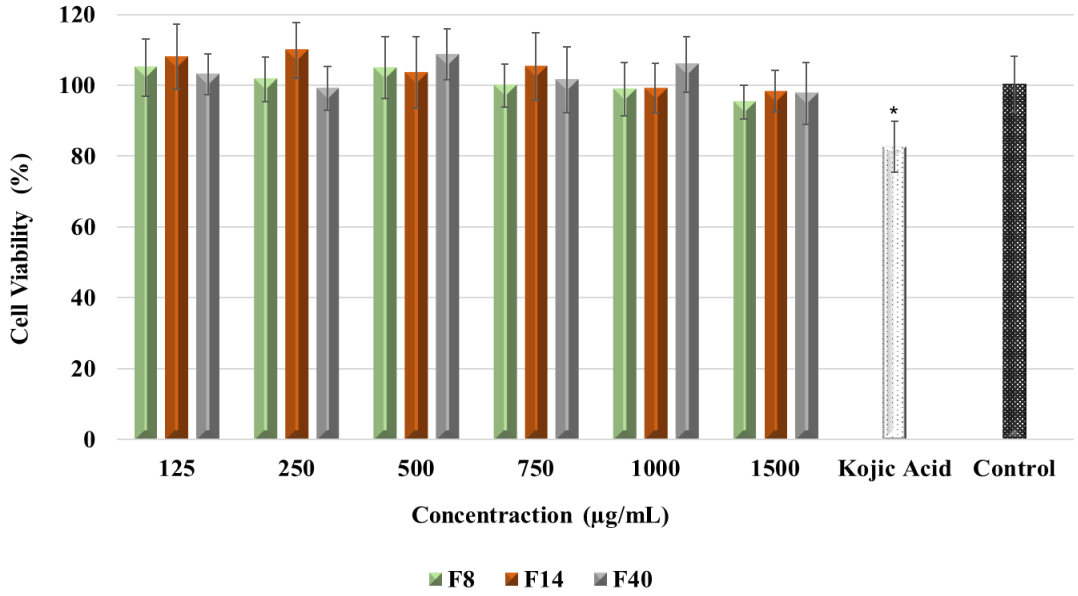

Figure 5.5. Effect of isolated peptides (F8, F14, and F40) on cell viability in mouse melanoma cells (B16F10). Kojic acid: positive control. Data represents means of three independent experiments ± SD (standard deviation). (*) indicates the significant change at ($p<0.05$) compared to the control.

Table 5.1. The highest scored MS/MS searched peptide sequence, mass, and location of the peptide fractions isolated by RP-HPLC from WSPP of SSF-HDRB and identified by MALDI-TOF-TOF-MS

RP-HPLC Fraction	Peptide Sequence MS/MS	Mass (M+H)	Calculated Mass	Score	Gene
F8	SCADGGF	656.2	655.2272	0.4	H0801D08.5 H0402C08.15
F14	FPLHF	660.2	659.3431	10.5	rpl5
	SYVFGCF	822.3	821.3418	15.6	H0716A07.10
F40	TSFTL	568.3	567.2904	6.6	H0613H07.7
	APDLPML	756.6	755.3887	21.5	OSIGBa0148A10.11

RP-HPLC: revers phase-high performance liquid chromatography. WSPP: water-soluble protein fractions and peptides. SSF-HDRB: solid-state fermented HDRB.

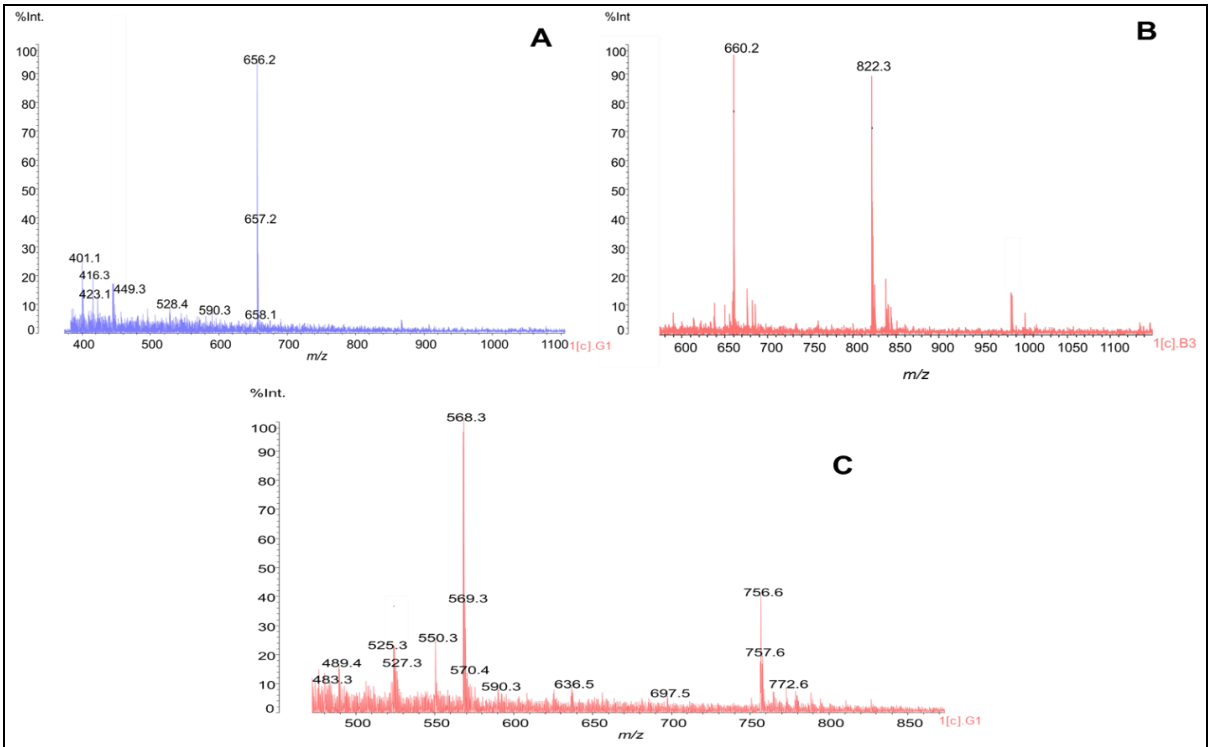

Figure 5.6. MALDI TOF-TOF-MS profile of the RP-HPLC isolated peptides. (A) Spectrum of peptide fraction (F8). (B) Spectrum of peptide fraction (F14). (C) Spectrum of peptide fraction (F40). Y-axis: intensity; X-axis: mass to charge ratio (m/z);

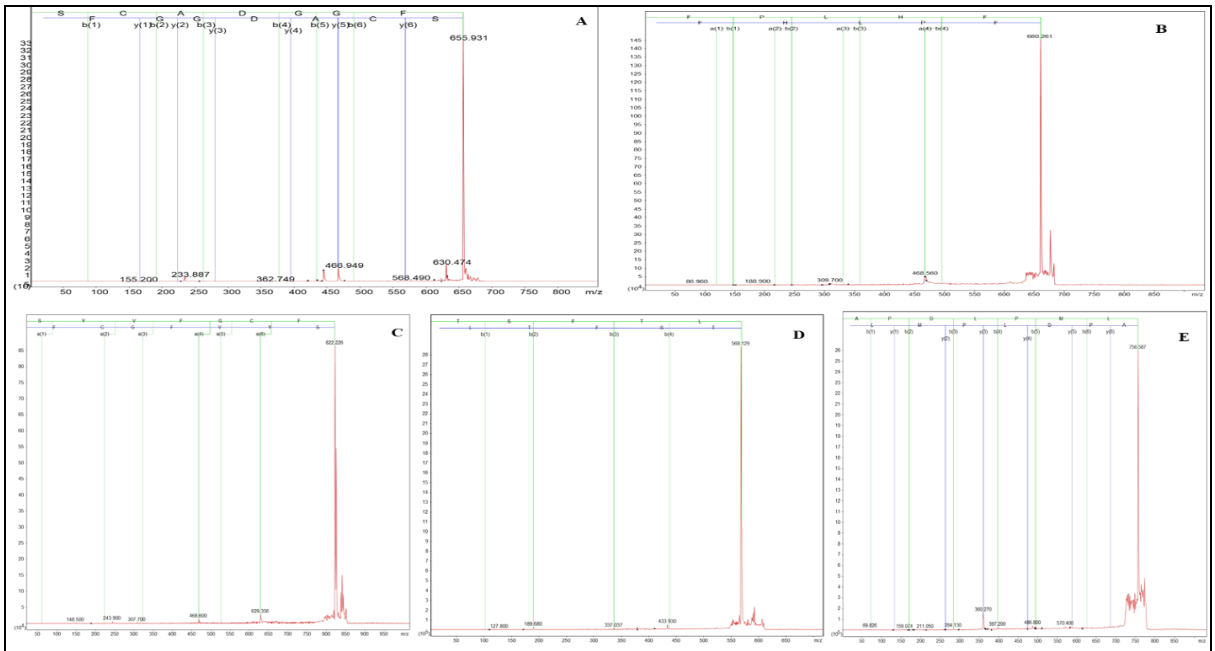

Figure 5.7 Tandem MS-MS fragmentation profile of the candidate peptides by Mascot in the analysis of isolated fractions after searching in rice protein database. (A) Peptide m/z 655.931 Da from F8, sequence: SCADGGF, (B) Peptide m/z 660.261 Da from F14, sequence: FPLHF, (C) Peptide m/z 822.226 Da from F14, sequence, SYVFGCF, (D) Peptide m/z 568.129 Da from F40, sequence, TSFTL, (E) Peptide m/z 756.587 Da from F40, sequence, APDLPML

CHAPTER 6

Antioxidant activities of solid-state fermentation derived proteins and peptides from heat-stabilized defatted rice bran

(Part of the chapter published in the *Journal of American Oil Chemists' Society,* November 2021 with co-authors: Hettiarachchy, NS, Kumar, T.K.S, Lay, JO)

Abstract

Rice bran as an abundant by-product is a valuable source of different nutritious components including proteins which can be a good source of bioactive peptides. Solid state fermentation is an emerged approach to produce bioactive compounds such, as antioxidant peptides. The water-soluble protein fractions and peptides (WSPP) from solid-stat fermented heat-stabilized defatted rice bran (SSF-HDRB) showed significant radical scavenging activities (56, 44.4, and 84.5 %) in comparison to that from HDRB (12.5, 9.3, and 35.5%), of 2–2-diphenyl-1-picrylhydrazyl radical (DPPH) free radical, superoxide radicals, and hydroxyl radicals scavenging activities, respectively. Also, there was a strong positive correlation between the three antioxidant evaluation methods, DPPH, SOS, and HRS, and the content of protein hydrolysates and phenolic compounds. The SSF method is an efficient method to generate protein hydrolysates and peptides from HDRB with activity of scavenging different free radicals and can find application as an ingredient in suitable products. Furthermore, the higher antioxidant activity is indicative of its potential function in controlling the oxidative stability in food products and as potential skin-antiaging component in the cosmetic products.

Introduction

Solid state fermentation has been correlated with the improvement in the bioactivities of plant material including rice bran through increasing of bioactive compounds. Fermentation has been used to increase antioxidant capacity of protein isolate prepared from different sources

including plant proteins and proteins extracted from agricultural by-products (Chai et al., 2020), such as SSF of soybean flours (Fernandez-Orozco et al., 2007), wheat germ extract (Liu et al., 2017), and corn gluten meal (Jiang et al., 2020). Antioxidnt compounds play an important role in skin aging. As a result of the ageing and different environmental stresses including UV radiation exposure, the levels of antioxidant enzymes and internal, cellular, antioxidants suffer from reduction. Thus, accumulation of reactive oxygen species (ROS) becomes the main cause to initiate oxidative stress which advances skin aging characterizing by atypical pigmentation and skin wrinkles when ROS produce in the dermal fibroblasts. Therefore, it is important to replenish the decrease of the antioxidants by using external compounds with antioxidant properties including protein fractions and peptides, in cosmetic formulations as topical applications. This method is considered as an effective mean for antiaging benefits by preventing of the photo-induced aging symptoms and skin aging disorders (Buranasudja et al., 2021; Masaki, 2010).

Solid state fermentation using *B. subtilis* variants showed promising results in term of production protein fractions and peptides with free radical scavenging activities. Soybean flours fermented by *B. subtilis* presented high antioxidant capacity in comparison to non-fermented flour (Fernandez-Orozco et al., 2007), Solid-state fermentation with *B. subtilis MTCC5480* was successfully used to convert corn gluten meal into a product with high quantity of peptides having high in vivo antioxidant capacity (Jiang et al., 2020). Furthermore, *B. subtilis* fermentation of defatted wheat germ (DWG) for 48h resulted in an increase in the content of peptides about 6.5 folds which positively corelated with high antioxidant activity (80 %) of the fermented DWG extract (Liu et al., 2017). Likewise, antioxidant activity effectively can be improved in rice bran by SSF technique. Defatted rice bran (DRB) was fermented using *Grifola frondose* as a starter fungus in SSF, and the water extract of fermented DRB exhibited potential

scavenging activity of hydroxyl radical at concentration of (1mg/ml). also, the FRB extract at 2mg/ml showed 87.8% scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH), a synthetic free radical (Liu et al., 2017). In another study, *Rhizopus oryzae* mediated fermentation of FRB showed up to 50% scavenging activity against DPPH at concentration of 100µL/mL of fermented DRB extract which also had peroxidase inhibition activity (Schmidt et al., 2014). Lactic acid bacteria, *Pediococcus. acidilactici*, was inoculated in DRB, and the phenolic compound extraction of fermented DRB showed 82.6% DPPH radical scavenging activity while scavenging activity of the extracts that obtained from DRB fermented with *Lactobacillus lactis* and *P. pentoseous* were about 77.2% and 71.5% respectively (Abd Rashid et al., 2015).

Solid-state fermentation (SSF) using *B. subtilis* (natto) Takahashi can be an efficient method to generate protein fractions and peptides having radical scavenging activity from HDRB because this microorganism is able to produce variety of enzymes that can degrade cell wall components and proteins and liberate proteins and peptides in a single economically viable process. This study aimed to evaluate the antioxidant bioactivities of the bacterial assisted extraction of protein and protein hydrolysates from heat-stabilized defatted rice bran using *B. subtilis* (natto) Takahashi in solid-state fermentation, and demonstrate the correlation between phenolic compounds, protein hydrolysates, and their antioxidant activities of the fermented HDRB extracted broth.

Materials and Methods

Materials

Heat-stabilized defatted rice bran (HDRB) from Arkansas rice variety was supplied by Riceland Foods, Inc. (Stuttgart, AR, U.S.A.) that was subjected to heat stabilization at (~120 °C) through steam expander prior to the oil extraction. *B. subtilis* (natto) Takahashi was purchased as

a natto starter from Yuzo Takahashi laboratory Co., (Yamagata, Japan), and 2–2-diphenyl-1-picrylhydrazyl radical (DPPH) was purchased from Alfa Aesar (Ward Hill, Mass., U.S.A.). Phenanthroline and pyrogallol were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA). All the other analytical-grade chemicals and supplies were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Sigma Chemical Co. (St. Louis, MO, USA).

Methods

Preparation of protein hydrolysates from SSF-HDRB

The extract of protein fractions and peptides was prepared from HDRB using *B. subtilis* (natto) Takahashi under optimized conditions of solid-state fermentation as described previously in chapter three.

Determination of protein content

The protein content of the supernatants was quantified using the Pierce Rapid Gold BCA Protein Assay Kit (A53225, Thermo Fisher Scientific) based on Bicinchoninic acid (BCA) method. Bovine serum albumin was used to establish the standard curve.

Determination of total phenolic content of SSF-HDRB extract.

The total phenolic content in the supernatant of the SSF-HDRB and HDRB samples were determined by the Folin–Ciocalteu method as described by (Ainsworth and Gillespie, 2007) with minor modifications. The diluted extract solution (0.2 mL) was reacted with 0.8 mL of 0.2N Folin–Ciocalteu reagent for 5 min. Then, 2 mL of 0.7 M sodium carbonate solution was added to the solution and incubated in the dark for 2h at ambient temperature. The absorbance was determined at 765 nm using a Shimadzu UV-1601 spectrometer (ShimadzuInc., Kyoto, Japan). Since the ferulic acid is the main phenolic in the rice bran, the results were expressed as mg Ferulic acid equivalents (FAE) per 100 g HDRB on dry basis using Ferulic acid standard curve.

Determination of antioxidant activities

The WSPP from SSF-HDRB and HDRB were evaluated for free radical scavenging activities utilizing the following methods:

a) DPPH radical scavenging assay

This method determines the proton radical-scavenging activity of antioxidant compounds by donating protons to electron deficient free radicals. The method of (Tang et al., 2010) with some modification was used to determine the DPPH• scavenging activity. The WSPP from SSF-HDRB or HDRB (100 µL of 0.5% w/v of extract) was added to 100 µL of a 100 mM DPPH solution in a 96-well microplate. After incubation in dark at 37°C for 30 min, the decrease in absorbance of each solution was measured at 517 nm using UV-Vis. microplate reader spectrophotometer (Epoch, Bio-Tek Instruments, Inc., VT, USA)). Absorbance of blank sample containing the same amount of dH_2O and DPPH solution was prepared and measured. 100 µL Glutathione (GSH) at 1mg/mL concentration was used as positive control. Radical scavenging activity was calculated by the following formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(Ab - As)}{Ab}\ x\ 100$$

Where, (*As*) absorbance of samples and (*Ab*) absorbance of blank.

b) Superoxide radical scavenging activity:

This method determines the ability of WSPP in the SSF-HDRB and HDRB extracts to remove superoxide radicals, a type of reactive oxygen species (ROS) produced from a normal biological process, due to cellular defense mechanisms. Excess production of these radicals triggers various diseases related to DNA mutations as well as cellular and genome instability (Sarmadi and Ismail, 2010). Superoxide radical ($O_2^{\bullet -}$) scavenging activity was determined spectrophotometrically by observing the inhibition of the autoxidation rate for the assay's

reagent, pyrogallol, based on method of (Alrahmany and Tsopmo, 2012) with slight alterations. The WSPP from SSF-HDRB and HDRB (80 µL of 0.5% w/v of the extract in 50 mM Tris-HCl Buffer-EDTA 1 mM, pH 8.3) was added into a 96-well clear bottom microplate with 80 µL of 50 mM Tris-HCl buffer followed by the addition of 40 µL of pyrogallol (15 mM in 10 mM HCl). Immediate measurements at 420nm of the mixtures were conducted to determine the reaction rate through kinetic reading for 5 min with an interval of 1 min at ambient temperature. The blank solution (Ab) contained only the buffer and pyrogallol. Ascorbic acid at 0.1 M was used as positive control. To calculate the percentage of the superoxide scavenging activity, the following equation was used:

$$\text{Superoxide radical scavenging activity (\%)} = \frac{(\Delta Ab/\min - \Delta As/\min)}{\Delta Ab/min}\ x100$$

Where *Ab* is the absorbance of the control group, and *As* is the absorbance of sample group.

c) Hydroxyl (•OH) radical scavenging activity:

In this method, the role of WSPP from SSF-HDRB and HDRB to prevent the formation of hydroxyl radicals, which are highly reactive and have destructive ability in physiological systems, was evaluated by determining the ability of peptides to scavenge and neutralize the hydroxyl radicals generated from hydrogen peroxide (H_2O_2). In this method, increase in the development of a red-orange color complex between Fe^{2+} and 1, 10 phenanthroline reflects the potential activity of the protein hydrolysates and peptides toward scavenging hydroxyl radicals. Oxidation of Fe^{2+} to Fe^{3+} by H_2O_2 prevents the formation of the complex, so addition of the antioxidant compound decreases the conversion of the ferrous ion to ferric ion and leading to increase the formation of the red-orange complex which has maximum absorbance at 536nm.

Hydroxyl radical scavenging assay was tested by the method of (Mu et al., 2018) with minor modification. The WSPP from SSF-HDRB and HDRB (50 µL of 0.5 % of the extract) was

added to 96-well microplate followed by the addition of 50 µL of 1,10-phenanthroline (3 mM in 0.1 M PBS, pH 7.4) and 100 µL of 3 mM $FeSO_4$ in water. Before incubation of the mixture for one hour at 37 °C with constant shaking, 50 µL of hydrogen peroxide H_2O_2 (0.5%) was added to initiate the Fenton reaction. The absorbance was read at 536 nm using UV-Vis. microplate reader spectrophotometer. Glutathione at 1mg/mL was used as a positive control. The following formula was used to calculate the percentage of scavenging activity:

$$\text{Hydroxyl radical scavenging activity (\%)} = \frac{(As - Ac)}{(Ab - Ac)}\, x100$$

Where, *As*: absorbance of the sample solution; *Ac*: absorbance of the control solution containing 1,10-phenanthroline, $FeSO_4$ and H_2O_2; *Ab*: absorbance of the blank solution containing 1,10-phenanthroline and $FeSO_4$.

Statistical analysis

All experiments were performed in triplicate determinations. The results are presented as means ±SD. For statistical analysis of data, JMP®, a statistical program, (Ver.15.1. SAS Institute Inc., Cary, NC, USA) was used. One-way analysis of variance (ANOVA) was performed for comparison of means with statistical significance at level $p < 0.05$. Pearson correlation test was used to perform the correlation analyses between antioxidant activities and the content of protein and peptides and total phenolic compounds (TPC)

Results and Discussion

Free Radical Scavenging Activities of WSSP from SSF-HDRB and HDRB

Production of food with antioxidant capacity is of remarkable interest in the research field related to the development of functional products. The antioxidant compounds including phenolics and amino acid moieties of peptides can deactivate free radicals and reactive oxygen species (ROS) in three main mechanisms, by hydrogen atom transfer (HAT), by single-electron

transfer (SET), and by chelating of transition metals such as Fe or Cu ions. In these reactions, the direct reaction between the antioxidant compounds and the reactive radicals can neutralize the radicals, and the antioxidant molecules become more stable new free radicals. The antioxidant compounds having aromatic ring in their structures delocalize the unpaired electron by resonance stabilization after donating of H-atoms from hydroxyl group (-OH) to free radicals. Other antioxidant molecules may stop the ROS-induced impairment via terminating the chain reaction of free radicals and ROS after reaction with intermediates induced by these radicals (Lü et al., 2010). In this study, based on different kinds of radical oxidative species (free, superoxide, and hydroxyl radicals), three different methods of *in vitro* free radical scavenging capacity were conducted to estimate antioxidant capability of WSPP from SSF-HDRB and HDRB, and these methods were DPPH radical scavenging, superoxide radical scavenging, and hydroxyl radical scavenging activities.

Results shown (Figure 6.1) indicated that the SSF-HDRB extract before the PVPP treatment (SSF-HDRB-PC) showed higher antioxidant activities of 71.0±1.5%, 51.7 ±1.7%, and 92.1±1.3% in scavenging of DPPH, Superoxide, and hydroxyl radicals, respectively. Hence, decreasing in the antioxidant activities of WSPP from SSF-HDRB after removing of the phenolic compounds denoted the interaction of phenolic compounds with the scavenging activities of protein fractions and peptides. This interaction can cause overestimation of antioxidant activities of WSPP. Also, phenolic compounds are considered as a main source of impurities in protein extraction contributing to the dark color and undesirable taste of the extract (Xu and Diosady, 2002).

As illustrated in (Figure 6.1), WSPP from SSF-HDRB observed scavenging activity of 56.0±0.9% against DPPH (as a free radical) which is higher significantly ($P<0.05$) from that of

WSPP from HDRB (12.5 ±0.7%) at concentration of 0.5% w/v of the extract. However, the DPPH scavenging activity of WSPP from SSF-HDRB was lower than that of the positive control (GSH) (77.0±0.5%). The WSPP from SSF-HDRB contains higher amount of low molecular weight fractions and peptides (<5KDa) as monitored by size exclusion chromatography (Figure 3.6). This implied that the antioxidant activity of WSPP from SSF-HDRB can be dependent on the molecular weights of its protein and peptides. This result agreed with data from other studies. It was reported that rice bran albumin hydrolyzed with Alcalase showed 49.4% DPPH radicals scavenging activity which was the highest activity among other rice bran albumin hydrolysates generated by using other proteases (Uraipong and Zhao, 2016).

The highest activity obtained in our study could be explained by the activity of different proteases excreted by *B. subtilis natto* Takahashi during SSF leading to more hydrolysis of different types of HDRB proteins including albumin, glutelin, globulin, and prolamin, in the fermented media (HDRB) generating a pool of high variation of peptides. Peptide fractions obtained by using pure enzymes from hemp protein hydrolysate, barley hordein, and canola meal protein have demonstrated trends and activities which are like what was noticed in this study (Alashi et al., 2014; Bamdad and Chen, 2013). However, in this study, bacterial solid-state fermentation by *B. subtilis* (natto) Takahashi not only assisted in extracting bound proteins from HDRB but also in hydrolyzing the proteins and generating peptides with molecular sizes <5KDa (Figure 3.6) in one step of processing.

In addition to the molecular weight, the amino acid composition of protein fractions and amino acid sequence of the peptides can contribute to the antioxidant properties of the peptides. Protein hydrolysates and peptides that contain higher amounts of aromatic and hydrophobic amino acid can efficiently scavenge the DPPH radicals (Ketnawa et al., 2018). The main

protease that *B. subtilis* excretes is a serine protease (subtilisin) (Razzaq et al., 2019) which cleaves peptide bonds at C-terminal of aromatic amino acids in the innate protein (Kato et al., 1992). Consequently, most of the peptides in WSPP from SSF-HDRB might contain one or more of aromatic and/or hydrophobic amino acids (Y, F, L, V). Hence, the protein fractions and peptides in WSPP from SSF-HDRB can neutralize DPPH radical by providing a hydrogen atom by the aromatic and hydrophobic amino acids.

Superoxide radicals, one of the oxygen species, are generated by many biological reactions in normal and stressed physiological activities. Superoxide radicals are indirectly responsible from initiation of lipid oxidation through generating a very harmful reactive radical, hydroxyl radical, which cause damage in different cellular organelles including DNA, enzymes, and cell membrane lipids and proteins (Engwa, 2018). Therefore, the scavenging of superoxide anion radicals is crucial. Furthermore, determining the antioxidant capability of hydrophobic protein fractions and peptides can increase the potential of using them as lipid-soluble antioxidant peptides which can play an essential role in inhibition of the lipid oxidation. The results of superoxide radical scavenging activity (Figure 6.1) showed that WSPP extract from SSF-HDRB had a higher value of 44.4±1.0% in comparison to non-fermented HDRB extract (9.3±1.1), and it corresponds about 50% of the positive control, ascorbic acid, scavenging activity (97.7±1.5) against the same radical species. The peptide content in WSPP from fermented HDRB is higher than non-fermented HDRB as evidenced from the size exclusion HPLC chromatogram (Figure 3.6). Alcalase-treated canola and zein protein hydrolysates were reported to have superoxide scavenging ability of about 20% and 11.5%, respectively (Alashi et al., 2014; Tang et al., 2010). In The higher superoxide radical scavenging activity of WSPP from SSF-HDRB in comparison to that of canola and zein protein hydrolysate could be due to the

difference in the size and structure of peptides generated by SSF than that of using only the pure protease.

Hydroxyl radical is very active in comparison to other radicals. It reacts with most of the cellular biomolecules leading to chronic diseases and ageing disorders and triggers peroxidation of lipid. Fenton reaction is an established method to generate hydroxyl radicals (Ajibola et al., 2011). The hydroxyl radical scavenging activity of SSF-HDRB protein fractions and peptides was significantly (P<0.05) higher (84.5 ±1.2) at a maximum concentration of 0.5% w/v of the extract than that of non-fermented HDRB extract (35.5±1.6) at the same concentration and closer to the activity of GSH (98.7 ±0.7 %), the positive control. The high OH• scavenging activity noted for WSPP from SSF-HDRB could be due to the peptides with low molecular weight, as reported in several studies (Ajibola et al., 2011; Cheung et al., 2012). It has been shown to scavenge hydroxyl radicals by donating electrons or converting to stable compounds, hydroxy-cyclohexadienyl radicals, by forming double bond with HO• (Lee et al., 2004). The sum of the aromatic amino acids, Cys, and Met in rice bran protein is about (141mg/g protein) (Wang et al., 1999), so these amino acids could have also contributed to the OH• scavenging activity of SSF-HDRB extract. The additive and synergistic effects of peptide fractions in this pool of hydrolyzed proteins might contribute to the higher activity of the WSPP from SSF-HDRB. This data is comparable with results obtained from other plant protein hydrolysates. Barley glutelin hydrolyzed by alcalase for 4h showed hydroxyl scavenging activity of 58% at 1mg/mL (Xia et al., 2012). Protein hydrolysates at 2mg/mL of black soybean (germinated for 4h) and Mung bean meal had activity against HO• radicals of 97.4% and 39.9%, respectively (Sefatie et al., 2013; Sonklin et al., 2018). The activity of WSPP extracted from SSF-HDRB against hydroxyl radicals is most effective among other studied antioxidant activities at the assayed concentration.

Therefore, SSF-HDRB extract has the potential in a food or living system protection against OH• radical-induced oxidation.

Correlations between the antioxidant activity methods and HDRB and SSF-HDRB extracts of protein fractions and total phenolic compounds

Since the extracts from HDRB and SSF-HDRB contain protein fractions, peptides**,** and phenolic compounds, the protein and peptide content (PPC) and total phenolic compounds (TPC) content of the extracts indicated a positive correlation (r=0.797), implying that TPC participates considerable amount in the extracts before removing TPC by PVPP treatment. The relationship between antioxidant activity and the protein and peptide content (PPC) or TPC content of the HDRB and SSF-HDRB extracts is coherent with the Pearson correlation analysis between PPC or TPC and various antioxidant activities (Table 6.1).

Protein and peptide indicated a strong and significant positive correlation ($P<0.01$) to DPPH ($r=0.902$), superoxide radical scavenging (SOS) ($r=0.909$), and hydroxyl radical scavenging (HRS) ($r=0.867$) activities. Also, TPC showed higher significant ($P<0.01$) and positive correlation to DPPH ($r=0.713$), but there was a moderate positive correlation between TPC and SOS ($r=0.671$, $P<0.05$), or HRS ($r=0.0.650$, $P<0.05$).

These differences could be due to DPPH radical scavenging assay methanolic environment which is more suitable for compounds that have higher solubility in methanol in comparison to SOS and HRS methods that are based on aqueous environment (Kim, 2015). Based on the data (Table 6.1), the three methods, DPPH, SOS, and HRS, were positively correlated with one another, despite the antioxidant activity mechanisms based on, hydrogen atom transfer (HAT), single-electron transfer (SET) reactions, or chelating of transition metals such as Fe or Cu ions.

Conclusion

The effects of solid-state fermentation on the HDRB and the improvement of the antioxidant activities of WSPP after fermentation of HDRB were investigated in this study.

The results from this study established that SSF of HDRB with B. subtilis (natto) Takahashi is effective in enhancing the antioxidant activities of HDRB. The SSF with B. subtilis (natto) Takahashi technology can be used as an effective processing method to add value to HDRB as a natural antioxidant ingredient in healthy and nutritious food and cosmetic products.

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Tables and Figures

Table 6.1. Correlation coefficients between antioxidant activities in (%) evaluated by different methods and the contents of extracted protein and peptide in (mg/g HDRB) or total phenolic compounds in (mg FAE/g HDRB)

	DPPH	SOS	HRS	PPC	TPC
DPPH	1.000	0.832*	0.930*	0.902*	0.713*
SOS	-	1.000	0.755*	0.909*	0.671**
HRS	-	-	1.000	0.867*	0.650**
PPC	-	-	-	1.000	0.797*
TPC	-	-	-	-	1000

* Significant at $P< 0.01$; ** Significant at $P< 0.05$. **FAE**, Ferulic acid equivalent; **HDRB**, heat-stabilized defatted rice bran; **TPC**, total phenolic content; **PPC**, protein and peptide content; **DPPH**, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity; **SOS**, superoxide radical scavenging activity; **HRS**, hydroxyl radical scavenging activity.

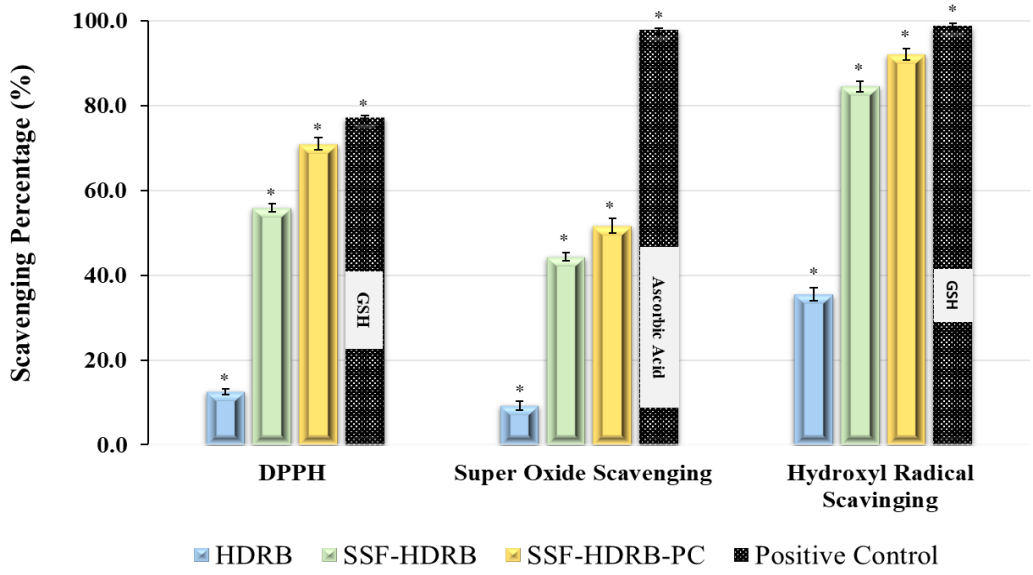

Figure 6.1. Antioxidation activities of WSPP from SSF-HDRB and HDRB before and after removing phenolic compounds (PC). SSF-HDRB-PC: Before removing PC. GSH: Glutathione. Bars represent means ± standard deviation. (*) represents the significant difference at $P \leq 0.05$ between the samples in each group.

Conclusion

This research has presented the basis for developing a novel approach to improve extracting water-soluble proteins and peptides from heat-stabilized de-fatted rice bran (HDRB), an inexpensive and underutilized co-product of rice processing using a natural and economical technique, solid-state fermentation (SSF) under optimized condition and using B. subtilis (natto) Takahashi as a fermenting microorganism. Hence, SSF is a feasible method to valorize different agro-industrial by-products.

Heat-stabilized de-fatted rice bran supported fast growth of B. subtilis (natto) Takahashi without introducing external carbon and nitrogen sources.

The water-soluble protein and peptides extracted from fermented HDRB contained high amount of low molecular weight of protein hydrolysates and peptides which is a good source for bioactive peptides and other beneficial compounds, such as phenolic compounds.

This research has demonstrated that SSF of HDRB using B. subtilis (natto), extraction of protein hydrolysates and peptides, fractionation by ultrafiltration, and testing for skin-aging enzymes inhibition, elastase and tyrosinase, and antioxidation activities is novel of this research and advantageous in deriving effective bioactive peptides which have potential for application in the cosmetic industries as a potential skin-antiaging component in the cosmeceutical products supporting skin health.

Five short peptides with anti-melanogenesis activities and non-cytotoxicity were isolated and characterized using established proteomic tools. The information related to the structural components of the peptides is important for synthesizing the peptides for applications cosmeceutical products.

Appendixes

Appendix 1: Research safety committee approval letter

UNIVERSITY OF ARKANSAS

Office of Research Compliance

July 17, 2020

MEMORANDUM

TO: Dr. Navam Hettiarachchy

FROM: Ines Pinto, Biosafety Committee Chair

RE: New Protocol

PROTOCOL #: 21001

PROTOCOL TITLE: Preparation of Proteins and Peptides from Heat-Stabilized Defatted Rice Bran (HDRB) Via Solid State Fermentation and Investigating for Skin Health

APPROVED PROJECT PERIOD: **Start Date** July 9, 2020 **Expiration Date** July 8, 2023

The Institutional Biosafety Committee (IBC) has approved Protocol 21001, "Preparation of Proteins and Peptides from Heat-Stabilized Defatted Rice Bran (HDRB) Via Solid State Fermentation and Investigating for Skin Health". You may begin your study.

If modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

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