Functional and Biological Properties of Sang Yod Rice Bran Hydrolysate Prepared by Enzymatic Hydrolysis and Its Application in Rice Pudding Product

Natcha Phantuwong

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Functional Food and Nutrition

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I hereby certify that this work has not already been accepted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ สมบัติเชิงหน้าที่และสมบัติทางชีวภาพของสารสกัดรำข้าวสังข์หยดและการนำไปใช้ประโยชน์ในผลิตภัณฑ์พุดดิ้งข้าว
ชื่อผู้เขียน นางสาวณัชฌา พันธุ์วงษ์
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บทคัดย่อ

การผลิตไฮโดรไลเสทรำข้าวสังข์หยดที่เตรียมด้วยการใช้เอนไซม์โปรติเอสและเอนไซม์อะไมโลกลูโคซิเดส โดยประยุกต์ใช้สภาวะการทำงานเอนไซม์สองชนิดประกอบด้วย การย่อยแบบเอนไซม์เพียงชนิดเดียวและการย่อยแบบเอนไซม์สองชนิด โดยการย่อยด้วยเอนไซม์สองชนิดใช้แบบย่อยตามลำดับและแบบย่อยร่วมกัน การย่อยทุกสภาวะที่ความเข้มข้นของเอนไซม์ 3, 4 และ 5% w/v และเวลาในการย่อย 30, 60 และ 120 นาที พบว่าเมื่อย่อยโดยใช้เอนไซม์สองชนิดแบบย่อยตามลำดับและย่อมแบบร่วมกันในทุกสภาวะการย่อยให้ปริมาณโปรตีน เบตากลูแคน สารประกอบกลุ่มฟีโนลิคในปริมาณที่มากกว่าการย่อยแบบใช้เอนไซม์เพียงชนิดเดียว การย่อยด้วยเอนไซม์สองชนิดแบบย่อยตามลำดับที่ความเข้มข้น 5% เป็นเวลา 30 นาทีได้ไฮโดรไลเสทที่มีปริมาณโปรตีนสูง เบตากลูแคนสูงสุด ในขณะที่มีผลกับคุณสมบัติเชิงหน้าที่ด้านความสามารถในการจับน้ำและไขมันเพิ่มขึ้นด้วย และในทุกสภาวะการย่อยเมื่อเพิ่มเวลาย่อยมีผลให้สีของไฮโดรไลเสทเพิ่มขึ้น ไฮโดรไลเสทรำข้าวที่ผลิตโดยการย่อยโดยใช้เอนไซม์สองชนิดแบบย่อยตามลำดับที่ระดับความเข้มข้น 5% w/v ที่ใช้ระยะเวลาการย่อยสั้นให้ปริมาณโปรตีน เบตากลูแคน คุณสมบัติเชิงหน้าที่และคุณสมบัติด้านสีที่ดี

ไฮโดรไลเสทรำข้าวสังข์หยดที่ผลิตโดยใช้เอนไซม์สองชนิดแบบย่อยตามลำดับและแบบย่อยร่วม แสดงกิจกรรมการเป็นสารต้านการเกิดออกซิเดชั่นในระบบหลอดทดลองได้สูงในทุกการทดสอบ ได้แก่ การต้านอนุมูลอิสระ ABTS, อนุมูลอิสระ DPPH, อนุมูลอิสระรีดิวซิ่งพาวเวอร์ และความสามารถในการจับโลหะ นอกจากนั้นไฮโดรไลเสทที่ได้จากการย่อยด้วยเอนไซม์สองชนิดแบบตาลำดับที่ 5% เป็นเวลา 60 นาที แสดงการต้านการเกิดออกซิเดชั่นสูงสุด ไฮโดรไลเสทจากทุกสภาวะไม่พบการเป็นพิษต่อการทดสอบความเป็นพิษต่อเซลล์แมคโครฟาจน์ RAW 264.7 เมื่อใช้ความเข้มข้นสูงสุดที่ 2000 ไมโครกรัมต่อมิลลิลิตร พบว่าไฮโดรไลเสทจากที่ได้จากสภาวะการย่อยด้วยเอนไซม์สองชนิดแบบตามลำดับและย่อยร่วมที่ 5% เป็นเวลา 60 นาที มีฤทธิ์ในมีฤทธิ์ยับยั้งการสร้างไนตริกออกไซด์ดีที่สุด โดยมีค่า IC50 เท่ากับ 408 และ 973 ไมโครกรัมต่อ

มิลลิลิตร ตามลำดับ ซึ่งในสภาวะการย่อยนี้พบว่าไฮโดรไลเสทมีฤทธิ์ลดการหลั่งสารไซโตไคน์ TNF-α, IL-1β และ IL-6 ดังนั้นรำข้าวสังข์หยดจึงสามารถใช้ผลิตไฮโดรไลเสทที่มีฤทธิ์ต้านการเกิดออกซิเดชั่นและต้านการอักเสบได้

การนำไฮโดรไลเสทที่มีคุณสมบัติเชิงหน้าที่และมีฤทธิ์ต้านการเกิดออกซิเดชั่นและต้านการอักเสบมาใช้เป็นส่วนผสมที่มีประโยชน์ในผลิตภัณฑ์พุดดิ้งข้าว พบการสำรวจผู้บริโภคต้องการผลิตภัณฑ์พุดดิ้งข้าวเป็นแบบพร้อมบริโภคในภาชนะถ้วยพลาสติกและผสมธัญพืชหรือผลไม้ ผลจากการนำไฮโดรไลเสทรำข้าวใช้เป็นส่วนผสมฟังก์ชั่นนอลในพุดดิ้งข้าวบนคุณสมบัติทางกายภาพ, คุณค่าทางโภชนาการและการการยอมรับของผู้บริโภค พบว่าผลิตภัณฑ์พุดดิ้งข้าวได้รับคะแนนความชอบที่ระดับชอบปานกลางและสามารถเติมไฮโดรไลเสทสูงสุดที่ระดับ 5% พุดดิ้งข้าวเสริมไฮโดรไลเสท 100 กรัมให้พลังงาน 120 กิโลแคลอรี่และมีอายุการเก็บ 10 วันในอุณหภูมิแช่เย็น การนำไฮโดรไลเสทเสริมในพุดดิ้งข้าวเป็นการเติมส่วนผสมชังก์ชั่นที่มีฤทธิ์ทางชีวภาพซึ่งเป็นผลดีต่อสุขภาพผู้บริโภค

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Major Program Functional Food and Nutrition

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ABSTRACT

Sang Yod rice bran (SYRBH) was prepared by enzymatic hydrolysis of Protease G6 and Amyloglucosidase. Application of these two enzymes were either single, sequential or combined digestion. Effects of concentration of the enzyme extract (3, 4 and 5% w/v) and hydrolysis duration (30, 60 and 120 min) on catalytic process and hydrolysate characteristics were investigated. The sequential and combined hydrolysis yielded the hydrolysate with high protein and β-glucan content as well as great value of total phenolic and total anthocyanin contents, relative to that of the process using single enzyme. This condition also improved functional properties of the RBH (water holding, swelling capacity and fat binding capacity). The treatments of extension of hydrolysis time caused the darkening of RBH. The hydrolysate exhibiting the high protein and highest β-glucan content was prepared by using sequential hydrolysis of 5% enzyme concentration and 30 min. The results thus suggest that SYRBH has great composition of bioactive compound with two enzymes sequential hydrolysis as well as short hydrolysis time.

Effect of sequential and combined enzymatic hydrolysis yielded the hydrolysate with the high *in vitro* anti-oxidative activities. The hydrolysis using 5% concentration for 60 min showed highest antioxidant activities. All obtained RBHs exhibited no cytotoxicity on RAW 264.7 cell lines at the maximum concentration of 2000 µg/ml. The RBHs obtained by using sequential and combined with 30 min hydrolysis time showed the best nitric oxide inhibitory in the RAW 264.7 cell with an IC_{50} of 408 and 973 µg/ml, respectively. Furthermore, The hydrolysis using 5% concentration with 60 min hydrolysis time exhibited the strongest reduction of TNF-α,

IL-1β and IL-6 cytokines. Thus, Sang yod rice bran by-product could be used for preparation of RBH strong anti-oxidative and anti-inflammatory activities.

SYRBH application as a functional food ingredient to develop the functional rice pudding. The most preference product profile of functional rice pudding as ready-to-eat in plastic cup mixed with cereal/multi-grain and fresh fruit. From consumer sensory evaluation the sample fortified with 5 % SYRBH received the moderately like. The functional rice pudding of one serving size (100g) provide energy at 120 Kcal. Stability of functional rice pudding is being investigated by microbial counts and sensory test during 10 days storage in refrigerator. Fortification of SYRBH in rice pudding thus has the potential to serve as a functional food ingredient that can offer health benefits to consumer.

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I would like to express my appreciation to Suan Dusit University and Graduate School of Prince of Songkla University, Hat-Yai campus, for their fully research grant. I would like to thank you to all volunteers, who came for product sensory evaluation. There are many other people without whom I would have never complete my study. During conducted field research, I am also grateful to Dr. Tatchaporn Chaijaroen, Dr. Sureeporn Kangsanant, Dr. Worraphanit Chansuwan, Ms. Korawan Chakree, Ms. Vijitra Plongbunjong and Ms. Anchana Ampai for their field work assistant. Unforgettably.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Rice (*Oryza sativa* L.) is the main staple food in all Asian countries. In Thailand, rice is of special importance as it is the staple food for 65 million people. White and brown rices are crops that are mostly consumed world-wide. For pigmented wild rice species, there are a largh variety. These varieties produce rice with colors ranging from red, black and purple. The colors of these pigmented rice are the differences in anthocyanine contents in the outer layers (Oki *et al*., 2002). The major by-products during milling of rice are husk (20%), rice bran (8%) and rice germ (2%) (Norhalzan *et al*., 2013). In recent years, rice bran by-products have gained much attention the having high amounts of vitamin, minerals, protein, antioxidant compounds and fiber, with several health benefits (Borresen and Ryan, 2014). One of the active compounds is a water soluble dietary fiber. (Aoe *et al*., 1993). And β-glucan is a polysaccharide fraction as a composition of soluble dietary fiber (Ahmad *et al*., 2009). Meanwhile, rice bran contains protein (12-20%) which is very digestible and hypoallergenic (Wang *et al*., 1999). In addition, rice bran also contain lysine approximately 3-4%, which is higher than that of rice endosperm protein and protein from other cereal bran (Yeom *et al*., 2010). Therefore, rice bran protein thus holds a great promise as an alternative source for as bioactive compounds and function food ingredients.

Sang Yod rice is a red-violet pigmented rice grown in the southern part of Thailand. Rice production areas in the South are Songkhla lake basin covering 3 provinces Phatthalung, Nakhorn Si Thammarat and Songkhla. Sang Yod rice is the first rice variety which has been registered for geographical indication (GI) product of Thailand. Production of Sang Yod rice can produce yield around 7,000 tons (Thai rice exporters association, 2016). The rice milling processing of Sang Yod rice generates a great amount of by-products, Previously, Sang Yod rice bran has been used as a low-

cost animal feed or discarded as waste, it has not been utilized for oil extraction. Nowadays numerous reports mention biological activities of rice bran, colored rice bran. Health benefits of rice bran have also been reported (Gunaratne *et al.*, 2013; Sompong *et al*., 2011). Sang Yod rice bran consists of high content of polyphenol compound exhibited antioxidant activity. Moreover, The various bioactive compound from pigmented rice bran (protein, anthocyanin, oil extract, vitamin and mineral) showed pharmacological activities such as anti-diabetic and antioxidant effects (Yodmanee *et al*., 2011; Uttama *et al*., 2014; Itharat *et al*., 2016).

Extracting method of rice bran hyrolysate are acid or alkali extraction and enzyme extraction. Nevertheless, the large portions of rice bran protein cannot be solubilized by regular solvents such as salt, alcohol and acid due to extensive disulfide bonding and aggregation (Silpradit *et al*., 2010). The most common method of isolating rice bran proteins is alkali extraction. Although this treatment with high temperatures and concentration of alkaline solutions solubilizes most of the rice bran proteins, it also leads to the occurrence of denaturation and hydrolysis of proteins (Wang *et al*., 1999). Enzymatic method has been used to enhance solubilization of rice bran protein and obtain a wide range of protein hydrolysates (Silpradit *et al*., 2010). Several enzymatic hydrolysis methods can be applied to alter the structural characteristic of rice bran in order to increase its accessibility to hydrolyze and to obtain the most potent bioactive compounds. The cell wall of rice bran is a complex three dimensional structures consisting of cellulose, polysaccharide and protein. One possible method of degrading the cell wall to further release bioactive compounds such as protein, dietary fiber and phenolic is complex enzyme hydrolysis using protease, cellulase and glucoamylase (Liu *et al*., 2017). Several studies have been carried out on rice barn protein (RBP) hydrolysates. For example, Kokkeaw and Thawornchinsombut (2007) prepared RBP hydrolysates using commercial proteolytic enzymes, Protex 6L, The product's maximal radical scavenging activity was 27%. Chanput *et al*. (2009) reported that KDML 105 rice bran protein fractions digested with pepsin and subsequently with trypsin exhibited high antioxidant activity.

Functional food from rice is becoming increasingly popular in Thailand and throughout the world. The products based on rice or rice isolate e.g. porridge or rice pudding, cereal grain drink and bakery products are commercially available in

Japan and European countries. Moreover, Development of value-added rice products is important to encourage rice consumption especially rice products of traditional rice.

In this study, the chemical and functional properties and bioactive properties of Sang Yod rice bran hydrolysate were investigated. The optimized enzymatic extraction methods were used for extraction of bioactive compounds from Sang Yod rice and its fraction. Additionally, functional rice pudding fortified with Sang Yod rice bran hydrolysate were formulated.

1.2 Review of literature

1.2.1 Rice and Structure

Rice (*Oryza sativa* L.) is the most important cereal crop and the staple food for over 50% of the world's population. Rice has more than 120,000 varieties. Nowadays, about 90% of the world's rice production occurs in Asia. Rice grown in Asia is *O. sativa*, which can be grown under a broad climate and geographic conditions extending from 53^0north latitude to 35^0south latitude and from sea level to an altitude of 2,500meters. In addition, the optimum temperature is 30^0C to 34^0C. The species *O. sativa*, can be categorized into three subspecies, Japonica (planted in tropical are such as India and the Philippines, including Thailand) (Juliano and Bechtel, 1985). The majority of rice varieties grown in Thailand are long grain. In Thailand, rice varieties are divide into 3 types according to custom and usage, such as waxy rice, low amylose rice and high amylose rice. In 2015-2016, Thailand produced 27.42 million metric tons of rice and exported 9.88 million metric tons (Thai rice exporter association, 2016). Food ingredient usage of rice accounts for 22% of domestic rice sales. The use of rice as a food ingredient has also increased by 3.7% due to the rising popularity and availability of snack, frozen dish, rice pudding, package and beverage. More research on rice functionality, physical and chemical properties is needed in order to meet the food industry's demand for low-fat, hypoallergenic and nutritious food ingredient.

Rice is classified by amylose content into waxy (0-2%) and non-waxy low (12-20%), intermediate (20-25%), and high (25-33%). In addition, rice is also classified into three types by grain varieties that has low amylose content (12-20%) and

some long grain varieties have intermediate amylose content (20-25%) and others have high amylose content(>25%) (Juliano and Bechtel, 1985). The rice grain consists of hull, pericarp, seed coat, nucellus, embryo, aleurone layer and endosperm as shown in Figure 1. Comprises the hull (16-28% dry mass basis) and the caryopsis. The mass distribution of the rice caryopsis is 1-2% pericarp, 4-6% aleurone plus seed coat and nucellus, 2-3% embryo and 89-94% starchy endosperm (Hoseney, 1990). Further milling to remove the pericarp, seed coat, testa, aleurone layer and embryo to yield milled or white rice results in a disproportionate loss of lipid, protein, fiber, reducing sugars and total sugars, ash and minor components including vitamins, free amino acid and free fatty acid (Park *et al*., 2001)

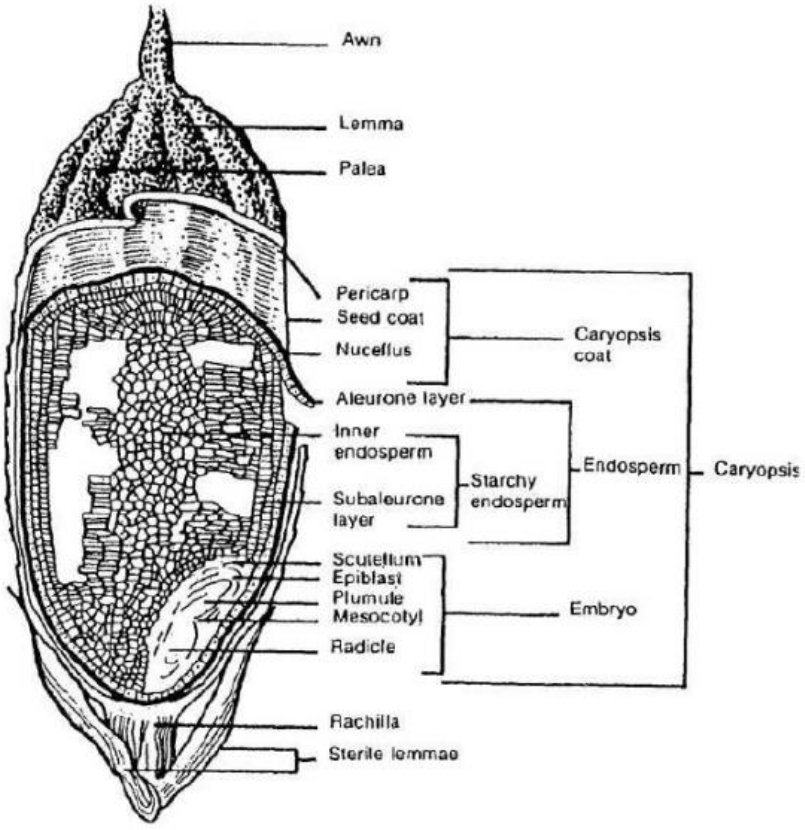


Figure 1 A detailed structure of rice grain

Source: Blakeney (1984)

1.2.1.1 Hull

The hull is the outer covering for the caryosis (brown rice). It serves a as protective function against insect infestation and rapid changes in moisture content of the grain due to large humidity fluctuation in the external environment. Hull are low in protein, fat and starch but high in crude fiber, crude ash and dietary fiber (Table 1).

Table 1 Proximate compositions of rice grains

Rice	Protein	Fat	Fiber	Ash	Starch	Dietary fiber
Rough	6.7–8.3	2.1–2.7	8.4–12.1	3.4–6.0	62.2	19.1
Brown	8.3–9.6	2.1–3.3	0.7–1.2	1.2–1.8	77.2	4.5
Milled	7.3–8.3	0.4–0.6	0.3–0.6	0.4 – 0.9	90.2	2.7
Hull	2.3–3.2	0.4–0.7	40.1–53.4	15.3–24.4	1.8	77.3
Bran	13.2–17.3	17.0–22.9	9.5–13.2	9.2–11.5	16.1	27.6–33.3
Embryo	17.7–23.9	19.3–23.8	2.8–4.1	6.8–10.1	2.4	-
Polish	13.0–14.4	11.7–14.4	2.7–3.7	6.1–8.5	48.3–55.4	-

Source: Juliano and Bechtel (1985)

1.2.1.2 Caryopsis

Removal of the hull from paddy grain by dehulling exposes the rice caryopsis, which are the pericarp, seed coat (tegmen), nucellus and aleurone (Figure 1). Along with much of embryo (germ), these layers comprise the bran portion of the rice grain. The bran portion accounts for 8-10% of the brown rice weight. The bran is the most nutritious part of the caryopsis. Bran contains protein, fat (rice oil), carbohydrate (starch) and dietary fiber (Table1).

1.2.1.3 Subaleurone layer and starchy endosperm

Further milling of the caryopsis removes the subaleurone layer and a small part of the starchy endosperm. This milling fraction is referred to as polish. The end result of bran and polish removal is milled (white) rice. The polish comprises 3-4% by weight of brown rice. The starchy endosperm is rich in starch granules, contain some protein bodies, especially in the outer endosperm layers, and almost no lipid bodies, Polish contains only slightly less protein and lipid but considerably more starch than bran (Table1). The polish fraction is only somewhat less nutritious than the bran fraction, primarily because it contains lower levels of minerals and vitamins than the bran.

1.2.2 Rice bran and health benefits

Rice bran is the hard outer part of the grain that consists of aleurone, a form of protein found in the protein granules of maturing seeds, as well as pericarp, the outer and edible layer of the rice kernel (Figure 2). Apart from these two, it also contains germs and endosperm of the rice kernel. Rice bran is obtained as a by-product during the rice milling process and the outer layers are removed at the time of whitening or polishing (the conversion of brown rice to white rice) of the husked rice.

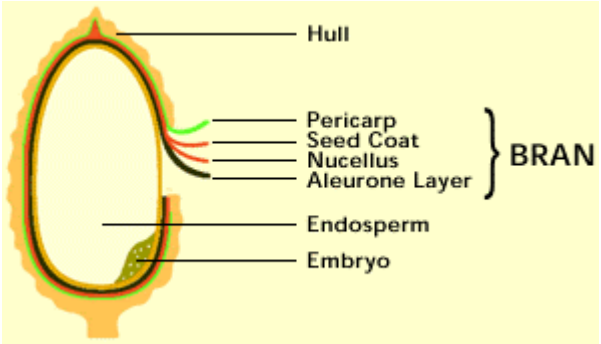


Figure 2 Structure of rice bran

Rice bran contains appreciable amount of protein (11-17%), fat (12-22%), dietary fiber (6-14%) like β-glucan, pectin and gum, moisture (10-15%) and ash (8-17%). Also it is rich in vitamins including vitamin E, thiamin, niacin and minerals

like aluminum, calcium, chorine, iron, magnesium, potassium, zinc and sodium (Saunders, 1990). A major rice bran fraction contains 12%-13% oil and highly unsaponifiable components (44.3%). This fraction also contains tocotrienols (a form of vitamin E), gamma-oryzanol, and beta-sitosterol; all these constituents may contribute to the lowering of the plasma levels contained in the lipid profile. Its prospects are the presence of antioxidants like tocopherol, tocotrienol and γ-oryzanol for utilization of humans as functional ingredients to reduce threatening disorders (Monsoor *et al*., 2003; Gong and Yao, 2001). Rice bran is composed of many nutritious substances like protein, fat, fiber, various antioxidants, etc. that have a beneficial effect on human health. Because of its multi-nutritional properties, rice bran has being consumed by humans for thousands of year. The nutritional composition of rice bran is shown in Table 2.

Table 2 The nutritional composition of rice bran

Proximate composition of rice bran	
Energy content	399 – 467 Kcal
Crude fat	15.0 – 19.7 g
Available carbohydrate	34 – 62 g
Crude ash	6.6 – 9.9 g
Amino acid composition of rice bran (g)	
Crude protein g N*5.95	11.3 – 14.9
Isoleucine	2.7 – 4.1
Lycine + Crysteine	4.8 – 5.4
Metthionine + Typtophan	4.2 – 4.8
Histidine	2.7 – 3.3
Leucine	6.9 – 7.6
Phynylalanine	7.7 – 8.0
Threonine	3.8 – 4.2
Valine	4.9 – 6.0
Tryptophan	0.6 – 1.2

Table 2 Cont.

Vitamins and minerals contents of rice bran (mg)	
Thaiamine	1.2 – 2.4
Niacin	26.7 – 49.9
Calcium	30 - 120
Zinc	4.3 – 25.8
Riboflavin	0.18 – 0.43
α-Tocopherol	2.6 – 13.3
Phosphorus	1.1 – 2.5
Iron	8.6 – 43.0

Source: Juliano and Bechtel (1985); Pedersen and Eggum (1983)

Rice bran contains many important nutrients that are essential for the body. The various health benefits associated with the use of rice bran are antioxidants and phytochemicals. Other health benefits of rice bran are:

1. Antioxidant: The antioxidant at cellular and molecular levels are known to deactivate the natural byproduct of the oxidative metabolism that are free radicals. The components of rice bran that is γ-Oryzanol, tocols and phytosterol conjugates are known to have antioxidant properties. Rice barn polysaccharides exhibited good potential for scavenging effects of DPPH and hydrogen peroxide (Zha *et al*., 2009). Phytosterols are the chemical compounds which are synthesized by plants including sterols and stanols. Phytosterols cannot be synthesized by human hence supplied thought the diet. The major phytosterols in rice bran that have hypocholesterolemic effects (Jong *et al*., 2003).

It helps in reducing the level of cholesterol, thereby reducing the risks of heart attacks (Othman *et al*., 2011). The presence of dietary fibers and whole grains helps in preventing the occurrence of Type II diabetes (Liu, 2003). Rice bran also helps in reducing high blood pressure as well as intestinal cancer (Most *et al*., 2005).

2. Coronary Heart Disease: The consumption of dietary fiber that is present in rice bran have shown to reduce the risk of coronary heart disease mortality by lowering blood pressure, lowering blood cholesterol levels and by improving insulin sensitivity (Truswell, 2002; Whelton *et al*., 2005; Issara and Rawdkuen, 2016). It helps

in reducing the level of cholesterol, thereby reducing the risks of heart attacks in human beings (Othman *et al*., 2011). Moreover, Kahlon coworker studied the regulation of plasma cholesterol levels which is performed by the liver, hence liver cholesterol level also provides a measure of influence of diet on cholesterol metabolism. In hamsters, diet containing 10% total dietary fiber or a 5:5 total dietary fiber combination of rice bran and a 1-3-glucan enriched barley fraction in diets containing 0.25% significantly lowered cholesterol (Kahlon *et al*., 1993) Rice bran also contains a high level of dietary fibers like beta-glucan, pectin, and gum. The dietary fibers composition of cereal bran is showed in Table 3.

Table 3 Percent dietary fibers of whole barley, β-glucan-enriched barley fraction (GEB), rice bran, and oat bran.

Cereal	**Total dietary fiber**	**Soluble dietary fiber**	**β-glucan**
De-hulled whole Barley	17.2	6.0	5.7
GEB	43.8	19.8	18.9
Rice bran	22.9	1.4	1.8
Oat bran	18.6	8.0	8.3

Source: Kahlon *et al*. (1993)

Diabetes Melitus: The fiber in rice bran has a laxative effect withch increase fecal output and stool frequencies. The postprandial blood glucose in normal and diabetic persons can be reduced by soluble fibers. Dietary fibers of rice bran acts like asponge and absorbs water in the intestine, mixes the food into gel and thereby slows down the rate of digestion and absorption. The blood glucose, total cholesterol and triglycerides can be decreased by rice bran (Qureshi *et al.,* 2002). The presence of dietary fibers and whole grains helps in preventing the occurrence of Type II diabetes (Liu, 2003).

Anticancer: The anticancer effects of rice bran are mediated thought the ability of these agents to induce apoptosis, inhibit cell proliferation and alter cell cycle progression in malignant cells. These protect against tissue damage though the scavenging of free radicals and the blocking of chronic inflammatory responses.These have been shown to activate anticancer immune responses as well as affecting the

colonic tumor micro-environment in favor of enhanced colorectal cancer chemoprevention (Raghav *et al*., 2016). Several, the dimethylhydrazine (DMH) and axoxymethane (AOM) induced preneoplastic lesions are inhibited by rice bran-derived sphingolipids in the colon of rat (Sunagawa *et al*., 2009)

Other benefits: Many studies about health benefits of rice bran have found anti-aging, Osteoporosis and Post-menopausal sysdromes. Oryzanol can impede the progress of melanin pigmentation by intercepting the UV rays at the skin's surface and hindering its transmission. Rice bran oil such as γ-oryzanol, tocotrienols, these protect the skin against UV induced skin damage and skin ageing (Nobor and Yusho, 1970; Eitenmiller, 1997). Rice bran was found to be an effective adsorbent of para-dicholorobenzene in a board pH rang of 1-12.The properties of rice bran was attributed to the uptake by the intracellular particles called spherosomes (Adachi *et al*., 2007).

1.2.3 Sangyod Phatthalung rice

Sangyod Phatthalung rice is a traditional rice variety grown in the area of Phatthalung province for more than a hundred years. Since 1987 plant breeder of Phatthalung Rice Research Center had in improved Sang Yod rice variety called "pure-line selection" with this rice improvement process, which acquired the best Sang Yod and named it Sang Yod Phatthalung. It is the first rice variety which has been registered for geographical indication (GI) that has been supported by Her Majesty Queen Regent Sirikit (Burean of rice research and development, 2006) of Thailand. Source of production of Sangyod rice is under the national resource of the plain area between Ban-Tad Mountains Rang and Songkhla-Phatthalung Lake which is in abundance of many agricultural biodiversity resources at this specific area. The typically grain is small, long and slender, dark red pericarp, soft and aromatic of cooked rice. Pigmented rice had the highest antioxidant activitys (Nam *et al*., 2006). Sangyod rice contains various nutritive substances, including vitamin B1, B2, B6, carbohydrate, fibers and protein. It is also rich in various minerals such as iron, calcium and phosphorus (Thai food composition organization, 1999). Red rice had higher total phenolic, flavonoid than pigmented rice their higher pro-anthocyanidins and anthocyanins contents, respectively (Min *et al*., 2012) and Nam *et al*. (2005) studied pigmented rice extracts and found they can inhibit phorbol ester-induced tumor promotion in marmoset

lymphoblastoid cells B95-8 in vitro. Sangyod rice is nutritional enrich (high niacin), oryzanol and γ-aminobutyric acid(GABA), which provided to reduce the risk of cancer as well as preventing Alzheimer's disease.

Figure 3 Sangyod rice grain
Source: Burean of rice research and development (2006)

1.2.4 Extraction of rice bran

Methodology of hydrolysis of plant and rice bran have various methodology.

1.2.4.1 Extraction of dietary fiber with enzymatic method

There is no single analytical method which meets all the requirements for the nutritional or chemical components of dietary fiber in foods. Dietary fiber methodology can be classified into three major categories that is non-enzymatic-gravimetric, enzymatic-gravimetric and enzymatic-chemical methods. An enzymatic-gravimetric method was developed in which the sum of soluble and insoluble polysaccharides and lignin were measured as a unit and considered to be total dietary fiber. That method is detailed in the AOAC International Official Methods of Analysis (FAO, 2011). The AOAC method (991.43) requires enzymatic digestion of protein and non-resistant starch, followed by precipitation of soluble fiber with 95% alcohol and weighing (IFST, 2007). Salehifar and Fadaei (2011) reported that dietary fiber content from Iranian rice bran extracted with enzymatic method higher than chemical method. Fadaei and Salehifar (2012) extracted dietary fibers from commercially available rice

bran and husk of Alikazemi variety by enzymatic method. Protein content of rice bran and rice husk fibers was 2.53%, and 2.30%, respectively.

Daou and Zhang (2011) extracted dietary fiber from defatted rice bran using the enzymatic-gravimetric method with termamyl (heat stable amylase 20,000 U/g) at 100ºC for 1 h to give gelatinization, hydrolysis and depolymerization of starch after it was digested with alcalase (Alcalase 2.4 AU/g) at 60ºC for 1 h to solubilize and depolymerize proteins and enzymatic treatment was complete with incubation with amyloglucosidase (100,000 U/g) at 60ºC for 1 h to hydrolyze starch fragments to glucose. Abdul-Hamid and Luan (2000) extracted dietary fibers from defatted rice bran using a modified enzymatic-gravimetric method, and obtained 27.04% total dietary fibers and 2.25% soluble dietary fibers.

1.2.4.2 Extraction of rice bran protein hydrolysate

Proteins, as a result of the cleavage of peptide bond, are broken down into peptides of different sizes and free amino acids. This procedure namely hydrolysis can be achieved by enzyme, acids or alkali. Acid and alkali processes are difficult to control and provide product with meager nutrition. Chemical hydrolysis can transform L-form amino acids to D-form amino acids and this process can generate toxic substance such as lysino-alanine (Clemente, 2000). Enzymatic hydrolysis is performed under a mild condition pH (6-8) and temperature (40-60 ºC). The overall amino acid composition of enzymatic hydrolysis is similar to that of the starting material. These released peptides may exhibit various regulatory effects such as antioxidative, immune defense, and antihypertensive activities, called bioactive peptide (Lee *et al.*, 2010). To develop commercial protein hydrolysates with defined physical, chemical and nutrition characteristics, many different factors must be taken into account. Among them, choice of suitable protein source, enzymatic hydrolysate process, and the development of post-hydrolysis processes have special relevance. Although the process of protein hydrolysis is relatively simple, there are a number of factors that need consideration before, during, and after hydrolysis. The nature and quality of the raw material can have a great impact on the hydrolysis process and the quality and functionality of the final product (Kristinsson, 2006).

Different techniques subsist for extracting or hydrolyzing protein from plants and other sources. These include using organic solvents to hydrolyzed proteins and concentrated acid or alkali to extract and hydrolyze proteins at elevated temperatures. These methods normally adversely affect the functional and organoleptic properties of protein hydrolysate and may produce toxic by-products. The use of enzymes allows greater control of the hydrolysis and there by the properties of the resulting product. Biological processes using endogenous or exogenous enzymes are now far more frequently employed in industrial practices to produce protein hydrolysate (pH), because enzyme hydrolysis is usually a gentle process that results in products of high functionality, good organoleptic properties and excellent nutritional value. The process of preparation of pH consists of proteolytic digestion of plants or plants waste at optimum conditions of pH and temperature of enzymes. The enzymes can be from plant (papain, ficin), animal (trypsin, pancreatin), or microbial (pronase, Alcalase and Flavourzyme) sources (Venugopal, 2009). The hydrolysis condition is controllable; it may be possible to produce hydrolysates with desirable activities. When producing hydrolyzed proteins, it is important to determine the degree of hydrolysis (DH). The DH, which is expressed as the percentage of α-amino nitrogen in the soluble fraction, is important in optimizing the process parameter. Increasing in DH increased the amount of low molecular weight peptides (Raghavan and Kristinsson, 2009). There are several advantages and disadvantages of acid and enzymatic hydrolysis, which are listed in Table 4. Using enzymes enables better control of the hydrolysis process and results in high functionality products, good organoleptic properties and excellent nutritional value. The material that results from a proteolytic digestion is a mixture of amino acids and polypeptides of varying lengths

Table 4 Comparison between acid and enzymatic hydrolysis.

Comparing variable	Acid hydrolysis	Enzymatic hydrolysis
Mild hydrolysis condition	No	Yes
High yield of hydrolysis	No	Yes
Product inhibition during hydrolysis	No	Yes
Formation of inhibitory by-products	Yes	No
Low cost of catalyst	Yes	No
Short time of hydrolysis	Yes	No

Source: Taherzadeh and Karimi (2007)

1.2.5 Enzyme of hydrolysis

1.2.5.1 Protease

Proteases can be classified, their similarities to well characterized proteases, as trypsin like, chymotrypsin-like, etc., their pH activity profiles as acid, neutral or alkaline proteases, substrate specificity and mechanism of catalysis (Haard and Simpson, 1994). Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are of commercial value and find multiple applications in various industrial sectors. Proteases are classified according to their source (animal, plant and microbial), their catalytic activity of endopeptidase or exopeptidase and the nature of the catalytic site. Endopeptidases cleave the polypeptide chain at particularly susceptible peptide bonds distributed along the chain, whereas exopeptidases hydrolyze one amino acid from N-terminus (amino peptidase) or from C-terminus (carboxypeptidases).

Endopeptidases or endoproteinases are proteolytic peptidases that break peptide bonds of nonterminal amino acids (e.g. within the molecule). Endopeptidases cannot break down peptides into monomers. A particular case of endopeptidase is the oligopeptidase, whose substrates are oligopeptides instead of proteins. They are usually

very specific for certain amino acids. Examples of endopeptidases include trypsin, chymotrypsin, elastase, thermolysin and pepsin.

Exopeptidases are proteolytic peptidases enzyme that catalyze the cleavage of the terminal (last) or next-to-last peptide bond from a polypeptide or protein, releasing a single amino acid or dipeptide. Examples of exopeptidases include carboxypeptidase, metallo carboxypeptidase and cysteine carboxypeptidase.

The classification of proteases from fish and aquatic invertebrates could be classified into four major groups (Simpson, 2000) these include acid proteases, serine proteases, cysteine proteases and metalloproteases.

1. Acid proteases; The acid or aspartyl protease have been described as a group of endopeptidase characterized by high activity and stability at acid pH. They are referred to as "aspartyl" proteinases (or carboxyl proteinases) because their catalytic sites are composed of the carboxyl group of two aspartic acid residues (Whitaker, 1994).

Among these acid proteases, pepsin is the major enzyme for this group found in fish viscera. Pepsin is secreted as a zymogen (pepsinogen) activated by acid in stomach (Clarks *et al.*, 1985). Pepsin prefers specifically the aromatic amino acids phenylalanine, tyrosine and tryptophan. Pepsin activity is very dependent on pH values, temperatures and type of substrate. Pepsin from polar cod stomach exhibited a maximal activity against hemoglobin at pH 2.0 and 37 °C (Arunchalam and Haard, 1985). Gildberg *et al.* (1990) reported that the optimal pH of Atlantic cod pepsin for hemoglobin hydrolysis was 3.0. Pepsin is quite stable from pH 2 to about 6 but it rapidly loses activity at pH above 6 due to the denaturation (Simpson, 2000).

2. Serine proteases; The serine proteases have been described as a group of endopeptidase with a serine residue together with the imidazole group and an aspartyl carboxyl group in their catalytic site (Simpson, 2000). Serine protease exhibits high activity under alkaline rather than neutral pH and sensitivity to serine protease inhibitors (Simpson, 2000). The common serine proteases have been recovered from digestive glands of marine animals are trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and elastase (EC 3.4.21.11) (Klomklao, 2008).

Trypsin has a very narrow specificity for the peptide bonds on the carboxyl side of arginine and lysine, while chymotrypsin has a much broader specificity for amino acids with bulky side chains and nonpolar amino acid such as tyrosine, phenylalanine, tryptophan and leucine. Trypsins from marine animals tend to be more stable at alkaline pH, but are unstable at acidic pH. On the other hand, mammalian trypsins are most stable at acidic pH (Simpson, 2000; Klomklao *et al.*, 2006). Trypsins from marine animals resemble mammalian trypsins with respect to their molecular size (22-30 kDa), Their pH optima for the hydrolysis of various substrates were from 7.5 to 10.0, while their temperature optima for hydrolysis of those substrates ranged from 35 to 65°C (De Vecchi and Coppes, 1996). The stability of trypsins at a particular pH might be related to the net charge of the enzyme at that pH (Castillo-Yanez *et al.*, 2005). Trypsin might undergo the denaturation under acidic conditions, where the conformational change took place and enzyme could not bind to the substrate properly (Klomklao *et al.*, 2006).

Chymotrypsins have been isolated and characterized from marine species such as anchovy (Heu *et al.*, 1995) and Monterey sardine (Castillo-Yanez *et al.*, 2006). In general, these enzymes are single-polypeptide molecules with molecular weights between 25 and 28 kDa. They are most active within the pH range of 7.5 to 8.5 and are most stable at around pH 9.0 (Simpson, 2000). Chymotrypsin has a much broader specificity than trypsin. It cleaves peptide bonds involving amino acids with bulky side chains and nonpolar amino acids such as tyrosine, phenylalanine, tryptophan, and leucine (Simpson, 2000).

3. Cysteine proteases; Cysteine or thiol protease are a group of endopeptidase that have cysteine and histidine residues as the essential groups in their catalytic sites. The sample of cysteine protease from the digestive glands of marine animals is cathepsin B (EC 3.4.22.1) (Simpson, 2000).

4. Metalloproteases; The metalloproteases are hydrolytic enzymes whose activity depends on the presence of bound divalent cations. The metalloproteases have been studied from marine animals such as rockfish, carp, and squid mantle but have not been found in the digestive glands except in the muscle tissue (Simpson, 2000).

1.2.5.2 Amylase

Amylases are starch degrading enzymes that catalyzes the breakdown of starch into sugars. They are widely distributed in microbial, plant and animal kingdoms. They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes. Initially the term amylase was used to designate enzymes capable of hydrolyzing α-1,4-glucosidic bonds of amylose, amylopectin, glycogen and their degradation products (Aiyer, 2005). They act by mean of hydrolyzing bonds between adjacent glucose units, yielding products characteristic of the particular enzyme involved. Amylases are divided into endo-amylase (α-amylase, EC 3.2.1.1) and exo-amylase (β-amylase, EC 3.2.1.2 and γ-amylase, EC 3.2.1.3) and are widely distributed in animals, plants and microbes. They can hydrolyze starch on non-reducing ends into mono-, di-, tri- and oligo-dextroses, and are usually used to produce syrup, wine and fermented foods.

The amylase family of enzymes play an important role in the enzyme industry due to its wide range of application. Amylases are used in a number of industrial process such as food fermentation, textiles, paper industries, etc. α-amylase splits randomly α-1,4-glucosidic bonds between adjacent glucose units in linear amylose chain, while glucoamylase hydrolyses single glucosidic residue from the non-reducing ends of amylose and amylopectin in a stepwise manner. GA also hydrolyses the 1,6 α-linkages in the branching points of amylopectins, although at a slower rate than the α-linkages. Starch saccharification is a multi-step process involving several enzymes such as α-amylase, β-amylase, GA, pullulanase and isoamylase. Liquefaction is the first step, where α-amylase distrubts the granular structure of starch and also causes thinning by breaking down amylose and amylopectin. β-amylase is used in the saccharification step only if maltose is needed as a product. Pullulanase is required to obtain a higher dextrose/maltose concentration. Amylases have replaced the used of acid hydrolysis of starch as they offer potential benefits. Amyloglucosidase or γ-amylase hydrolyses the 1,3,1,4, and 1,6 α-linkages in the branching points, it can hydrolyze into β-D-glucose,glucan and limit of dextrin. (Chaplin and Bruck, 2006)

1.2.6 Antioxidants and Oxidation

1.2.6.1 Antioxidants

Antioxidants are substances, synthetic or naturally occurring, that can delay the onset or slow the rate of oxidation. Antioxidants are regarded as compounds capable of delaying, retarding or preventing autoxidation processes. The activity of antioxidants is strongly influenced by numerous factors. Thus, compounds that are effective antioxidants in one system may be unsuitable in other systems. Antioxidants may decrease oxygen concentration, intercept singlet oxygen, prevent first-chain initiation by scavenging initial radical such as hydroxyl radicals, bind metal iron catalysts, decompose primary products of oxidation to non-radical species and break chains to prevent continued hydrogen abstraction from substrates (Shahidi, 2002). Antioxidants is of great interest to identify antioxidants from many natural sources to replace the use of synthetic antioxidants. There are numerous proteins and peptides derived from hydrolyzed food proteins that have been found to possess significant antioxidant activity against lipid peroxidation including milk casine(Blanca et al., 2007), soybean (Li *et al.*, 2006), rice (Adebiyi *et al.*, 2009), and wheat (Wang *et al.*, 2009).

In general, antioxidants act by reducing the rate of initiation reaction in the free radical chain reactions. According to their mechanism of action, antioxidants can be classified as primary or secondary antioxidants.

Primary antioxidants are chain breaking antioxidants and can inhibit lipid oxidation by interfering at the propagation or initiation phase or in β-scission reactions by accepting free radicals to form stable free radicals. (Chaiyasit *et al*., 2007). The primary antioxidant includes phenolic compounds such as vitamin E(α-tocopherol), flavonoids, butylated hydroxyanisole (BHA), galletes, butylhydroxyl toluene(BHT), tertiary butyl hydroxyquinone (TBHQ), and ester of gallic are the major synthetic primary antioxidants. These compounds are consumed during the induction period.

Secondary antioxidants are considered preventative antioxidants, such as chelators (metal ions), oxygen scavengers and singlet oxygen quenchers. These antioxidants decrease the rate of oxidation thought numerous mechanism; however,

they do not convert free radicals into more stable products (Chaiyasit *et al*., 2007). Synergistic antioxidant can be broadly classified as oxygen scavengers and chelators. Transition metals, such as iron, copper, cobalt etc in foods affect both the rate of autoxidation and the breakdown of hydroperoxide to volatile compounds. Transition metal ions react very quickly with peroxides by acting as one electron donors to form alkoxyl radical (Godon, 2001). The secondary antioxidants such as ascorbic acid which are effective in presence of tocopherols or other primary antioxidants, peroxide decomposers such as thioethers, methionine, metal chelaters, glutathaione peroxidase, and catechins (Venugopal, 2009). Peptides in hydrolysate could chelate the pro-oxidant, leading to decreased lipid oxidation. Therefore, chelation of transition metal ions by antioxidant or anti-oxidative peptides retard the oxidationreaction (Sherwin, 1990).

1.2.6.2 Oxidation in biological system

An oxidation and reduction (Redox) reaction is a type of chemical reaction that involves a transfer of electrons from an electron donor (reducing agent) to an electron acceptor (oxidizing agent). The cellular redox environment is influenced by the production and removal of reactive oxygen species (ROS) (Sarsour *et al*., 2009). ROS are chemically reactive molecules containing oxygen that are highly reactive in redox reaction such as oxygen ions and peroxides. This is a collective term and includes oxygen free radicals and several non-radical agents (Table 5).

Table 5 Reactive oxygen species (ROS).

Oxygen radicals/Symbol	**Non-radical oxygen derivatives/Symbol**
Superoxide radical, $O_2^{\bullet-}$	Hydrogen peroxide, H_2O_2
Hydroxyl radical, $HO^{\bullet}$	Hypochloric acid, HOCl
Peroxyl radical, $RO_2^{\bullet}$	Singlet oxygen, O_2^1
Alkoxyl radical, $RO^{\bullet}$	Organic peroxides, ROOH
Nitric oxide radical, $NO^{\bullet}$	Ozone, O_3
	Aldehydes, HCOR
	Peroxynitrite, ONOOH

Source: Kohen and Nyska (2002); Abrahamsen (2009)

ROS generation procedures in biological system are illustrated in Figure 4. The partial reduction of molecular oxygen results in the production of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). $O_2^{\bullet-}$ and H_2O_2 react with transition metal iron (ferrous and cuprous iron) thought Fenton and Haber Weiss chemistry, generating the highly reaction hydroxyl radical ($HO^{\bullet}$). ROS were traditionally thought of as toxic byproducts of living in an aerobic environment because they are known to damage cellular macromolecules, which could subsequently lead to cell death (Sarsour *et al*., 2009). In addition, oxidation can generate free radicals and these free radicals are known as a major factors causing various diseases, such as cancer multiple sclerosis, coronary hearth, cardiovascular disorders, atherosclerosis and inflammation (Willcox *et al.,* 2004. In generally aerobic cell, ROS usually exist in balance with biological antioxidant. Nevertheless, disruption of this critical balance could result in oxidative stress, due to many diseases. (Raghavan *et al*., 2008).

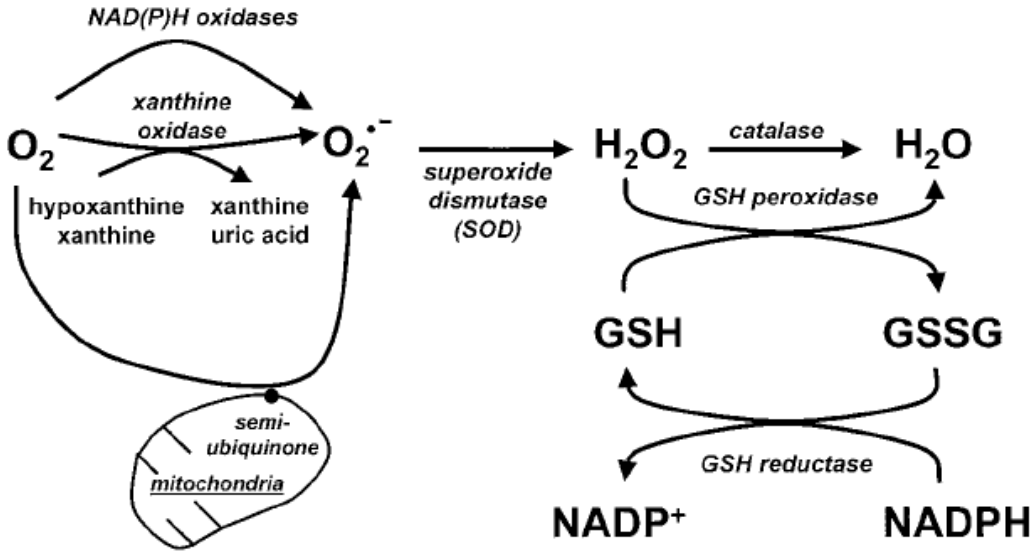


Figure 4 Generation of major from of ROS and their metabolism in biological system. Source: Droge (2002)

1.2.6.3 Antioxidative activity of rice bran hydrolysate

Numerous recent researches have heen published on anti-oxidative properties of rice bran and hydrolysate. The operational conditions employed in the process of protein and bioactive compound isolates are a type of enzyme and process of hydrolysis. Enzymatic hydrolysis is used as a tool to control hydrolysis condition to obtain various bioactive compounds of rice bran hydrolysate with exerted oxidative activity. Some of these studies further fractionated the hydrolysates in order to isolate and identify individual antioxidative peptides. The antioxidant activity of rice bran protein isolate and bioactive compounds from rice bran may be the result of scavenging of specific radicals formed during peroxidantion, scavenging of compounds containing oxygen and the chelating capacity of metals (Kristinsson and Rasco, 2000). However, anti-oxidative activity of rice bran bran is dependent on their amino acid composition of protein and type of compounds obtained.

Rice bran protein has high nutritional value and nutraceutical properties. Kokkeaw and Thawornchinsombut (2007) prepared rice bran protein hydrolysates using the commercial proteolytic enzyme, Protex 6L. It’s radical scavenging activity

was 27.08%. Chanput *et al.* (2009) reported high antioxidant activity from Khao Dawk Mali (KDML) 105 rice bran proteins fractioned through digestion of pepsin followed by trypsin. The phenolic compounds of pigmented rice have been found as a major active component for antioxidant activity (Zhang *et al*., 2006; Yawadio *et al*., 2007; Tabart *et al*., 2009). Pigmented rice is also an important source of vitamins and mineral (iron, calcium, copper and manganese) have reported antioxidant properties (Meng *et al.*, 2005; Yodmanee *et al*., 2011). Ngamdee *et al.* (2016) reported antioxidant activity from black glutinous rice bran as phytochemical and mineral contents.

Rice bran polysaccharides exhibited good potential for reducing power, chelating ferrous irons and scavenging effects of 2-2-azinol-bis(3-ethylbenzthiazoline -6-sulphonate),1,1-diphenyl-2-picrylhydrazyl (DPPH), and hydrogen peroxide (Zha *et al*., 2009).

1.2.7 Bioactive compound in rice bran

Natural products obtained from plants have been used as a prominent source of prophylactic agent for prevention and treatment of disease in humans and animals. Rice bran contains many various bioactive compounds including phytochemicals (anthocyanins and flavonoids, polymeric carbohydrates, phenolic compound and steroidal compounds) with promising health benefits. All of the important bioactive components presented in rice bran are shown in Table 6.

Table 6 Bioactive compound in rice bran. (Modified from Friedman, 2013)

Bioactive compounds	
Anthocyanins and flavonoids	Anthocyanin monomers, dimers and polymers Apigenin, Cyaniding glucoside Cyanidin rutinoside, Epicatechins, Eriodtyol, Hermnetins, Hesperetin, Isohamnetins, Luteolin, Peonidin glucoside, Tricin
Polymer carbohydrates	Arabinoxylans, Glucans, Hemicellulose
Phenolic and cinnamic acids	Caffeic acid, Coumaric acid, Catechins, Ferulic acid, Gallic acid, Hydroxybenzoic acid, Methoxycinnamic acid, Sinapic acid, Syringic acid, Vanilla acid
Steroidal compounds	Acetylated steyrl glucosides, Cycloartenol ferulate, Campesterol ferulate, 24-methyleancycloartenol ferulate, γ-oryzanol, β-sitosterol ferulate, Tocopherols, tocotrienol

1. γ-oryzanol ; γ-oryzanol is a mixture of ferulic acid esterified with normal sterols or triterpean alcohols, called α, β and γ-oryzanol, of which γ-oryzanol has been the most commonly mentioned. The sterol components of γ-oryzanol are primarily campesterol and sitosterol, and the triterpene alcohol components are cycloartenol and 24-methylean cycloartenol (Dey and Harborne, 1991).

The pathway of terpenoids biosynthesis is enzymatically controlled and starts from acetyl-CoA, to movalonate, to 2E, 6E-farnesyl pyrophosphate, to squalean, and then to triterpenoids, with a final chair-boat-chair-boat configuration as shown in Figure 5. Phytosterols are further formed from triterpenoids or cycloartenol (Dey and Harborne, 1991). The initial pathway from acetyl-CoA to squalene is common to all organisms, but the sequence of modifications of the sterol ring system and side chain can differ from species to species or even in various tissues or during different developmental stages in a plant. Sterol production supposedly occurs in the cytosolic

and microsomal compartments of the plant cells (Huang, 2003). The three major components of γ-oryzanol from rice bran are cycloartenyl ferulate, 24-methylean cycloartanyl ferulate and campesteryl ferulate.

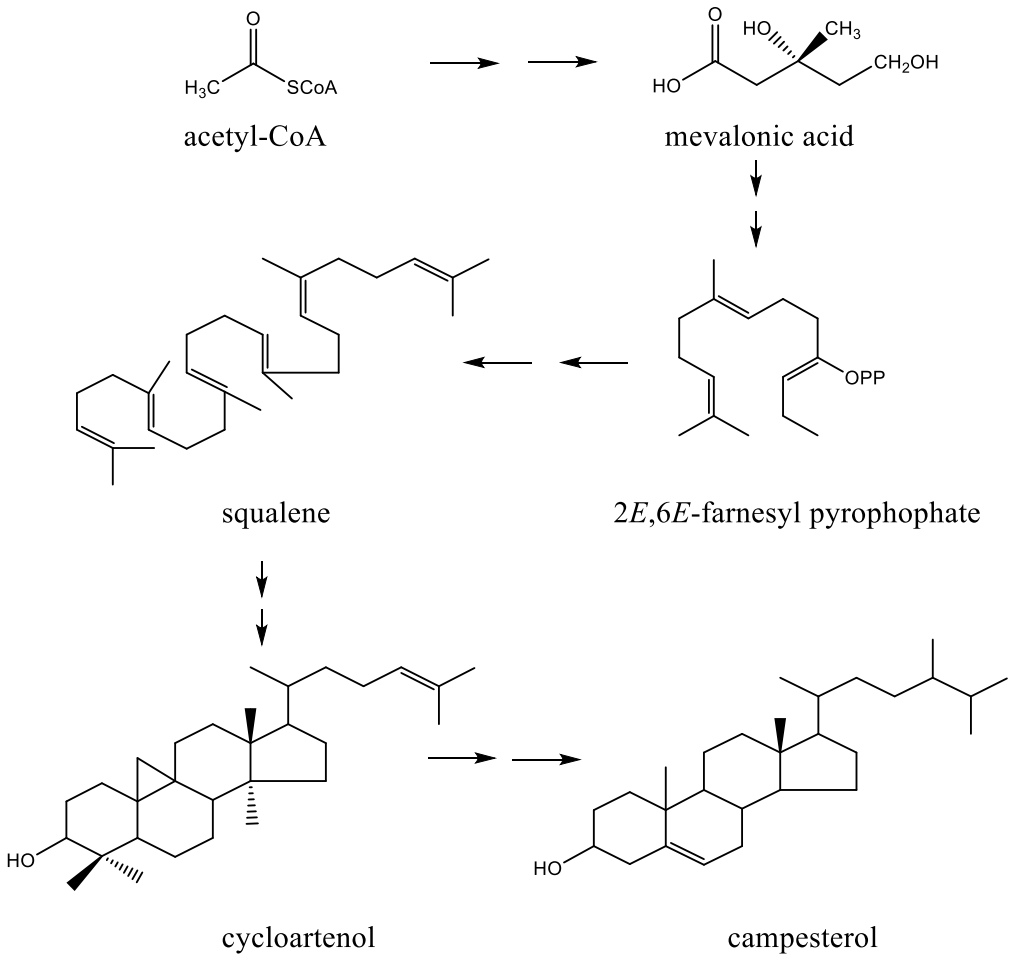


Figure 5 Pathways for biosynthesis of terpenoids in plants
Source: Hung (2003)

The content of γ-oryzanol from rice bran varies between different rice varieties. The high content of γ-oryzanol were found in bran layers of the kernels and varies approximately between 56-474 mg/100g in rice bran. γ-oryzanol has been suggested to have potential functionality such as antioxidant activity (Xu *et al.*, 2001), inhibition of tumor promotion (Yasukawa *et al*., 1998).

2. Ferulic acid; Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a ubiquitous phenolic compound in plant tissues. Ferulic acid occurs as esters in many plants, which is one of the metabolites of the biosynthesis of lignin from phenylalanine and tyrosine in plants, may imply the pathway further undergoes conjugation with other molecules as shown in Figure 6 (Zhao and Moghadasian, 2008). The biological roles of ferulic acid has been presented to crosslink to some cell components and lower their availability to hydrolytic degradation by endospermic enzymes and inhibit germination. Cereal grains contain unique free phenolic compound and their glycoside, which exist in solution, and amount of insoluble phenolic compounds, most of which are bound to polysaccharides in the cell wall (Miller *et al.*, 2000).

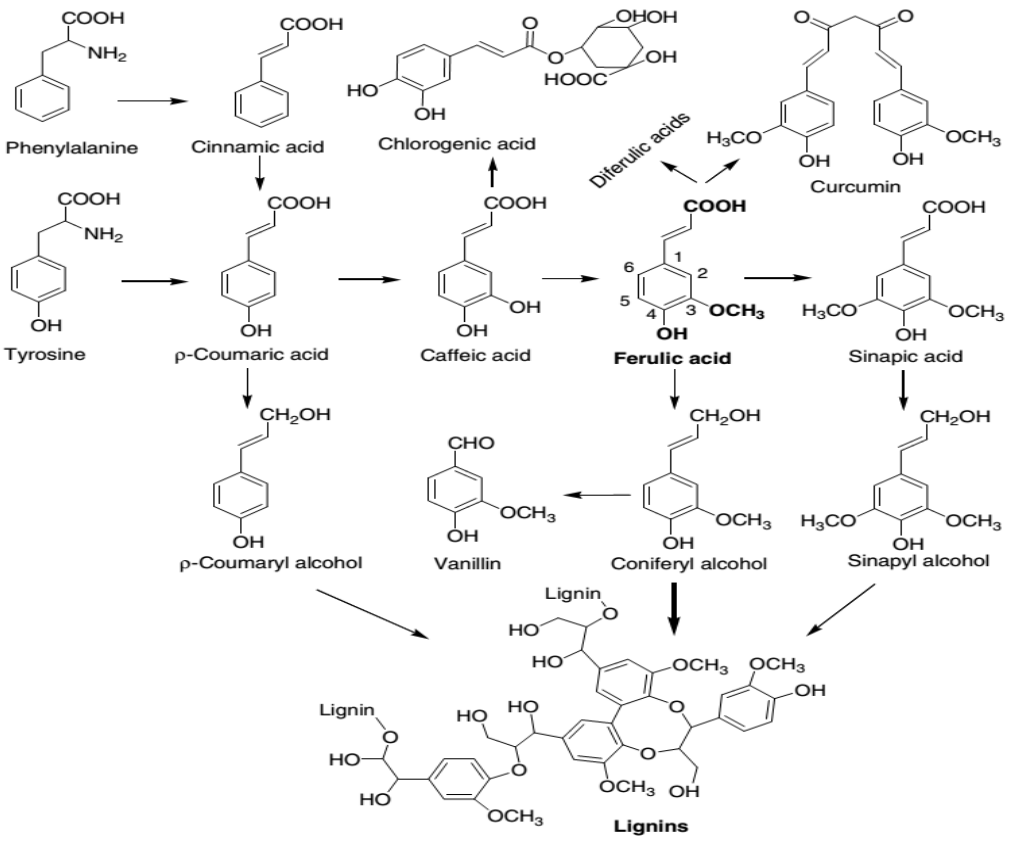


Figure 6 Chemical structure and synthesis of ferulic acid formation in plants
Source: Zhao and Moghadasian (2008)

Ferulic acid is a bio-active compound that have been reported for its cholesterol-lowering properties as well as for its antioxidant capacity. The antioxidant properties of ferulic acid is primarily based on hydrogen donation from the ferulic acid hydroxyl group (Nystrom *et al.,* 2007). This enables ferulic acid to protect DNA and lipid aginst oxidation though reactive oxygen species (ROS). The strong link between inflammation and oxidative stress suggest that it may be effective against inflammatory diseases (Zhao and Moghadasian, 2008).

3. Phytic acid; Phytic acid (inositol hexaphosphate, IP_6) is the principal storage from of phosphorus in many plant tissues, most cereal, nuts, oilseed, legumes especially bran. The structure of phytic acid is a hexaphosphate of myo-inositol. It imparts a potential to bind positively charged molecules such as cations, proteins and carbohydrate. Due to its multiplicity of reactive phosphate groups, phytic acid can complex a cations within phosphate group itself, between two phosphate groups of molecule, or between phosphate groups of different phytic acid molecules (Harland and Morris, 1995).

Phytic acid constitutes about 1-2% by weight of various cereal seeds. Deposition of phytic acid found in small-grained cereals, about 90% of the seed. Phytic acid is found in the aleurone layer and the remaining 10% in scutellum (Brinch-Peddersen *et al.,* 2007). The highest phytic content was found in rice bran whereas the lowest amount of phytic acid was found in rice rootlet and shoot. Phytic acid has antioxidant functions, some anticancer and prevents coronary disease. It prevents the buildup of superoxide, as well as to boost the immune system. It was recently found able to prevent cardiac infraction, diabetes and control anemia (Kayahara, 2004). Serveral other mechanisms by which the phytic acid exerts its anti-cancer and chemopreventive properties include gene alteration, cell cycle inhibition, increased natural killer cell activity and antioxidant function. Phytic acid enacts effects at the genetic level by affecting signal transduction pathways, cell cycle regulatory genes and tumour suppressor genes. Hence, phytic acid may cause greater differentiation among malignant cell and complete reversions to normal phenotypes (Shamsuddin *et al.,* 1997).

4. Dietary fiber; Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine

with complete or partial fermentation in the large intestine (Camire *et al.*, 2001). Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation. There are two categories of dietary fiber: soluble, and insoluble. Soluble fiber includes gums, mucilages, pectin and some hemicelluloses. Insoluble fibers are cellulose, lignin, and the rest of the hemicelluloses; these fibers provide structure to plants (Caprita *et al.*, 2011). Chemically, dietary fiber consists of non-starch polysaccharides such as arabinoxylans, cellulose, and many other plant components such as resistant starch, resistant dextrins, inulin, lignin, waxes, chitins, pectins, beta-glucans, and oligosaccharides. Rice bran is reported to contain 25.5-40.0% total dietary fibers and 2.3-4.3% soluble dietary fibers (Aoe *et al*., 1993; Abdul-Hamid and Luan, 2000; Sudha *et al.*, 2007). Dietary fibers extracted from defatted rice bran using enzymatic-gravimetric method are mainly composed of hemicelluloses (Abdul Hamid and Luan, 2000). Glucose, arabinose, xylose and galactose were the main monosaccharides found in rice hemicelluloses. Xylan provides as the backbone of rice bran hemicelluloses and other sugars act as side chains (Harada *et al.*, 2005).

Insoluble dietary fiber,that will not dissolve in liquid. . Insoluble fiber will absorb liquid and expand in the tract, gently but effectively speeding the process of moving bulk through the system while scraping the interior walls of the tract clean. Cellulose, a major dietary insoluble fiber source, is a linear polymer of between 1000 and 10,000 β-D-glucose molecules in which adjacent glucose molecules are joined covalently through β (1-4) glycosidic bonds. The β (1-4) bonds cause the polymer to assume a non-helical, straight structure, which is different from the helical structure imposed on starch molecules by the α (1-4) bonding. The non-helical structure of cellulose also promotes hydrogen bonding between cellulose molecules. Cellulose polymers associate with one another through a vast number of hydrogen bonds to form microfibers. Microfibers interact to form cellulose fibers. A typical fiber contains roughly 500,000 cellulose molecules. The high tensile strength of cellulose fibers reflects the massive number of hydrogen bonds involved in its structure (Bowen, 2006).

Soluble dietary fiber, this type of fiber dissolves in water to form a gel-like material. Soluble fiber will turn into a gel in the digestive tract and works to keep the rate of food passing through the system from progressing too quickly. This allows

the nutrients derived from food to be absorbed into the system before the bulk is excreted. Soluble fiber is thought to also help with maintaining healthy cholesterol levels, which may indirectly also promote a healthier cardiovascular system. Almost all water-soluble polysaccharides produce viscous solutions. The viscous properties of dietary fiber are determined by several factors, including their chemical composition, molecular size, and composition of the extraction media (Caprita *et al.*, 2011). The predominant water soluble dietary fiber in wheat, triticale and rye are arabinoxylans, while β-glucans are the predominant water soluble dietary fiber in barley and oat. β-glucans are linear polymers of glucosewith β-(1,3)(1,4) glycosidic links.(Lineback and Rasper, 1998).

Beta-glucan (β-Glucan) is a polysaccharides that contain only glucose as structural components, and is linked with β-glycosidic bonds (Mason, 2001). β-glucans are a diverse group of molecules that can vary with respect to molecular mass, solubility, viscosity, and three-dimensional configuration. They occur most commonly as cellulose in plants, the bran of cereal grains, the cell wall of baker's yeast, certain fungi, mushrooms and bacteria. Some forms of β-glucan are useful in human nutrition as texturing agents and as soluble fiber supplements. For decades scientists have known β-glucan as a food constituent, and they knew it was abundant in the foods. It is extremely difficult to extract and purify. However, oat bran contains about 7% β-glucan, and is inexpensive, but only good as a food.

β-glucan have 2 forms structure that are (1→3),(1→4)-β-D-glucan and (1→3),(1→6)-β-D-glucan. The name of (1→3),(1→4)-β-D-glucan is mixed-linkage, belongs to a family of polymers which are heterogeneous with respect to the molecular size and fine structure, varying with tissue, stage of maturity and source (Bacic *et al*., 1988). Mixed-linkage β-glucans are exclusively found in cereal grains (Ford, 1965). On 21 January 1997, The U.S. federal authority Food and Drug Administration (FDA) published health claim on food-product packages stating that "A diet high in soluble fibre from whole oats and low in saturated fat and cholesterol may reduce the risk of heart disease" (FDA, 1996; FDA, 1997). The beneficial effect of oat products is primary attributed to the soluble dietary fibre compound that is mixed-linkage β -glucans (FDA, 1997). Mixed-linkage β -glucans is a linear molecule of partially water-soluble polysaccharide consisting of glucose linked composed of (1→4) linked glucopyranosyl

(Glcp) residues (approx. 70%) substituted at position 3 or 4 and (1→3)-linked Glcp residues (approx. 30%) substituted at position 4 (Aspinall and Carpenter, 1984) (Figure 7).

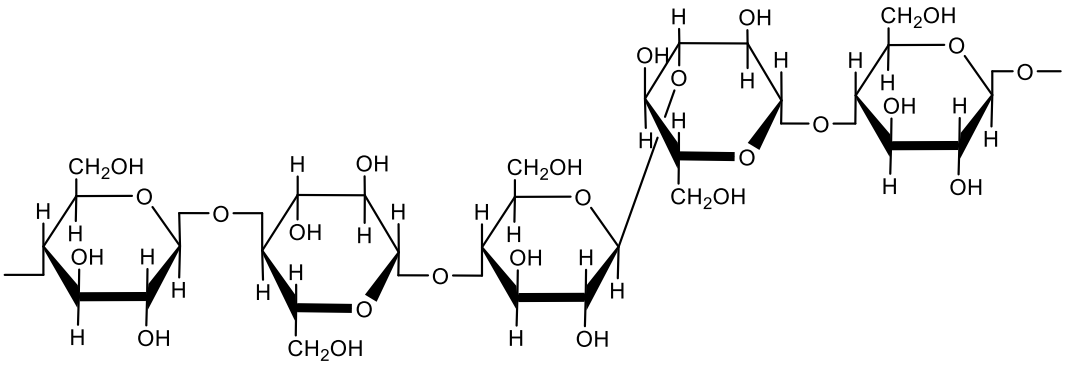


Figure 7 Mixed-linkage β –glucans structure.
Source: Roubroeks *et al*. (2001).

Mixed-linkage β-glucan is known to reduce blood cholesterol levels. The Food and Drug Administration of the USA has accepted a health claim in which it is stated that a daily intake of 3 g of soluble oat β-glucan can lower the risk of coronary heart disease (FDA, 1997). Oats also reduce the glucose and insuline responses (Johansson, 2006). Ripsin *et al*. (1992) showed in a meta-analysis that oat products in a diet cause a modest reduction in blood cholesterol level. They concluded that a daily intake of 3 g of oat soluble fiber was needed to reduce cholesterol 0.13-0.16 mol^{-1}/l. Cereal β-glucan showed positive physiological effects on the cardiovascular system but also their antibacterial, anti-tumural, immunomodulant, and radio-protective properties are mentioned (Havrlentová *et al*., 2011). β–Glucans from cereal and other sources have the characteristics of both dietary fiber and insoluble dietary fiber, therefore development of β–Glucans rich products could potentially reduce the incident or slow progression of chronic diseases (Ahmad *et al*., 2012).

Epidemiological data has suggested that the consumption of dietary fiber, especially the mixture of soluble and insoluble fibres, is inversely associated with the risk of cancers, such as colon cancer. Therefore, the presence of dietary fiber in high amounts in rice bran might partly explain its effects on the reduction of cancer.

5. Rice bran protein (RBP); Rice bran contains about 10-15% of high quality proteins and possesses a powdery consistency. RBP is of this is of high nutritional value and has nutraceutical properties. It has a comprising amino acid composition and protein qualities (e.g. essential amino acids, protein efficiency ratio and net protein utilization). A protein formulation based on rice bran can help overcome protein related nourishment disorders. It has also been reported to have anti-cancer and di-peptidyl peptidase IV inhibitory activity (Hatana *et al.,* 2012) Additionally, the hypoallergenic and anticancer properties make rice bran proteins superior to cereal proteins (Fabian and Ju., 2011). Several studies have been carried out on RBP hydrolysates. For example, RBP hydrolysates prepared using commercial proteolytic enzymes, the product's presented radical scavenging activity (Kokkeaw and Thawornchinsombut., 2007). Chanput *et al.* (2009) reported that KDML 105 rice bran protein fractions digested with pepsin and subsequently with trypsin revealed high antioxidant activity. Protein from Japanese rice bran was fractionated and hydrolyzed with protease resulting in peptides of 6-30 amino acid residues, showed high antioxidant activity (Adebiyi *et al.,* 2009)

Bioactive peptides are specific and small protein fragment that are inactive within the sequence of their parent protein. These peptides are 2-9 amino acids in size and typically possess specific amino acid sequences, mainly comprised of hydrophobic groups in addition to proline, arginine and lysine. Moreover, several researches reported about bioactive peptide exhibit antioxidant, anti-hypertensive and anti-obesity and the ACE inhibitory activity are often shown by antioxidant peptides simultaneously (Xiuming *et al.,* 2016). Moreover, Zhang *et al.* (2014) reported bran protein hydrolysate from enzymatic hydrolysis is an excellent source of protein and provides bioactive such as antioxidant activity, thus the phenolic hydroxyl group of tyrosine, acidic and basic amino acids and hydrophobic amino acids could contribute to the antioxidant activity. The hydroxyl groups bonded to the aromatic ring provide hydroden atoms which terminated the radical chain reaction by scavenging free radicals. The acidic and basic amino acid could bind metal irons preventing the oxidation. The hydrophobic amino acids could increase the presence of the peptides at water-lipid interface, which improve scavenging activity with hydrophobic radicals or free radicals generated in lipid phase.

6. Anthocyanins and flavonoids; in rice, phytochemicals are concentrated in the rice bran layer (bran and germ), the outer layer of whole grain rice that gives the color of the whole grain. The black, red and some purple rice varieties contain anthocyanin and proanthocyanin and have several fold higher antiradical capacity than light brown bran rice (Min *et al.*, 2012). Thus, rice with high pro-anthocyanin has the potential to be sold as specialty rice or as its PA extract as a dietary supplement. Shen *et al.* (2009) reported various ranges of total phenolic concentration and antioxidant capacity of rice with red bran.

Proanthocyanidins (PAs) condensed tannins, are a major subgroup of flavonoids that are oligomers and polymers of flavan-3-ol units. The molecular weights on the number of monomeric units (i.e.(+)-catechin and/or (-)-epicatchin) that are linked via an interflavan bond of C4 → C6 or C4 → C8 (B-type) or doubly linked by a C4 → C8 bond and C2 → O7 ether bond (A-type) (Gu *et al.,* 2002). PAs are capable of modulating inflammatory responses, which are involved in the development of cardiovascular disease and some cancers, as well as providing protective effects against type 2 diabetes. Studies have suggested that PAs might have prebiotic-potential by modulating the gut microbiota and providing health benefit without being absorbed (Ou and Gu., 2014).

1.2.8 Application in food products

Currently, people are concerned about health and nutrition. Rice bran is important nutritious and thus is used as a food additive and functional food. The primarily use of rice bran as an additive in food is due to its high fiber content. From a commercial marketing view point, the most commonly available rice bran-derived products is oil. Rice bran oil has an impressive nutritional quality that makes it suitable for nutraceutical products. Additionally, it has the potential to be used as an additive to improve the storage stability in food due to its antioxidant activity (Kim and Godber, 2001). Rice bran fiber can be used as both a nutritional and functional ingredients. The nutritional and functional properties of rice bran are suitable for baked products, including cookies, crackers, muffins, breads, pastries and pancakes (Sharif *et al.*, 2009). The addition of rice bran into wheat flour increased the protein, lysine and dietary contents in bread and cookies. The color, flavor, protein extractability and solubility of

bran, as well as other properties, such as water and fat absorption, foaming capacity and emulsifying, have presented improvements that further enlighten us on the potential use of bran in foods. Due to its natural occurring enzymatic activity (lipase) and inhibit hydrolytic rancidity, rice bran needs to be stabilized in order to control these undesirable reactions (Sharma and Chauhan, 2002). The process destroys the fungi, bacteria and insect infestations, thus enhancing the shelf life of rice bran derivative.

Rice bran was incorporated in up to 20% of the production of yeast bread because the hygroscopicity of rice bran may improve its moisture retention in the baked products, while its ability to foam improved the air incorporation and leavening process. Defatted rice bran can be used to substitute up to 10 to 20% of wheat flour used for making cookies without adversely affecting the quality (Shairf *et al.,* 2009). Biscuits produced with broken rice powder were highly acceptable in terms of taste and smell (Ashaf *et al.,* 2012).

Also, rice bran has a 10-15% proein content, consisting of 37% water-soluble, 31% salt-soluble, 2% alcohol-soluble and 27% alkali-soluble storage proteins. Rice bran proteins have been found to be of high quality and application in food and pharmaceutical industries. Its unique properties, hypoallergenicity (Tsuji *et al.,* 2001) and anticancer effects make it a superior cereal protein with a wide range of possible applications. Nevertheless, as of now, commercial rice bran protein is still unavailable on the market (Fabian and Ju., 2011). Rice bran protein concentrate (RBPC) from defatted rice bran was incorporated at 5, 10 and 15% in biscuits. The phytochemical, fracture strength, sensory attributes were analyzed in biscuits to assess their acceptability (Yadav *et al.,* 2011). The fiber in rice bran has been reported to contain high amounts of functional proteins and fats along with antioxidants, vitamins and trace minerals, in addition to being a concentrated source of fiber. The presence of these nutrients allows rice bran fiber to be used as both nutritional and functional ingredient. A number of studies have been carried out to evaluate rice bran as a functional ingredient in various foods to improve the nutritional quality. Its therapeutical potential, its addition can contribute to the development of value added foods or functional foods that currently are in high demand (Table 7.).

Table 7 Application of functional food products with rice bran. (Modified from Khalid, 2015)

Product enriched	**Purpose of addition**	**References**
Noodle and Pasta	Effect on textural and antioxidant properties (higher polyphenols,flavonoids and anthocyanins and higher antioxidant activity)	Kong *et al*.,2012 Kaur *et al*.,2012
Gluten-free bread with dietary fiber fraction	Acceptable structural and textural quality	Phimonsiripol, Mukprasirt and Schoenlechner, 2012
Cookies with rice bran	Effect on cooking quality (thickness and spread increased)	Younas *et al.*, 2011
Flake products with rice bran	Effects of chemical, physical and antioxidation properties	Wanyo, Chomnawang and Siriamornpun, 2009
Bread with rice bran hemicellulose	Effect on chemical and functional properties	Hu *et al.*, 2009
Cookies	Fiber and mineral enrichment	Sharif, Butt and Nawaz, 2009
Pan bread with defatted rice bran	Enrichment with fiber and minerals	Ajmal *et al.*, 2006
Pizza with high content dietary fiber rice flour	Effects of chemical, physical and anti-oxidation	Delahaye *et al.*, 2006

1.2.9 Relationship with inflammation

1.2.9.1 Inflammation

Inflammation is a normal protective response to tissue injury caused by physical trauma, allergen, noxious chemicals or microbiological agents (Vijayalakshmi *et al.,* 2011) involving a wide variety of physiological and pathological processes to inactivate or destroy invading organism, to remove irritants and to set stage for tissue repair (Paschapur *et al.,* 2009). **Inflammation is the body's attempt at self-protection; the aim being to remove harmful stimuli, including damaged cells, irritants, or pathogens and begin the healing process.** When something harmful or irritating affects a part of our body, there is a biological response to try to remove it, the signs and symptoms of inflammation, specifically acute inflammation, show that the body is trying to heal itself. Inflammation does not mean infection, even when an infection causes inflammation. Infection is caused by a bacterium, virus or fungus, while inflammation is the body's response to it (Gould, 2002; Gupta *et al*., 2003). Inflammation is characterized by several familiar signs, redness, swelling, heat, fever, pain and loss of function. Therefore, the main purpose of inflammation is to identify and eliminate injurious agents and to repair the surrounding tissue. The inflammation response involves several stages which include dilation of capillaries to increased blood flow, microvascular structural change and escape of plasma proteins from the blood stream, leukocyte-adhesion cascade, and elimination of possible pathogens and resolution of inflammation.

Common acknowledge, inflammation plays an important role in the initiation and progress of many diseases including cancer in multiple organ sites (Schetter *et al*., 2010). Inflammation, classified either acute or chronic, has been described as a link to many age related health problems. Acute inflammation occurs from minutes to hours and days following tissue damage caused by physical force or an immune response. Chronic inflammation occurs over a longer period of time and is caused by pro-inflammation mediators. Chronic inflammation in linked to rheumatoid arthritis, diabetes, atherosclerosis, and cancer (Guo *et al*., 2008). Thus, inhibition of the production of pro-inflammatory mediators is an important point in the treatment of various inflammatory diseases. Inflammation is triggered by a release of chemical

mediators form the injured tissues and migrating cells. Role of mediators in different reactions of inflammation are also summarized in Table 8.

Table 8 Chemical mediator of inflammation and their actions

Mediator	Principal Sources	Actions
CELL-DERIVED (Performed)		
Histamine	Mast cells, Basophils, Platelets	Vasodilation, increased vascular permeability, endothelial activation
Serotonin	Platelets	Vasodilation, increased vascular permeability
CELL-DERIVED (Newly synthesized)		
Prostaglandins	Mast cells, Leukocytes	Vasodilation, pain, fever
Leukotriene	Mast cells, Leukocytes	Increased vascular permeability, chemotaxis, leukocyte adhesion and activation
Platelet-activation factor	Mast cells, Leukocytes	Vasodilation, increased vascular permeability, degranulation oxidative burst, leukocyte adhesion and activation, chemotaxis
Reactive oxygen species	Leukocytes	Killing of microbes, tissue damage
Nitric oxide	Endothelium, Macrophages	Vascular smooth muscle relaxation, killing of microbes
Cytokines (TNF, IL-1, IL-6)	Macrophages, Endothelial cells, Mast cells	Local endothelial activation (expression of adhesion molecules), decreased vascular resistance (shock), fever/pain/anorexia/hypotension
Chemokines	Activated macrophages, Leukocytes	Chemotaxis, leukocyte activation
PLASMA PROTEIN-DERIVED		
Component products (C5a, C3a, C4a)	Plasma (product in liver)	Leukocyte chemotaxis and activation, vasodilation (mast cell stimulation)
Kinins (Bradykinin)	Plasma (product in liver)	Increased vascular permeability, smooth muscle contraction, vasodilation, pain
Proteases activated during coagulation	Plasma (product in liver)	Endothelial activation, leukocyte recruitment

IL-1- interleukin-1; TNF- tumor necrosis factor; IL-6- interleukin-6. Source: (Kurmar *et al.,* 2011)

1.2.9.2 Nitric oxide and nitric oxide synthase

Nitric oxide (NO) is a major mediator of inflammation. It is a soluble and short-lived free radical gas produce by many cell types and capable of mediating a variety of functions. Nitric oxide (NO), a colorless gas, has been considered as an important biological regulator which is a fundamental component in the fields of neuroscience, physiology and immunology (Derosa *et al*., 2008; Jiang *et al*., 2012). Nitric oxide (NO) or nitrogen oxide also known as nitrogen mon-oxide, is a free radical and is a member of the labile radical entities knows as reactive oxygen species (ROS). It is a gaseous signaling molecule that regulates various physiological and pathophysiological responses in the human body (Alderton *et al*., 2001). These include circulation and blood pressure, platelet function, host defense and neurotransmission in central nervous system and in peripheral nerves (Korhonen and Pihlanto, 2006). However, NO can act as a cytotoxic reagent in pathological processes, particularly in inflammatory disorder (Alderton *et al*., 2001).

Nitric oxide synthase (NOS); Nitric oxide is a pancreatine mediator that is released by endothelial cells and by certain neurons (Sharma *et al.,* 2007). NO is produced by various group of enzyme termed as nitric oxide syntheses (NOS) which are present in the body (Brero *et al*., 2010). The formation of NO is synthesized form L-arginine in a reaction catalyzed by a family of nitric oxide synthase (NOS) enzyme. Active NOS is a tetramer formed by two NOS proteins and two calmodulin molecules. Conversion of L-arginine to NO and L-citrulline requires also NADPH (nicotinamine adenine dinucleotide phosphate with extra hydrogen) and Oxygen as necessary co-factors. FDA (flavin adenine di-nucleotide), (6R) tetrahydrobioprotein (H_4B), FMN (flavin mono-nucleotide), and iron protoporphyrin IX (heam) are also co-factors. Moreover, three isoforms of NOS are present whose names are termed on the basis of their activities, which include following neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). The mechanism of NO formation by NOS was illustrated in Figure 8 (Korhonen and Pihlanto, 2006).

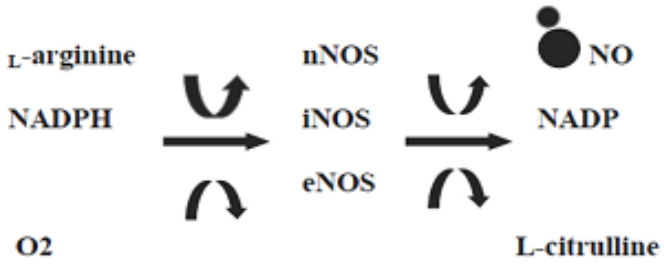


Figure 8 Mechanism of NO formation by NOS
Source: Sharma *et al.*, (2007)

In animal tissue, NO is generated enzymatically by systhases (NOS). Three different isomer of NOS have been characterized (Guzik *et al.*, 2003).

1. NOS I or nNOS is bound to plasma membranes and known to be strongly activated by the entry of calcium though membrane-bound receptors (Novo and Parola, 2008). Whereas the reactivity of NOS isoform depend upon their binding with calmodulin (Bath *et al*., 2002). When intracellular calcium level leads to increased production of calmodulin ultimetly leading to augmented binding of calmodulin to eNOS and nNOS which further leads to enhanced production of NO by their enzymes (Sánchez *et al*., 2012). The neural nitric oxide synthase (nNOS) is mainly present in neural tissue and serves as a neurotransmitter.

2. NOS II or iNOS is incudible nitric oxide synthase. Inducible NO systhase iNOS), which was first identified in macrophages and then in other cells, including hepatocytes, is known to be up-regulated by pro-inflammatory cytokines and/or lipopolysaccharide (LPS), and is able to generate low levels of NO compared with the other NOS isoforms (Novo and Parola, 2008). iNOS derived nitric oxide has also been reported in certain T (Taylor-Robinson, 1997) and B cell lines (Koide *et al*., 2003), optic nerve astrocytes (Neufeld and Liu, 2003), hepatocytes, neutrophils, vascular smooth muscle cells, and endothelial cells (Bogdan, 2000). Infected or activated macrophages by such as bacterial lipopolysaccharide (LPS) produce high levels of iNOS-derived nitric oxide. iNOS is induced upon activation (of mostly macrophages or monocytes) in response to inflammatory cytokines such as IFN-γ, IL-1β, and TNF-α (Bogdan, 2001).

3. NOS III or eNOS is a constitutive enzyme primarily discovered in endothelium, which is known to play an important role in the dynamic control of vascular tone. eNOS and nNOS are constitutive isoforms of NOS and are also known as cNOS.

1.2.9.3 Nitric oxide (NO) and inflammation

NO is a reaction molecule that has a variety of effect depending on the relative concentrations of NO and the surrounding environment in which NO is produced. There are both direct effects of NO that are mediated by the NO molecules itself, and direct effect of NO that are mediated by reactive oxygen species produced by the interaction of NO with superoxide anion or with oxygen (Korhonen and Pihlanto, 2006). The molecular mechanism that mediate the biological activities of NO can be divide into three categories.

1. Nitric oxide reacts readily with transition metals, such as iron, copper and zinc. These metals are abundantly present in prosthetic groups of enzymes and other proteins, and by that mechanism, NO regulates the activity of various enzymes.

2. NO is able to induce the formation of S-nitrosothiols from cysteine residues in a reaction called S-nitrosylation. Nitrosylation has been shown to modify the activity of several protein involved in cellular regulatory mechanism.

3. NO reacts very quickly with superoxide anion (O_2^-), resulting in the formation of peroxynitrite ($ONOO^-$). Peroxynitrite is a nitrating agent and a powerful oxidant that is able to modify protein, lipids and nucleic acids.

Inflammation is a defense mechanism initiated by invasion of pathogens or by tissue injury caused by biological, chemical or physical damage. The activation of macrophage is an important part of initiating defensive reactions, and macrophage-release inflammatory mediators such as nitric oxide, and proinflammatory cytokines such as tumor necrosis factor-α (TNF- α), interleukine-6, interleukine -1β are released to enhance defense capacity (Ahn *et al.,* 2012). NO gives an anti-inflammatory effect under normal physiological conditions. Conversely, NO is considered as a pro-inflammatory mediator that induces inflammation due to over production in abnormal situations (Sharma *et al.,* 2007). Over production of NO and proinflammatory cytokines

is associated with many disease, including asthma, atherosclerosis, rheumatoid arthritis and endotoxin-induced multiple organ injury (Ahn *et al.*, 2012).

1.2.9.4 Cytokines and inflammation

Cytokines are small secreted proteins released by cells which have a specific effect on the interactions and communications between cells. Cytokine is a general name, other names include lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by one leukocyte and acting on other leukocytes). Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action) (Zang *et al*., 2007). Cytokines are made by many cell populations, but the predominant producers are helper T cells (Th) and macrophages pathological processes by resident and recruited macrophages, mast cells, endothelial cells, and Schwann cells. Following a peripheral nerve injury, macrophages and Schwann cells that gather around the injured site of the nerve secrete cytokines and specific growth factors required for nerve regeneration synthesized inside the spinal cord (DeLeo *et al*., 1996) the DRG soma (Schafers *et al*., 2003) or the inflamed skin (Heijmans *et al*., 2006). Moreover, cytokines may be transported in a retrograde fashion from the periphery, via axonal or non-axonal mechanisms, to the DRG and dorsal horn, where they can have profound effects on neuronal activity (Ozaktay *et al*., 2006) and therefore contribute to the etiology of various pathological pain states.

Inflammation is usually initiated within minutes in any host with a functional innate immune system. As innate immune system is the major contributor to inflammation, immune cell such as macrophages, dendritic cells, mast cell, neutrophils and lymphocytes play important roles in inflammatory responds. Based on the nature of stimulants, inflammatory pathways very significantly and are their target tissues. In the case of bacterial infection, the immune cells though specific receptor immediately, activation of pathogen-specific receptors induces the production of inflammatory mediator such as inflammatory cytokines (e.g. tumor necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-6 (IL-6) and chemokines (Ahmad, 2011). Inflammation

responses are coordinated by the products of such gene, precisely proinflammatory cytokines such as TNF, IL-1β and IL-6 are expressed in response to bacterial infection.

1.2.9.5 Pro-inflammatory cytokines

Pro-inflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. There is abundant evidence that certain pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are involved in the process of pathological pain (Zang *et al*., 2007).

1. TNF-α ; Tumor necrosis factor alpha (TNF-α) also known as cachectin is a multifunctional cytokine that can regulate many cellular and biological processes for instance immune function, inflammation, cell differentiation, proliferation, apoptosis and energy metabolism (Bazzoni and Beutler, 1996; Cawthorn and Sethi, 2008). TNF-α is a potent pro-inflammatory cytokine released primarily from stimulated macrophages. TNF-α was originally identified and isolated for two known characteristic activities, the ability to induce hemorrhagic necrosis of certain tumors and the ability to induce cachexia during states of chronic infection. TNF-α now represents a key mediator of inflammatory responses. Many aspects of tissue damage following acute or chronic inflammatory reactions can be directly attributed to the concomitant induction of TNF biosynthesis and release, and provide the therapeutic rationale for developing TNF antagonists (Crisafulli *et al.,* 2009).

2. IL-1β ; Interleukin-1 beta (IL-1β) also known as catabolin, is A member of the interleukin 1 family of cytokines. This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The induction of cyclooxygenase-2 (PTGS2/COX2) by this cytokine in the central nervous system (CNS) is found to contribute to inflammatory pain hypersensitivity. This gene and eight other interleukin 1 family genes form a cytokine gene cluster on chromosome 2 (Dinarello, 1994). IL-1β has been described in endothelial cells and in keratinocytes (Nylander and Egelrud, 1997). Low-dose administration of cytokine induces local inflammatory responses followed by activation of protective immunity, whereas high-dose administration causes broad

inflammation accompanied by tissue damage and tumor invasiveness (Apte and Voronov, 2002).

3. IL-6 ; Interleukin-6 (IL-6) is a multifunctional cytokine that plays a key role not only in the immune system but also in a variety of biological processes. IL-6 acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine (Akira *et al*., 1990). IL-6 is a primary regulator of both acute and chronic inflammations. It has a dual effect; at some levels as it acts as a defense mechanism but in chronic inflammation it is rather pro-inflammatory (Gabay, 2006).

IL-6 is secreted by T cells and macrophages to stimulate immune response, e.g. during infection and after trauma, especially burns or other tissue damage leading to inflammation. IL-6 also plays a role in fighting infection, as IL-6 has been shown in mice to be required for resistance against bacterium *Streptococcus pneumoniae* (Van der Poll *et al*., 1997). IL-6 is a pro-inflammatory cytokine that signals via binding to a soluble or membrane bound receptor, while nitric oxide (NO), an oxidative stress molecule, diffuses through the cell membrane without a receptor. Both mediators signal through different mechanisms, yet they are dependent on nuclear factor kappa B (NFκB) (Maalouf *et al*., 2010). IL-6 is an important mediator of fever and of the acute phase response. It is capable of crossing the blood-brain barrier (Banks *et al*., 1994) and initiating synthesis of PGE_2 in the hypothalamus, thereby changing the body's temperature set point. In muscle and fatty tissue, IL-6 stimulates energy mobilization that leads to increased body temperature. IL-6 can be secreted by macrophages in response to specific microbial molecules, referred to as pathogen-associated molecular patterns (PAMPs).

The inflammatory response is often categorized according to the duration and kinetics of the reaction and can be classified as either acute or chronic.

Acute inflammation; In general terms, acute inflammation is the immediate and early host response to an injurious agent. The period of acute inflammation is relatively short, lasting from a few minutes to a few days. Acute inflammation has three major components: (1) alteration in vascular caliber that lead to an increase in blood flow, (2) structural changes in the microvasculature that permit plasma proteins and leukocytes to leave the circulation, and (3) emigration of the leukocytes from the microcirculation, their accumulation in the focus of injury, and

their activation to eliminate the offending agent (Kurmar *et al.,* 2011). Clinically, acute inflammation is characterized by 5 cardinal signs: rubor (redness), calor (increased heat), tumor (swelling), dolor (pain), and functio laesa (loss of function) (Ramzi *et al.,* 1999). Redness and heat are due to increased blood flow to the inflamed area; swelling is due to accumulation of fluid; pain is due to release of chemicals that stimulate nerve endings; and loss of function is due to a combination of factors. These signs are manifested when acute inflammation occurs on the surface of the body, but not all of them will be apparent in acute inflammation of internal organs.

Acute inflammation is mediated by an increase in blood flow into injured tissues primarily from arteriolar dilatation and the opening of capillary beds. There is also increased movement of leukocytes (in particular granulocytes, such as neutrophils) and increased leukocyte adhesion to the vascular endothelium (via adhesion molecules). Leukocytes phagocytose the injurious agent and release toxic metabolites and proteases, which potentially causes tissue damage. These processes result in a cascade of biochemical mediators, such as vasoactive amines (e.g., histamine and 5-hydroxytryptamine), plasma proteases (e.g., the complement, kinin and clotting systems) and arachidonic acid metabolites (e.g. prostaglandins, leukotrienes and lipoxins). A complex array of cytokines and chemokines are also produced by inflammatory cells and the vascular endothelium, which regulate lymphocyte function through activating (e.g., IL-2, IL-4, IFN-α and IFN-β) or inhibiting immune responses (e.g.IL-10 and TGF-β). Cytokines activate inflammatory cells (e.g., TNF-α, IL-1β, IFN-γ and IL-6) or stimulate hematopoiesis and leukocyte growth and differentiation (e.g., IL-3 and IL-7) (Winyard, 2003). Physiological gaseous mediators, such as nitric oxide (NO) and carbon monoxide (CO) have been proposed to induce and regulate the inflammatory process.

Chronic inflammation ; Chronic inflammation may progress from acute inflammation if the stimuli persist. A cronic inflammation is generally believed to develop if the elimination of the triggering stimulus fails to happen, such as any persistent infection or chronic cellular injury. It is inflammation of prolonged duration (weeks or months), in which active inflammation, tissue destruction and attempts at repair proceed simultaneously (Ramzi *et al.,* 1999) and lead to several distinct processes. A progressive shift in the types of cells present at the site of inflammation

(e.g. from granulocytes to mononuclear cells, such as macrophages, lymphocytes and plasma cells, which characterize the persistent reaction to injury). Tissue destruction mediated predominantly by the inflammatory cells. Connective tissue replacement of damaged tissue via micro-vascular proliferation (angiogenesis) and fibrosis (attempts at healing). Although chronic inflammation may follow acute inflammation, frequently, it has insidious beginnings as a simmering low grade and often asymptomatic response, so-called ‘subclinical inflammation. Types of chronic inflammation include some of the most common and debilitating human diseases, such as rheumatoid arthritis, tuberculosis, asthma, and inflammatory bowel disease.

1.3 Objectives

1. To investigate effect of enzymatic hydrolysis conditions on Sang Yod rice bran hydrolysate (SYRBH) and their properties.
2. To verify the composition and functional properties of SYRB hydrolysates.
3. To evaluate anti-oxidative activity and anti-inflammatory activity of SYRB hydrolysates.
4. To utilize SYRB hydrolysates as functional ingredients in preparation rice pudding product.

CHAPTER 2

SANG YOD RICE BRAN (SYRB) HYDROLYSATE PRODUCTION BY ENZYMATIC HYDROLYSIS

2.1 Abstract

Sang Yod rice bran hydrolysates (SYRBH) were prepared by enzymatic hydrolysis of Protease G6 and Amyloglucosidase. Application of these two enzymes were either single, sequential or combined digestion. Effects of enzyme concentrations (3, 4 and 5% w/v) and hydrolysis duration (30, 60 and 120 min) on catalytic process and hydrolysate characteristics were investigated. Protein content of the SYRBH was increased (0.59-3.37%) with the increase of enzyme concentrations and hydrolysis times. Their β-glucan content, however, decreased with those parameters. The hydrolysis using 5% w/w enzyme for 30 min yielded the hydrolysate with the highest β-glucan content. The sequential and combined hydrolysis yielded the hydrolysate with high protein and β-glucan content as well as great value of total phenolic content (TPC), total anthocyanin (TAC) and functional properties relative to those of the process using single enzyme. The hydrolysate exhibiting the highest protein and β-glucan content was prepared by using sequential hydrolysis of 5% w/w enzyme concentration for 30 min. The results thus suggest that SYRBH derived by using sequential hydrolysis has great composition of bioactive compounds and functional food.

2.2 Introduction

Sang Yod (SY) rice is a traditional rice cultivars that has long grain, the cooked rice is soft and tasty. Presently, pigmented rice is the most favorite consumer type of rice for consumption of this variety. Moreover, increasing interest to healthy rice consumption and support is growing for SY in other areas of Southern Thailand. SY rice bran consists of high content of polyphenol compound which contains

antioxidant activity (Naiyana and Nakanyapatthara, 2011). Additionally, various bioactive compound from pigmented rice bran (protein, anthocyanin, oil extract, vitamin and mineral) showed pharmacological activities such as anti-diabetic effects, antioxidant effect and hence they would have benefit in antioxidant prevention, against human cancer cell line (Yodmanee *et al*., 2011; Uttama *et al*., 2014; Itharat *et al*., 2016). Though, it is considered a less valuable product, rice bran still contains useful substances such as fiber and protein (Anderson *et al.,* 2009). Therefore, the current interest is to separate the benefit nutrient of rice bran before discarding as waste.

Rice bran is the most abundant and valuable by-product in the rice milling process (Wang *et al.,* 2015). It has been used as a feed stock for oil extraction and it is being used as a low-cost animal feed (Kannan *et al.,* 2008) and has a potential to be used as a food ingredient. Rice bran contains a non-significant amount of protein (12-20%), with fairly high nutritional quality (Saunders, 1990), containing many bioactive compound for instance 20-27% total dietary fibers, a rich source of iron and vitamin B, and small molecule (essential fatty acids, phytosterols, and antioxidants) (Borresen and Ryan, 2014). Rice bran proteins contain 37% albumin, 36% globulin, 22% glutelin and 5% prolamin (Tang *et al.,* 2003). The lysine content of rice bran protein is approximately 3-4%, which is higher than proteins from other cereal brans. Rice bran protein is also digestible and maybe hypoallergenic (Wang *et al.,* 1999). Rice bran protein shows great promise as an alternative protein source such as functional food ingredients (Yeom *et al.,* 2010). Also, it can be used as a dietary fiber source. One active component in rice bran is water soluble polysaccharide (Aoe *et al.,* 1993). β-glucan is soluble polysaccharide, which is a main composition of rice bran (Ahmad *et al.,* 2009). Health benefits associated with intake of soluble dietary fiber include better control of diabetes (Brennan and Tudorica, 2003), lowering of blood cholesterol and reduction of hypertension (Keogh *et al*., 2003), antioxidation and cancer (Sier *et al*., 2004), and promote growth of beneficial intestinal micro flora (Tungland, 2003).

In the present report, the various method of extracting rice bran hyrolysate are acid or alkali extraction, chemical and enzyme extraction. Several enzymatic hydrolysis methods can be applied to alter the structural characteristic of rice bran in order to increase its accessibility to hydrolyze and to obtain the most potent bioactive compounds (peptide and dietary fiber). One possible method of degrading the

cell wall to further release bioactive compound such as protein, dietary fiber and phenolic is complex enzyme hydrolysis using protease, cellulase and glucoamylase (Liu *et al*., 2017). Protease have been used to enhance recovery of rice bran protein from 60% to 93% and obtain a wide range of protein hydrolysates (Hamada, 2000). Alkaline extraction or strong acids can also be applied to break the peptide bond, which is a simple method, albeit harsh. Furthermore, this either destroys or modifies the essential amino acids, and creates toxic by-products and undesirable side reactions that reduce the quality of the protein (Humiski and Aluko, 2007). Nevertheless, enzymatic hydrolysis does not affect the nutritional value of the proteins. Additionally, enzymatic hydrolysis can improve the physicochemical, functional, and sensory properties of native proteins (Kristinsson and Rasco, 2000). Many reported enzymatic hydrolysis has been used for protein extraction from plant protein sources (Zhang *et al.,* 2012). Therefore, the work presented focuses on enzymatic release of bioactive compounds from rice bran.

The purpose of this study was to investigate the effect of enzymatic hydrolysis conditions on protein and β-glucan hydrolysate extraction efficiency and to investigate their properties (chemical and functional properties) of Sang Yod rice bran hydrolysates.

2.3 Materials and Methods

2.3.1 Materials and chemicals

Partially Sang Yod rice bran (SYRB), a by-product of rice milling process was obtained from Phatthalung Province in southern Thailand. SYRB was screened to pass a 40-mesh sieve, placed in popylean bags and stored at (-20) ^{o}C thoughout the study. It was thawed at 4 ^{o}C on the day before use.

Testing chemicals, including chemicals for analysis of degree of hydrolysis (DH), chemical composition and functional property were purchased from Sigma Chemical Co. (St. Louis, MO, USA). (1-3) (1-4) β-glucan Assay kit (Mccleary method) was purchased from Megazyme International Ireland Limited. All other employed chemicals and reagents were analytical grad and commercially available.

The enzyme used in the experiments was Protease G6 (Alcalase) and Amyloglucosidase. A commercial Protease G6 (alkali serine protease) was purchased as a brownish liquid preparation by Siam victory chemicals Co., Ltd (Thailand). Amyloglucosidase from *Aspergillus niger* (A7095) was purchased by Sigma-aldrich Co. Ltd., product from Denmark.

2.3.2 Analysis of chemical composition of Sang Yod rice bran

The chemical composition of defatted rice bran was performed include moisture content, protein, ash, fat, starch, crude fiber and total dietary fiber according to the method of AOAC (2000). Total β-glucan content was determined by the method of McCleary and Mugford (1997) using Megazyme (Megazyme International Ltd., Bray, Ireland) mixed linkage β-glucan assay kit. Reducing sugar was determined by 3,5-dinitro salicylic acid (DNS method) (Neureiter *et al*., 2002)

2.3.3 Preparation of Sang Yod rice bran

The preparation of SYRB was then dispersed in a 5-fold volume of distilled water. For each experiment, 100 g of rice bran was mixed with 500 mL distillation water. The mixture pH was adjusted to 8.0 by 1 NaOH, after it was immediately exposed to ultrasound at 70W power output for 60 min (pluse duration of

on time 10 s and off-time 20 s). This was performed by the immersion of the tip of the stanless stell probe of ultrasonic processor (Sonic, VCX 750, USA) into the center position of a 1000 ml beaker.

2.3.4 Extraction of Sang Yod rice bran by enzymatic hydrolysis

Enzymatic hydrolysis of SYRB was conducted using Protease G6 (P) and Amyloglucosidase (A) enzymes. After preparation with ultrasound, it was placed in a water bath set a desired temperature (optimum 55 ^{o}C) and was continuous stirred by overhead stirrer at 200 rpm. The experiment was conducted using a flask equipped with a mechanical stirrer and water bath with temperature control. The preparation of SYRB followed by adding of Amyloglucosidase and Protease G6. Enzyme concentration, incubation time were varied from 3.0%, 4.0% and 5.0% (3-5g/100g bran), 30, 60 and 120 min repectively. The process were single, sequential and combined hydrolysis. The enzyme activity was terminated by heating to 90 ^{o}C for 10 min. After enzyme treatment, the insoluble residues were separated by centrifugation at 15,000×g for 20 min at 4 ^{o}C. The hydrolysates was prepared into a powder using a freeze dryer and was used for analysis (Figure 9).

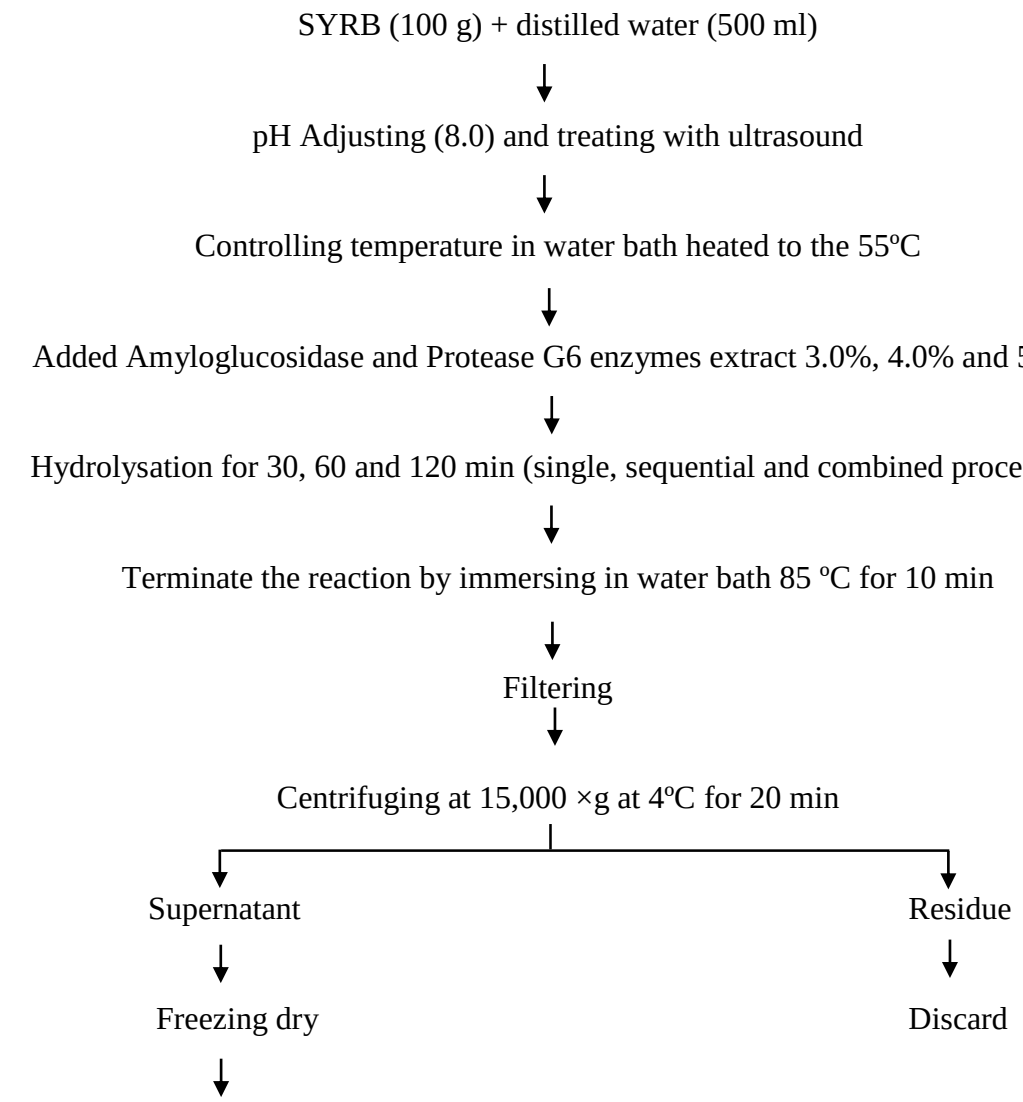


Figure 9 Sang yod rice bran hydrolysate production.

Experiment design was employed for three independent parameters, type of enzyme hydrolysis, concentration and incubation times to investigate the effect of enzyme treatment on protein and β-glucan content in hydrolysates. At three different levels each, were employed. The parameters chosen and their levels were based on preliminary experiments carried out in our laboratories (Table 9).

Table 9 Factors and levels of experiment design in SYRBH

Treatment	Coded parameter	Actual Parameters		
		Enzyme hydrolysis	Concentration (%)	Incubation time (min)
1	3 A 30 min	Single A	3	30
2	3 A 60 min	(Single enzyme)	3	60
3	3 A 120 min		3	120
4	4 A 30 min		4	30
5	4 A 60 min		4	60
6	4 A 120 min		4	120
7	5 A 30 min		5	30
8	5 A 60 min		5	60
9	5 A 120 min		5	120
10	3 P 30 min	Single P	3	30
11	3 P 60 min	(Single enzyme)	3	60
12	3 P 120 min		3	120
13	4 P 30 min		4	30
14	4 P 60 min		4	60
15	4 P 120 min		4	120
16	5 P 30 min		5	30
17	5 P 60 min		5	60
18	5 P 120 min		5	120
19	3 A→ 3 P 30 min	A followed by P A→P	3	30
20	3 A→ 3 P 60 min	(Sequential)	3	60
21	3 A→ 3 P 120 min		3	120
22	4 A→ 4 P 30 min	A followed by P A→P	4	30

Table 9 Cont.

Treatment	Coded parameter	Actual Parameters		
		Enzyme hydrolysis	Concentration (%)	Incubation time (min)
25	5 A→ 5 P 30 min		5	30
26	5 A→ 5 P 60 min		5	60
27	5 A→ 5 P 120 min		5	120
28	3A + 3P 30 min	A mixed with P A + P	3	30
29	3A + 3P 60 min		3	60
30	3A + 3P 120 min	(Combined)	3	120
31	4A + 4P 30 min		4	30
32	4A + 4P 60 min		4	60
33	4A + 4P 120 min		4	120
34	5A + 5P 30 min		5	30
35	5A + 5P 60 min		5	60
36	5A + 5P 120 min		5	120

2.3.5 Analysis of degree of protein hydrolysis

The degree of protein hydrolysis (DH) in the SYRBH solution was determined using ortho-phthalaldehyde (OPA) method with some modifications described by (Wanasundara *et al*., 2002). The degree of protein hydrolysis (DH) was based on the availability of the free amino acid group upon hydrolysis reaction with OPA. OPA reagent was prepared by dissolving 6 mM OPA and 5.7 mM DL-dithiothreitol in 0.1 M sodium tetraborate decabohydrate containing SDS 2%. Then 0.4 ml of the hydrolysate (at a suitable concentration) was added to 3 ml of OPA solution. The reaction mixture was incubated at room temperature for 20 min and its absorption at 340 nm was recorded by a spectrophotometer. The quantity of free amino acid groups

were calculated as L-serine-NH_2 group which was used as a reference. The blank value was obtained by adding only the buffer. The OPA test was validated using a serine standard solution (10% w/v). The amounts of free amino groups were calculated on basis of the serine standard.

The acid hydrolysis of SYRBH in 6 M HCl for 24 h at 110 ºC was carried out for determination of total free amino group of defatted rice bran. The DH value was calculated as the ratio of released free amino groups because of enzymatic hydrolysis to total free amino groups released due to complete acid hydrolysis per unit of protein and expressed as a percentage value:

$$\text{DH (\%)} = \frac{\text{free amino groups released due to enzymatic hydrolysis}}{\text{total free amino groups from acid hydrolysis}} \times 100$$

$$= [(NH_2)T_X - (NH_2)T_0) / (NH_2)_{Total} - (NH_2)T_0] \times 100$$

Where: $(NH_2)T_0$ = Number of free $-NH_2$ group at 0 min of hydrolysis

$(NH_2)T_X$ = Number of free $-NH_2$ group at x min of hydrolysis

$(NH_2)_{Total}$ = Number of free $-NH_2$ group (completed hydrolysis of Protein is assumed)

2.3.6 Chemical composition of SYRBHs

The chemical composition of SYRBH was performed including protein content using the standard Kjeldahl method and total dietary fiber (AOAC, 2000). The Kjeldahl method were used for determination of the nitrogen content with protein conversion factor of 5.59. Protein content were calculated as follows:

$$\text{Protein (\%)} = \frac{\text{vol of HCl x N of HCl x 14.4 x 5.59}}{\text{Weight of sample}} \times 100$$

Total β-glucan content was determined by the method of McCleary *et al*. (1997) using Megazyme (Megazyme International Ltd., Bray, Ireland) mixed linkage

β-glucan assay kit. Reducing sugar was determined by 3,5-dinitro salicylic salicylic acid (DNS method) (Neureiter *et al*., 2002)

2.3.7 Color of SYRBHs

Color of SYRBHs powder was determined by a Hunter-Lab (Miniscan XE Plus, Hunter-Lab, USA). The instrument was calibrated before color measurement with black glass and white calibration tile. Each SYRBH sample was put in a cuvette and replaced into the sample port, the color parameters (*a*, b** and *L**) were then read. Three values of L, a and b were measured, where L = 100 (white), L = 0 (black), +a = red, -a = green, +b = yellow and –b = blue.

2.3.8 Functional properties of SYRBHs

2.3.8.1 Water holding capacity (WHC) of SYRBHs

WHC was demonstrated following the methods of Robertson *et al*. (2000) and Daou and Zhang (2011). Distilled water (30 ml) containing sodium azide (0.02%) was added into SYRBH (1.0 g) and hydrated for 18 h. Then, the supernatant was then removed by allowing the wet SYRBH to drain in a sieve. The hydrated SYRBH was removed, weighed and dried to stable weight (±0.05 mg) in a hot-air oven at 110ºC. WHC was expressed as the amount of water retained per gram dry sample (g/g dry weight).

$$\text{WHC (g/g)} = \frac{\text{(Hydrated residue weight - Dry residue weight)}}{\text{Dry residue weight}}$$

2.3.8.2 Swelling capacity (SC) of SYRBHs

SC was investigated following Robertson *et al.* (2000). Distilled water (10 ml) containing sodium azide (0.02%) was added into dry sample (0.2 g). After that tubes were allowed to stand for 18 h at room temperature. The volume was recorded and SC was calculated as ml per gram of dry SYRBH for 18 h, then the final volume attained was evaluated:

$$\text{SC (ml/g)} = \frac{\text{Volume occupied by sample}}{\text{Original sample weight}}$$

2.3.8.3 Fat binding capacity (FBC) of SYRBHs

FBC was examined by the method from Lin *et al*. (1974) and Zhang *et al*. (2012) with some modifications. The SYRBH (5 g each) was mixed with soybean oil (20 ml) in centrifuge tube. Then, stirred for 30 min in every 5 min and after 30 min centrifuged at 1600×g, 25 min. Free oil was gradually poured and absorbed oil was calculated by difference and illustrated as ml (oil) per gram sample.

$$\text{FBC (ml/g)} = \frac{\text{(Precipitation weight - Dry weight)}}{\text{Dry Weight}}$$

2.3.8.4 Emulsifying capacity (EC) of SYRBHs

EC was evaluated following Yasumatsu *et al*. (1972) method. Mix aqueous dispersion of SYRBH (1.5 g) with soybean oil (20 ml) and mixed for 5 min with high speed blender. Then, centrifuged at 3000×g for 5 min. The total mixture (%) that remained after centrifugation was presented as stability index. The stability index of a good emulsion is greater than 94% while a poor emulsion is lower than 50% agreeable with Yasumatsu *et al.* (1972).

2.3.9 Determination of total phenolic content (TPC)

Total phenolic content was determined according to the modified method of Liu and Yao *et al*. (2007). Dried hydrolysate was dissolved in distilled water at 1.0 mg mL^{-1}. To 200 µL sample solution, 1 mL 1N Folin-Ciocalteu's reagent was added and mixed . The mixture was neutralized with 800 µL of 10% Na_2CO_3 and final volume was made up to 5 ml with distilled water and allowed to mix for 2 h. Absorbance at 760 nm was done using Microplate reader. Phenolics content in each extract and was calculated using a calibration curve of gallic acid standard. The results were expressed

as mg gallic acid equivalents (GAE) per g of sample on dry weight basis (mg GAE equiv.g^{-1}). Analyses were performed at least in triplicates.

2.3.10 Determination of anthocyanin content

The analysis method for anthocyanin content was modified from the method used by Hosseinian *et al.* (2008). SYRBH was dissolved in distilled water 1.0 mg mL^{-1}. The sample (20 μL) was added into 2 mL of potassium chloride buffer (0.03 mol/L, pH 1.0) and 2 mL of sodium acetate buffer (0.4 mol/L, pH 4.5). Each of them was left for 15 min before taking an absorption measurement using spectrophotometer at 550 nm and 700 nm. Distilled water was used as a blank. The anthocyanin concentration (mg/L) of sample was calculated according to the following formula and expressed as Cyanidin-3-glucoside equivalents:

$$\frac{A \times MW \times DF \times 1000}{\varepsilon \times 1}$$

Where: A = (A λ700- λ550) pH 1.0 - (A λ700- λ550) pH 4.5

MW = the molecular weight of Cyanindin-3-glucoside (449.2 g/mol)

DF = the dilution factor (20 μL sample is diluted to 2 mL, DF = 1000)

Ɛ = the extinction coefficient (L x cm^{-1} x mol^{-1}) = 26,900 for Cyanindin-3-glucoside, where L (path length in cm) = 1

2.3.11 Statistical analysis

All experiments were run in triplicate. Results were expressed as the mean value ± standard deviation from three replicates. A completely randomized design was used throughout this study. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's Multiple Range Test (Steel and Torrie, 1980) that were significantly different at p-value ≤ 0.05.

2.4 Results and Discussion

2.4.1 Chemical composition of Sang Yod rice bran

The proximate composition of Sang Yod rice bran (SYRB) is shown in Table 11. SYRB to be composed of 11.60 % protein which Esa *et al*. (2013) reported that the protein content of rice bran by-product ranged from 10.6 to 16.9 % depended on rice varieties (Oszvald *et al*., 2008). Moomgngarm *et al.* (2012) reported that red-brown color rice bran containing protein content from 12.93%. Ash content of SYRB was 8.98%, which represented that rice bran may be a good source of minerals and trace minerals. The crude fiber content of SYRB used in this study was 9.35% slightly lower than value of 12.11 % reported by Moomgngarm *et al.* (2012). Moreover, Raghav *et al*. (2016) reported that 6-14% of dietary fiber like β-glucan, pectin and gum was found in rice bran. In addition, SYRB also contains β-glucan 7.02% and reducing sugar 0.013%. Norhaizan *et al.* (2013) reported that protein, crude fat, crude fiber and ash content of rice bran range of 10.6-16.9, 5.1-19.7, 7.0-18.9, 8.8-28.8, respectively. This variation might be due to the different rice milling processing equipment and conditions that may remove rice bran from rice with more or less endosperm (Wan *et al.*, 2011).

Table 10 Chemical composition of Sang-yod rice bran.

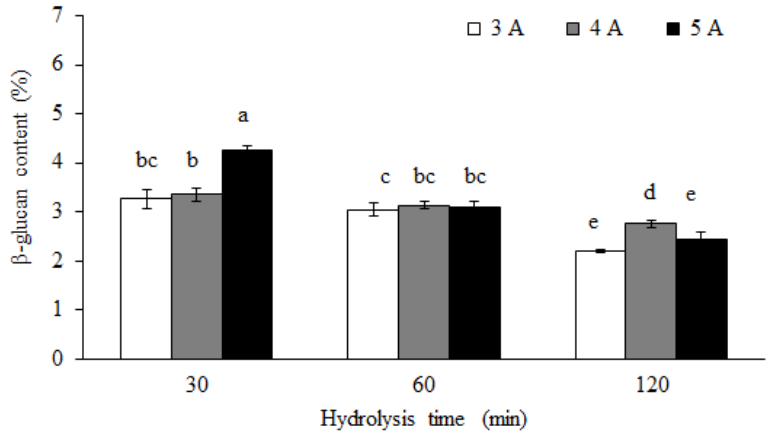
Compositions	Values (%)
Moisture	9.96 ± 1.33
Protein	11.60 ± 0.48
Ash	8.98 ± 0.73
Fat	9.01 ± 1.24
Crude fiber	9.35 ± 0.51
Starch	50.43 ± 0.81
Total β-glucan	7.02 ± 0.21
Reducing sugar	0.013 ± 0.25

The data are mean ± SD (n=3)

2.4.2 Effect of single enzyme hydrolysis conditions on protein and β-glucan content of SYRBHs.

The protein and β-glucan from the rice bran hydrolysate were prepared by using single enzyme Amyloglucosidase or Protease G6 at various hydrolysis conditions and are shown in Figure 10. The β-glucan content of SYRBH was in the range of 0.73-4.26 % (w/w) (Figure 10 (a) and (b)). The highest content (4.26 % w/w) of β-glucan was obtained by using high concentration Amyloglucosidase (5% v/w) and mildest hydrolysis condition (30 min). At specific hydrolysis duration, the β-glucan content was increased with increasing of Amyloglucosidase concentration. Incontrast, extension of hydrolysis duration caused a reduction of β-glucan content. The β-glucan content obtained by Protease G6 hydrolysis was less than that of the SYRBH obtained by Amyloglucosidase hydrolysis. This may be due to primary activity of protease G6 and the subsequent breakdown of rice bran protein. Amyloglucosidase has exo-amylase enzyme. Thus, it can hydrolyze glucan and limit of dextrin. The primary hydrolysis of starch by Amyloglucosidase into oligosaccharides and further to short-chain, water-soluble dextrins is sufficient for easier removal of starch from hard surfaces. On the other hand, Amyloglucosidase enzyme specifically cleaves the (1-4)-β-linkage next to a (1-3)- β-linkage at the reducing end and products are (1-4)-link-oligosaccharide with one (1-3)-linked glucose unit (Sivaramakrishnan *et al.*, 2006). Thus, release of β-glucan from rice bran was more profound by Amyloglucosidase than that of Protease G6. Activity of Amyloglucosidase may be responsible for reduction of β-glucan due to the severe hydrolysis of lengthen hydrolysis time. The protein content of SYRBH was in the range of 0.43-1.16% (w/w) (Figure 11 (a) and (b)). This result was interesting to note that significant amount of soluble protein was co-released with β-glucan after the amyloglucosidase treatment (Fabian and Ju, 2011). As soluble protein is accounted for a major part of rice bran protein it thus may readily leach out by destruction of rice bran by amyloglucosidase activity.

(a)



(b)

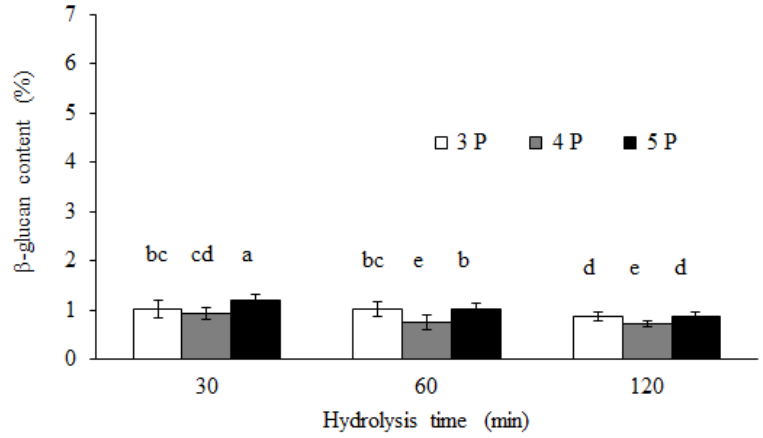
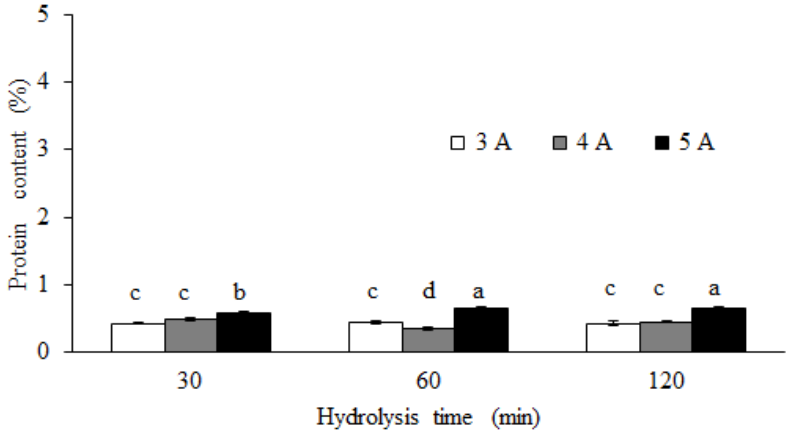


Figure 10 Effect of single enzyme hydrolysis and their concentrations and hydrolysis times on β-glucan (a), (b) of SYRBH. Values are as mean ± SD of triplicate determinations. Bars with different alphabet are significantly different ($p \leq 0.05$)). The SYRBH were prepared from using enzymatic hydrolysis of Amyloglucosidase (A) or Protease G6 (P) either at concentration of 3, 4 or 5 % v/w (3A, 4A and 5A or 3P, 4P and 5P).

(a)



(b)

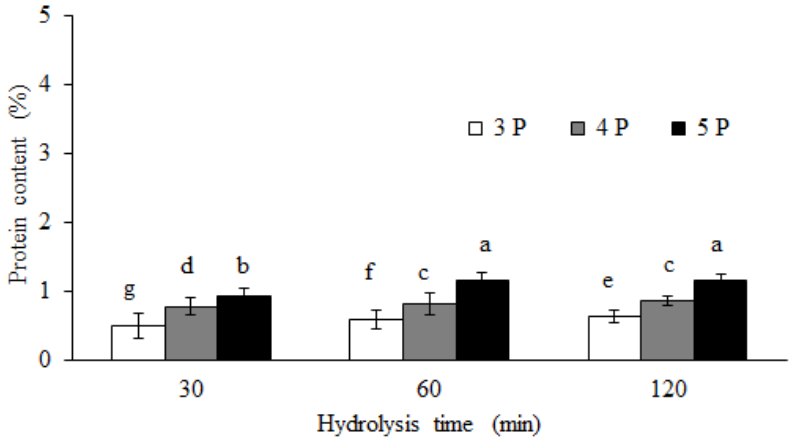
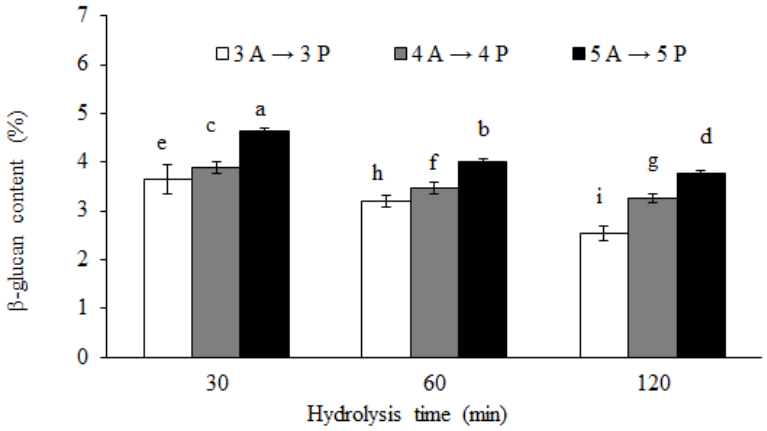


Figure 11 Effect of single enzyme hydrolysis and their concentrations and hydrolysis times on protein contents (a), (b) of SYRBH. Values are as mean ± SD of triplicate determinations. Bars with different alphabet are significantly different ($p \leq 0.05$)). The SYRBH were prepared from using enzymatic hydrolysis of Amyloglucosidase (A) or Protease G6 (P) either at concentration of 3,4 or 5 % v/w (3A, 4A and 5A or 3P,4Pand5P).

2.4.3 Effect of co- enzyme hydrolysis conditions on protein and β-glucan content of SYRBHs.

The protein and β-glucan contents of the rice bran hydrolysates prepared by sequential and combined enzyme hydrolysis of Amyloglucosidase (A) and Protease G6 (P) and various hydrolysis conditions are shown in Figure 12. The β-glucan content of SYRBH was in the range of 2.54-4.63% (w/w) which corresponds well with the study of oat bran (Sibakov *et al*., 2013). The highest content (4.63 % w/w) of β-glucan content was obtained by using the sequential process using high concentration of enzymes (5A→5P) for 30 min. All processes showed significantly ($p \leq 0.05$) increased of β-glucan content with increasing enzyme concentration. The sequential enzyme hydrolysis showed β-glucan content more than that of the combined enzyme hydrolysis. Moreover the β-glucan content was decreased with extension of hydrolysis duration. This is likely caused by over catalytic of amyloglucosidase. The decreasing of β-glucan contents might be due to degradation of β-glucan during hydrolysis reaction. The process related degradation of β-glucan is based on several mechanisms or condition (Kivela, 2009).

(a)



(b)

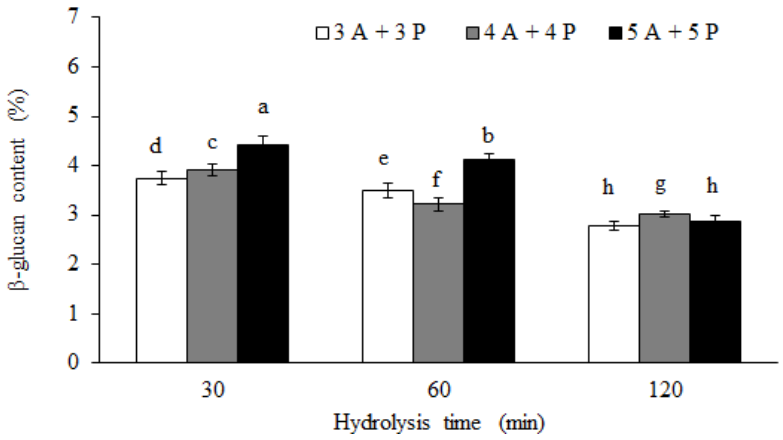
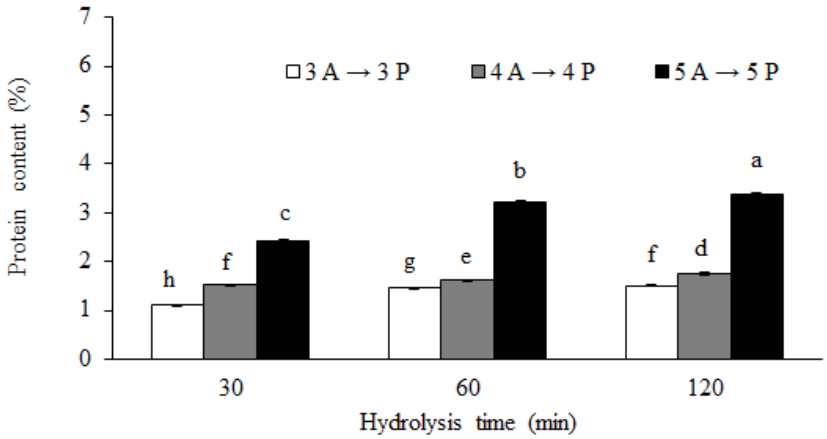


Figure 12 Effect of co-enzymatic hydrolysis and their concentrations and hydrolysis time on β-glucan (a), (b) of SYRBH. Values are reported as mean ± SD of triplicate determinations. Bars with different alphabet are significantly different ($p \leq 0.05$)). Amyloglucosidase (A) and Protease G6 (P) were used for preparation of the SYRBH either by using sequential (A→ P) or combined(A + P) hydrolysis process. The enzyme concentrations were at 3, 4 or 5% w/w.

(a)



(b)

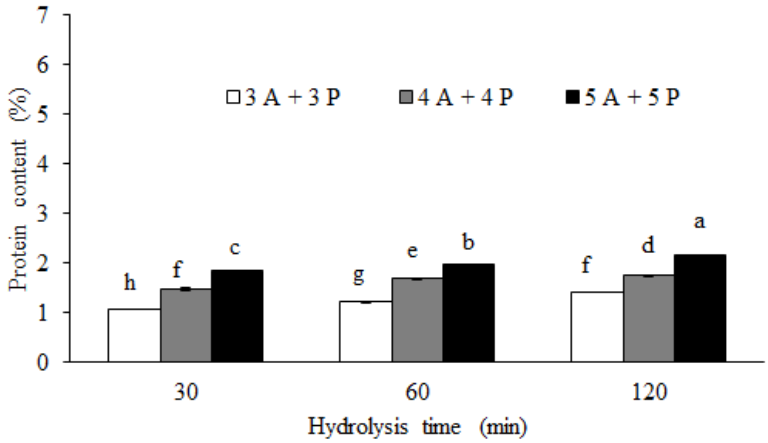


Figure 13 Effect of co-enzymatic hydrolysis and their concentrations and hydrolysis time on protein contents (a), (b) of SYRBH. Values are reported as mean ± SD of triplicate determinations. Bars with different alphabet are significantly different ($p \leq 0.05$). Amyloglucosidase(A) and Protease G6(P) were used for preparation of the SYRBH either by using sequential (A→ P) or combined (A + P) hydrolysis process. The enzyme concentrations were at 3, 4 or 5% w/w.

The protein content of SYRBH was in the range of 1.08-3.37 % (w/w) Figure 11 (a) and (b)). The highest protein content (3.37% w/w) was obtained by sequential of high concentration of enzymes (5A→5P) for 120 min. The protein content of the SYRBH depended on the concentration of enzyme as well as the hydrolysis time. Thereafter gradual reaction of protein content was found with extension of hydrolysis time. Enzymatic hydrolysis involved hydrolyzing the cell wall components and increasing the protein extractability. The alkaline protease was used to hydrolyze the protein into short peptide (Zhang *et al*., 2012). Protease have been used to enhance the recovery of rice bran proteins from about 60% to 93% and to obtain a wide range content of protein hydrolysates. Commercial protease are mainly endoprotease, thus they attack peptide bonds in the interior of the polypeptide chain producing a range of polypeptides, which differ in molecular weight, depending on the extent of hydrolysis (Hamada, 2000). However, this is a significant difference in amounts of proteins obtained by the two enzyme treatments ($p \leq 0.05$). Nonetheless, the protein content tended to increase by increasing of hydrolysis time in contrast, it was effected by β-glucan content decrease. However, the sequential hydrolysis of high concentration for 60 min showed high content of both compounds.

2.4.4 Effect of hydrolysis conditions on degree of hydrolysis and reducing sugar of SYRBHs.

A selected hydrolysate samples exhibiting high protein and/or β-glucan content was determinated in degree of hydrolysis (DH) and reducing sugar (RS). Result demonstrated that amount of enzyme and hydrolysis time as well as hydrolysis process were the major factors affecting the DH and RS. As show in Table 11.

Table 11 Effect of hydrolysis condition on DH and RS of SYRBH.

Hydrolysate	Protein (%)	DH (%)	β-glucan (%)	RS (%)
5 A 30 min	0.59 ± 0.04^{m}	6.30 ± 0.14^{gh}	4.26 ± 0.10^{b}	0.33 ± 0.05^{cd}
5 P 30 min	0.93 ± 0.02^{l}	4.56 ± 0.19^{i}	1.20 ± 0.10^{h}	0.55 ± 0.07^{a}
3 A→3 P 30 min	1.10 ± 0.01^{k}	6.29 ± 0.22^{gh}	3.64 ± 0.30^{de}	0.41 ± 0.09^{abcd}
3 A→3 P 60 min	1.46 ± 0.02^{i}	7.53 ± 0.13^{g}	3.21 ± 0.12^{f}	0.47 ± 0.11^{abc}
4 A→4 P 60 min	1.62 ± 0.01^{h}	18.21 ± 0.77^{d}	3.48 ± 0.10^{e}	0.44 ± 0.06^{abc}
5 A→ 5 P 30 min	2.43 ± 0.02^{c}	24.94 ± 0.92^{a}	4.63 ± 0.05^{a}	0.25 ± 0.05^{d}
5 A→5 P 60 min	3.23 ± 0.03^{b}	25.94 ± 1.03^{a}	4.02 ± 0.05^{c}	0.39 ± 0.09^{abcd}
5A→5 P 120 min	3.37 ± 0.04^{a}	23.41 ± 1.02^{b}	3.75 ± 0.07^{d}	0.40 ± 0.04^{abcd}
3 A + 3 P 30 min	1.08 ± 0.01^{k}	6.05 ± 0.41^{h}	3.75 ± 0.12^{d}	0.41 ± 0.05^{abcd}
3 A + 3 P 60 min	1.22 ± 0.02^{j}	7.28 ± 0.36^{gh}	3.50 ± 0.15^{e}	0.42 ± 0.11^{abcd}
4 A + 4 P 60 min	1.69 ± 0.02^{g}	22.26 ± 0.83^{bc}	3.22 ± 0.13^{f}	0.47 ± 0.08^{abc}
5 A + 5 P 30 min	1.85 ± 0.01^{g}	11.47 ± 0.64^{f}	4.42 ± 0.18^{b}	0.30 ± 0.01^{cd}
5 A + 5 P 60 min	1.98 ± 0.01^{e}	16.63 ± 0.09^{e}	4.13 ± 0.10^{bc}	0.36 ± 0.06^{bcd}
5 A +5 P 120 min	2.16 ± 0.02^{d}	21.24 ± 0.84^{c}	2.88 ± 0.10^{g}	0.52 ± 0.09^{ab}

Values are expressed as mean ± standard deviation from triplicate determinations.
Columns with different letters indicate statistical differences (P≤0.05).

The DH of freeze dried SYRBH by sequential enzymatic hydrolysis (A→ P) as high concentration of enzyme showed higher DH (23.41-25.94%) than other hydrolysates. Furthermore, it demonstrated that SYRBHs obtained high protein content (2.43-3.37%). Higher DH indicated higher amount of peptide bonds, which were cut (Ma *et al.*, 2013). The result revealed that both enzymes contents and hydrolysis time affected DH of the enzymatic process. The DH increased with increasing hydrolysis time of SYRBH form all hydrolysis processes. The DH value was significantly ($p<0.05$) increased when enzyme concentration was increased from 3% to 5%. The significant increase of DH was observed by using 5% (v/w) enzyme suggesting that at concentration at 3% enzyme content is considerably low relative to available peptide bond. This result is similar to the report of Haslaniza *et al*. (2010) which stated that significant improvement of DH occurred after increase enzyme concentration at certain

level. Overall, the DH ranged from 0.59 to 3.37%. The variation was depending on protein substrate and hydrolysis condition. Compared with other studies, DH of albumin and grobulin hydrolysate from rice bran using protease, which was below 15% (Uraipong and Zhao, 2015). DH of barley protein from Flavourzyme was over 16% after 4 h. of hydrolysis time (Bamdad *et al.*, 2011).

The RS content associated with hydrolysis level of dietary fiber as well as reducing sugar increased with increasing of hydrolysis duration and enzyme concentration. The result related with the result of β-glucan content (Table 11). The RS showed range 0.25 to 0.55%. The opposite result of RS and β-glucan when SYRBH presented high level of β-glucan related low level of RS. The β-glucan was decreased by increasing hydrolysis time of the same concentration of enzyme. The highest content (4.63% w/w) of β-glucan was obtained by using the mildest hydrolysis condition (5 A→ 5 P 30 min). The Amyloglucosidase enzyme has Amylase or Lichenase enzyme. The Lichenase enzyme specifically cleaves the (1-4)-β-linkage next to a (1-3)-β-linkage at the reducing end and products are (1-4)-link-oligosaccharide with one (1-3)-linked glucose unit (Johansson *et al.*, 2004). Activity of this enzyme may be responsible for reduction of β-glucan after extension of the hydrolysis. This was supported by an increase of reducing sugar with increasing hydrolysis duration. The report of the hydrolysis of β-glucan from oat bran by Sibakov *et al.* (2013) showed the β-glucan concentration was reduced from 16.4 to 11.6% when the hydrolysis time was prolonged from 60 to 240 min.

2.4.5 Total phenolic and total anthocyanin content of SYRBHs

SYRBH samples including hydrolysate from single and co-enzymatic hydrolysis that presented high protein and β-glucan content. Total phenolic content (TPC) and total anthocyanin (TAC) of all selected hydrolysates are in Figure 14 (a) and (b) respectively. TPC was 2.33-4.48 mg GAE/g (a) with hydrolysates by co-enzymatic hydrolysis (5 A → 5 P 120 min, 5 A + 5 P 120 min) with slightly higher TPC (4.34-4.48 mg GAE/g). It demonstrates that when enzyme was used as the extractant at about 5% with longer time hydrolysis increase in the concentration of these phytochemicals was achieved in the enzymatic hydrolysis under the extractions employed. TPC was

significantly greater for the co-enzymatic hydrolysis with longer time hydrolysis including high concentration of enzyme than single enzymatic hydrolysis. Numerous studies have shown the TPC for rice bran are close to that reported for Thailands long-grain rice bran (2.2-3.2 g GAE/kg bran), TK9 rice bran extracts (1.2-2.5 g GAE/kg) with MeOH and pigmented rice bran extract (8-15 mg GAE/g) (Goffman and Bergman, 2004 ; Chotimarkorn *et al.*, 2008), respectively. Moreover, Moongngarm *et al*. (2012) reported the TPC in red rice bran (4.39 mg GAE/g) and black rice bran (6.65 mg GAE/g). It was found that the rice bran layer of black pigmented rice contained higher level of TPC than red pigmented rice

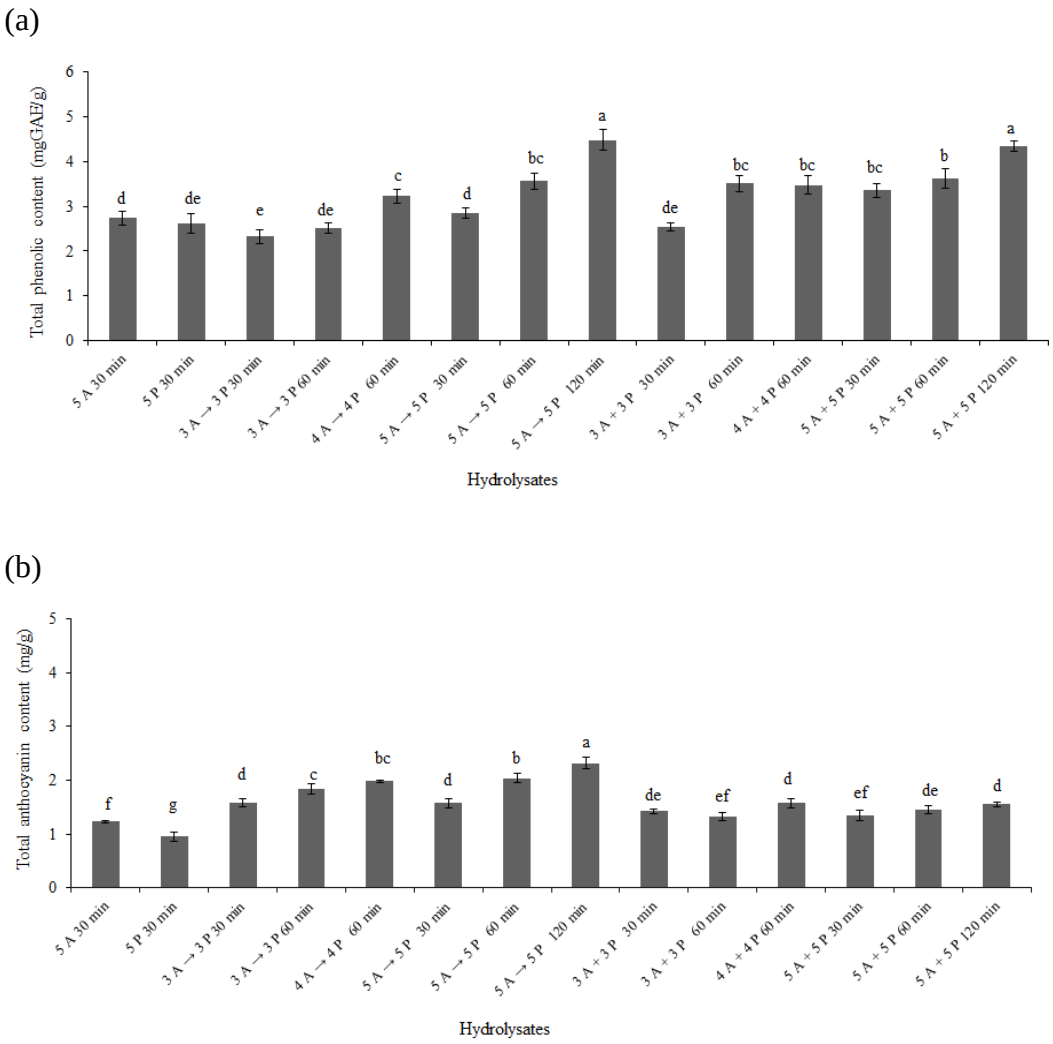


Figure 14 Total phenolic content (a) and total anthocyanin (b) of SYRBH using different enzymatic hydrolysis, mean ± SD of triplicate determinations. Bars with different alphabet are significantly different ($p \leq 0.05$).

TAC of hydrolysates was 0.95-2.32 mg/g (Figure 14 (b)). TAC of hydrolysate by sequential enzymatic hydrolysis (5 A → 5 P 120 min) had the highest value 2.32 mg/g whilst hydrolysate by single enzymatic hydrolysis (5 P 30 min) had lowest TAC (0.95 mg/g). All hydrolysate of sequential enzymatic hydrolysis showed the greatest TAC compared to other hydrolysis. This might be due to similarities to the long period time of hydrolysis. These results demonstrate the trend to increase of TPC and TAC with increasing enzyme concentration and hydrolysis time. A slight increase in TPC by enzymatic hydrolysis was reported in hydrolysates from rice bran protein by Flavourzyme and Alcalase with highest TPC when used 2 h for hydrolysis (Thammarathip *et al.*, 2016). Normally, phenolic compounds interact with protein by both covalent and noncovalent bonds (Yurdemir and Yemenicioglu, 2013). The contents of phenolic compounds could be released during enzymatic hydrolysis; hence , the highest TPC of hydrolysate by Protease G6 or Alcalase could be due to the highest efficiency in protein extraction. Inaddition , anthocyanins are concentrated in the pigmented layer of purple, red and black rice accouted for 85% of the anthocyanin content in whole rice. Anthocyanin is glycoside compounds or glycoside of phenolic compound in outer membrane (pericarp) and inner membrane or aleurone layer (Hu *et al.*, 2003). During hydrolyzation, the anthocyanin content intensity increased because of anthocyanin dissolving in water. However, some reports of TAC have been found TAC in black, purple and red color rice (11.13-245.30 mg Cy-3-glc/100g) , Moreover dark purple rice has showed higher TAC than red-brown rice (Yodmanee *et al.*, 2011).

2.4.6 Effect of hydrolysis condition on color of SYRBHs

Table 12 shows the color parameters (L^*, a^* and b^*) of the SYRBH. RBH samples including the different hydrolysis process. L^* value which expresses the lightness were in the range of 30.00-35.25. The value of a^* (redness) and b^* (yellowness) were in the range of 15.40-19.18 and 14.48-20.90, respectively. The color of SYRBH, lightness (L^*) decreased slightly but redness (a^*) increased marginally by increasing hydrolysis time and concentration of enzyme. It can be noticed that Amyloglucosidase and Protease G6 extract content have effect on L^*, a^* and b^* values of the SYRBH. Moreover, duration of hydrolysis reaction has effect. Increasing of hydrolysis time cause decreased in L^* and a^* and b^* value but increased in a^* value.

It demonstrates that the SYRBH turns a darker-red with longer hydrolysis time. Redness in SYRB is embedded in the structure of rice bran. When the rice bran is hydrolyzed, the color is darker in appearance. During hydrolysation, the color intensity increased because anthocyanin dissolving in water resulting in change in color particularly *a** (Chung *et al.*, 2012). The heat-moisture treatment resulted in decrease in lightness and a increased in redness and yellowness for SYRBH. It has been reported that heat-moisture process results in a darker color for flours from rice (Lorlowhakarn and Naivikul, 2006). This was likely due to the higher reducing sugar content in longer time hydrolysis samples. Which was caused by the condensation of an amino group and reducing a reducing compound, resulting complex change in color of RBHs (Mailard, 1912). The Maillard reactions between the reducing sugars and proteins occurred during enzymatic hydrolysis and heat-moisture treatment, which could decrease the lightness.

Table 12 Effect of hydrolysis condition on color of SYRBH.

Hydrolysates	***L****	***a****	***b****
5 A 30 min	35.06 ± 0.07^{a}	15.40 ± 0.06^{h}	20.90 ± 0.16^{a}
5 P 30 min	35.15 ± 0.02^{a}	15.93 ± 0.09^{g}	20.78 ± 0.19^{a}
3 A→3 P 30 min	34.13 ± 0.12^{a}	16.88 ± 0.14^{d}	14.48 ± 0.03^{j}
3 A→3 P 60 min	31.81 ± 0.06^{d}	17.11 ± 0.04^{c}	15.41 ± 0.08^{h}
4 A→4 P 60 min	31.70 ± 0.14^{d}	17.20 ± 0.08^{c}	15.75 ± 0.07^{g}
5 A→ 5 P 30 min	33.90 ± 0.20^{b}	17.18 ± 0.04^{c}	15.11 ± 0.06^{i}
5 A→5 P 60 min	30.84 ± 0.42^{e}	17.81 ± 0.03^{b}	15.22 ± 0.06^{hi}
5A→5 P 120 min	30.00 ± 0.13^{f}	19.18 ± 0.09^{a}	16.17 ± 0.03^{f}
3 A + 3 P 30 min	34.95 ± 0.15^{a}	16.10 ± 0.05^{f}	20.19 ± 0.04^{b}
3 A + 3 P 60 min	31.81 ± 0.06^{d}	17.11 ± 0.04^{c}	15.41 ± 0.08^{h}
4 A + 4 P 60 min	33.94 ± 0.27^{b}	16.13 ± 0.03^{f}	18.24 ± 0.07^{d}
5 A + 5 P 30 min	35.25 ± 0.30^{a}	16.45 ± 0.10^{e}	19.93 ± 0.16^{c}
5 A + 5 P 60 min	34.05 ± 0.21^{b}	16.29 ± 0.06^{e}	20.18 ± 0.02^{b}
5 A +5 P 120 min	33.19 ± 0.10^{c}	16.42 ± 0.04^{e}	17.86 ± 0.12^{e}

The data are expressed as mean ± SD (n =3). Column with different letter indicate statistical differences ($p \leq 0.05$).

Where The maximum for L^* is 100, which represents a perfect reflecting diffuser.
The minimum for L^* is zero, which represents black.
a^* = Positive is red, Negative is green
b^* = Positive is yellow, Negative is blue

2.4.7 Effect of hydrolysis condition on functional properties of SYRBHs

The functional properties of SYRBHs by different enzymatic hydrolysis are shown in Table 13. The determination of hydration properties of SYRBH including water holding capacity (WHC), swelling capacity (SC), emulsifying capacity (EC) and fat binding capacity (FBC).

According to Table 13, the highest WHC value (13.56 g/g), SC value (11.62 ml/g), EC value (58.77%) and FBC value (6.20 ml/g) correspond to sequential enzymatic hydrolysis (5A→5P 60 min). The same trend was observed with all properties when hydrolysates using sequential enzymatic hydrolysis. The result revealed that increasing of hydrolysis by extension of hydrolysis duration caused reduction in water holding capacity (WHC), water binding capacity (WBC) and swelling capacity (SC) of the obtained SYRBH. Tounkara *et al*. (2013) suggested that water holding ability of protein hydrolysate may be determined by the difference in molecular weight of peptides within protein hydrolysates, type of amino acid, amino acid content and amino acid sequence. Moreover, SYRBH had high value of protein and β-glucan content that could be improved the greater functional properties than other. Hydration properties (WBC and SC) are determined by content in water soluble fiber components of foods and their values could have a similar evolution (Daou and Zhang, 2011). Thus, some components of SYRBH are β-glucan or (1-3)-(1-4) β-D-glucan that is the water soluble dietary fiber (Johansson, *et al.,* 2004).

Table 13 Effect of hydrolysis condition on function properties of SYRBH

Hydrolysates	Functional properties			
	WHC (g/g)	SC (ml/g)	EC (%)	FBC (ml/g)
5 A 30 min	8.92±0.11^{e}	8.96±0.43^{c}	54.65±1.91^{b}	4.43±0.10^{f}
5 P 30 min	4.48±0.07^{i}	3.40±0.28^{i}	45.30±0.85^{e}	5.42±0.27^{cd}
3 A→3 P 30 min	4.20±0.14^{j}	5.31±0.14^{fg}	53.95±0.64^{b}	4.97±0.32^{de}
3 A→3 P 60 min	6.20±0.09^{g}	5.51±0.12e^{fg}	45.94±0.13^{e}	4.57±0.20^{de}
4 A→4 P 60 min	9.28±0.03^{d}	5.82±0.38^{ef}	47.35±0.22^{d}	5.23±0.24^{cd}
5 A→ 5 P 30 min	13.21±0.11^{b}	11.34±0.37^{a}	58.77±0.47^{a}	5.49±0.30^{bc}
5 A→5 P 60 min	13.56±0.11^{a}	11.62±0.31^{a}	58.54±0.23^{a}	6.32±0.26^{a}
5A→5 P 120 min	2.21±0.09^{k}	3.31±0.51^{i}	47.67±0.22^{d}	6.20±0.10^{a}
3 A + 3 P 30 min	4.96±0.03^{h}	5.15±0.14^{g}	50.36±0.26^{c}	5.36±0.14^{cd}
3 A + 3 P 60 min	5.04±12^{h}	6.05±0.24^{e}	44.84±0.17^{e}	4.63±0.10^{de}
4 A + 4 P 60 min	6.22±0.11^{g}	7.21±0.17^{d}	48.23±0.25^{d}	4.72±0.23^{de}
5 A + 5 P 30 min	12.06±0.12^{c}	9.87±0.14^{b}	53.65±0.17^{b}	5.15±0.53^{cd}
5 A + 5 P 60 min	7.14±0.18^{f}	10.13±0.57^{b}	53.83±0.30^{b}	5.96±0.31^{ab}
5 A +5 P 120 min	1.43±0.18^{l}	4.06±0.28^{h}	47.73±0.14^{d}	5.64±0.08^{bc}

Values are expressed as mean ± standard deviation from triplicate determinations.
Columns with different letters indicate statistical differences (P≤0.05).

Emulsifying capacity (EC) of the SYRBH with 3.23% protein and 25.94% DH showed the highest value ($p < 0.05$). Mutilangi *et al*. (1996) supposed that higher MW peptides or more hydrophobic peptides contribute to the stability of the emulsion. Most SYRBH (EC 44.84-58.77%) are presented as poor emulsifier based on their stability indices was less than 50%. Prakash and Ramanathan (1995) showed that EC of protein concentrate from rice bran, range from 52 to 57 %, therefore the lower EC of SYRBH might be due to the lower protein level in the SYRBH. FBC of high concentration enzyme and hydrolysis time are greater than that of lower concentration and hydrolysis time (Table 10) all of hydrolysis process. Moreover, the result revealed that high value of protein and β-glucan content of SYRBH (Table 11) could be

enhanced FBC. Generally, the oil absorption is related to the nature of the surface and density or thickness of particles (Amado, 1994). It was reported that lignin-rich samples had higher FBC. So, insoluble dietary fiber had higher FBC levels than soluble dietary fiber because of their percentage of particles with large size, and for the lignin that can be found in their chemical composition (Daou and Zhang, 2011). Hydrolysis with high solubility and smaller molecular sizes should facilitate that diffusion and enhance the interaction between protein and lipid (Bandyopadhyay *et al.,* 2008). Hamada (2000) and Theerakulkait *et al.* (2006) showed that rice bran protein hydrolysates using enzyme extraction could show emulsification properties. The functional properties of SYRBHs which presented great capacity might be due to the value of protein and β-glucan content including other components in SYRBH.

2.5 Conclusion

The enzymatic hydrolysis conditions including enzyme content, hydrolysis type as well as hydrolysis time were important factor influencing the hydrolysate properties. The hydrolysate from co-enzymatic hydrolysis exhibited higher protein and β-glucan content than those of the single enzymatic hydrolysis. The β-glucan content decreased with increasing hydrolysis time. The hydrolysate obtained from the sequential hydrolysis with long hydrolysis time had high phenolic content and anthocyanin content. The high content of protein and β-glucan of hydrolysate exhibited excellence of functional properties (WHC, SC, RBH and FBC). Thus, SYRBH provide important information for food applications are most suitable for the protein and/or soluble fiber concentration. Moreover, the ability of protein and soluble fiber of SYRBH to interact with lipids and form stable emulsions is essential to yield a stable food product.

CHAPTER 3

ANTIOXIDATIVE AND ANTI-INFLAMMATORY PROPERTIES OF SANG YOD RICE BRAN HYDROLYSATE

3.1 Abstract

The selected hydrolysates (14 hydrolysates) based on its high amount of both protein and β-glucan content were used for determine action their antioxidant activities, cytotoxicity and NO inhibition. The antioxidant capacities of the SYRBHs as measured by either the DPPH and ABTS scavenging activities, reducing power and metal chelating activity were improved by the hydrolysis process and associated with their protein and β-glucan content. The sequential and combined hydrolysis yielded the hydrolysate with high anti-oxidative activity relative to those of the process using single enzyme. Selected SYRBHs were determined for anti-inflammatory by evaluation of pro-inflammatory factors (NO, TNF-α, IL-1β and IL-6 cytokines) in lipopolysaccharide (LPS) induced RAW 264.7 macrophage cells. All RBHs exhibited no cytotoxicity on RAW 264.7 cell lines at the maximum concentration of 1500 μg/ml. The selected SYRBHs from sequential and combined process showed high NO inhibition. The hydrolysates were selected based on its NO inhibitory effect to measure effect of measure pro-inflammatory activity. The SYRBHs exhibited pro-inflammatory inhibition (9.37-71.96%). The high potential of NO and pro-inflammatory mediators inhibition were related with the protein and β-glucan contents. SYRBH which have the benefitcial effect on anti-oxidation and anti-inflammatory property was obtained by sequential and combined hydrolysis process.

3.2 Introduction

Reactive oxygen species (ROS) are highly reactive ions and free radicals (chemicals containing atoms with an unpaired electron in its outer orbit) involving oxygen molecules, including the superoxide anion, hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\bullet}$). ROS consist of radical and non-radical oxygen species formed by the partial reduction of oxygen. ROS are normally generated during mitochondrial oxidative metabolism as well as in cellular response to xenobiotics, cytokines, and bacterial invasion (Ray *et al.*, 2012). Its occurrence is associated with chronic diseases such as cancer, coronary and Alzheimer's disease (Ahn *et al.*, 2012).

Nitric oxide (NO), a colorless gas, has been considered as an important biological regulator which is a fundamental component in the fields of neuroscience, physiology and immunology (Jiang *et al.,* 2012). NO is produced by various group of enzyme termed as nitric oxide syntheses (NOS) which are present in body (Brero *et al.,* 2010). Three isoforms of NOS are present whose names are termed on the basis of their activities which include following endothelial NOS(eNOS), neurons NOS (nNOS) and inducible NOS(iNOS) (Bath *et al.,* 2000). NO can be stimulation with bacterial lipopolysaccharide (LPS) many cells including macrophages express the iNOS which is responsible for the production of large number of NO. Low concentrations of NO produced by the constitutive and nNOS inhibit adhesion molecule expression, cytokine and chemokine synthesis as well as leucocyte adhesion and transmigration (Guzik *et al.,* 2003). The large number of NO primarily by iNOS can be pro-inflammatory and toxic that interacts between NO and ROS generate potentially cytotoxic agents which may mediate some of the pathology associated with cancer and chronic inflammation.

Inflammation is a biodefense mechanism against external stimuli such as bacterial infection or internal stimuli such as biometabolic product (Hye Kim *et al*., 2013) and many mediators are involved such as intracellular inflammatory controllers and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8), prostagrandin, lysosomal enzyme, and free radicals. In particular, transcription factors of inflammatory response become activated in the macrophage by stimuli such as cytokines, tumor necrosis factor (TNF-α), and lipopolysaccharide (LPS), inducible nitric oxide synthase(iNOS), and cyclooxygenase-2 (COX-2) are expressed, and nitric oxide (NO) and prostaglandin E2

(PGE2) are produced, resulting in inflammation (Kwqamata *et al*., 2000). In addition, excessive NO production causes exasperation in inflammatory response, septic shock by excessive blood vessels dilation, inhibited healing, and nerve tissue injury, suggesting harmful action on the body (Hye Kim *et al*., 2013). Dietary pigmented rice protects lipid peroxidation in rat kidneys (Toyokuni *et al.,* 2002) and suppressed ROS in vitro assay (Hu *et al.,* 2003).

Many studies have reported that antioxidant activity of protein hydrolysates prepared from sources have antioxidant activitys (Tang *et al.,* 2003; Park *et al.,* 2012; Pownall *et al.,* 2010). Protein hydrolysates are one of the most interesting candidates to be employed as a natural antioxidant. Many mechanism, e.g. free-radical scavenging, metal ion chelating, oxygen quenching or hydrogen donating (Moure *et al.*, 2006). The protein hydrolysates ability to prohibit oxidation is attributed to free radical scavenging and metal chelating abilities provided by some amino acid (Pena-Romos *et al.,* 2004). Furthermore, it has been reported that colored rice bran contains more bioactive compounds and exhibits more antioxidant activity than white rice bran (Muntana and Prasong, 2010; Sompong *et al.*, 2011). Arab *et al*. (2011) found that Iranian rice extract have high antioxidant activities. Chanput *et al*. (2009) have ever been reported to produce protein hydrolysates from rice bran by pepsin and trypsin that had high antioxidant activities. Given the potential health benefits of rice bran and its importance in local agriculture, studies of the biological activity of rice bran are warranted. As a consequence, rice bran by-products can be better utilized and obtained for further application.

The purpose of this study was to investigate the effect of hydrolysis condition on its protein and β-glucan contents on anti-oxidative activity and anti-inflammatory activity of the SYRBH.

3.3 Materials and Methods

3.3.1 Materials and Chemicals

Testing chemicals, including chemicals for analysis of antioxidative activity, anti-inflammatory activity were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Murine TNF, IL-1β and IL-6 ELISA Development Kit were purchased from Peprotech (Peprotech, Rocky Hill, USA).

The mouse macrophage cell lines, RAW 264.7, were obtained from **Nutraceutical and Functional Food Research and Development Center**, Prince of Songkla University, Songkhla, Thailand. The other materials required for culturing of the cells were purchased from Gibco BRL, Lift Technology (Thailand).

Rice bran hydrolysate (RBHs) was prepared by enzymatic hydrolysis with Amyloglucosidase and Protease G6 enzyme selected from chapter 2. Finally, the hydrolysates containing high value of protein and β-glucan as well as great functional properties were selected to investigate for anti-oxidant and anti-inflammatory activities.

3.3.2 Antioxidative activity of rice bran hydrolysates

3.3.2.1 ABTS radical scavenging activity assay

2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical cation decolorization assay was carried out using an improved method with some modifications by Khantaphant and Benjakul (2008). ABTS radical ($ABTS^{\bullet+}$) was generated by oxidation of 4.0 mM ABTS stock solution with 2.5 mM potassium persulfate at the ratio of 1:1 (v/v) for 16 h prior to use in the dark at room temperature (stock solution). For the study of the hydrolysate, the $ABTS^{\bullet+}$ solution was diluted with methanol to give an absorbance of 0.8 ± 0.02 at 734 nm before being used ($ABTS^{\bullet+}$ working solution). Different concentrations of the hydrolysate, along with the standard Trolox were mixed to $ABTS^{\bullet+}$ working solution. To initiate the reaction, 20 µl of hydrolysate was mixed with 300 µl of $ABTS^{\bullet+}$ solution. The higher the degree of decoloration of the $ABTS^{\bullet+}$ solution, the more efficient the scavenging ability of the tested compounds. The absorbance was then read at 734 nm after 2 h dark incubation

at room temperature. The results are expressed as Trolox equivalent antioxidant capacity.

3.3.2.2 DPPH radical scavenging activity assay

The method of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was described by (Orhan *et al.*, 2007) with a slight modification. The 100 µl selected RBH were dissolved in distilled water and mixed with 100 µl of 0.2 mM DPPH that was dissolved in methanol in a test tube. The mixture was then shaken and kept in the dark for 30 min. The absorbance of the resultant solution was recorded at 517 nm. Trolox(6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid) 10-150 µM was used as the standard for calibration. The scavenging activity was expressed as Trolox equivalent antioxidant capacity.

3.3.2.3 Metal chelating activity

The metal chelating activity of the RBH was assessed using the method of Dinis *et al.* (1994) with slight modification. 1 ml of selected RBH sample was mixed with 3.7 ml of distilled water. The mixture was then reacted with a solution containing 0.1 ml 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine (3-(2pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine). The control was prepared in the same manner except that distilled water was used instead of the sample. The absorption was measured at 562 nm and used to determine the Fe^{2+} chelating activity.

3.3.2.4 Reducing power assay

The reducing power of the RBH was measured according to the method described by Chen *et al*. (2007). Different concentrations of selected RBH were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (1.0% w/v). The mixtures were incubated at 50°C for 20 min. After incubation, the reaction was terminated by the addition of 10% trichloroacetic acid (TCA) and then followed by centrifugation at 5000×g for 10 min. The upper layer was mixed with distilled water and 0.1% (w/v) ferric chloride ($FeCl_3$) and then the absorbance of the mixture was

measured at 700 nm against a blank. Increase of absorbance of the reaction mixture at a wave length of 700 nm indicates an increase of reducing power.

3.3.3 Anti-inflammatory activity

3.3.3.1 Cell culture and viability determination

The mouse macrophage-like cell line RAW264.7 (the American Type Culture Collection, USA) was cultured and maintained in RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin G/streptomycin sulfate at 37 ^{0}C and 5% CO_2 in humidified incubator. For all experiments, the cells were sub cultured until a 70%-80% confluent monolayer was achieved.

The cell viability was determined by the 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay as described by Szliszka *et al*. (2011) and Bronikowska *et al*. (2012). This test is based on the cleavage of the tetrazolium salt MTT to a blue formazan dye by viable cells. Briefly, RAW264.7cells (1×10^6 cell/well) were seeded in a 96 well plate for 2 h and treated with RBH at the concentrations of 100, 300, 500, 1000, 1500 μg/ml. After 24 h the medium was removed, and 10 μL MTT solutions (5 mg/ml) was added to each well for 2 h. The resulting formazan crystals were dissolved in DMSO. The controls included native cells and mediumalone. The spectrophotometric absorbance was measured at 570 nm wavelength using a microplate reader. The cytotoxicityas percentage of cell death was calculated by the formula: (absorbance of sample/ absorbance of control)×100.

3.3.3.2 Inhibitory of NO production

The inhibition of NO production was assayed using the method of Wang *et al*., (2007) with slight modification. The effect of RBH on NO production by RAW 264.7 cell lines was assessed by Griess reaction. Briefly, RAW 264.7 cells were cultured in 96-well plates at a density of 1.0×10^6 cells/well and allowed to adhere for 2 h. After that the cell were pretreated with RBH (100, 300, 500, 1000, 1500 μg/ml) containing 0.5 μg/ml of LPS. NO production was determined after 48 h by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent (0.1% N-(1-naphthyl)-ethylenediamine, 1% sulfanilamide in 5% phosphoric acid). It responded

at the normal temperature for 10 minutes and its absorbance was measured at 570 nm using the micro plate reader. Cytotoxicity was also determined by MTT assay. The NO inhibition was calculated by the formula:

$$\text{Inhibition (\%)} = \frac{\text{ODcontrol} - \text{ODblank control}) - (\text{ODsample} - \text{ODblank sample})}{\text{ODcontrol} - \text{ODblank control}} \times 100$$

3.3.3.3 Inhibitory of cytokine (TNF-α, IL-1β, IL-6)

The hydrolysates containing great anti-oxidation and NO inhibition activities were selected to investigate for anti-inflammatory activities on inhibitory of cytokine.

RAW 264.7 cells were seeded in 96-well plates at $1x10^6$ cells/well and cultured for 2 h. After LPS media were treated for 6 h, The selected RBH sample were added and incubated until 48 h. Then cytokine (TNF-α, IL-1β, IL-6) content was determined by a quantitative sandwich enzyme-linked immune-sorbent assay (ELISA) using the Murine TNF, IL-1β and IL-6 ELISA Development Kit (Peprotech, Rocky Hill, USA) according to the manufacturer's instructions.

3.3.3.4 Statistical analysis

All experiments were run in triplicate. Results were expressed as the mean value ± standard deviation from three replicates. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's Multiple Range Test. Differences were considered to be significantly different at ≤ 0.05.

3.4 Results and Discussion

3.4.1 Anti-oxidative activity of SYRBHs

Four antioxidant assays were utilized to evaluate the free radical scavenging and anti-oxidant activities of RBHs. The ability to the ABTS radical, DPPH radical, reducing power and metal chelating activity of selected RBHs using different enzymatic hydrolysis is illustrated in Table 14. The hydrolysate derived from 5% sequential enzymatic hydrolysis for 60 min exhibited the highest anti-oxidant activity including ABTS radical (18.05±0.23 g Trolox/g sample), DPPH radical (17.49±0.28 g Trolox/g sample), reducing power (6.87±0.49 g Trolox/g sample) and metal chelating activity (7.61±0.30 g EDTA /g sample). The results were observed by increase of protein and β-glucan contents and co-enzymatic hydrolysis process with high concentration of enzyme as well no longer time of hydrolysis that show display abundant anti-oxidant activities. It is important to note that this condition showed the high protein and β-glucan contents, that the both components could improved the stronger activity. Therefore, it can be indicated that the RBHs with co-enzymatic hydrolysis have more ability to anti-oxidant activities than single enzymatic hydrolysis. However, Selected RBHs that gave high protein and/or β-glucan content showed no significant difference from DPPH radical-scavenging activity.

Table 14 Antioxidant activity of SYRBHs.

Hydrolysate	ABTS	DPPH	Reducing power	Metal chelating
5 A 30 min	14.50±0.18^{ef}	16.55±0.52^{abcd}	4.64±0.45^{d}	7.57±0.27^{bc}
5 P 30 min	12.25±0.47^{g}	15.24±0.45^{fgh}	3.29±0.25^{e}	7.06±0.16^{c}
3 A→3 P 30 min	13.75±0.25^{f}	15.64±0.87^{defgh}	4.47±0.25^{d}	6.37±0.06^{d}
3 A→3 P 60 min	14.54±0.42^{ef}	15.29±0.53^{efgh}	4.87±0.41^{cd}	6.16±0.32^{d}
4 A→4 P 60 min	15.13±0.33^{de}	16.28±0.78^{cdef}	5.61±0.31^{b}	7.61±0.30^{bc}
5 A→ 5 P 30 min	16.55±0.40^{bc}	17.38±0.31^{ab}	6.61±0.21^{a}	8.06±0.10^{ab}
5 A→5 P 60 min	18.05±0.23^{a}	17.49±0.28^{a}	6.87±0.49^{a}	8.29±0.31^{a}
5A→5 P 120 min	15.86±0.59^{cd}	16.37±0.24^{bcde}	6.49±0.27^{a}	7.28±0.14^{c}
3 A + 3 P 30 min	14.23±0.73^{f}	14.81±0.37^{h}	4.87±0.18^{cd}	6.10±0.34^{d}
3 A + 3 P 60 min	14.09±0.11^{f}	15.14±0.46^{gh}	6.53±0.33^{a}	6.24±0.27^{d}
4 A + 4 P 60 min	14.52±0.22^{ef}	15.76±0.49^{defgh}	5.49±0.08^{bc}	7.37±0.38^{c}
5 A + 5 P 30 min	15.46±0.29^{d}	16.21±0.27^{cdefg}	4.78±0.24^{d}	7.43±0.49^{c}
5 A + 5 P 60 min	16.78±0.34^{b}	16.89±0.19^{abc}	6.50±0.17^{a}	8.23±0.20^{a}
5 A +5 P 120 min	15.21±0.15^{de}	16.30±0.19^{cdef}	5.07±0.30^{bcd}	6.46±0.18^{c}

Values are expressed as mean ± standard deviation from triplicate determinations. Columns with different letters indicate statistical differences ($P \leq 0.05$).

Mean ABTS radical scavenging activity as g of Trolox equivalent/g sample; DPPH radical scavenging activity as g of trolox equivalent/g sample; Reducing power as mg of trolox equivalent/g sample; Metal chelating activity as g of EDTA equivalent/g sample

The reducing power and metal chelating activity of RBHs showed high ability by sequential and combined hydrolysis. Increasing of value of concentration of enzyme and protein and/or β-glucan content has an effect on the reducing power and metal chelating activity of RBHs. The activity decreased with hydrolysates using single enzymatic hydrolysis as well as presented lower protein and/or β-glucan contents. The data also showed that the activity was greatest with the hydrolysates from sequential enzymatic hydrolysis.

The results of this study were corresponded with good quality protein, high fiber content and nutritive elements of rice bran, they contain anti-oxidative compounds that have the ability to inhibit the formation or to reduce the concentration of reactive cell-damaging free radical. Some of these include water-soluble, fat-soluble and insoluble antioxidants, such as vitamin E, vitamin B1, B2, B3 and B6, tocotrionols, selenium and phytic acid (Sompong *et al*., 2011; Chotimakorn *et al*., 2008; Butsat *et al*., 2010; Huang and Ng. 2011). The previous document also reported that the anti-oxidant activity of various compounds of rice bran could be found in rice bran protein hydrolysates, dietary fiber, the phenolic compound and anthocyanin and alkaloid 4-carbomethoxy-6-hydroxy-2-quinolone (Thamnarathip *et al*., 2016; Arab *et al*., 2011; Chooklin, 2013; Muntana and Prasong, 2010; Zhang *et al*., 2010; Chotimakorn et al., 2008; Wang *et al*., 2015 ; Chung and Woo, 2001). Moreover, β-glucan as dietary fiber has been reported possess free radical scavenging activity and ability to alleviate inflammatory conditions (Du and Xu, 2014; Uskokovic *et al.*, 2013; Suchecka *et al.*, 2015).

Therefore, the antioxidant activity (ABTS, DPPH, reducing power and metal chelating) of RBHs was found to depend on the component of bioactive compound (protein and β-glucan contents) included. Extension of hydrolysis duration caused reduction on the anti-oxidative activity when the RBH was hydrolyzed with the similar process, which may have been due to expressive hydrolysis resulting in cleavage of proteins. The structure of active peptide was broken relating to a decrease in bioactivity. The result of this study was similar with the finding of (Li *et al*., 2014; Thamnarathip *et al*., 2016). However, all selected RBHs presented ability of anti-oxidative activity especially sequential hydrolysis that has the potential to be novel hydrolysis process of bioactive hydrolysates.

3.4.2 Cytotoxicity of RBH to RAW 264.7 cells

The RBHs with high protein and/or β-glucan contents prepared by using different process and conditions were selected and evaluated for their toxicity against RAW 264.7 cells. In this study, we used a hydrolysate concentration in the range of 100-2,000 µg/mL. It was found that all the selected RBHs at concentration as high as 2,000 µg/mL exhibited no cytotoxicity against RAW 264.7 cell lines (Figure 15 (a), (b)

and (c)). None of the hydrolysates exhibited any toxic effects the RAW 264.7 cell lines. The stimulus effect on the cell growth was likely decreased by increasing of concentration the RBH. At high concentration of samples of both process probably cause a rapid cell growth and due to limited resource thereafter may trigger cell death.Therefore, the RBH could be used for further analysis the intracellular nitric oxide inhibitory and anti-inflammatory activities. Kim and co workers reported that defatted rice bran ethanol extract at 1,000 µg/mL had found no cytotoxicity on RAW 264.7 macrophage cell line (Kim *et al*., 2013). Furthermore, Thai purple rice bran gamma-oryzanol rich hydrolysate at the maximum concentration, 100 µg/mL, had no effect on cell death (Chalermpong *et al*., 2012).

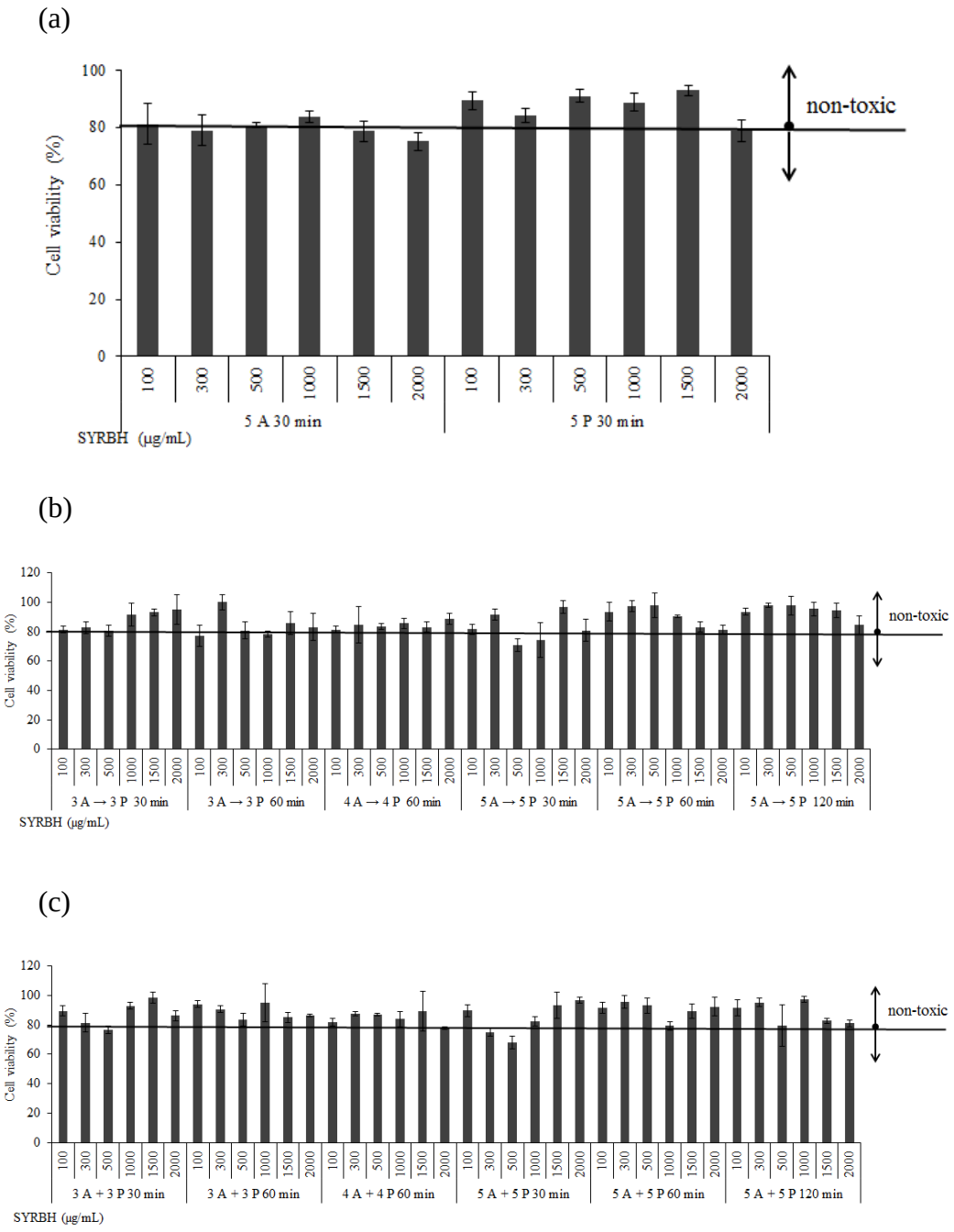


Figure 15 Effect of concentration of SYRBH on RAW 264.7 cell viability. Values are expressed as mean ± standard deviation from triplicate determinations. Columns with different letters indicate statistical differences ($P \leq 0.05$).

3.4.3 Inhibitory effect of RBH on nitric oxide production in LPS-stimulated RAW264.7 cell

In murine macrophage RAW 264.7 cells, LPS only induced the transcription and protein synthesis of iNOS (inducible nitric oxide synthase) and increased nitric oxide production (Yoon *et al*., 2009). The selected RBHs as high protein and β-glucan contents at a concentration of 1,500 μg/mL were used to verify their NO inhibition activity of RAW 264.7 macrophage cells as show in Table 15. It was found that RBHs by sequential hydrolysis showed higher anti-inflammatory activity than that of RBHs by combined and single hydrolysis. The RBHs by 5% concentration for 30 and 60 min exhibited the most promising anti-inflammatory activity (IC_{50} = 547.72 and 408.63 μg/ml, respectively). The RBH by 5% concentration for 60 min also exerted the highest inhibitory activity on NO production from RAW 264.7 macrophage cells. Most of the RBHs exhibited the stronget activity were prepared by using both sequential and combined process.

It is an unexpected result on finding that the activity of the RBH does not related with both protein and β-glucan content. It is proposed that the strong NO inhibitory activity is mainly due to the high value β-glucan and/or protein in the hydrolysates. The result also suggested that anti-inflammatory activity of RBHs was decreased with increasing hydrolysis time. The hydrolysates with an exerted NO inhibitory activity were normally produced by the catalytic with low DH. On the other hand, it is not straightforward to explain the relation on activity of hydrolysate with DH as there are some process condition having an exerted DH but produced a hydrolysate with low NO inhibitory activity on to opposite, other than a possible alteration of enzyme cleavage. The hydrolysate by enzymatic hydrolysis in previous results either in low or high reactive hydrolysate is likely an account for the observation made. Few studies have reported the NO inhibitory activity of rice bran hydrolysates. However, none of them mentioned the mechanism responsible for the reported activity. In generally, inhibition of the iNOS expression may be attributed to reactive hydrolysates (Chalermpong *et al.*, 2012).

The protein hydrolysate and β-glucan have been reported for their anti-inflammatory activity. Ndiaye *et al*. (2012) indicated that pea protein hydrolysate showed significant inhibition of NO production by activated macrophage up to 20%. Ciacci *et al*. (2014) reported that kiwi fruit peptide displays anti-inflammatory activity that was highly effective in preventing the increase of LPS-induced ROS levels in cell line. While, Xu *et al.* (2012) reported that β-glucan from the fruiting bodies of *Lentinus edodes* resulted in the striking inhibition of NO production in LPS-activated macrophage RAW 264.7 cells. Suchecka *et al.* (2015) reported that high β-glucan concentration from oat resulted in anti-inflammatory effect of LPS-induced chronic enteritis. Moreover, Black rice bran extract showed inhibition of nitric oxide by LPS-induced with RAW 264.7 cells. (Min *et al.*, 2010).

The RBHs containing sequential and combined by 5% concentration for 30 and 60 min that presented strong NO inhibitory activity were selected for further investigation for pro-inflammatory cytokine.

Table 15 Effect of RBH on NO inhibition in RAW264.7 cells of SYRBHs.

Hydrolysate	% Inhibition at concentration 1500 µg/ml	IC_{50} (µg/ml)
5 A 30 min	29.75 ± 3.13^{ef}	> 1500
5 P 30 min	24.82 ± 9.06^{ef}	> 1500
3 A→3 P 30 min	23.05 ± 6.51^{f}	976.92 ± 55.11^{c}
3 A→3 P 60 min	62.01 ± 8.69^{abc}	1068.61 ± 35.70^{cd}
4 A→4 P 60 min	67.31 ± 3.81^{ab}	1103.12 ± 45.98^{de}
5 A→ 5 P 30 min	58.76 ± 5.49^{bcd}	547.72 ± 55.56^{b}
5 A→5 P 60 min	71.63 ± 7.63^{a}	408.63 ± 14.78^{a}
5A→5 P 120 min	34.49 ± 2.58^{e}	1068.61 ± 35.70^{cd}
3 A + 3 P 30 min	28.18 ± 4.28^{ef}	1099.11 ± 21.46^{de}
3 A + 3 P 60 min	49.51 ± 2.20^{d}	1047.29 ± 38.17^{cd}
4 A + 4 P 60 min	53.36 ± 1.20^{cd}	1176.61 ± 33.34^{e}
5 A + 5 P 30 min	49.09 ± 3.36^{f}	980.36 ± 27.81^{c}
5 A + 5 P 60 min	68.75 ± 5.76^{ab}	973.36 ± 49.06^{c}
5 A +5 P 120 min	36.07 ± 2.71^{e}	1100.5 ± 32.60^{de}

Values are expressed as mean ± standard deviation from triplicate determinations. Columns with different letters indicate statistical differences ($P \leq 0.05$).

3.4.4 Effect of RBH on cytokines (TNF-α, IL-1β, IL-6) secretion

The RBHs with strong NO inhibitory activity were selected for their inhibition activity against secretion of pro-inflammatory cytokine. The result shown in Figure 16 illustrated that TNF-α is a major medium of LPS response followed by IL-6 and IL-1β respectively. Our data presented that TNF-α is a major medium of LPS response because of their highest level. Bessis *et al.* (1998) reported that TNF-α is one of the major pro-inflammatory cytokines involved in the pathogenesis of chronic inflammatory disease. TNF-α play an important part in an innate immune response (Kim *et al*., 2013). Moreover, TNF-α is a pleiotropic inflammatory cytokine and can stimulate the production or expression of IL-1β and IL-6 (Aggarwal and Natarajan,

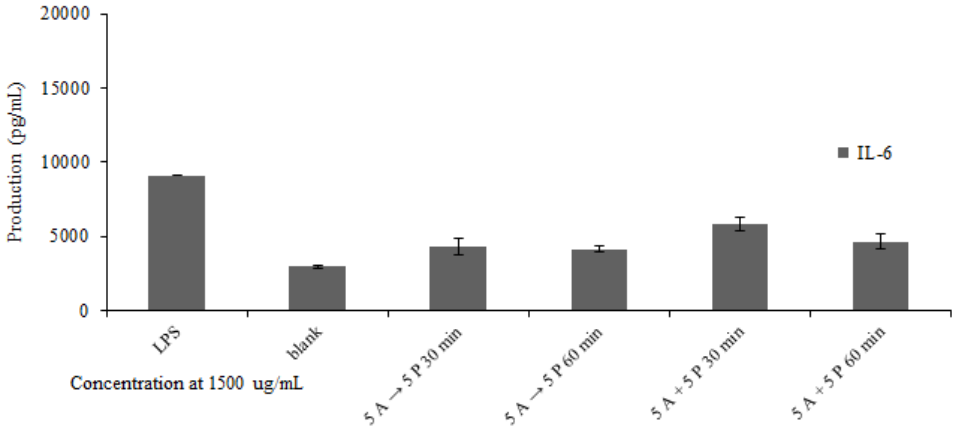
1996). And, secretion of these cytokines induced by LPS was inhibited differently by all the RBHs used. Regardless to samples, reduction on IL-1β secretion was outstanding with inhibition range of 45.58-71.96% followed by those of IL-6 and TNF-α inhibition range of 35.56-54.55% and 9.37-29.20% respectively.

Based on their inhibition against generation of all cytokines, the RBHs derived by using the sequential process was therefore the most outstanding anti-pro-inflammatory cytokines. From this result, it appears that the percentage of pro-inflammatory cytokine inhibition of sequential hydrolysis by 5% v/w concentration for 60 min was effected with the trend of pro-inflammatory cytokine inhibition likely by the commercial barley. This result was similar to the study by Wasaporn *et al*. (2013) which reported the anti-inflammatory properties of cereal crude β-glucan

Few studies have reported that anti-inflammatory activity of extraction though inhibition effect on nitric oxide production in combined LPS-IFN-γ activated RAW 264.7 murine macrophage cells (Chalermpong *et al*., 2012). Thai colored rice extracts, especially red color reduced pro-inflammatory cytokines (IL-6, TNF-α and NF-κB) and MMP-2 expression in LPS-induced HL-60 cells (Kitisin *et al*., 2015).

Moreover, Phangthip *et al*. (2013) found that rice-berry bran extract could reduce IL-6 and TNF-α in streptozotocin induced diabetes rats. Thus, this study suggests that Sang Yod rice bran hydrolysate, especially those high in protein and β-glucan can be used to reduce pro-inflammatory cytokine in RAW 264.7 murine macrophage cells via their anti-inflammatory activities.

(a)


20000
15000
10000
5000
0
Production (pg/mL)
IL-6
LPS
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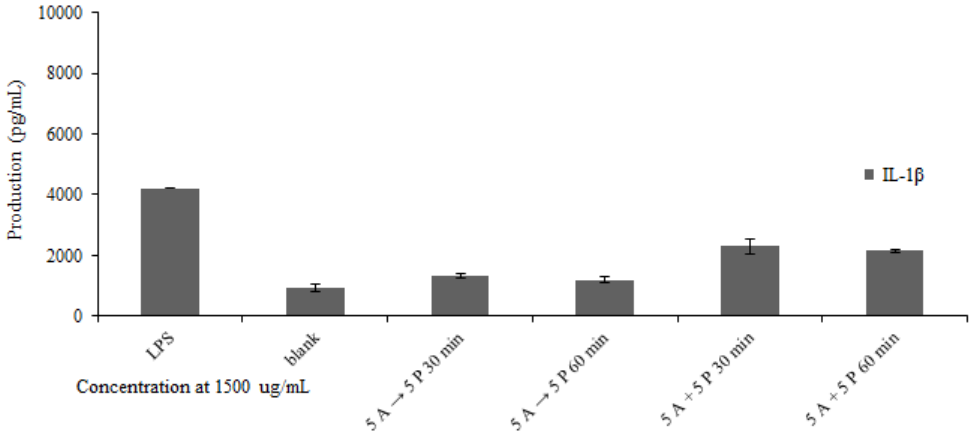
5 A → 5 P 60 min
5 A + 5 P 30 min
5 A + 5 P 60 min
Concentration at 1500 ug/mL

(b)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8000
6000
4000
2000
0
Production (pg/mL)
IL-1β
LPS
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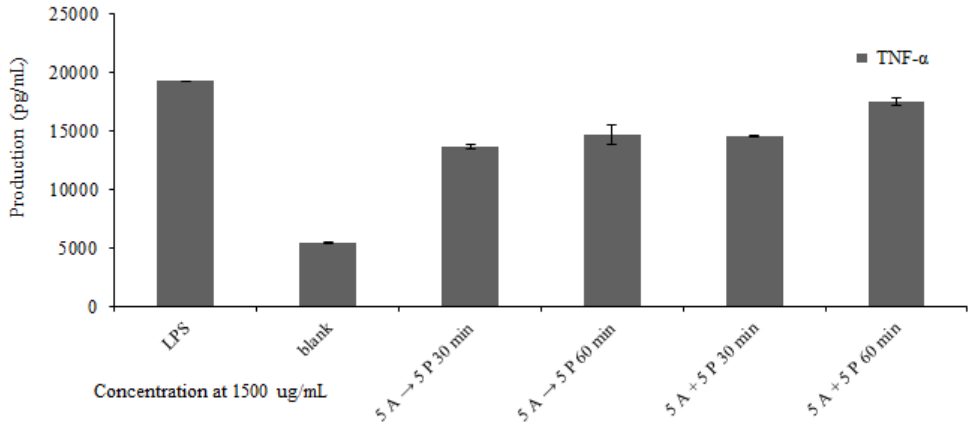
5 A → 5 P 60 min
5 A + 5 P 30 min
5 A + 5 P 60 min
Concentration at 1500 ug/mL

(c)


25000
20000
15000
10000
5000
0
Production (pg/mL)
TNF-α
LPS
blank
5 A → 5 P 30 min
5 A → 5 P 60 min
5 A + 5 P 30 min
5 A + 5 P 60 min
Concentration at 1500 ug/mL

(d)

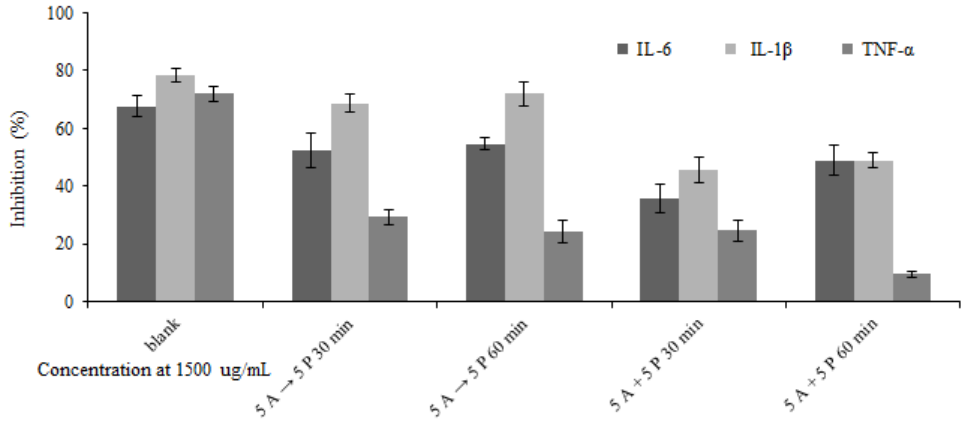


Figure 16 Inhibitory effects of the selected SYRBH on IL-6, IL- 1β and TNF-α in LPS-stimulated RAW 264.7 cells. The data are means ± SD (n =3) and shown as IC_{50} (µg/ml). Effect of SYRBH on cytokine (IL-6, IL-1β and TNF-α) secretion by RAW 264.7 cells induced with LPS (a, b and c). Inhibitory of the selected SYRBH on the production of IL-6, IL-1β and TNF-α (d).

3.5 Conclusion

The RBH produced with different conditions showed antioxidant activity and non-toxicity to RAW 264.7 cell line. The RBHs collected from the sequential hydrolysis process at 5% for 60 min contained a high amount of protein and β-glucan contents. They showed strong anti-oxidative and anti-inflammatory activities.

CHAPTER 4

DEVELOPMENT AND SENSORY EVALUATION OF FUNCTIONAL RICE PUDDING FORTIFICATION WITH SYRBH

4.1 Abstract

Sang Yod rice bran hydrolysate (SYRBH) with inhibitory for nitric oxide (NO) and pro-inflammatory generation was prepared using an enzymatic hydrolysis process and used for application as a functional food ingredient in functional rice pudding. Questionnaire was used to survey consumer behaviors and opinion on functional rice pudding. The results indicated that the respondents consume rice pudding at least 3-4 time/week mostly in morning. The two top ingredients mixed with rice pudding were cereal/multi-grain and fresh fruit or vegetable. Rice pudding is the most preferred product of the functional rice pudding as it is ready-to-eat in a plastic cup with natural flavor. Effect of SYRBH fortification range of 1-7% on the product preference was evaluated. The result revealed that the sample fortified with 5 % SYRBH received the highest acceptance score (≥7.0). The functional rice pudding of one serving size (100g) provide energy at 120 Kcal and added β-glucan 0.2 g and protein 0.16 g from SYRBH. Stability of functional rice pudding was investigated by microbial counts and sensory test thoughout 10 days storage in a refrigerator. Fortification of SYRBH in rice pudding thus has the potential to serve as a functional food that can offer health benefits to the consumer.

4.2 Introduction

Higher incidences of severe diseases have contributed to the evolution of novel food trend in the industry to develop products that meet the created demand. Consumers are taking a more proactive approach for their health status by practicing a healthy life style and turning toward healthy food options. There has been increasing

evidence for discovery of novel bioactive ingredients showing higher nutritive value and bioactive activity. These have beneficial effects for human health and encourage utilization in functional food, nutraceutical food or medical food. The protein and amino acid in rice bran are of high nutritional value and qualities (e.g. essential amino acid, protein efficiency ratio and net protein utilization) comparable to soy protein, rice endosperm protein, whey protein and casein (Han *et al.,* 2015). Enzymatic hydrolysis was used to improve protein extraction from rice bran. Tang *et al.* (2002) reported that a combination of enzyme with high pressure treatment prepared rice bran protein hydrolysate, exhibited anti-oxidant activity against ABTS and DPPH radicals. It also showed reducing power and metal ion chelating activity (Adebiyi *et al*., 2009; Chanput *et al.,* 2009; Zhang *et al.,* 2014). Rice bran also rich in anti-oxidant compounds (e.g. polyphenols, carotenoids, vitamin E and tocotrienol) which help in preventing the oxidative damage of body tissues and DNA. Rice bran is also rich in soluble fiber like β-glucan, pectin, gums which helpful in reduction of chronic ailments such as in serum cholesterol, certain forms of cancer and constipation (Anderson et al., 1990; Gordon, 2001; Dhingra and Jood, 2001). The Food and Drug Administration (1997) has recently allowed rolled oats, oat flour and oat bran to claim health benefits provided that they are give at least 3 g of soluble fibre[(1-3)(1-4)-beta-D-glucan] per day and used as a part of a low-saturated fat, low cholesterol diet, as the soluble fiber, β-glucan from oats and barley is recognized for its hypocholesterolemic value. More activities are based on using biologically active soluble fiber as nutraceuticals and functional food ingredients (Inglett, 1999).

In recent years, research has shown showed that rice bran serves as an important functional food. It has cholesterol lowing properties, cardiovascular health benefit an anti-tumor activity (Kawamura and Muramoto, 1993). In addition to its high nutritive value rice protein is hypoallergic, which is easily digestible and have an anticancerous properties. Rice bran or rice-derived bioactive compounds are effectively utilized as a supplement for bekery products like cookies, muffins, bread, crackers, pasteries, pancakes (Barber *et al.,* 1981). With increasing awareness of a healthy and function food, fortified bakery products has become the emerging market in the bakery industry. However, there are only a few studies of the fortification of other products espectially rice pudding. Up to date, in general, the substitution of milk by soy protein

concentrate or soy isolate, rice drink, pea isolate has been examined in pudding. Lim and Narsimhan (2006) studies on the pasting and rheological properties of soy protein-based pudding. Almprese and Mariotti (2011) examined the effect of milk substitution by soy and rice drink on rheological and texture properties of puddings. Moreover, the fortification of rice pudding by rice bran has not been explored yet.

Thai Sang Yod rice is a dark red-violet pigmented rice from the south of Thailand (Phatthulung province) that has been supported by Her Majesty Queen Regent Sirikit of Thailand (Burean of Rice Research and Development, 2006). Additional, Sang Yod rice is the first rice variety which has been registered for geographical indication (GI) product of Thailand. It contains various nutritive substances, including carbohydrate, fibers, protein and vitamin B1, B2, B6. It is also rich in various minerals such as iron, calcium and phosphorus (Thai Food Composition Organization, 1999). Nowadays numerous reports mentioned biological activities of rice bran, colored rice bran as biologically active compound. Thus, brown rice is the most favorite among consumers for consumption of this variety. The pigmented rice in Thailand has grown significantly over the past decade.

Rice pudding is a dish made from rice mixed with water or milk and other ingredients such as muti-grain, raisin and fruits. Different variants are used for either desserts or dinners. Such desserts are found on many continents, especially Asia. Rice pudding is a traditional popular product worldwide. They are high nutritive in carbohydrate, protein, fiber, vitamin and mineral which make it healthy for daily consumption. Because of its acceptability in all age group enhanced taste, soft texture and its position as dinners or deserts. It is considered as a good product for protein fortification and other nutritional improvement. As the main ingredient in rice pudding, rice starches as polysaccharides and milk proteins such as casein and whey contribute to the structural and textural properties of the pudding. Normally, when mixing proteins with polysaccharides, the interactions of those polymers can be segregated or associative depending on types and concentrations of the biopolymers and conditions of the system (Corredig *et al.*, 2011). Rice pudding is an uncommon and unpopular product to Thais due to its slow development, while although rice pudding shows many beneficial ingredient. It is suitable for general consumes and special consumers as a modified texture food. Rice pudding is usually milk protein-based rice starch paste but

it is uncommon to Thais. The compositions of rice pudding are milk or skim milk, rice grain or flour, sugar, egg and salt. With a rough texture from rice grains and strong egg taste, rice pudding is not ly accepted by Thais. Thais rice and rice milk containing unique fragrance and persisting soft texture is a preferable choice to improve this product. Thus applying rice and cereal grain as an eastern raw material as well as fortification with bioactive compound from RBH in order to improve the acceptance of rice pudding is an alternative way for such functional product development.

The objective of this study was to investigate the possibility of fortifying the SYRBH to formulate functional rice pudding product on properties, shelf life and sensory acceptability.

4.3 Materials and Methods

4.3.1 Materials and Chemicals

The SYRBH from 5% w/w of sequential hydrolysis for 60 min (5A→5P 60 min) was analyzed for their chemical properties and physical properties. The chemical composition of RBH performed include moisture content, protein, ash, fat, starch, and crude fiber according to the method of AOAC (2000). Pigmented rice (Sang yod rice, Rice berry and Hom-nil rice), muti-grains (mungbean, job's tears, seasame), low fat milk, rice/cereal milk, honey, sugar, cinnamon, nutmeg, raisin, almond and fresh fruits were purchased from a local market.

4.3.2 Development of functional rice pudding fortified with SYRBH

4.3.2.1 Consumer survey of functional rice pudding

The objectives of this survey were 1) to study the behavior, attitude and perception of consumers to functional rice pudding. 2) to study the factors that affect behavior and 3) to understand the consumer requirement for developing functional rice pudding fortified with SYRBH. The questionnaire consisted of 3 parts; the consumer attitude and behavior toward rice pudding product, the development functional rice pudding product and personal data. The questionnaire was pre-tested by 15 consumers

and then it was corrected before giving to a further 15 consumers to make sure that any defects in the questionnaire had been corrected. The corrected questionnaire can be found in Appendix A. The consumer survey was conducted at university, government offices and food shop/canteen at Suan Dusit School of Culinary Arts, Suan Dusit University in Bangkok and Suphanburi campus, Thailand. This survey focused on 3consumer age group; 15-25, 26-45 and over 45 years old. The profile of an ideal functional rice pudding fortified with SYRBH as bioactive compounds was used in a further study.

4.3.2.2 Mock-up of functional rice pudding

Mock-up of rice pudding was selected from three standard formulation witch gave the highest of sensory score. Three standard formulation rice pudding were evaluated by nine-point hedonic scale, Panelists (n=15) were recruited from food specialists, staff and students of the Suan Dusit School of Culinary Arts, Suan Dusit University in Bangkok and Suphanburi campus, Thailand. The panelists evaluate the overall acceptability, appearance, color, odor, texture and taste. A nine-point hedonic scale was used where 1= dislike extremely and 9 = like extremely (Peryam and Pigrim, 1957). The standard formulation that gave the highest sensory score was selected for formulation of subsequent studies.

4.3.2.3 An application of SYRBH as bioactive compounds fortified with functional rice pudding

The experiments were conducted according to the ratio given by the standard formulation which were composed of pigmented brown rice 50 g, muti-grains 30 g, honey 25 g, sugar 10 g, cereal milk 700 ml and other ingredients. Pigmented brown rice and multigrain were washed and soaked in water to water ratio of 1:3 at room temperature for 4 hours. The hydrated grains were drained, rinsed and heated until pre-cooked. Then brown rice and multi-grains were mixed with water and heated at 95 °C in streaming pot for 40 min in order to make rice and multigrain porridge. Then, honey, all other ingredients including raisin cinnamon and rice milk were added to the mixed porridge rice and heated at 70 °C to dissolve the ingredient completely. After

adding SYRBH powders in the rice pudding all ingredients were gently mixed in a mixing pot and heated at 65 °C for 30 min. The final functional rice pudding was poured into polypropylene plastic cups and cooled down at room temperature to let the pudding set. The pudding was kept in refrigerator at 5 °C before further analysis.

The SYRBH was chosen by means of the previous experiment (Part 2 and Part 3). The SYRBH powder was added to the rice pudding formulations at 0-7% based on weight of rice pudding(w/w) as shown: Treatment: T1- 0% SYRBH: rice pudding 100%, T2-1% SYRBH: rice pudding 99%, T3-2% SYRBH: rice pudding 98%, T4-3% SYRBH: rice pudding 97%, T5-4% SYRBH: rice pudding 96%, T6-5% SYRBH: rice pudding 95%, T7-6% SYRBH: rice pudding 94%, T8-7% SYRBH: rice pudding 93%, respectively.

4.3.2.4 Sensory evaluations

Panelists (n=50) were recruited from food specialists, staff and students of the Suan Dusit School of Culinary Arts, Suan Dusit University in Bangkok and Suphanburi campus, Thailand. The panelists noted the appearance and smell and then ate each sample to evaluate the overall acceptability, appearance, color, viscosity, texture, order and test. A nine-point hedonic scale was used where 1= dislike extremely and 9 = like extremely (Peryam and Pigrim, 1957).

Statistical analysis

The questionnaire was used to facilitate data entry. The percentages of responses of the personal data and attitudinal data were calculated. The results were statistically analyzed using one way analysis of variance (ANOVA). Mean were compared by Ducan multiple rang test with mean square error at 5% probability

4.3.3 Study on the properties of functional rice pudding

Functional rice pudding coded with SYRBH from part 1 was prepared and analyzed for their physical properties, nutrition composition as the follow;

4.3.3.1 Physical properties

The chemical composition of pudding include moisture content, protein, ash, fat, starch, and crude fiber were determined according to the method of AOAC (2000).

pH: A pH meter (Schott, Germany) was used to measure the pH of samples.

Color: The color of the rice pudding was evaluated using a Hunter Lab CIE spectrophotometer (Reston, USA). The samples were placed in 6 cm diameter cups fitted with a 1-mm black rubber ring. Each sample cup was filled up to the level of the ring at room temperature. A black cover was placed over the cup prior to measure. The measurements were recorded as: *L**, *a** and *b** values which represent the light-dark spectrum with a range from 0 (black) to 100 (white) : a green-red spectrum.

Nutrition compositions: Nutritional compositions of rice pudding analyzed for total fat, cholesterol, protein, total carbohydrate, dietary fiber, sugar, sodium (AOAC, 2000). The total energy was calculated as amount per serving (100 g).

4.3.4 Consumer acceptability of functional rice pudding

One hundred consumers participated to evaluate the acceptability of functional rice pudding. The surveys were conducted at the university, government offices and food shop/canteen at Suan Dusit School of Culinary Arts, Suan Dusit University in Bangkok and Suphanburi campus, Thailand. The questionnaire consisted of 4 parts, the demographic information, the acceptability test and the product information parts and the personal data (Appendix B). Panelists evaluated the developed functional rice pudding fortified with SYRBH using a nine-point hedonic scale with 1= dislike extremely and 9 = like extremely (Peryam and Pigrim, 1957) for overall acceptability, appearance, color, texture, odor and taste.

4.3.5 Study on the stability of functional rice pudding

Functional rice pudding samples was prepared and packed in plastic cup. The products were stored at refrigerator (4-8 ^{0}C) for 10 Days. The following quality characteristic of functional rice pudding were daily analyzed. Microbial, pH, color were

analyzed. Sensory evaluations were evaluated the overall acceptability, appearance, color, texture, odor and taste. A nine-point hedonic scale with 1= dislike extremely and 9 = like extremely (Peryam and Pigrim, 1957).

Statistical analysis

All experiments were carried out using three replicates and all results were expressed as replicate of means. The results were statistically analyzed using one way analysis of variance (ANOVA). Mean were compared by Ducan multiple rang test with mean square error at 5% probability.

4.4 Results and Discussion

4.4.1 Chemical and physical properties of the selected SYRBH

The chemical and physical properties of SYRBH (5A→5P 60 min) are shown in Table 16. SYRBH composed of moisture 7.36%, protein content 6.70%, ash content 19.98%, crude fat 6.38%, crude, fiber 10.35% and β-glucan content 4.12%. Which had the color with *L*, a* and b** values of 43.85, 14.18 and 12.12, respectively. The brown red-colored SYRBH had lower *L** (darker), higher *a** (more red) and *b** (more blue). Thamnarathip *et al*. (2016) reported that protein hydrolysates from rice-berry showed the color with *L*, a* and b** values of 36.0, 11.50 and 9.50, respectively. The pH value was 6.32, Feature of partial SYRBH is shown in Figure 17. Norhaizan *et al.* (2013) reported that the protein content of rice bran hydrolysates ranged from 10.6 to 16.9 % depended on rice varieties (Oszvald *et al*., 2008). Moomgngarm *et al*. (2012) reported that red-brown color rice bran containing protein content from 12.93%. Ash content of SYRB was 8.98%, which represented that rice bran was a good source of minerals and trace minerals. Morover, Paghav *et al*. (2016) reported that 6-14% of dietary fiber like β-glucan, pectin and gum in rice bran, Therefore, this variation might be due to the different rice milling processing equipment and conditions hydrolysis processes that may effect to chemical composition and properties of rice bran hydrolysates.

Table 16 Chemical and physical of SYRBH powders.

Compositions	Values (%)
Moisture	7.36 ± 0.33
Protein	6.70 ± 0.28
Ash	19.98 ± 1.63
Fat	6.38 ± 0.24
Crude fiber	10.35 ± 0.62
Total β-glucan	4.12 ± 0.21
pH	6.32 ± 0.25
Color(*L**,*a**,*b**)	*L**43.85±1.49, *a**14.18± 0.32, *b**12.12± 0.28

The data are mean ± SD (n=3)

Figure 17 Feature of Sang Yod rice bran hydrolysates

4.4.2 Development of functional rice pudding fortified with SYRBH

Consumer survey of rice pudding product

Demographic data and functional rice pudding behavior

The age groups were selected in the original design of the surveys and included 15-25, 26-45 and 45 years old respondents. The sample was divided into these particular age groups as it was though that they would have quite different rice pudding and attitudes due to their life style, circumstances and experience. The minimum age for respondents was set at 15 because at this age starting to gain a basic understanding of nutrition and understanding of how various basic nutrients affect their healthy bodies. Moreover, they can taste and answer the questionnaire.

The demographic profiles of one hundred respondents are shown in Table 17. There were 75% females and 25% males. Marital status of respondents were single (60%) and married (34%). The highest education indicated for most of them was bachelor degree (42%). Most of them had income of 10,000-20,000 Bath/month (46%).

Table 17 The demographic profiles of the respondents in consumer survey

Respondent characteristic	Response (%)
Gender	
Female	65.0
Male	35.0
Age	
15-25 years old	50.0
26-45 years old	37.0
Over 45 years old	13.0
Marital status	
Single	65.0
Married	32.0
Other	3.0
Occupation	
Government official	27.0
Student	50.0
Employee	10.0
Other	13.0
Education	
Lower secondary school certificate	15.0
Vocational certificate/2years of collage	12.0
Bachelor degree	53.0
Higher than Bachelor degree	20.0
Personal income(Bath per month)	
Less than 10,000 Bath	32.0
10,000-20,000 Bath	45.0
More than 20,000 Bath	23.0

Consumers were asked a number of questions relating to their functional rice pudding product consumption behavior as a healthy product. The consumer behaviors on rice pudding of the respondents are shown in Table 18. The respondents ate rice pudding at 3-4 times/week (36%), less than 1 time/week (28%), 1-2 times/weak

(22%) and daily (14%). Mostly eaten in the morning (47%) and the evening (32%). The objectives for eating functional rice pudding(cereal grain) were improving their health (75%) and curing disease (32%). Uutama-ang (2006) reported that health is one of the frequently mentioned motivations behind food choices.

Table 18 The consumer behavior on rice pudding of respondents.

Respondent characteristic	Response (%)
Eating frequency	
Daily	14.0
3-4 times/week	36.0
1-2 time/ week	22.0
< 1 time/week	28.0
Eating time	
Morning	47.0
Morning break	12.0
Noon	13.0
Afternoon break	6.0
Evening	32.0
Objective of eating rice pudding (cereal grain)	
Healthy benefit	75.0
Refreshing	12.0
Curing the disease	32.0
Control diet	16.0
Anti-aging	7.0

Note: Rice pudding is like rice porridge, The objective of eating rice pudding, the consumer could be selected more one reason.

The attitudes of consumers to Consumer were healthy/functional rice pudding are shown in Table 19. The respondents thought that healthy/functional rice pudding should be made from the ingredients from mix cereal or multi-grain (65%), then fresh fruit or vegetable (55%) the last was dried fruit or vegetable (24%). The most

favorite taste functional rice pudding was natural taste (43%). They also though that ready-to-eat and fresh (72%) was the best type of functional rice pudding.

Table 19 The attitudes to healthy rice pudding of respondents.

Attitudes	Response (%)
Ingredient for mixing	
Cereal or multi- grain	65.0
Fresh fruit or vegetable	55.0
Dried fruit or vegetable	24.0
Dairy product	15.0
Dried herb	8.0
Tea or coffee	4.0
Syrup or jam	13.0
From/type of rice pudding	
Ready-to-eat(fresh)	72.0
Ready-to-cook(dried)	11.0
Commercial (canning or pouch bag)	17.0
Taste	
Sweet	13.0
Milk	21.0
Natural	43.0
Vanilla	14.0
Other	9.0

In summary, the product profile of functional rice pudding being ready-to-eat with fresh mixed with cereal and multi-grains in plastic cup with natural taste was preferred.

Mock-up of rice pudding

Mock-up of rice pudding was selected from standard formula rice pudding which had difference style pudding formula. Formula 1 (Asia-style : S-1) consisted of cereal milk and without butter and egg yolk, formula 2 (US-style : S-2)

consisted of egg yolk and double cream , that baked after cooked and formula 3 (EU-style : S-3) consisted of double cream. The sensory scores of standard formula rice pudding are show in Table 20. The result showed that formula 1 (Asia-style) had highest sensory score which was then selected for the development of functional rice pudding.

Table 20 Sensory scores of standard formula rice pudding.

Attributes	Formula		
	S-1	S-2	S-3
Appearance	7.77 ± 0.55^{a}	6.79 ± 0.52^{b}	7.21 ± 0.73^{ab}
Color	7.98 ± 0.91^{a}	6.71 ± 1.04^{b}	7.70 ± 0.64^{a}
Odor	7.61 ± 0.95^{a}	6.73 ± 0.84^{c}	7.28 ± 0.70^{b}
Taste	8.02 ± 0.45^{a}	6.40 ± 0.93^{c}	7.52 ± 1.11^{b}
Texture	7.94 ± 0.54^{a}	5.93 ± 1.04^{b}	7.29 ± 0.70^{a}
Overall liking	8.13 ± 0.49^{a}	6.52 ± 0.74^{c}	7.52 ± 0.44^{b}

The different superscripts letters under the same row were significantly different ($p<0.05$).

The formula of mock-up rice pudding consisted of pigmented rice grain mixed, multi-grain mixed, cereal milk, milk and without butter, double cream and egg yolk as well as steam cooking. Therefore, the formula of functional rice pudding will fortify Sang Yod rice bran hydrolysate to be developed.

An application of SYRBH as bioactive compounds fortified with functional rice pudding

Eight formulas of functional rice pudding fortified with SYRBH (0-7% SYRBH-fortified) were evaluated for sensory characteristics by food specialist (30 panelists) and the results are shown in Table 21. The result showed that control formula 1 (0% SYRBH-fortified) had the highest sensory score while formula 8 (7% SYRBH-fortified) had the lowest. Based on the mean sensory score of all attributes the functional rice pudding fortified with SYRBH could be presented slightly (score ≥ 6). It was found that all attributes of formula 6.0 (5% SYRBH-fortified) scored more than 6.0. Moreover, they rated moderately for attributes of color, texture and overall liking and

slightly for attributes of appearance, odor and taste. Whereas the formula 7 and 8 having the level of 7% and 8% SYRBH-fortified were rated neither like nor dislike and dislike slightly. However, the high level of SYRBH-fortified, the attributes of taste and odor were decreased. This indicated that the levels of SYRBH rang 7-8% were required to mask the favorable taste and odor.

Overall consumer panelist liked the overall, odor and taste attributes, their hedonic impression of odor and taste were slightly low due to unpalatable bitter taste caused by the presence of peptide. (Amanda *et al*. (2016) reported that developed orange juice incorporate with rice bran pentapeptide, the sensory score for flavor attribute was low when incorporate at high level. Mishra and Chandra (2012) developed of functional biscuit substitute from soy flour and rice bran. The selected sensory score, overall acceptability 6.80 and all attribute more than 6.0 were selected for optimum formula of functional biscuit. Since information relating proper amount of RBH that should be used in rice pudding formula is not available, it is difficult to compare result of this study to others.

In this study we wanted this product to contain the maximum SYRBH-fortified value and have great sensory attributes. Thus, from the sensory result, the optimum of level SYRBH-fortified at 5% selected to prepare functional rice pudding for consumer acceptability of the product.

Table 21 Hedonic mean score of sensory evaluation of rice pudding containing different amounts of SYRBH

Attributes	% SYRBH-fortified							
	F1-0%	F2-1%	F3-2%	F4-3%	F5-4%	F6-5%	F7-6%	F8-7%
Appearance	7.44±0.76^{a}	7.33±0.73^{ab}	7.22±1.07^{abc}	7.15±1.78^{d}	6.80±0.94^{d}	6.85±0.95^{cd}	7.15±1.46^{bcd}	6.90±0.81^{cd}
Color	7.30±1.01^{a}	6.87±0.90^{bc}	6.68±1.02^{c}	7.20±0.94^{ab}	7.06±0.93^{abc}	7.12±1.13^{abc}	6.69±0.96^{c}	6.91±1.05^{abc}
Odor	7.70±1.10^{a}	7.43±0.86^{ab}	7.20±1.13^{b}	6.77±0.97^{c}	6.26±0.90^{d}	6.09±0.83^{d}	5.88±0.74^{de}	5.67±0.79^{e}
Taste	7.87±0.93^{a}	7.42±0.93^{b}	7.35±1.43^{bc}	7.15±1.43^{bc}	6.99±0.81^{c}	6.32±0.79^{d}	5.60±1.48^{d}	5.28±0.70^{e}
Texture	7.66±0.86^{a}	7.45±0.78^{ab}	7.23±1.14^{b}	7.29±0.92^{b}	7.40±0.63^{ab}	7.18±0.68^{b}	7.13±0.77^{bc}	6.81±0.85^{c}
Overall liking	7.82±0.94^{a}	7.68±0.88^{a}	7.21±1.05^{b}	7.35±0.95^{b}	7.16±0.67^{b}	7.05±0.78^{b}	6.59±0.67^{c}	6.00±1.09^{d}

The different superscripts letters under the same row were significantly different ($p < 0.05$).

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4.4.3 Properties of functional rice pudding fortified with SYRBH

The optimum formula of functional rice pudding was poured into plastic cup (100 gm per cup) with cover off. The processes of functional rice pudding are shown in Table 22, which indicated that the appropriate processing temperature of functional rice pudding was processed at 90^0C. Feature of functional rice pudding is shown in Figure 18. Which had the color with *L**, *a** and *b** values of 50.3, 9.05 and 9.26, respectively. The pH value was 6.50, total soluble solid was 16^0Brix.

Table 22 Process factor and properties of functional rice pudding

Process/properties	
Plastic cup size (mL/gm)	120
Maximum filling weight(gm)	100
Net weight(gm)	100
Heat temperature(^{0}C)	90
Packing temperature(^{0}C)	75
Storage temperature(^{0}C)	4-8
Color(*L**,*a**,*b**)	Brown to dark red : 50.3, 9.05 ,9.26
pH	6.5
Total soluble solid(^{0}Brix)	16

Figure 18 Feature of functional rice pudding

The nutrition compositions of functional rice pudding shown in Table 23. One serving size (100 g) was containing of 120 Kcal of energy, protein 12 g, fat 4 g, carbohydrate 14 g, dietary fiber 5 g, sodium 13.58 mg and sugar 3 g. However, there are not information of fortification of RBH that should be used in rice pudding formula is not available, it is therefore difficult to compare results of this study to others.

Table 23 Nutritive value of functional rice pudding

Nutritive value	
Total energy (kcal)	120
Protein(gm)	12
Fat (gm)	4
Carbohydrate (gm)	14
Fiber(gm)	5
Sodium(gm)	13.58
Sugar(gm)	3

Data was calculated by each of consume at 100 g of rice pudding.

4.4.4 Consumer acceptability of functional rice pudding

One hundred consumers participated in consumer acceptability in the test of functional rice pudding. The surveys conducted were at the university, government offices and food shop/canteen at Suan Dusit School of Culinary Arts, Suan Dusit University in Bangkok and Suphanburi campus, Thailand. The demographic profiles of the respondents are shown in Table 24. There were 65% females and 35% males. The ages were divided into three group which included 50% of 15-25 years old, 37% of 26-45 years old and 13% of older than 45 years old. Marital status of respondents were single (65%) and married (32%). Most of them (53%) learned a bachelor degree and higher than bachelor degree (20%). Most of them had an income of 10,000-20,000 Bath per month (45%).

Functional or healthy rice pudding behavior of consumer were evaluated and the results shown in Table 25. The first question asked about if the consumer had ever heard functional rice pudding. The results showed that 87% of respondents ever had heard or known about functional rice pudding. Newspaper, television or radio was

the main source of information. Most of respondent (98%) showed interest in functional rice pudding because of its nutritious (87%).

Table 24 The demorgraphic profiles of the respondents in consumer test.

Respondent characteristic	Response (%)
Gender	
Female	65.0
Male	35.0
Age	
15-25 years old	50.0
26-45 years old	37.0
Over 45 years old	13.0
Marital status	
Single	65.0
Married	32.0
Other	3.0
Occupation	
Government official	27.0
Student	50.0
Employee	10.0
Other	13.0
Education	
Lower secondary school certificate	15.0
Vocational certificate/2 years of collage	12.0
Bachelor degree	53.0
Higher than Bachelor degree	20.0
Personal income (Bath per month)	
Less than 10,000 Bath	32.0
10,000-20,000 Bath	45.0
More than 20,000 Bath	23.0

Table 25 The consumer behavior on functional rice pudding of the respondents.

Respondent characteristic	Response (%)
Have you ever heard healthy rice pudding	
Yes	13.0
No	87.0
Source of information	
Newspaper/TV/radio	48.0
Friend or personal contract	38.0
Internet/E-mail	34.0
Food exhibition	8.0
Interest in healthy rice pudding	
Yes	98.0
No	2.0
Reason for interested in healthy rice pudding	
Nutritious	87.0
Hearsay	21.0
Popularity/in trend	11.0
Advertisement	8.0
Have you even ate healthy rice pudding	
Yes	41.0
No	59.0
Product acceptance	
Acceptance	82.0
Non acceptance	18.0
Decision of buying	
Buy	68.0
Not ensure	25.0
Not buy	7.0

The consumer acceptance test of developed functional rice pudding was conducted by 100 respondents and sensory score are shown in Table 26. Overall acceptability of the product was rated at like very much range (7.60). The other attributes were rated like very much range such as color rating (7.36), Odor rating (5.55), taste rating (7.63) and texture rating (7.51). Resurreccion (1998) pointed that hedonic rate should be more than 6 for acceptance of food products. Therefore, functional rice pudding fortified by SYRBH was accepted by the consumer. Confirmation was obtained by the acceptance question and decision of buying , 82% and 68% of consumer accepted this product by itself that means it has a high potential in the market.

Table 26 Sensory scores of functional rice pudding by consumers.

Attributes	Sensory score
Appearance	7.74 ± 1.46
Color	7.36 ± 0.93
Odor	7.55 ± 1.00
Taste	7.63 ± 1.13
Texture	7.51 ± 0.90
Overall liking	7.60 ± 1.07

Consumer acceptance ratings are based on a 9-point hedonic scale

4.4.5 Stability of functional rice pudding

This experiment was conducted to investigate the quality of functional rice pudding during storage in refrigerator at 4-8 °C for 10 days. The properties (pH, total soluble solid, color) microbial count and sensory evaluations were measured. The properties of developed functional rice pudding are shown in Table 27. Changes of pH, total soluble solid were ranged from 6.50 to 6.44, 16.0 to 16.7 ^{0}Brix respectively. The color changed to brown dark- red when increasing time storage. The brown red colored RBH had lower *L**(darker), higher *a** (more red) and *b** (more blue). The darker color may be due to the non-enzymatic reaction (Maillard reaction) between reducing sugar molecules and lysine protein. Soybean and rice bran are reported to be rich in lysine

which produces darker shades of brown color (Tsuji *et al.*, 2001). The darker color of the fortified breads and biscuits have been reported by Best. (1987) and Akubor *et al.* (2005). The microbial count of functional rice pudding were found to be of lower standard microbial count of ready to eat food during 10 days. However, change of pH and total soluble solid not significantly differ ($p<0.05$) during storage at day 7.

Table 27 Properties and microbial count of functional rice pudding during 1-10 days.

Days	pH	Color			Total soluble solid	Microbial count			
		*L**	*a**	*b**	^{0}Brix	TPC (CFU/g)	Yeast (CFU/g)	Mold (CFU/g)	*E.Coli* (MPN/g)
						(<10,000)	(<100)	(<100)	(<10)
1	6.50±0.02^{abc}	50.31±0.32^{a}	9.12±0.02^{f}	9.09±0.15^{d}	16.00±0.06^{d}	ND	ND	ND	
3	6.51±0.05^{abc}	50.35±0.19^{a}	9.21±0.07^{f}	9.16±0.08^{d}	16.30±0.17^{c}	ND	ND	ND	Day 1-10 < 10
5	6.53±0.04^{ab}	50.16±0.28^{a}	9.40±0.18^{ef}	9.20±0.06^{d}	16.30±0.10^{c}	ND	ND	ND	
6	6.55±0.04^{ab}	48.58±0.94^{a}	9.62±0.10^{e}	9.36±0.04^{d}	16.33±0.12^{c}	ND	ND	20	
7	6.57±0.03^{a}	46.51±0.70^{b}	10.24±0.25^{d}	9.52±0.11^{c}	16.40±0.10^{bc}	300	10	35	
8	6.49±0.02^{abc}	43.47±0.69^{c}	10.86±0.25^{c}	10.01±0.09^{c}	16.60±0.10^{ab}	500	25	40	
9	6.47±0.04^{bc}	42.80±1.02^{c}	10.75±0.24^{b}	10.45±0.41^{b}	16.63±0.15^{a}	1035	35	50	
10	6.44±0.02^{c}	42.73±1.62^{c}	12.22±0.18^{a}	11.09±0.24^{a}	16.70±0.10^{a}	3300	50	65	

The different superscripts letters under the same row were significantly different ($p<0.05$).

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Sensory evaluation of functional rice pudding during storage were also investigated and result showed a decrease in attributes of sensory especially odor and taste ($p<0.05$) which varied and changed from 7.70 to 6.56, 8.50 to 6.99, respectively (as shown in Figure 19., Moreover, change of all attributes significantly differed ($p<0.05$) during storage at 8 to 10 day. Change of the color affected the appearance, color and overall acceptability score from sensory evaluation. Based on the results of this experiment, overall acceptability and all attributes were chosen as the score more than 6. However, the use of different sensory tests, that utilize different score of measurements and the product-specific reduction in sensory quality perceived to be tolerated by consumers had resulted in the use of different cut-off points to mark an end of stability or shelf-life of foods. On a nine-point hedonic scale, scores of 4.5 in sunflower kernels (Fritsch *et al.*, 1997), score of 5 in fruit juice drinks (Alves *et al*., 2005) were used to mark sensory failure. In this study, a score less than 6.0 for each attributes was used to indicate product failure. Thus, the greatest of all attributes at day 7 presented the sensory score of more than 6.0.

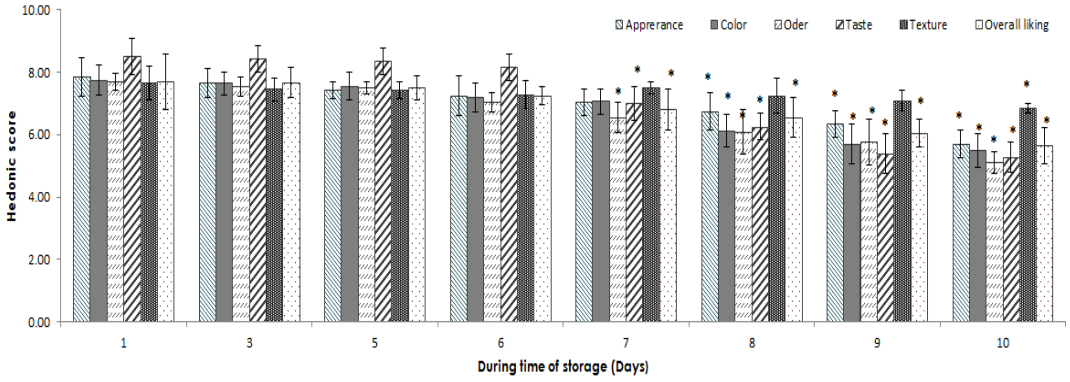

Figure 19 Sensory acceptant of functional rice pudding during storage at 1-10 days

In conclusion, the stability study, based on selection of functional rice pudding, the properties (color, pH, microbial) and sensory evaluation relationship proved useful to measure quality and decay during the time of storage of functional rice pudding.

4.5 Conclusion

The functional rice pudding fortified with SYRBH was developed using a consumer affective sensory evaluation study. The optimum formula was 5% of SYRBH-fortified received which 82% of consumer acceptance. Most of respondent (98%) showed interest in functional rice pudding. The SYRBH was successfully fortified into rice pudding along with a stable 7 day refrigerated shelf life and likability. The functional rice pudding made from pigmented rice and multi-grain added SYRBH seemed to give the best properties in nutritious (protein and fiber) more than original pudding. In addition, incorporation of bioactive compounds such as SYRBH in rice pudding has the potential to serve as a functional food that can offer health benefits to consumer.

CHAPTER 5

SUMMARY AND FUTURE WORKS

5.1 Summary

1. Different of Hydrolysis processes have affected on the properties of SYRBH. The sequential and combined hydrolysis, high enzyme content and short hydrolysis time, results in better functional properties and bioactive hydrolysate.

2. The optimum extraction by Protease G6 and Amyloglucosidase for high content of protein and β-glucan were sequential and combined hydrolysis, also the highest protein content of SYRBH and β-glucan content were obtained when extracted with, 5% concentration sequential for 120 min, 5% concentration sequential for 30 min, respectively.

3. The rice bran hydrolysates exhibit biological activities from with potential free radical scavenging, nitric oxide inhibition, anti-inflammatory and anti-proinflammatory cytokine (TNF-α, IL-1β and IL-6) secretion properties. The hydrolysate could be prepared by using 5% (v/w) of Protease G6 and Amyloglucosidase with the sequential hydrolysis.

4. The functional rice pudding in formula fortified with SYRBH was developed by using a consumer affective sensory evaluation study. The fortified level of 5% w/w of SYRBH, gave a 82% of consumer acceptance with sensory score gave of like very much range.

5.2 Future works

1. Crude hydrolysate with anti-oxidative or anti-inflammatory activities should be study for primary component of hydrolysate and scaled up for commercial production.

2. Anti-oxidative and anti-inflammatory activities of SYRBH in animal trial should be study. The amino acid profile of peptide should be determined with bioactive compound such as di-peptide and tri-peptide.

3. Development of other functional food products from RBH and bio-activity of the development product in human clinical trial should be studied.

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APPENDIX

Appendix A

Consumer Survey

This survey a part of research for Miss Natcha Phantuwong, Graduate student in the function food and nutrition , Interdisciplinary Graduate School of Nutraceutical and Functional Food , Prince of Songkha University. Please feel to answer the questions. Your name and personal identification in not required.

Instruction sections

Please read each of the following questions. Then place a tick in the box that best reflects your opinions the particular question. Please answer any sections where you are asked to give reasons for your selection or actions. With some questions you may be asked to tick more one box. The instruction to tick more than one box will be provided after the question.

Part I: Rice pudding (as rice porridge in Asia) behavior

Rice pudding are solf diet for dinder or dessert ,a dish made from rice mixed with milk or water and other ingredients such as multigrain, cereal, fruit, raisine and cinnamon that are formulated to provide nutrients for healthy propose such as dietary purposes, healthy food for children and althetes, energy meal for sport and meal for general adults and specific soft diet for patients.

1. Do you normally eat rice pudding ?

☐ Yes (Please continue with the next question)

☐ No (Please state reason and go to Part II)

Why do you not eat rice pudding?

……

……

2. How often did you eat rice pudding?

☐ Daily ☐ 3-4 times/weak

☐ 1-2 times/week ☐ Less than one a week

3.What time of the day do you normally eat rice pudding?

(You may tick as many boxes as you like)

☐ Morning ☐ Morning break

☐ Noon ☐ Afternoon break

☐ Evening ☐ Before bed time

☐ Other (Please specify)……

4.What is your purpose for eating rice pudding ?

☐ Health benefit ☐ Refreshing

☐ Curing the disease ☐ Beauty or Diet

☐ Anti-aging ☐ Other (Please specify)……

5. What is your favorite Ingredient for mixing in rice pudding?

☐ Cereal or multi- grain ☐ Fresh fruit or vegetable

☐ Dried fruit or vegetable ☐ Dairy products

☐ Dried herb ☐ Tea or coffee

☐ Syrup or jam

6. What type of rice pudding you want?

☐ Ready-to-eat ☐ Ready-to-cook (Dry power)

☐ Commercial (canning or pouch bag) ☐ Other (Please specify)…

7. What taste of functional rice pudding do you want?

☐ Sweet ☐ Natural

☐ Milk ☐ Vanilla

☐ Other (Please specify)

Part II: Consumer attitudes to functional rice pudding (fortified with Sang-yod rice bran Hydrolysate)

Sang-yod rice bran hydrolysate (SYRBH) is hydrolysate powder produced by enzymatic hydrolysis. SYRBH graet of nutrients such as protein,dietary fiber(β-glucan), ferulic acid ,magnesium,phenolic compounds that showed antioxidation and anti-inflammatory properties.

1.What is your favorite rice pudding?

(You may tick as many boxes as you like)

Part I : Demographic data

1. Gender

☐ Male ☐ Female

2.Age

☐ 15-25 years old ☐ 26-45 years old ☐ over 45 years old

3.Maritol statue

☐ Single ☐ Married ☐ Other

4.Occupation

☐ Student ☐ Government official

☐ Business ☐ Agricultural/Farmer

☐ Housewife ☐ Employee

☐ Unemployed ☐ Other (Please specify)……

5. Education

☐ Lower secondary school certificate

☐ High school ☐ Vocational certificate/2 years of collage

☐ Bachelor degree ☐ Higher than Bachelor degree

6. Your monthly income

☐ Less than 10,000 Bath ☐ 10,000-20,000 Bath

☐ More than 20,001 Bath

Appendix B

Consumer Acceptance Test

This survey a part of research for Miss Natcha Phantuwong, Graduate student in the neutraceutical and function food ,Interdisciplinary …..school, Prince of Songkha University. Please feel to answer the questions.Your name and personal identification in not required.

Instruction

Please read each of the following questions. Then place a tick in the box that best reflects your opinions the particular question. Please answer any sections where you are asked to give reasons for your selection or actions. With some questions you may be asked to tick more one box. The instruction to tick more than one box will be provided after the question.

Part I : Functional rice pudding behavior

Functional rice pudding is rice pudding fortified with Sang-yod rice bran hydrolysate as bioactive ingredients and nutrient compound.

1.Have you ever heard about “functional rice pudding”?

☐ Yes ☐ No

If yes, what is the source of your information about “functional rice pudding”?

(You may tick as many boxes as you like)

☐ Newpaper/TV/Radio ☐ Internet/E-mail

☐ Food exhibition ☐ Friend or personal contact

☐ Other (Please specify)……

2. Do you interest in the “functional rice pudding”?

☐ Yes (Please continue in question No.3)

☐ No (Please continue in question No.4)

3.Why you are interested in “functional rice pudding”?

(You may tick many boxes as you like)

☐ Nutritions ☐ Hearsay

☐ Popularity/in trend ☐ Advertisement

☐ Other (Please specify)……

4.Why you don’t interest in “functional rice pudding”?

☐ Don’t like the new product ☐ Don’t like ensure in nutrition

☐ Difficult to available ☐ Don’t known the product

☐ Other (Please specify)……

5.Do you ever ate about “functional rice pudding”?

☐ Yes ☐ No

Part II : Acceptance test of functional rice pudding .

I am going to you taste functional rice pudding fortified with Sang-yod rice bran hydrolysate then I want you to tell me how you like its appearance, its color, its odor, its taste, its texture and then how much you like the product.I am going to use a liking score(dislike extreamely-like extreamely) for describing the product.

Appearance : Anything of product you can see such as color, viscosity,etc.

Color : color of product when you see.

Oder : Oder of product when you sniff.

Taste : felling you can fell when you taste product such as sweetness or bittes.

Texture : feeling you can feel when you taste product such as smoothly, sticky or viscosity.

Overall : overall liking.

Please test the functional rice pudding sample and weight your linking score (9-point hedonic scale) by put score (0-9) in the box which is directly to you opinion.

Liking score	Appearance	Color	Oder	Taste	Texture	Overall liking
Like extremely						
Like very much						
Like moderately						
Like slightly						
Neither like nor dislike						
Dislike slightly						
Dislike moderately						
Dislike very much						
Dislike extremely						

Part III : Information of functional rice pudding

Functional rice pudding fortified with Sang-yod rice bran hydrolysate

Functional rice pudding is produce from standard formula rice pudding and fortify with Sang-yod rice bran hydrolysate which bioactive ingredient and mixes with multi-grain and fruit for added value. The product was pasteurized and can keep at refrigerator during 10 days.

These functional rice pudding have protein …. And dietary fiber(β-glucan) Sang-yod rice bran hydrolysate is a functional ingredients. It can promote anti-oxidation and anti-inflammation, and has the benefit for human.

After reading this information, please answer the questions.

1.Do you accept this product?

☐ Yes ☐ No

If no, why do you not accept this functional rice pudding

……

……

2.Do you want to buy this product?

☐ Yes ☐ No

VITAE

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Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Agricultural Industry)	King Mongkut's Institute of Technology Ladkrabang	2001
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List of Publication and Proceeding

Natcha Phantuwong, Chakree Thongraung and Chutha Saewong. 2015. Nitric-oxide inhibition and Anti-oxidant activity of Sang Yod Rice Bran Hydrolysate. In Proceeding of 19th International Conference of Functional Food Centre (FFC): Functional and Medical Foods, Bioactive Compounds and Biomarkers: Longevity and Quality of Life. Kobe University, Kobe, Japan. 17-18 November 2015.