CHEMICAL AND ANTIOXIDANT PROPERTIES OF ARABINOXYLAN HYDROLYSATES FROM RICE BRAN

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คุณสมบัติเคมีและต้านออกซิเดชันของอะราบิโนไซแลนไฮโดรไลเซทจากรำข้าว

นางประชิต อยู่หว่าง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ประชิต อยู่หว่าง : คุณสมบัติเคมีและต้านออกซิเดชันของอะราบิโนไซแลนไฮโดรไลเซท จากรำข้าว (CHEMICAL AND ANTIOXIDANT PROPERTIES OF ARABINOXYLAN HYDROLYSATES FROM RICE BRAN) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.สุนันทา ทองทา, 108 หน้า.

การศึกษาอะราบิโนไซแลนส่วนที่ไม่สามารถสกัดด้วยน้ำจากรำข้าว (WUAX) ด้วยเอนไซม์ไซลาเนส (*Bacillus subtilis* endoxylanase GHF 11) กระทำโดยนำรำข้าวปราศจากไขมันผ่านการกำจัดสตาร์ชและโปรตีนออกด้วยเอนไซม์ จากการศึกษาปัจจัยสำคัญที่มีผลต่อการสกัด คือ การให้ความร้อนด้วยออโตเครฟ ปริมาณเอนไซม์และเวลาในการย่อย พบว่าการให้ความร้อนโดยออโตเคฟที่อุณหภูมิ 121 องศาเซลเซียส นาน 4 ชั่วโมง ก่อนนำมาย่อยด้วยไซลาเนสจำนวน 5 ยูนิต/กรัม เป็นเวลา 4 ชั่วโมงให้ผลผลิตสูงสุดเป็นปริมาณน้ำตาลทั้งหมด 9.14 เปอร์เซ็นต์ การตกตะกอนอะราบิโนไซแลนจาก WUAX ด้วยเอทานอลสามารถแยกออกเป็น 3 แฟรกชัน คือ แฟรกชันที่ตกตะกอนด้วยเอทานอลความเข้มข้น 0-60 เปอร์เซ็นต์ (F60) 60-90 เปอร์เซ็นต์ (F6090) และมากกว่า 90 เปอร์เซ็นต์ (F90)

การให้ความร้อนโดยออโตเคฟเพิ่มปริมาณฟีนอลลิกยึดเหนี่ยวรวมและลดปริมาณฟีนอลลิกอิสระในรำข้าว และกรดฟีนอลิกหลักที่พบคือกรดเฟอรูลิกชนิดยึดเหนี่ยว การวิเคราะห์อะราบิโนไซแลนที่ได้จากการตกตะกอนด้วยเอทานอล พบว่าทุกแฟรกชันยกเว้น F90 มีกรดฟีนอลลิกชนิดยึดเหนี่ยวมากกว่ากรดฟีนอลลิกชนิดอิสระ F6090 มีปริมาณกรดฟีนอลิกมากที่สุดตามด้วย F60 และ F90 โดย F60 และ F6090 มีกรดเฟอรูลิกเป็นองค์ประกอบหลัก ส่วน F90 มีปริมาณกรดเฟอรูลิกและกรดคูมาริกใกล้เคียงกัน การวิเคราะห์น้ำหนักโมเลกุลของ F60 F6090 และ F90 ด้วยวิธีโครมาโตกราฟีแบบแยกขนาด พบว่ามีค่าเท่ากับ 5.786×10^{4}, 4.137×10^{4} และ 1.525×10^{3} กรัม/โมล ตามลำดับ การวิเคราะห์น้ำตาลด้วยวิธีเมทาโนไลซิส (methanolysis) พบว่าน้ำตาลหลักใน F60 และ F6090 คือไซโลสและอะราบิโนส โดยปริมาณอะราบิโนไซแลนของทั้งสองแฟรกชันมีค่าเท่ากับ 70.3 และ 73.8 เปอร์เซ็นต์ตามลำดับ ส่วนน้ำตาลหลักที่พบใน F90 คือ กลูโคสและไซโลส การศึกษาโครงสร้างของทั้งสามแฟรกชันพบน้ำตาลอะราบิโนสที่ส่วนกิ่งเชื่อมกับน้ำตาลไซโลสด้วยพันธะ α-1,3 เป็นส่วนใหญ่และมีปริมาณประมาณ 29-33 เปอร์เซ็นต์โมล

การศึกษาฤทธิ์ต้านอนุมูลอิสระ 2,2-Diphenyl-1-picrylhydrazyl (DPPH) พบว่า F6090 มีฤทธิ์ต้านอนุมูลอิสระสูงที่สุดอย่างมีนัยสำคัญ ($p<0.05$) โดยมีค่า EC_{50} เท่ากับ 389.5 ไมโครกรัม/มิลลิลิตร อันดับรองลงมาคือ F60 และ F90 การทดสอบคุณสมบัติรีดักชันด้วยวิธี Ferric reducing ability power assay (FRAP) แสดงผลที่มีแนวโน้มเดียวกันคือ F6090 และ F60 มีประสิทธิภาพสูงกว่า F90 การทดสอบการออกฤทธิ์ต้านอนุมูลอิสระภายในเซลล์ HepG2 ด้วยวิธี DCFH DA assay

พบว่า F60 F6090 และ F90 มีประสิทธิภาพสูงสุดในการต้านอนุมูลอิสระที่ความเข้มข้น 5, 10, และ 10 ไมโครกรัม/มิลลิลิตร ตามลำดับ ทั้งสามแฟรกชันมีความเป็นพิษต่อเซลล์ HepG2 ที่แตกต่างกัน F60 มีความเป็นพิษต่ำ (12-35%) ที่ความเข้มข้นระหว่าง 50-2,500 ไมโครกรัม/มิลลิลิตร ส่วน F6090 ไม่แสดงความเป็นพิษต่อเซลล์ที่ความเข้มข้น 500-12,500 ไมโครกรัม/มิลลิลิตร แต่กลับมีผลส่งเสริมการเจริญของเซลล์ ส่วน F90 มีฤทธิ์ยับยั้งการเจริญเติบโตของเซลล์สูงสุดที่ความเข้มข้น 5,000-11,500 ไมโครกรัม/มิลลิลิตร โดยมีค่า IC_{50} เท่ากับ 8,132 ไมโครกรัม/มิลลิลิตร

สาขาวิชาเทคโนโลยีอาหาร
ปีการศึกษา 2556

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

PRACHIT YUWHANG : CHEMICAL AND ANTIOXIDANT PROPERTIES OF ARABINOXYLAN HYDROLYSATES FROM RICE BRAN. THESIS ADVISOR : ASST. PROF. SUNANTA TONGTA, Ph.D. 108 PP.

RICE BRAN/ARABINOXYLANS/ENZYME-EXTRACTED ARABINOXYLANS/ PHENOLIC ACID/FERURIC ACID/ANTIOXIDANT ACTIVITY/CELL-BASE DCFH DA ASSAY

The extraction of water-unextractable arabinoxylans (WUAX) from rice bran with *Bacillus subtilis* endoxylanase GHF 11 was studied. Removal of starch and proteins from defatted rice bran was conducted by enzymatic treatment. The important factors affecting extraction, namely autoclave condition, enzyme concentration, and incubation time were optimized. The autoclave of destrach and deprotein rice bran (DSDPB) at 121 °C for 4 h before hydrolysis with 5 Unit endoxylanase for 4 h provided the highest yield of 9.14% total sugars. Ethanol precipitation could fractionate the rice bran WUAX into 3 fractions: 0-60% ethanol (F60), 60-90% ethanol (F6090), and above 90% ethanol (F90).

The autoclave treatment increased the total bound phenolic and decreased the total free phenolic contents of DSDPB. The major phenolic acid of all autoclaved DSDPB fractions, except F90, was bound ferulic acid. The analysis of ethanol-fractionation of WUAX showed that F6090 fraction had the highest phenolic acid content followed by F60 and F90. F60 and F6090 fractions contained mostly bound ferulic acid, while F90 had comparable amounts of ferulic acid and coumaric acid. The molecular weights of F60, F6090 and F90, determined by HPSEC-MALLS, were

5.786×10^4, 4.137×10^4, and 1.525×10^3 g/mol, respectively. The sugar analysis by methanolysis method revealed that the major monosaccharides of F60 and F6090 fractions were xylose and arabinose in which arabinoxylans of both fractions were 70.3 and 73.8%, respectively. In addition, the majority of sugars found in F90 were glucose and xylose. Structural characterization of all three fractions of WUAX indicated that the majority of the arabinose residues were α-1, 3-linked to monosubstituted xylose and was about 29-33 % molar ratio

The study of scavenging against 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radicals revealed that F6090 fraction exhibited the highest antioxidant activity ($p<0.05$) with an EC_{50} value of 389.5 μg/mL, followed by F60 and F90. The F6090 and F60 fractions also possessed higher total reducing power in the FRAP assay than that of F90. In the cell-based antioxidant assay with HepG2 using DCFH DA probe, the most effective concentrations of F60, F6090 and F90 in scavenging intracellular ROS were 5, 10 and 10 μg/mL, respectively. The cytotoxicity against HepG2 of these fractions was varied. F60 fraction only slightly induced cell death (12-35%) at the concentration range of 50-2,500 μg/mL. At 500 to 12,500 μg/mL, F6090 fraction had no cytotoxic effect but could slightly enhance the growth of HepG2 cells. F90 fraction exhibited the most potent cytotoxic activity against the HepG2 with the IC_{50} of 8,132 μg/mL.

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CHAPTER I

INTRODUCTION

1.1 Background and significance of the study

Under normal physiological conditions, there is a balance between generation of reactive oxygen species (ROS) and antioxidant defense mechanisms consisting of enzymic and non-enzymic systems in living organisms. If ROS, which is composed of superoxide anions, hydroxyl radicals and hydrogen peroxide, are not adequately removed or formed additionally in the cells by exogenous sources, oxidative stress may occur (Aherne and O'brien, 2000). Oxidative stress has been defined as a disturbance to the equilibrium status of prooxidant and antioxidant systems in favor of prooxidation due to excess formation of free radicals and decreased activity of antioxidant defense systems (Kennedy et al., 2005). Excess ROS are toxic as they can attack and damage cellular constituents such as DNA, proteins, carbohydrate, nucleic acid, and membrane lipids leading to cell death and tissue injure. Oxidative stress and ROS are associated with a variety of chronic health problems, such as cardiovascular disease, cancers, arthritis, diabetes, Alzheimer disease, other neurological disorders and also aging process. Epidemiological studies strongly suggested that diets rich in cereals played a crucial role in the prevention of chronic diseases such as coronary heart disease, certain types of cancers, diabetes, and Alzheimer's disease (Babior, 2000; Diaz, Frei, Vita, and Keaney, 1997; Halliwell and Aruoma, 1991). The beneficial health effects derived from the intake of diets rich in cereals are mainly

attributed in part to dietary fiber and nonnutrient phytochemicals that are located in the bran fraction. Among these interesting phytochemicals are arabinoxylans (AX) arabinoxylo-oligosaccharides (AXOS) or feruloyl arabinoxylo-oligosaccharides and phenolic acids such as *p*-coumaric acid and ferulic acid (Andreasen et al., 2001; Wang, Sun, Cao, Song, and Tian, 2008, Wang, Sun, Cao, and Tian, 2009).

Arabinoxylans (AX) are the main nonstarch polysaccharides in rice bran (Shibuya and Iwasaki, 1985). The AX from rice and wheat bran have been reported for high ability to scavenge reactive oxygen species both *in vitro* and *in vivo* studies (Katapodis et al., 2003; Rao and Muralikrishna, 2006; Yuan, Wang and Yao, 2005). The studies focused on the activity of feruloyl arabinoxylo-oligosaccharides are much stronger than the free ferulic acid and the activity increased with the increasing number of sugar moieties. Several studies showed that the basis of molecular characteristics had an effect to the antioxidant activity (Ishii, 1997; Ohta, Yamasaki, Egashira, and Sanada,1994; Ohta, Semboku, Kuchii, Egashira, and Sanada, 1997). While an increase in the activity might be small in low molecular weight AXOS (Kikuzaki, Hisamoto, Hirose, Akiyama, and Taniguchi, 2002; Ohta, Yamasaki, Egashira, and Sanada, 1994; Ohta, Semboku, Kuchii, Egashira, and Sanada, 1997), it could be very high in case of feruloyl arabinoxylans having high molecular weight (Rao and Muralikrishna, 2006).

Antioxidant AX can be obtained by enzymatic hydrolysis of rice bran (Ghoneum and Gollapudi, 2003; Ghoneum, Hamilton, and Gollapudi, 2004; Gollapudi and Ghoneum, 2008; Noaman, El-Din, Bibars, Mossallam and Ghoneum 2008), wheat bran (Wang, Sun, Cao, Song, and Tian, 2008; Wang, Sun, Cao, and Tian, 2009; Yuan, Wang, Yao, and Chen 2005), Wheat flour water unextractable

arabinoxylans (WUAX) (Katapodis et al., 2003). Water was used to extract heteropolysaccharides from rice bran (Wang, Zhang, Zhang, and Chen, 2008). For corn bran, feruloyl arabinoxylans can be extracted by acid hydrolysis (Ohta, Yamasaki, Egaehira, and Sanada, 1994; Ohta, Semboku, Kuchii, Egashira, and Sanada, 1997; Zhao, Egashira, and Sanada, 2003).

Rice (*Oryza sativa*) bran is a by-product produced in the rice milling industry. It possesses approximately 10% weight of the total rice grain. In Thailand, 32.02 million tons of rice is produced annually (Office of Agricultural Economics, 2010). The rice bran is a natural resource of oil, proteins, fibers, vitamins, and antioxidants. Although rice bran is high in nutrients and phytochemicals, it is largely used as an animal feed (Luh, 1991). Several reports indicated that cereal grains contain special phenolic compounds, such as ferulic acid and diferulate. Ferulic acid and *p*-coumaric acid are the major phenolic compounds in rice and exist in the form of free, soluble conjugated, and insoluble bound (Sosulski, Krygier, and Hogge, 1982). Most of these compounds are bound to arabinoxylans in the cell wall (Shibuya, 1984; Shibuya and Iwasaki, 1985).

For the enzymatic hydrolysis of AX-rich substrates such as bran, the major enzyme requirement is depolymerising xylanase which can hydrolyze the β- (1→4)-linkages between xylopyranoside residues in xylan. Endo-β-(1, 4)-xylanase (EC 3.2.1.8), referred to as endoxylanase, are used to improve the AX functionality. Endoxylanases are in the glycoside hydrolase families (GHF) 10 and 11. The GHF10 and 11 endoxylanases are produced from *Aspergillus aculeatus* (XAA) and *Bacillus subtilis* (XBS), respectively. The former enzyme has lower substrate specificity towards water-unextracable arabinoxylan than the latter one. The results of a

comparative study of these two endoxylanase indicated that a GHF11 xylanase is more useful than a GHF10 xylanase for the upgrading of wheat bran into soluble oligosaccharides. While the GHF10 xylanase was the most active on soluble arabinoxylans, the GHF11 xylanase can better penetrate the cell-wall network (Beaugrand et al., 2004; Swennen, Courtin, Lindemans and Delcour, 2006; Trogh, Croes, Courtin, and Delcour, 2005). The XBS is useful for the conversion of insoluble fibre to soluble fibre. (Maes, Vangeneugden, and Delcour, 2004).

WUAX are the major AX of cereal bran and their fine structure is different among cereal (Izydorczyk, and Biliaderi, 1995). As the function is correlated with the structure, investigations of the free radical scavenge ability and chemical compositions of arabinoxylans from enzyme hydrolysis will be considerably interesting. Within this study, many chemistry methods and cell culture-based system, which are successfully applied to evaluate plant-derived bioactive for their health effects, will be used to evaluate both properties. The successful finding of effective AX will expectedly be great benefit to either food or pharmaceutical industry.

1.2 Research objectives

The objectives of this study were:

1.2.1 To prepare arabinoxylans from rice bran by enzymic preparation with endoxylanase.

1.2.2 To characterize rice bran arabinoxylans composition obtained from endoxylanase hydrolysis

1.2.3 To assess *in vitro* antioxidant activity of arabinoxylan hydrolysates with chemical assays using FRAP and DPPH methods and cell-based antioxidant activity (CAA) assay using DCFH-DA probe.

1.2.4 To assess cytotoxicity of arabinoxylan hydrolysates against human liver cancer HepG2 using MTT method.

1.3 Research hypothesis

The endoxylanase could hydrolyze rice bran WUAX to produce antioxidant arabinoxylans. In addition, the extracted arabinoxylans possess antioxidant activity in both chemical and cell-based assays.

1.4 Expected results

Results from this research will lead to fully utilize rice bran, a by-product from rice milling industry and increase the value of this by-product. In addition, more knowledges about antioxidant arabinoxylan hydrolysates, especially its mode of action, will be gained. This research will also lead to more understandings about the cytotoxic effect of antioxidant arabinoxylan hydrolysates on the HepG2 cancerous cells. The resulting arabinoxylan hydrolysates would be a promising functional food ingredient or a supplement.

CHAPTER II

LITERATURE REVIEWS

2.1 Rice bran arabinoxylan structure

Rice bran, a by-product of rice milling, consists of many nutrients which come from the outer layers of the kernel and the germ. It composes of protein (12.0-15.6%), fat 15.0-19.7%, crude fibre (7.0-11.4%) and carbohydrate 31.1-52.3%, in addition to several valuable phytonutrients, antioxidants, vitamins and minerals (Connor, Saunders, and Kohler, (1976); Cherukuri *et al*., (2007)

Cereal AX consist of a chain backbone of (1→4)-linked β-D-xylopyranosyl (Xylp) residues to which α-L-arabinofuranose (Araf) units linked as side branches. The α-L- Araf residues are attached to some of the β-D-Xylp residues at O-2, O-3, and/or at both O-2, 3 positions, resulting in four structural elements in the molecular structure of arabinoxylans: monosubstituted Xylp (mXyl) at O-2 or O-3, disubstituted Xylp (dXyl) at O-2,3, and unsubstituted Xylp (uXyl) (Figure 2.1) (Izydorczyk, and Biliaderi, 1995; Izydorczyk and Dexter, 2008).

AX in cereal grains has two forms: water-extractable AX (WEAX) and water-unextractable AX (WUAX). WUAX, a large portion of cereal arabinoxylans cannot be extracted from the cell wall material with water. Differences in the water extractability of these non- starch polysaccharides might be related to differences in substitution patterns and in the extent of physical entanglement, covalent ester

bonding between carboxyl groups of uronic acids and the hydroxyl groups of arabinoxylans, as well as the formation of diferulic acid bridges between adjacent arabinoxylan chains. WUAX have been studied after alkaline extraction. The structure of alkaline extractable AX (AEAX) is basically the same as that of WEAX with only small differences in average molecular weight, which is higher for AEAX than for WEAX, and slightly higher A/X ratio due to the higher proportion of arabinose side branches (Ordaz-Ortiz, and Saulnier, 2005).

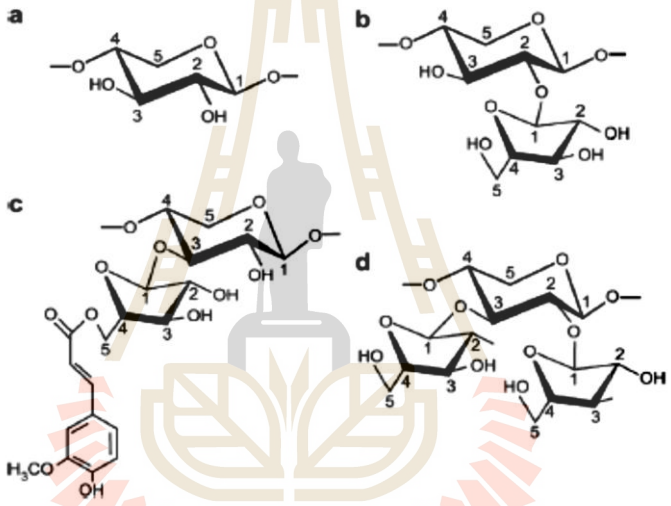

Figure 2.1 Structural elements present in arabinoxylans: (a) unsubstituted Xylp; (b) monosubstituted Xylp at O-2; (c) monosubstituted Xylp at O-3 with ferulic acidresidue esterified to Araf and (d) disubstituted Xylp at O-2, 3 (Izydorczyk and Dexter, 2008).

AX extracted from rice husks, oat spelts, corn cobs and barley husks presented of uncommon side chain 2-O-β-D-Xylp -α-L- Araf after Shearzyme® (GH10 endo-1,4-β-D-xylanase) hydrolysis (Pastell, Virkki, Harju, Tuomainen, and Tenkanen, 2009). In general, arabinoxylans from rice and sorghum seems to consist of more

highly branched xylan backbones than those from wheat, rye, and barley, and they may contain galactose and glucuronic acid substituents, in addition to the pentose sugars. The ratio of arabinose to xylose (Ara/Xyl) in endosperm arabinoxylan may vary with cereal types but it is usually lower than that found in bran (Izydorczyk, and Biliaderi, 1995). Rice bran WEAX which was extracted by hot water contained 86.7% polysaccharide of arabinose, xylose, glucose and galactose with a molar ratio of 4:2:1:4 and. It had a backbone consisting of β-(1→3)-linked D-galacopyranosyl residues substituted at *O*-2 with glycosyl residues composed of α-D-xylose-(1→4)-α-D-arabinose-(1→ and α-D-glucose-(1→4)-α-D-arabinose-(1→ linked residues (Wang, Zhang, Zhang, and Chen, 2008). Rice bran WUAX had more complicated structural which was 74-79% of (1→4)-linked xylose are branched. Their side chain contains appreciable amounts of non-reducing end xylose and galactose, and also (1→2)-, (1→3)- or (1→5)-linked arabinose residues (Shibuya and Iwasaki, 1985).

2.2 Phenolic acids

Two families of phenolic acids, hydroxybenzoic acids and hydroxycinnamic acids are found in plant materials. Hydroxycinnamic acids and their derivatives are widely distributed in cereals, legumes, oilseeds, fruits, vegetables and beverages. The common hydroxycinnamic acids include coumaric, caffeic, ferulic, and sinapic acids are revealed in Figure 2.2. The occurrences of hydroxycinnamates in different cereals are presented in Table 2.1 (Shahidi and Chandrasekara, 2010).

Ferulic acid (3-methoxy-4-hydroxycinnamic acid) is the most abundant member among hydroxycinnamic acids. In plant tissue, ferulic acid can act as cross-

linking agents between polysaccharides, or between polysaccharides and lignin which promoting tissue cohesion and restricting cell expansion. Phenolic compounds in cereal grains exist in the free, soluble esters or conjugates and insoluble-bound forms. It is reported that 74% and 69% of total phenolic was present in rice and corn, respectively, and they are in the insoluble-bound form. Ferulic acid is usually concentrated in the bran of grains, peel of fruits, and roots and peel of vegetables (Zhao and Moghadasian, 2008).

Table 2.1 Cereal sources of hydroxycinnamic acids and derivatives.

Cereal	**Hydroxycinnamates**
Barley	Ferulic, *p*-coumaric, caffeic, chlorogenic acids, 8-O-4 diFA, 5-5 diFA, 8-5 diFA (benzofuran) 8-5 diFA
Corn	Ferulic, *p*-coumaric, 8-5 diFA, 8-8 diFA (cyclic), 8-O-4 diFA
Oat	Ferulic, caffeic, *p*-coumaric, sinapic, *O*-coumaric acids, avenanthramides
Rice	Ferulic, *p*-coumaric, caffeic acids, 5-5 diFA, 5-8 diFA (benzofuran form), 8-5 diFA, 8-8 diFA, 8-O-4 diFA, 4-O-5 diFA, 60-O-(E)-feruloylsucrose, 60-O-(E)-sinapoylsucrose, feruloyl esters of triterpene alcohols and sterols, o-methoxycinnamic acid, arabinoxylan ferulate, disinapates
Rye	Ferulic, caffeic, *p*-coumaric, sinapic acids, 8-O-4 diFA
Wheat	Ferulic, caffeic, *p*-coumaric, sinapic acids, campestanyl, sitostanyl (steryl ferulates), 8-8 diFA (cyclic), 5-8 diFA, 5-5 diFA, 8-O-4 diFA, 8-5 diFA (benzofuran)

Modified from: Shahidi and Chandrasekara, 2010.

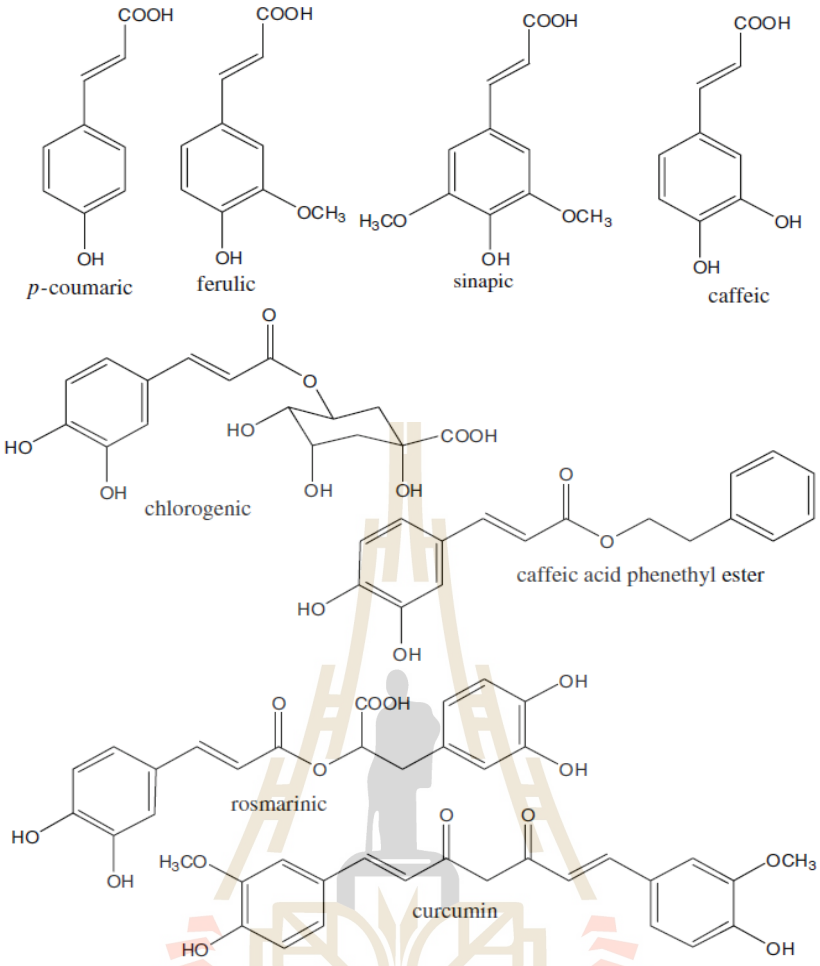

Figure 2.2 Structures of hydroxycinnamic acids and related compounds (Shahidi, and Chandrasekara, 2010).

The percentage of free ferulic acid in cereals only accounts for 0.1–0.5%. A significant proportion of these phenolic acids are known to be linked to lignans and arabinoxylans. The levels of ferulic acid in different parts of grains are summarized in Table 2.2 (Zhao and Moghadasian, 2008; Adom and Liu, 2002). The different dehydrodimer and trimer of ferulic acid found in plant tissues is revealed in Figure 2.3. The amount and type of hydroxycinnamic acids are greatly varied according to the different tissues in cereal grains. Clearly, the outer tissue of kernel and the

aleurone layer are very rich in ferulic acid and dehydrodimers. Irrespective of the tissue, 8- O-4′ and 5-8′ benzofuran dehydrodimers were mainly found but the 5-5′, 8-5′, and 8-8′ form was also detected in durum and bread wheat. Ferulic acid is highly concentrated in the cell walls of aleurone layer that is rich in arabinoxylan while the dehydrotrimer was only detected in the pericarp tissue of wheat (Saulnier, Sadoa, Branlard, Charmet, and Guillon, 2007).

Table 2.2 The levels of ferulic acid in grains**.**

Ferulic acid content [a] **Grains**	**(mg /100 g)**
Refined corn bran	2,610-3,300
Barley extracts	1,358-2,293
Soft and hard wheat bran	1,351-1,456
Rice endosperm cell wall	910
Fine wheat bran	530-540
Rye bran	280
Corn dehull kernels	174
Whole wheat kernels	64-127
Whole wheat flour	89
Whole grain rye flour	86
Whole brown rice	42
Corn flour	38
Whole oats	25-35
Whole grain barley flour	25-34
Oat bran	33

[a] The contents were calculated by 100 g fresh edible part of foods.

Modified from: Zhao and Moghadasian, 2008.

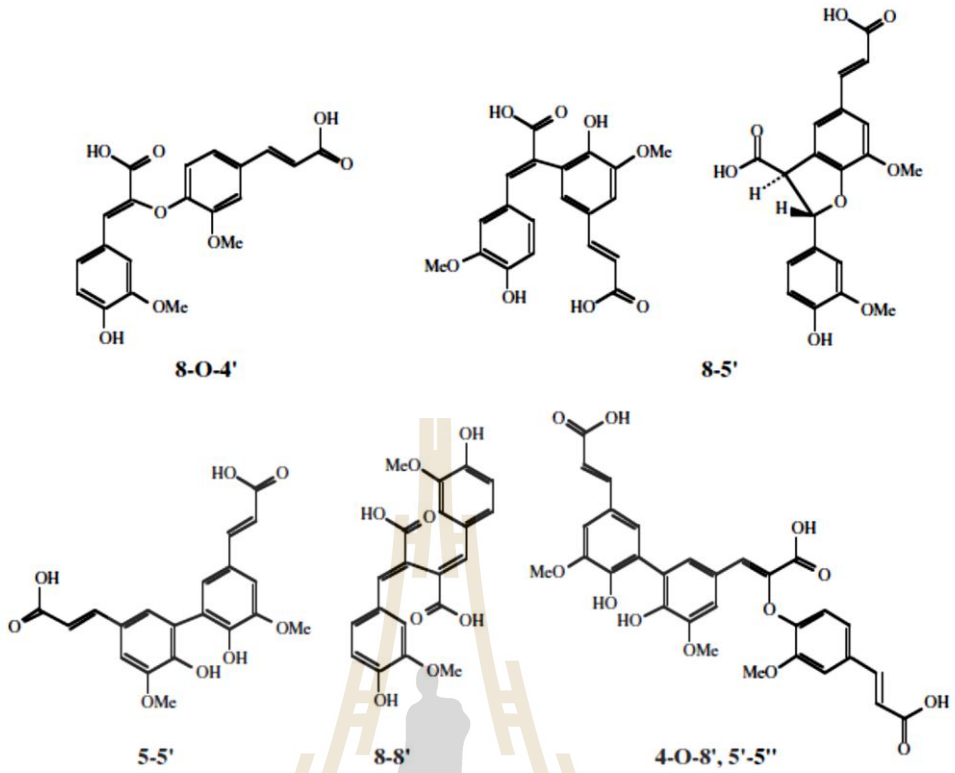

Figure 2.3 Structure of dehydrodimers and dehydrotrimer of ferulic acid identified in cereal grains.

Source: Saulnier, Sadoa, Branlard, Charmet, and Guillon, 2007.

2.3 Antioxidant activity and oxidative stress

Epidemiological studies have shown that large intakes of fruit and vegetables protect against a range of chronic diseases and problems associated with aging. This is often attributed to a high intake of phytochemicals with antioxidant activity, as this is thought to be the mechanism underpinning many of these protective effects. Antioxidants are phytochemicals, vitamins and other nutrients that protect cells from damages caused by free radicals (Babich *et al.*, 2011). *In vitro* and *in vivo* studies have shown that antioxidants can prevent the free radical damage that is associated

with cancer and heart disease. Antioxidants can be found in fruits and vegetables, culinary herbs and medicinal herbs, grains, bark of trees and others. The study on several culinary and medicinal herbs reported that the antioxidant level of herbs can be as high as 465 mmol Fe^{2+} per 100 g (dry weight) (Dragland *et al.*, 2003).

The consumption of smaller quantities of multiple phytochemicals may result in more health benefits than the consumption of larger quantities of fewer phytochemicals. Numerous studies with plant phytochemicals showed that phytochemicals with antioxidant activities may reduce risk of cancer and improve heart health. Antioxidants inactivate free radicals by donation of electrons and converting them into harmless molecules. Antioxidants can play a significant role as agents that prevent or inhibit oxidation. They are both natural substances and synthetic substances that can protect cells from the damaging effects of oxygen free radicals (Bagchi *et al.,* 2000). A number of nutrients have antioxidant properties, for example, vitamin E, manganese, glutathione, Co Q, vitamin C, selenium, carotenoid compounds and phenolic compounds. These antioxidants appear to be involved in the elimination of carbon-centered radicals and peroxyl radicals (Gropper *et al*., 2005).

Free radicals, highly unstable molecules, are formed as part of natural metabolism. They are also formed in the body due to external sources such as environmental factors, smoking, pesticides, pollution and radiation. Free radicals react easily with the essential molecules of body, including DNA, fat and proteins. All organic and inorganic materials consist of atoms, which can be bound together to form molecules. Each atom has a specific number of positively charged protons, and negatively charged electrons. Free radicals are atoms that possess an unpaired electron, and therefore are highly unstable. To regain stability they are prompt to

receive electrons from other atoms, thereby forming neutral molecules. To regain the stability, free radicals try to steal electrons from other molecules, thereby changing their chemical structure. As a result, the molecule becomes a free radical itself, causing a chain reaction which can result in the destruction of a cell. Antioxidants have the property to neutralize free radicals without becoming free radicals themselves because they are stable in both forms. In other words, antioxidants are chemicals offering their own electrons to the free radicals, thus preventing cellular damage. However, when the antioxidant neutralizes a free radical it becomes inactive. Therefore, we need to continuously supply our body with antioxidants. This is of importance because the action of free radicals could increase the risk of diseases such as cancer and heart problems and could accelerate aging.

The existence of free radicals in a human body at a low level is a part of the homeostasis. However, accumulation of free radicals at high levels creates an oxidative stress that is responsible for aging and a number of chronic diseases due to the damage to cell organelles such as lipids of the cell membrane, proteins and DNA. The damaged cells may eventually initiate mutation and formation of cancer (Klaunig and Kamendulis, 2004). The studies have shown that oxidative damage to cells and tissues are induced in the development of various chronic diseases such as cancers, aging, cataract, myocardial infraction and atherosclerotic cardiovascular disease (Halliwell, 2007). The authors reported that antioxidants inhibited oxidative stress by acting at different stages in the oxidation reaction and may have multiple mechanisms of action.

In biological systems, an antioxidant can be defined as any substance that, in low concentration compared with the oxidisable substrate, significantly delays or

prevents oxidation of that substrate. The substrate such as the oxidisable compound, is usually a lipid, but can be also protein, DNA, or carbohydrate. In the case of lipid oxidation, the main mechanism of antioxidants is to act as radical chain-breakers. The preventive antioxidant oxygen scavenging or blocking the pro-oxidant effects by binding proteins that contain catalytic metal sites is the other form of mechanism. The complexity of antioxidants needs to be taken into account for testing of antioxidant activity. The complexity of a multi-component oxidative biological material is often overlooked compared to oxidation model systems that are models of lipids in their real environment. There are a large number of methods to determine the antioxidant activity of compounds. The antioxidant activity may be varied widely depending on the environment of the lipid substrate. For antioxidant evaluation using a radical scavenging test, it should be recalled that this method can evaluate only the radical scavenging activity of the compound, and not the other antioxidant mechanisms, such as metal chelating. In addition, the antioxidant action is more complex in real foods and biological systems where several mechanisms become effective (Frankel and Meyer, 2000).

2.4 Antioxidant activity of phenolics and arabinoxylans

Previous studies have strongly suggested that phenolic acids and AX rich in cereals play a great antioxidant activity. Madhujith and Shahidi (2009) revealed that the insoluble-bound phenolic fraction contributed the highest proportion towards total antioxidant capacity, followed by soluble conjugated and free phenolic compound. The chain-breaking antioxidant mechanism of ferulic acid was investigated. It was revealed that radical oxidation reaction of a large amount of ethyl linoleate in the

presence of the methyl ester of ferulic acid produced four types of peroxides as radical termination products. A radical scavenging reaction occurred at the 3'-position of the ferulate radical. The produced peroxides subsequently underwent intramolecular Diels-Alder reaction to afford stable tricyclic peroxides (Masuda, Yamada, Maekawa, Takeda, and Yamaguchi, 2006). Ferulic acid displayed the highest inhibitory activity against deoxyribose degradation. Gallic acid and rosmarinic acid were the most potent antioxidants among the simple phenolic and hydroxycinnamic acids, respectively (Soobrattee, Neergheen, Luximon-Ramma, Aruoma, and Bahorun, 2005).

The antioxidant activity of ferulic acid arabinoxylan ester from refined corn bran which was hydrolyzed by oxalate and fractionated on gel filtration was investigated by Ohta, Semboku, Kuchii, Egashira, and Sanada (1997); Zhao, Egashira, and Sanada (2003). It was shown that the antioxidant activity of ferulic acid sugar esters such as 5-*O*-feruloyl-L-arabinofuranose and ferulic acid arabinoxylan ester were stronger than free ferulic acid for a low density lipoprotein (LDL) oxidation system,. This fact indicated that affinity of LDL particle and free or bound ferulic acid are important for the antioxidative effect. Ohta, Yamasaki, Egaehira, and Sanada (1994) produced antioxidant compounds from refined corn bran by hydrolysis with oxalic acid, separated on Amberlite XAD-2, further hydrolyzed by hemicellulases and purified on Amberlite XAD-2 by eluting with 60% methanol. The antioxidant activity of these solubilized compounds: 5-O-feruloyl-L-arabinofuranose and O-(5 - O -feruloyl-α-L-arabinofranosyl)-(1→3)-O-β-D-xylopyranosyl-(1→4)-D-xylopyranose were slightly stronger than ferulic acid.

Katapodis *et al*., (2003) produced feruloyl arabinoxylotrisaccharide (FAX_3) from wheat flour WUAX by treatment with a *Thermoascus aurantiacus* family 10 endoxylanase (100 U/ g WUAX, 30 min at 50 ^{o}C) and purified by an anion-exchange and size-exclusion chromatography. FAX_3 showed profound antioxidant activity in 2,2-diphenyl-1-picrylhydrazyl (DPPH) reduction assay exhibiting an EC_{50} of 1288 (g antioxidant/kg DPPH) antiradical which is lower than ferulic acid (EC_{50} = of 214 g antioxidant/kg DPPH). In the copper-mediated oxidation of human LDL test, antioxidant activity of FAX_3 was stronger than ferulic acid and showed a dose-dependent inhibition with almost complete inhibition at 32 μM. The abundant ferulic acid dehydrodimer found in rye, 8-*O*-4-diferuric acid, was a slightly better antioxidant than ferulic acid and *p*-coumaric acid.

From the above studied, structure has a great role in oxidative activity of AX. In addition to ferulic acid, the presence of sugars and degree of glycan-polymerization were observed to influence antioxidant activity of the polysaccharides. In the case of feruloyl arabinoxylo-oligosaccharides, the activity was much stronger than the free ferulic acid, and the activity was higher with increasing the number of sugar moieties. While the presence of ferulic acid is important for the activity, glycosyl group by itself showed no activity (Ishii, 1997; Kikuzaki *et al*., 2002; Ohta Yamasaki, Egaehira, and Sanada, 1994).

2.5 Effect of feruloyl arabinoxylo-oligosaccharides to lymphocyte and erythrocyte

Feruloyl oligosaccharides suppressed depletion of reduced glutathione (GSH), lipid peroxidation, and methaemoglobin and protein carbonyl group formation of

erythrocytes in concentration-and time-dependent manners, remarkably delayed 2, 2′-azobis-2-amidinopropane dihydrochloride (AAPH) -induced erythrocyte hemolysis (Wang, Sun, Cao, and Tian, 2009). Yuan, Wang, Yao, and Chen (2005) evaluated radical scavenging activity and capacity on inhibition of rat erythrocyte hemolysis of feruloyl oligosaccharides, which were released from wheat bran treated with xylanase from *Bacillus subtilis* and further purified with Amberlite XAD-2. The EC_{50} value of the feruloyl oligosaccharides against the DPPH radical was 0.52 mg/mL and showed 91.7% of inhibition of rat erythrocytes hemolysis at a concentration of 4 mg/mL. The erythrocyte hemolysis could be retarded for more than 120 min. Feruloyl oligosaccharides, from wheat bran, showed no cytotoxicity and genotoxicity to normal human lymphocytes at the concentrations of 10–500 μmol/L. In addition, DNA damage in human lymphocytes, which was induced by 100 μmol/L H_2O_2, was inhibited by feruloyl oligosaccharides in a concentration dependent fashion with 91.1% inhibition of lymphocyte DNA damage at 500 μmol/L (Wang, Sun, Cao, Song, and Tian, 2008).

2.6 Cell culture based and clinical study of AX

A heteropolysaccharide obtained from defatted rice bran prepared by hot water extraction, ethanol precipitation, and purified by gel chromatography exhibited potent anti-complementary activity. Its average molecular weight was 90,000 Da and contained 86.7% polysaccharide and 8.7% protein. Gas chromatography result indicated that it contained arabinose, xylose, glucose and galactose with a molar ratio of 4:2:1:4. It had a backbone consisting of β-(1→3)-linked D-galacopyranosyl residues substituted at *O*-2 with glycosyl residues composed of α-D-xylose-(1→4)-α-

D-arabinose-(1→ and α-D-glucose-(1→4)-α-D-arabinose-(1→ linked residues (Wang, Zhang, Zhang, and Chen, 2008). Noaman, El-Din, Bibars, Mossallam, and Ghoneum (2008) reported an antioxidant activity of MGN-3, an arabinoxylans extracted from rice bran with enzyme extracted from Shiitake mushrooms, enhanced the activity of the endogenous antioxidant scavenging enzymes: superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione-S-transferase (GST) in blood, liver, and tumor tissue. This resulted in a suppression of the tumor growth which was associated with normalization of the lipid peroxidation levels and augmentation of glutathione (GSH) contents. A supplement of 15 g/day of AX-rich fiber isolated from refined corn bran can significantly improve glycaemic control in people with Type II diabetes (Zhao, Egashira, and Sanada, 2003).

The information of corn bran ferulic acid and ferulic acid sugar ester metabolism and excretion *in vivo* was revealed by Zhao, Egashira, and Sanada (2003) in that the bioavailability is dependent on the absence or presence of the saccharide moiety and its structure. It was shown that ferulic acid-sulfoglucuronide (ferulic acid-diconjugate with sulfate and glucuronide) is the main metabolite in the plasma of rats administered ferulic acid or its sugar esters (5 - O -feruloyl-L-arabinofuranose and feruloyl-arabinoxylan). The wheat bran AX is relatively well absorbed in human (Garcia et al., 2007).

2.7 Determination of antioxidant capacity

The definition of antioxidant with more biologically relevant is “synthetic or natural substances added to products to prevent or delay their deterioration by action of oxygen in air. Dietary antioxidants are defined as “a substance in foods that

significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans" (Huang, Ou, and Prior, 2005). According to their mode of action, antioxidants can be classified into two classes: primary or chain-breaking antioxidants, which can react directly with lipid radicals and convert them into stable products, and secondary or preventative antioxidants, which can lower the rate of oxidation by different mechanisms. Primary antioxidants most often act by donating a hydrogen atom, while secondary antioxidants may act by binding metal ions able to catalyze oxidative processes, by scavenging oxygen, by absorbing UV radiation, by inhibiting enzymes or by decomposing hydroperoxides (Eklund *et al.*, 2005). Chain-breaking mechanisms are represented as follows:

$$L^{\bullet} + AH \rightarrow LH + A^{\bullet} \quad (1)$$

$$LO^{\bullet} + AH \rightarrow LOH + A^{\bullet} \quad (2)$$

$$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet} \quad (3)$$

Thus, radical initiation (by reacting with a lipid radical: $L^{\bullet}$) or propagation (by reacting with alkoxyl: $LO^{\bullet}$ or peroxyl: $LOO^{\bullet}$ radicals) steps are inhibited by the antioxidant or any H donor: AH.

On the other hand, secondary antioxidants retard the rate of oxidation. For example, metal chelator (e.g., iron-sequesterants) may inhibit Fenton-type reactions that produce hydroxyl radicals:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-} \quad (4)$$

An important function of antioxidants toward free radicals such as $^{\bullet}OH$, $O_2^{\bullet-}$, and $ROO^{\bullet}$ is to suppress free radical mediated oxidation by inhibiting the formation of free radicals and/or by scavenging radicals. The formation of free radicals may be inhibited by reducing hydroperoxides and hydrogen peroxide and by sequestering metal ions through complexation/chelation reactions (Niki, 2002). Radical scavenging action is dependent on both reactivity and concentration of the antioxidant. In a multiphase medium such as an emulsion, the localization of the antioxidant at the interphases may be important (Apak *et al*., 2007).

Antioxidant capacity assays. Depending upon the reactions involved, antioxidant capacity assays can be classified into two types: assays based on hydrogen atom transfer (HAT) reactions and assays based on electron transfer (ET). Both HAT- and ET-based assays are intended to measure the radical (or oxidant) scavenging capacity instead of the preventive antioxidant capacity of a sample.

HAT-based methods measure the ability of an antioxidant to quench free radicals (generally peroxyl radicals) by hydrogen donation as shown in the reaction (1), (2), and (3). In the case of phenolic compound, the HAT mechanisms of antioxidant action in which the hydrogen atom ($H^{\bullet}$) of a phenol (Ar-OH) is transferred to an $ROO^{\bullet}$ radical can be summarized by the reaction:

$$ROO^{\bullet} + AH/ArOH \rightarrow ROOH + A^{\bullet} / ArO^{\bullet} \qquad (5)$$

Where the aryloxy radical ($ArO^{\bullet}$) formed from the reaction of antioxidant phenol with peroxyl radical is stabilized by resonance (Apak *et al*. 2007).

For an example of HAT assays, oxygen radical absorbance capacity (ORAC) assay applies a competitive reaction scheme in which antioxidant and substrate

kinetically compete for thermally generated peroxyl radicals through the decomposition of azo compounds such as ABAP (2,2'-azobis(2-aminopropane) dihydrochloride) (Huang, Ou, and Prior, 2005). Other HAT assays include total peroxyl radical-trapping antioxidant parameter (TRAP assay) using R-phycoerythrin as the fluorescent probe. Relative reactivity in HAT methods is determined by the bond dissociation energy of the H-donating group in the potential antioxidant. HAT reactions are solvent and pH independent and are usually quite rapid, typically completed in seconds to minutes. The presence of reducing agents, including metals, brings about a complication on HAT assays and can lead to erroneously high apparent reactivity. (Prior, Wu, and Schaich, 2005)

ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced. The degree of color change (either an increase or decrease of absorbance at a given wavelength) is correlated with the sample's antioxidant concentrations. These assays include the total phenols assay by Folin-Ciocalteu reagent (FCR), 2,2'-azino – bis (3-ethylbenzothiazoline – 6 – sulfonicacid)/Trolox equivalence antioxidant capacity (ABTS/TEAC) (Re et al., 1999), ferric ion reducing antioxidant power (FRAP) (Benzie and Strain,1996; Benzie and Szeto, 1999), cupric reducing antioxidant capacity (CUPRAC, assay using a Cu(II) complex as an oxidant), *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD) assay, and 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH). Each method use different chromogenic redox reagents with different standard potentials. These methods involve two components in the reaction mixture, antioxidants and oxidant (also the probe) (Huang, Ou, and Prior, 2005). The ET mechanism of antioxidant action is based on the reactions:

$ROO^{\bullet} + AH/ArOH \rightarrow ROO^{-} + AH^{\bullet+}/ArOH^{\bullet+}$ (6)

$AH^{\bullet+}/ArOH^{\bullet+} + H_2O \leftrightarrow A^{\bullet}/ ArO^{\bullet} + H_3O^{+}$ (7)

$ROO^{-} + H_3O^{+} \leftrightarrow ROOH + H_2O$ (8)

Where the reactions are relatively slower than those of HAT– based assays, and are solvent–and pH–dependent. The aryloxy radical ($ArO^{\bullet}$) is subsequently oxidized to the corresponding quinone (Ar=O). The more stabilized the aryloxy radical is, the easier will be the oxidation from ArOH to Ar=O due to reduced redox potential. The reaction equations of various ET−based assays can be summarized as follows:

Folin: Mo (VI) (yellow) + e^{-} (from AH) → Mo(V) (blue) (9)

λmax = 765 nm

Where the oxidizing reagent is a molybdophosphotungstic heteropolyacid comprised of $3H_2O{\cdot}P_2O_5{\cdot}13WO_3{\cdot}5\ MoO_3{\cdot}10H_2O$, in which the hypothesized active center is Mo (VI).

FRAP: $Fe(TPTZ)_2^{3+} + ArOH \rightarrow Fe(TPTZ)_2^{2+} + ArO^{\bullet} + H^{+}$ (10)

λmax = 595 nm

Where TPTZ: 2, 4, 6-tripyridyl-s-triazine ligand.

Prussian Blue: $Fe(CN)_6^{3-} + ArOH \rightarrow Fe(CN)_6^{4-} + ArO^{\bullet} + H^{+}$ (11)

$Fe(CN)_6^{4-} + Fe^{3+} + K^{+} \rightarrow KFe[Fe(CN)_6]$ (12)

λmax = 700 nm

ABTS/TEAC: $ABTS + K_2S_2O_8 \rightarrow ABTS$ (13)

λmax = 734 nm

$$ABTS^{\bullet+} + ArOH \rightarrow ABTS + ArO^{\bullet} + H^{+} \quad (14)$$

Where ABTS is 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and TEAC is Trolox®-equivalent antioxidant capacity (also the name of the assay). Although other wavelengths such as 415 and 645 nm have been used in the ABTS assay, the 734 nm peak wavelength has been predominantly preferred due to less interference from plant pigments.

$$DPPH: DPPH^{\bullet} + ArOH \rightarrow DPPH + ArO^{\bullet} + H^{+} \quad (15)$$

Where DPPH is the [2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl] stable radical with $\lambda max = 515$ nm.

$$CUPRAC: n\ Cu(Nc)_2^{2+} + Ar(OH)_n \rightarrow n\ Cu(Nc)_2^{+} + Ar(=O)_n + n\ H^{+} \quad (16)$$

Where the polyphenol is oxidized to the corresponding quinone, and the reduction product, i.e., bis (neocuproine) copper (I) chelate, shows maxinum absorption at 450 nm (Apak *et al*., 2007).

2.8 Cereal bran arabinoxylans extraction

Arabinoxylans and feruloyl oligosaccharides can be extracted from cereal bran by enzymic or chemical extraction. For the enzymatic hydrolysis of AX-rich substrates such as bran, the major enzyme requirement is that of a depolymerising xylanase capable of hydrolysing the β- (1→4)-linkages between xylopyranoside residues in xylan. Endo-β-(1, 4)-xylanase (EC 3.2.1.8), referred to endoxylanase, are

used to improve the AX functionality. It hydrolyses the internal β- (1, 4)-linkages in the AX backbone in a random manner. In general, endoxylanase can hydrolyze water-unextractable AX (WUAX, insoluble dietary fiber), resulting in the release of solubilized AX (S-AX) and arabino-xylooligosaccharides (AXOS). Endoxylanases are in the glycoside hydrolase family (GHF) 10 and 11. The GHF10 family members display an average molecular mass of approximately 40 kDa. The GHF11 members are generally smaller (approximately 20 kDa). The enzymes from both families hydrolyze β- (1→4)-linkages between adjacent xylopyranoside residues. Additionally, the enzymes from both families show endo-action and thus exhibit extended active site clefts that are generally composed of three, five or more subsites (Beaugrand *et al*., 2004; Trogh, Croes, Courtin, and Delcour, 2005; Swennen, Courtin, Lindemans, and Delcour, 2006).

Treatment of hull-less barley flour WUAX with GHF 10 and 11 endoxylanases of *Aspergillus acul*eatus (XAA) and *Bacillus subtilis* (XBS) respectively, was studied by Trogh, Croes, Courtin, and Delcour (2005). For both XAA and XBS, the enzymic degradability of AX and apparent specific endoxylanase activity decreased with increasing arabinose to xylose ratio (A/X). The hydrolysis end products of lower average degree of polymerization were obtained from XAA than with XBS. The results of a comparative study of two endoxylanases indicated that a GHF11 is more useful than a GHF10 for the upgrading of wheat bran into soluble oligosaccharides. Upon xylanase treatment, the diferulic acid levels in residual bran were not altered. In addition, nearly 40% and 30% of the initial *p*-coumaric acid content and 50% and 36% of initial ferulic acid content were removed by GHF11 and GHF10. While the GHF10 xylanase was the most active on soluble arabinoxylans but

GHF11 xylanase can better penetrate the cell-wall network (Beaugrand *et al*., 2004). The XBS is useful for the conversion of insoluble fibre to soluble fibre. The A/X ratios of solubilized AX which was released by XBS were decreased with increasing enzyme levels (Maes, Vangeneugden, and Delcour, 2004).

Wheat bran WUAX was obtained as the residue was treated with thermostable α-amylase and protease and two consecutive extractions with alkaline hydrogen peroxide (2.0%, pH 11.5, 4 h, 60 °C). Graded ethanol precipitation with 20-80% ethanol concentration of these hydrolysates was studied. It showed a lowly substituted fraction (A/X = 0.40; 0–40% ethanol) of high molecular weight AX and a fraction of highly substituted AX (A/X>0.95; 40–70% ethanol) containing both low molecular weight as well as high molecular weight AX. At an ethanol concentration of 70% or more, only low molecular weight AX precipitated (Maes and Delcour, 2002). Regard to the work of Swennen, Courtin, Bruggen, Vandecasteele, and Delcour (2005), graded ethanol precipitation and ultrafiltraion were used to fractionate an AXOS with different structures from enzymically produced arabinoxylan hydrolysate. Gradual ethanol precipitation yielded AX containing fractions with a relatively narrow molecular mass (MM) distribution. At low ethanol concentrations, high average degree of polymerization (DP) and high degree of substitution (DS) AX precipitated, while lower average DP and DS components precipitated with increasing solvent concentrations. The obtained fractions from the ultrafiltration had less strict separation and more overlap between the different ultrafiltration fractions. The arabinoxylo-oligosaccharides could be enzymically produced from wheat bran. It was prepared by enzyme-based removal of starch and proteins, and further incubation with a *Bacillus subtilis* endoxylanase converting AX into AXOS. The recovered wheat

bran AXOS had a purity of 72% and an average DP and DS of 15 and 0.27, respectively. The hydrolysates were fractionated by means of graded ethanol precipitation with 0-90% ethanol concentration, which yielded nine different fractions. These fractions: F0–20%, F20–30%, F30–40%, F40–50%, F50–60%, F60–70%, F70–80%, F80–90% and F>90% had DP: 37, 21, 18, 59, 43, 30, 16, 10, and 4, respectively. Fractional precipitation with ethanol was effective in separating the wheat bran AXOS into fractions with different structure (Swennen, Courtin, Lindemans, and Delcour, 2006).

Extraction of hemicellulose with both enzymic and chemical methods was reported by Shibuya and Iwasaki (1985). Defatted rice bran was extracted with ethylenediaminetetraacetic acid (EDTA), digested with pronase and pancreatic amylase, extracted with NaOH, dialyzed against H_2O, and precipitated with ethanol. Then, crude hemicellulose was obtained. Antioxidant compounds from refined corn bran were produced by hydrolyzing with oxalic acid, separated on Amberlite XAD-2, further hydrolyzed by hemicellulases and purified on Amberlite XAD-2. By this method, the antioxidant 5 - O - feruloyl - L - arabinofuranose and O - (5 - O – feruloyl - α - L - arabinofranosyl) - (1→3) - O - β - D – xylopyranosyl - (1→4) – D - xylopyranoside were obtained (Ohta, Yamasaki, Egaehira, and Sanada, 1994).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

Ascorbic acid, 2,2′-azobis (2-methylpropionamide) dihydrochloride (AAPH), 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP), amyloglucosidase A7095**,** arabinose**,** bovine serum albumin (BSA)**,** N,O-bis trimethylsilyl trifluoroacetamide (BSTFA), 2′,7′-dichlorofluorescin diacetate (DCFDA), dimethyl sulfoxide (DMSO), 2,2-diphenyl- 1-picrylhydrazyl (DPPH˙), 4-dimethylaminocinnamaldehyde, ferulic acid, glucuronic acid, galactose, galacturonic acid monohydrate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), hanks' balance salt solution (HBSS), phenol, quercetin, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), sulfuric acid, trimethylchlorosilane (TMCS) and trimethylchlorosilane, trysin (EDTA) were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). Alcalase P4860 and α-amylase (Termamyl SC) were obtained from Novozyme, (Denmark). *tert*-Butyl hydroperoxide, mannose, 2-4- 6-Tripyridyl-s-triazine and potassium hydroxide were purchased from Acros Organics, (Geel, Belgium). Di-sodium hydrogen phosphate anhydrous, ethyl alcohol, methanol and sodium bicarbonate (Na_2CO_3) were obtained from Carlo Erba Reagents, (Milano, Italy). 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) and pencillin and streptomycin were purchased from Invitrogen (California, USA). Endoxylanase

Bacillus subtilis GHF 11 (Grindamyl Powerbake) was a gift from Danisco A/S (Thailand). Fetal bovine serum albumin (FBS) was purchased from Gibco (New York, USA). Iron (III) chloride ($FeCl_3$), iron (II) sulphate ($FeSO_4$) and 2, 4, 6-Tripyridyl-s-triazin (TPTZ) were obtained from Merck, KgaA, (Germany). All chemicals were of analytical, HPLC or molecular biology grade, where required.

3.1.2 Raw materials

Defatted rice bran was a gift from Surin Bran Oil Industry, Thailand. The bran was autoclaved at 121^{o}C for 45 min in order to destroy endogenous enzymatic activities followed by milling (Ika-Werke M20), passing through a 0.5 mm sieve, and stored at – 20^{o}C.

3.1.3 Mammalian cell lines and culture media

The HepG2 (hepatocellular carcinoma) cell line was from the American Type Culture Collection (ATCC, USA) and was cultured at 37 °C in a humidified 5% carbon dioxide (CO_2) atmosphere. HepG2 was cultured in Dulbecco's modified Eagle's medium *(*DMEM*)* media containing 10% fetal bovine serum (FBS, Gibco, NY, USA), 1.5% 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES), 100 µg/mL streptomycin and 60 µg /mL penicillin (Invitrogen, CA, USA). Experiments were conducted at cell line passages less than 20.

3.2 Preparation of rice bran water unextractable arabinoxylans

3.2.1 Preparation of destarched, deproteinised and autoclaved destarched, deproteinised rice bran

Destarched, deproteinised rice bran (DSDPB) was prepared as described earlier by Swennen *et al.*, 2005 and Yuan *et al.*, 2005 with slight modification. The

rice bran was weighed for 12.5 g and then a 30-fold volume of deionised (DI) water was added and swollen at 60^{o}C for 16 h with continuous stirring. Then, 937.5 μL of α-amylase was added. The flask was covered with aluminum foil and incubated for 40 minutes at 95^{o}C with gentle agitating. After that, the mixture was cooled to room temperature, adjusted pH to 7.5 ± 0.2, added 375 μL alcalase, and mixed well. The flask was covered with aluminum foil and incubated in water bath with continuous agitation at 60^{o}C for 30 min. The solution was cooled to room temperature and adjusted pH to 4.0-4.6. The 437.5 μL of amyloglucosidase was added and incubated for 30 min at 60^{o}C. The suspension was centrifuged at 10,000 g for 10 min (Thermo Scientific Sorvall Legend Mach 1.6 R, USA).The residue was transferred to a beaker, stirred with hot distilled water, washed repeatedly by decantation with large volumes of hot water, and then washed with DI water until no cloudiness was noticed. Then, it was washed twice with hot DI water. Finally, the residue was washed with 95% (v/v) ethanol and acetone respectively. The residue was dried in a vacuum oven (WTB Binder, Germany) at 40^{o}C for 12 h to obtain destarched, deproteinised bran (DSDPB). The DSDPB was ground (IKA -Werke M20, Germany) and sieved to pass a 250 μm sieve.

Autoclaved DSDPB was prepared by autoclaving 2% of DSDPB at 121^{o} C for 1-8 h (Hirayama HVA -110, Heidolph, Germany). It was centrifuged at 14,000 g, 10 min and the residue of autoclaved DSDPB (ADSDPB) was freeze dried (Heto FD8, South Africa) and stored at -20^{o}C.

3.2.2 Preparation of ethanol fractionated rice bran water unextractable arabinoxylans

3.2.2.1 Enzymatic hydrolysis of rice bran WUAX

Rice bran WUAX was prepared as described earlier by Swennen *et al.*, 2005 and Yuan *et al.*, 2005 with modification. *Bacillus subtilis* endoxylanase activity was determined using insoluble azurine crosslinked-arabinoxylan (AZCL-AX) tablets as described in Megazyme Data sheet T-XAX200. The endoxylanase was diluted in sodium acetate buffer (25 mmol /L, pH 4.7). Appropriate enzyme dilutions were incubated with an AZCL-AX tablet. The absorbance measurement at 590 nm was conducted using an UV/Visible spectrophotometer (Specto - Biochrom Libra S22, UK). One unit (U) of enzyme activity was the amount of enzyme required to yield an absorbance of 1.0 at 590 nm under the conditions of the assay. The enzyme activity was expressed as the Unit/g under the assay conditions (50^{o}C, pH 4.7)

The experiments were carried out to assess the enzyme dosage that was necessary to obtain a high amount of water unextractable arabinoxylans (WUAX). The 1, 2 and 5 Unit of *Bacillus subtilis* endoxylanase were incubated with DSDPB for 0.5 – 17.5 h at 50^{o} C and the hydrolysate was collected at 0.5, 2.5, 7.5, and 17.5 h. The solution of endoxylanase (2 Unit/mL) was initially prepared in which it consisted of 2.17 g endoxylanase powder and 25 mL of 25 mmol/L acetate buffer (pH 4.7). Then, the solution was stirred for 30 min at 150 rpm and centrifuged at 3,000 g for 30 min. The supernatant was collected and stored at 4^{o}C. The lyophilized powder of DSDPB (10 g) was dissolved in DI water (500 mL) and added with endoxylanase solution. After incubation in an incubator shaker (New Brunswick Scientific Excella E24 series, UK) at 50^{o} C for 0.5-17.5 h, the activity of enzyme was

inactivated by boiling for 30 min. The hydrolysate was cooled down to room temperature, and centrifuged at 10,000 g for 20 min. The rice bran WUAX supernatant was collected and stored at 4^{o}C before fractionation with ethanol.

3.2.2.2 Fractionation by ethanol precipitation

The enzymatically prepared rice bran WUAX was separated into three fractions by graded ethanol precipitation. Aliquots of ethanol were added under continuous stirring to a final concentration of 60% (v/v). The mixtures were then stirred for 30 min and kept overnight at 4°C. Precipitated materials were recovered by centrifugation at 4 °C, 10,000 xg for 20 min, (Thermo Scientific Sorvall Legend Mach 1.6R, USA), dissolved in deionized (DI) water and lyophilized (Heto FD8, South Africa) to obtain fraction F60. The ethanol concentration of the supernatant was further increased to 90% (v/v) and the precipitated fraction F6090, fraction F60–90% was recovered as described above. Ethanol was removed from the remaining supernatant by rotary evaporation (Buchi Rotavapor R-114, Buchi, Switzerland). The remaining fraction was lyophilized and recovered as fraction F90.

3.3 Analysis of rice bran and rice bran arabinoxylans

3.3.1 Proximate composition

A moisture content of all samples was determined according to AOAC (Horwitz, 2000). Protein of rice bran, DSDPB and ADSDPB were determined according to AOAC Official method 984.13 by determining the total nitrogen using the copper catalyst Kjeldahl method and multiplying by 5.75 for conversion from nitrogen to protein content (Horwitz, 2000).

3.3.2 Determination of protein (Bradford assay)

The protein of 3 fractions of ethanol fractionated rice bran WUAX was investigated using the Bradford assay as described by Bollag, Rozycki, and Edelstein (1996). The appropriated dilution of 3 ethanol fractionated rice bran WUAX were performed by mixing 40 μL of sample with 200 μL of Bradford working buffer in 96-well plates. After covered with aluminum foil and put in zip bag, 96-well plates were shaken in shaking water bath at 25ºC for 10 min, and absorbance was measured at 595 nm against Hanks' balance Salt Solution (HBSS) as blank (Specto - Biochrom Libra S22, UK). The protein content of the samples was calculated and expressed as percent of dry weight based on bovine serum albumin (BSA) standard curve.

3.3.3. Determination of total sugar content

Total sugar content was measured using the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Pebers, and Smith, 1956). The sample solution (0.5 mL) was transferred to a 10 mL test tube. Then, 500 μL of 4% phenol and 2.5 mL of 96% sulfuric acid were added and mixed. It was stood for 30 min and measured the absorbance at 490 nm using a spectrophotometer (Specto - Biochrom Libra S22, UK). The total sugar content of the samples was calculated and expressed as percent of dry weight based on standard curve of mixed standard sugars (Ara: Xyl, 0.93:1).

3.3.4 Determination of reducing sugar

The reducing sugar content was determined using the Nelson-Somogyi assay according to Somogyi (1952). The sample (0.5 mL) was added to alkaline copper tartrate reagent (0.5 mL) and boiled for 15 min in a shaking water bath. The mixture was cooled and added 0.5 mL of arsenomolybolic acid reagent. After incubation for 30 min, distilled water (5 mL) was added. The absorbance was

measured at 520 nm using a spectrophotometer (Specto-Biochrom Libra S22, UK). The reducing sugar of samples was calculated and expressed as percent of dry weight based on standard curve of mixed standard sugars (Ara: Xyl, 0.93:1).

3.3.5 Phenolic compounds

Quantification of phenolic compounds in the rice bran and of ethanol fractionated water unextractable arabinoxylans fraction was conducted according to Mattila *et al.*, (2005).

3.3.5.1 Phenolic compound extractions

Phenolic compounds of rice bran, DSDPB, ADSDPB and the three fractions of ethanol fractionated rice bran WUAX were extracted by mixing 0.1-0.5 g of sample with 7 mL of 80% methanol in cold dark room for 30 min. After centrifugation at 4,000 rpm for 5 min, the supernatant was removed and extraction was repeated one more time with 5 mL of 80% methanol. Supernatants were pooled and filtered for analysis of total free phenolic by Folin-Ciocalteu assay and free phenolic acids by HPLC. The bound phenolic compounds were extracted from the residue. The DI water (3 mL), 5 M NaOH (5 mL) and 10 M NaOH (5 mL) were added into the test tube of the residue, sealed, and stirred for 16 h. The solution was then adjusted to pH 2, and the liberated phenolic acids were extracted 3 times with ethyl acetate (12 mL). The organic layers were combined, evaporated to dryness, dissolved in 4 mL of 50% methanol, sonicated, filtered, and analyzed of total bound phenolic by Folin-Ciocalteu assay and bound phenolic acids by HPLC.

3.3.5.2 Total phenolic content (Folin-Ciocalteu assay)

Total free and bound phenolic content of the rice bran arabinoxylans were investigated using the Folin-Ciocalteu assay as described by

Singleton, Orthofer, and Lamuela-Raventos (1999). The extracts, which were diluted (1:10) in distilled water, were added to Folin-Ciocalteu reagent diluted (1:10) in distilled water. After 2 min, 0.8 mL NA_2CO_3 solution was added and shaken at 50 °C for 5 min in a water bath. The solution was cooled down to room temperature and measured the absorbance at 760 nm. The total phenolic content (TPC) of sample was expressed as mg of ferulic acid equivalent (FAE) per 100 g of dry weight of sample.

3.3.5.3 Phenolic acids

The analytical HPLC system consisted of SPD-M10AVP diode array detector, CTO-10ACVP column oven, DGU-14A degasser, LC-10ADVP solvent delivery module, SIL-10ADVP autoinjector, and SCL-10AVP system controller (Shimadzu Corporation, Kyoto, Japan) equipped with a Luna 250 x 4, 6 mm, 5µm C18 column (Phenomenex, Torrance, CA, USA).The temperature of column oven was set at 30 °C. A gradient elution with a flow rate of 1.0 mL/min was employed with a mobile phase consisting of 0.05% triflouroacetic acid (TFA) in water (solution A) and 95% acetonitrile and 0.05% TFA in water (solution B) as follows: isocratic elution 90% A, 0-3 min, linear gradient from 90% A to 87.5% A, 3-15 min, linear gradient from 87.5% A to 82.5% A, 15-25 min, linear gradient from 82.5% A to 70% A, 25-30 min, linear gradient from 70% A to 40% A, 30-36 min, linear gradient from 40% A to 20% A, 36-40 min, linear gradient from 20% A to 65% A, 40-43 min, linear gradient from 65% A to 90% A, 43-53 min, and post-time, 6 min, before the next injection.

Analytical HPLC was run at 30°C and monitored at 3 wavelengths: 260 nm for protocatechuic acid, 4-OH-benzoic acid, and vanilic acid, 270 nm for *O*-coumaric acid and gallic acid, and 280 nm for caffeic acid, *p*-coumaric

acid, and ferulic acid. The phenolic acids were quantified as μg of each of phenolic acid per gram of dry weight of sample.

3.3.6 Determination of molecular weight

The $NaNO_3$ (0.01 M) mobile phase solutions was prepared by dissolving the salts in water followed by vacuum filtration through a 0.2 μm vacuum filter (VacuCup Supor membrane, Pall life science, USA). The ethanol fractionated WUAX rice bran (2.5 mg) was dissolved in 10 mM $NaNO_3$ (1.0 ml) and filtrated through a 0.2 μm pore size polytetrafluoroethylene (PTFE) syringe filter. The high-performance size exclusion chromatography (HPSEC) system (Agilent 1200 series, USA) was performed using a pump equipped with an online degasser (Dionex DG-1210, USA) and autosampler (Agilent Technologies G1367C, USA). The samples were chromatographed on two GPC columns (Agilent PL Aquagel-OH Mixed-H, pore size, 8μm) and monitored by high-performance size exclusion chromatography - multiangle laser-light scattering (HPSEC-MALLS) detector (Wyatt Technologies DAWN HELEOS, Santa Barbara, USA with 120 mW solid-state laser operating at 658 nm), Dynamic light scattering (DLS) detector (Wyatt Technologies DynaPro NanoStar,USA detection angle-90^o, USA) and RI detector (Shodex RI-101, Japan). Measurements were performed at room temperature. The following system parameters were used: 0.8 mL/min flow rate, 100 μL injection volume, 30 min run time. Data collection and molecular weight calculation were performed by ASTRA software Version 6.0.1 (Wyatt Technologies, California, USA).

3.3.7 Determination of Monosaccharides and linkage

3.3.7.1 Determination of sugars (HPAEC-PAD)

The monosaccharide content was determined using high-performance anion-exchange chromatography with pulsed amperometric gold electrode detection (HPAEC-PAD) according to Beaugrand *et al*., (2004). The rice bran WUAX from endoxylanase hydrolysis (1.5 mL) was hydrolysed for 2 h at 100°C in the presence of 1 M H_2SO_4 (45 μL). Samples were then diluted with 15 μL of DI water, filtered through a 0.22 μm pore size polytetrafluoroethylene (PTFE) syringe filter before injection by HPAEC-PAD. The equipment consisted of a Dionex ICS 3000 system with a CarboPac PA 20 column (150 mm × 3 mm, Dionex, Sunnyvale, CA). Separation of saccharides was performed with a mobile phase consisting of DI water (solution A) and 250 mM sodium acetate (solution B) as follows: linear gradient from 96% A to 20% A, 0-30 min; isocratic elution 20% A, 30-40 min; linear gradient from 20% A to 96% A, 40-60 min. The flow rate of the mobile phase was 0.25 mL/min, and the injection volume was 20 μL. Chromelon software version 6.80 was used for data analysis.

3.3.7.2 Determination of sugars (GC)

Monosaccharide composition and structural linkage of arabinoxylans was investigated using methanolysis and methylation analysis as follows.

Methylation analysis: Methylation of samples (rice bran, DADPB, ADSDPB, and three fractions of ethanol fractionated rice bran WUAX) was performed according to Asres and Perreault (1997); Ciucanu and Kerek (1984). Subsequent acidic methanolysis was performed according to Sundberg, Sundberg,

Lillandt, and Holmbom. (1996). Briefly, The 2 M acidic methanol (2 mL) was added to polysaccharide sample (1-2 mg), mixed and placed in an oven at 100^{o}C for 3 h. Sample was cooled to room temperature, added pyridine (150 μL) to neutralize excess HCl and mixed. Sorbitol solution (1 mL) as internal standard was added and mixed. Evaporation the liquid part was performed under nitrogen stream in the water bath at 50^{o}C. The residual methanol was dried in desiccator connected to a high vacuum pressure pump for 20 min.

Silylation: Samples were equilibrated in 400 μL of pyridine for 1 h. BSTFA [N,O-bis (trimethylsilyl) trifluoroacetamide] (200 μL) containing 10% TMCS (trimethylchlorosilane) was added, and the reaction mixture was incubated at 70°C for 2 h. Samples were diluted with 600 μL of ethyl acetate, filtered through a 0.45 μm pore size polytetrafluoroethylene (PTFE) syringe filter, and analysed by gas chromatography.

Gas chromatography: A silylated sample (1 μL) was injected (260°C; split ratio, 1:50) into a HP-5 column (30 m x 0.25 mm, film thickness, 0.25 μm) in an Agilent 6890N Series gas chromatography (GC) system with an Agilent 5973 Series mass selective detector (Agilent Technologies, Santa Clara, USA). The temperature program was 100°C (2 min), 4°C /min; 220°C (2 min), 15°C /min, and 300°C (2 min).The carrier gas was helium (1 ml/min, constant flow). Detector conditions were 70 eV with a scan range of 40 to 600 Da. Data were acquired and processed with MSD Chemstation E.2.01.1177 software from Agilent Technologies.

Peaks were identified by comparison to the standards methylated xylose (2,3,4-linked xylose), methylated xylotriose (terminal xylose and 4-linked xylose), and nonmethylated xylose and arabinose (terminal xylose and

terminal arabinose) as well as wheat arabinoxylan (2,4-linked and 3,4-linked xylose) according to the procedure described above. Relative retention times were in accordance with those in published literature employing a comparable setup (Laine, Tamminen, Vikkula, and Vuorinen, 2002).

Molar response factors were calculated from the standards listed above. Since standards for 2, 4-linked and 3,4-linked xylose were not commercially available, their response factors were inversely calculated from the arabinose-to-xylose ratio of arabinoxylan after subtraction of the remaining constituents. Response factors for 2,4-linked and 3,4-linked xylose were assumed to be identical to those previously reported (Laine *et al*., 2002). The total ion count was used for calculations with the exception of terminal xylose whose data were obscured by silylation reagent products and whose value was calculated from its characteristic ion 101.

3.4 Antioxidant activity

3.4.1 Reagent- based assay

3.4.1.1 Ferric ion reducing antioxidant power (FRAP) assay

Total reducing capacity was determined using the FRAP assay conducted according to Katalinic, Milos, Modun, Music, and Boban (2004). The FRAP reagent was initially prepared consisting of 10 ml of 300 mmol/L acetate buffer, 10 mL of 20 mmol/L $FeCl_3$ and 1 mL of 10 mmol/L TPTZ (2,4,6-Tripyridyl-s-triazin) solution. The acetate buffer (pH 3.6) consisted of 3.1 g of sodium acetate and 16 mL acetic acid per litre of water. The TPTZ solution consisted of 31.2 mg of TPTZ in 10 mL HCl. Initially, the samples, which were diluted in water (0.1 mL), were

added to FRAP reagent (3 mL) and shaken for 15 sec. After incubation for 8 min, the absorbance was measured at 593 nm using a spectrophotometer (Specto-Biochrom Libra L22, UK). The reducing capacity of the samples was expressed as micromoles of Iron (II) per gram dry weight of the lyophilized powder (µmol Fe^{2+}/g) based on an Iron (II) sulphate standard curve, and standardized against a blank control in triplicate.

3.4.1.2 Free radical scavenging activity (DPPH assay)

The $DPPH^{\cdot}$ scavenging activity was determined following the method of Sánchez-Moreno *et al.* (1998). Sample solution of 0.1 mL at different concentration was added to 3.9 mL of 6.34×10^{-5} M methanolic DPPH solution. After incubation for 30 min in the dark, the absorbance (ABS) was measured at 515 nm. The DPPH radical remaining was calculated against DPPH standard curve. The percentage of DPPH radical scavenging was calculated as compared with control as follows:

DPPH scavenging effect (%) = [(ABS control-ABS sample)/ABS control] × 100

Antioxidative activity of the sample was defined as the amount of antioxidant necessary to reduce the initial DPPH radical concentration by 50% (Medium effective concentration, EC50) and expressed as µg/mL of a reaction assay.

3.4.1.3 Cell viability using MTT assay

The effect of the three fractions of ethanol fractionated rice bran WUAX upon proliferation of HepG2 hepatocellular carcinoma cell lines was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983). HepG2 cells were grown in a 96-well plate for 24 h at 3.0×10^4 cells/well in 100 µL media. The cells were allowed to attach for 24 h, then treated

with different concentrations of the extracts (100 µL/well) for 24 h. The cultured medium was removed and 200 µL of 5 mg/mL MTT were then added to each well and the cultures were further incubated for 4 h. The formazan crytal formed was dissolved by adding 50 µL of absolute DMSO. The plate was shaken for 10 min and the absorbance was measured at 540 nm using a spectrophotometer (Benchmark Plus, Bio-Rad, Japan). The results were expressed as the percentage of cell viability with respect to the control. The cell viability (%) was plotted against the various concentrations of samples. The concentration of sample efficiency to inhibit 50% cell viability (IC50) and 95% confidence intervals were obtained from best-fit nonlinear regression (PRISM program). At least 4 measurements were conducted for each treatment and repeated twice.

3.4.1.4 Cellular Antioxidant Activity (CAA) assay

The cellular antioxidant assay (CAA) utilizes 2',7'-dichlorofluorescin diacetate (DCFH-DA) as a probe incorporated into cultured human HepG2 liver cancer cells. HepG2 cells absorb non-polar DCFH-DA by passive diffusion and are deacetylated by cellular esterases. As a result, polar 2',7'-dichlorofluorescin (DCFH) is formed and trapped within the cells. Peroxyl radicals ($ROO^{\bullet}$) produced from 2, 2'-azobis (2-amidinopropane) (ABAP) lead to the oxidation of DCFH to form a fluorescent compound dichlorofluorescein (DCF). The level of fluorescence generated in the system is proportional to the level of oxidation. If antioxidant compounds or phytochemicals obtained from plant extracts can scavenge intracellular ROS, then the oxidation of DCFH to DCF will be inhibited resulting in decreased fluorescence intensity. A decrease in cellular fluorescence compared to the control cells indicates the antioxidant capacity of the compounds (Wolfe and Liu, 2007).

Intracellular reactive oxygen species (ROS) production was measured by modified method of Girard-Lalancette, Pichette, and Legault (2009). Briefly, The HepG2 cells were plated in a 96-well microplates, black plate and clear bottom, (Corning Incorporated, New York, USA) at 2.5×10^4 cells/well and then incubated at 37ºC for 24 h in 5% CO_2. Subsequently, the medium was then removed and the wells were gently washed with 200 µL phosphate buffer saline (PBS). Cells were treated for 1 h with 200 µL of treatment medium (without serum) containing various concentrations of extract. Then, medium was removed, washed with 150 µL PBS and 100 µL of 20 µM DCFH-DA solution were then added to each well and incubated for 30 min. After washing twice with 150 µL PBS, the cells were incubated with 200 µL of 200 µM *tert*-butyl hydroperoxide (*t*-BuOOH) (as inducer for ROS production) in Hank's balance salt solution (HBSS).The amount of ROS production in cells were evaluated by spectrofluorometer (Spectra MAX Gemini EM, Molecular devices, California) with an excited wavelength at 485 nm and emitting wavelength at 535 nm at 37 °C and was measured every 30 min for 4 h. The results were expressed as mean of DCF-fluorescence intensity ±SD for triplicate set of data obtained from the same experiment.

3.5 Statistical analysis

The experiment was conducted in replicate. All measurements were conducted in triplicate. Data were analyzed by ANOVA, followed by Duncan's multiple range tests. Results were presented as mean ± SD. Data analysis and correlations were analyzed using Statistical Package for Social Sciences (SPSS) program for Windows, V.17.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Preparation of rice bran water unextractable arabinoxylans.

The experiments were carried out to assess the optimum enzyme dosage that provided a high amount of water unextractable arabinoxylans (WUAX) with a board range of molecular size. Table 4.1 represents total sugar and reducing sugar contents of rice bran WUAX as a function of enzyme concentration after incubation of destarched, deproteinised (DSDPB) with 1.0, 2.0 and 5.0 Unit of *Bacillus subtilis* endoxylanase for 0.5, 2.5, 7.5 and 17.5 h. The total sugar to reducing sugar ratio was used to estimate an average size of hydrolysate.

The total sugar content was increased with increasing enzyme dosage and incubation time. However, it was increased in a low extent. The reducing sugar content did not change for the first period (2.5 h) of incubation while the total sugar was continued to increase. Thus, then a high molecular size of hydrolysate was obtained. It indicated that enzyme released longer chain WUAX during the earlier of incubation. The smaller WUAX molecules were obtained at the last period of incubation (17.5 h). Maes, Vangeneugden and Delcour (2004) explained that the degradation of WUAX by *Bacillus subtilis* endoxylanase was comprised of two steps which were solubilization, and subsequent degradation of solubilized fragments yielded a wide range of molecular mass profiles.

Table 4.1 Total sugar and reducing sugar after incubation of destarched, deproteinised rice bran with endoxylanase during 0.5 to 17.5 h.

Incubation Time (h)	**0.5**			**2.5**			**7.5**			**17.5**		
Enzyme concentration	**1 U**	**2 U**	**5 U**	**1 U**	**2 U**	**5 U**	**1 U**	**2 U**	**5 U**	**1 U**	**2 U**	**5 U**
TS (%)	0.23±0.03	0.38±0.05	0.60±0.05	0.44±0.06	0.54±0.06	1.42±0.06	0.58±0.19	0.76±0.03	1.86±0.26	0.67±0.16	1.24±0.1	2.61±0.34
RS (%)	0.04±0.01	0.04±0.01	0.04±0.01	0.04±0.01	0.05±0.01	0.07±0.02	0.04±0.0	0.07±0.01	0.1±0.01	0.15±0.02	0.18±0.02	0.23±0.0
TS/RS	5.62	11.75	15.11	10.41	12.73	19.74	13.52	10.5	17.13	4.50	7.0	11.40

Data are expressed as mean ±SD, (n = 3). TS, total sugar; RS, reducing sugar; TS/RS: total sugar to reducing sugar ratio; U, Unit.

Rice bran arabinoxylan had highly branched and complicated side chains (Shibuya and Iwasaki, 1985). Schooneveld-Bergmans, Beldma, and Voragen, (1999) observed that a highly substituted arabinoxylans resisted to enzymatic degradation. The above result illustrates that although the 5 Unit enzyme dosage was used, a low total sugar yield was still obtained. Then, a pretreatment of DSDPB before enzyme hydrolysis was studied.

A preliminary experiment was conducted by autoclaving 2% DSDPB at 121^{o}C for 1 hour before hydrolyze the residues with 2 Unit/g endoxylanase for a half of hour. The result revealed that endoxylanase could release about 2.5 times more WUAX of autoclaved destarched, deproteinised (ADSDPB) than that of DSDPB without heat treatment (Table 4.2). In order to obtain higher yield of WUAX from DSDPB, autoclave time, enzyme dosage, and incubation time were investigated. Figure 4.1 presents the total sugar content obtained from WUAX after autoclaving for 0, 1, 2, 4 and 8 hours with 2 Unit/g endoxylanase for 30 min. The total sugar content of rice bran WUAX was highly affected by autoclave time. It was considerably increased as the autoclave time increased from 1 to 8 hours. The 1.14 to 11.2% total sugar content was released when autoclave time were 1 to 8 hours. From this result, the autoclave time of 4 hours were chosen for preparing destarched, deproteinised rice bran for subsequent study on optimal enzyme dosage and incubation time because it is a practical time.

Table 4.2 Chemical component of WUAX of autoclaved DSDPB and DSDPB without heat treatment after hydrolysis with endoxylanase for 0.5 h.

Sugar (%)	Enzyme concentration (Unit/g)/ autoclave time (h)/ incubation time (h)		
	1U/0h/0.5 h[a]	2U/0h/0.5 h[a]	2U/1h/0.5 h[b]
Total sugar	0.20 ± 0.02	0.42 ± 0.04	1.0 ± 0.02
Reducing sugar	0.03 ± 0.0	0.04 ± 0.01	0.07 ± 0.0
Xyl	0.02 ± 0.0	0.03 ± 0.0	0.14 ± 0.0
Ara	0.12 ± 0.0	0.19 ± 0.0	0.38 ± 0.02
Glu	ND	ND	0.11 ± 0.0
AX	0.12	0.19	0.46
Ara/Xyl	6.0	6.33	2.71
TS/RS	6.7	10.5	7.13

[a] DSDPB without heat treatment; [b] autoclaved DSDPB at 121°C for 1 h followed by incubation with XBS for 0.5 h; ND, not determined.

AX, arabinoxylan = 0.88 x (%arabinose + %xylose); Ara/Xyl, arabinose to xylose ratio; TS, total sugar; RS, reducing sugar; TS/RS, total sugar to reducing sugar ratio.

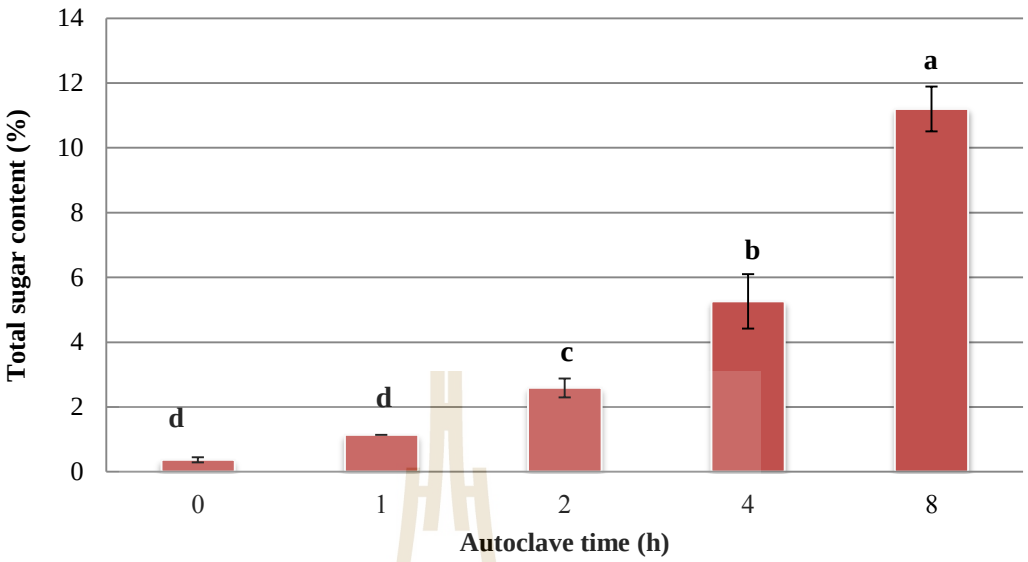

Figure 4.1 Total sugars content (% of ADSDPB) obtained after autoclaving DSDPB for 1- 8 h and then incubated with endoxylanase 2 Unit for 30 min. Mean with different letters are significantly different ($P < 0.05$).

The quantity of total and reducing sugar obtained of incubation of the ADSDPB with 5 and 10 Unit endoxylanase for 0.5 to12 h is shown in Figure 4.2. For the first period of 4 h incubation, total sugar content was significantly increased with a time - and enzyme dose-dependent manner but after 4 h, it was increased in a lower extent. The total sugar obtained during incubation with 5 and 10 Unit endoxylanase for 0.5 to 4 h was 6.62- 9.14 % and 7.62 - 11.74%, respectively. After incubation for 12 h, 9.89 and 13.56 % total sugar were obtained from hydrolysis with 5 Unit and 10 Unit endoxylanase, respectively. The reducing sugar got from 5 Unit endoxylanase incubation was slowly increased and was not significantly different in the first 4 h while it was significantly increased during 0.5-12 h for 10 Unit incubation.

The result in Table 4.1 suggested that the average size of hydrolysate was decreased with increasing of incubation time. Beaugrand *et al*., (2004) and Leguart *et al*., 1999 reported that hydrolysis of endoxylanase GHF 11 could degrade WUAX into small molecules of xylooligosaccharides. Acorrding to Maes *et al*., (2004), wheat bran arabinoxylans that released at low concentration of endoxylanase GHF 11 had a higher molecular mass than those releasing at higher enzyme concentrations. With respected to these previous studies and the large AX molecules were desirable for our objective, incubation of ADSDPB bran with 5 Unit endoxylanase for 4 h was chosen for producing rice bran arabinoxylans.

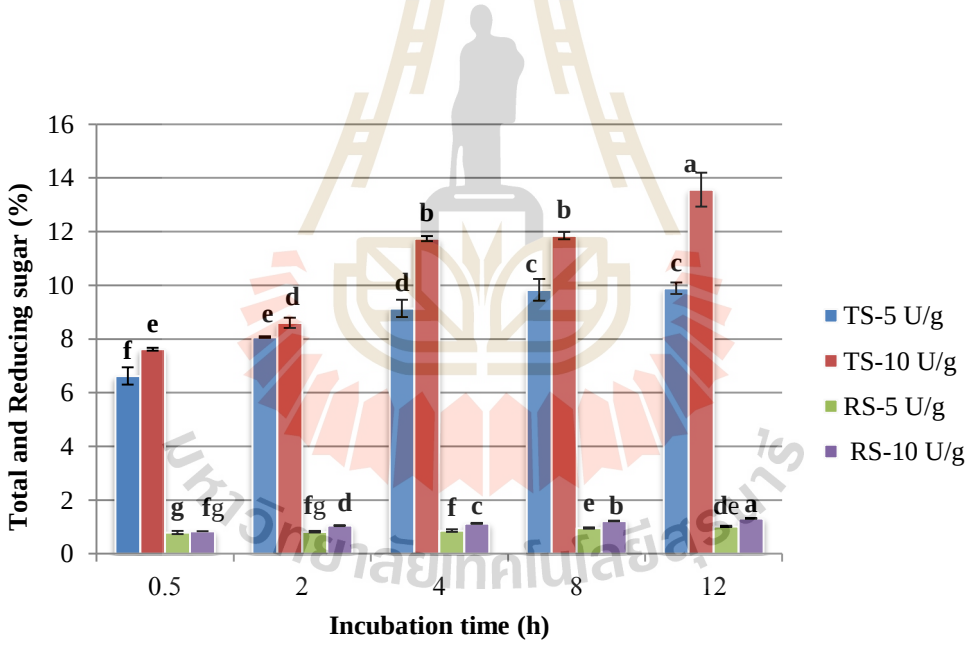

Figure 4.2 Total sugar (TS) and Reducing sugar (RS) content (%) obtained after incubation of ADSDPB bran with 5 and 10 Unit (U) *Bacillus subtilis* endoxylanase for 0.5 to 12 h; Means with different letters of the same concentration of endoxylanase are significantly different ($P < 0.05$).

4.2 Composition of raw material

4.2.1 Monosaccharide compositions and protein content

The monosaccharide compositions and protein content of three different samples, which are defatted rice bran, destarched, deproteinised rice bran (DSDPB), and autoclaved destarched, deproteinised rice bran (ADSDPB), are shown in Table 4.3. The yield of DSDPB was 26% after enzymatic removal of free sugar, starch, and protein from defatted rice bran. The ADSDPB yield was 20.9% of rice bran. ADSDPB yielded 2.52% WEAX, which was fractionated to F60, F6090 and F90 (9.5, 17.9 and 72.6%, respectively). The methanolysis revealed that glucose was the majority sugars of rice bran (70.68 mg/g, Table 4.3). After elimination of starch, free sugar and protein by enzyme-based method, the monosaccharide and protein content were affected by this treatment. Glucose content was reduced by 96% from 70.68 mg/g to 3.02 mg/g of defatted rice bran. Protein content was reduced by 80%, from 179.1 mg/g to 36.4 mg/g of defatted rice bran. It indicated that this enzymatic treatment was an effective procedure to remove starch and protein. Acidity of DSDPB suspension was 6.32±0.2 and 4.74±0.1 by before and after autoclaving.

As compared between DSDPB and ADSDPB, all monosaccharides of ADSDPB showed higher content than those of DSDPB. A considerably higher content of arabinose (94.69 mg/g) and xylose (153.79 mg/g) was hydrolyzed. Consequently, the arabinoxylan content of ADSDPB was increased around 190%, from 80.52 mg/g to 218.66 mg/g. Moreover, other monosaccharides and uronic acids contents were also raised. It indicated that an autoclave of DSDPB for 4 h could break or disengage or disentangle a complicated structure of rice bran cell wall. For production of high-value bioproducts, aqueous hydrothermal treatment (with hot,

compressed water or steam) was used to degrade of xylan from plant lignocellulosic materials into soluble carbohydrates. For examples, xylo-oligosaccharides were produced from rice hulls by hydrothermal processing at temperatures in the range of 40–225 ^{o}C (Vegas, Alonso, Dominguez, and Parajo, 2004; Parajo, Garrote, Cruz, and Dominguez, 2004); two-stage hydrothermal processing was used to produce feruloylated arabinoxylooligosaccharides from wheat bran (Rose and Inglett, 2010). When the hydrothermal treatment of xylan-containing lignocellulosic materials is carried out under suitable operational conditions, the hemicellulosic chains are progressively broken down by the hydrolytic action of hydronium ions, which generated from water autoionization and from *in situ* generated organic acids, yielding soluble products (mainly oligosaccharides), and leaving both cellulose and lignin in solid phase with little chemical alteration (Moure, Gullon, Dominguez, and Parajo, 2006).

This study showed that the glucose content was raised from 3.02 mg/g to 18.00 mg/g after autoclaving the DSDPB for 4 hours. Therefore, it was possible that a higher glucose of ADSDPB was resulted from hemicellulose molecules. Galactose, rhamnose, mannose, galacturonic acid, and glucuronic acid were found in the minority. They are terminally linked to the xylan backbone (Shibuya and Iwasaki, 1985; Rao and Muralikrishna, 2007). Arabinose to xylose ratio of rice bran and DSDPB was relatively high as it exhibited about 1: 1. This ratio was decreased to 1: 2 after autoclaving the DSDPB. This indicated that xylan chain could be affected by high temperature and high pressure of autoclaving.

Table 4.3 Compositions of rice bran, DSDPB and ADSDPB.

Compositions (mg/g)	Rice bran	DSDPB	ADSDPB
Ara	13.56± 0.60	39.40 ± 2.18	94.69 ± 0.51
Xyl	14.72 ± 0.66	52.11 ± 3.65	153.79 ± 0.71
Glu	70.68 ± 1.90	3.02 ± 0.07	18.00 ± 1.32
Gal	2.67 ± 0.09	6.45 ± 0.65	32.49 ± 1.55
Rham	ND	4.21 ± 0.94	4.73 ± 0.18
Man	0.60 ± 0.06	1.41 ± 0.11	12.69 ± 0.50
Glucuronic acid	ND	ND	10.59 ± 0.90
Galacturonic acid	2.55 ± 0.07	0.82 ± 0.06	6.31 ± 0.77
Protein[a]	179.1 ± 10.0	140.0 ± 10.0	140.0 ± 10.0
AX	24.89	80.52	218.66
Ara/Xyl	0.9	0.8	0.6
Yield[b] (%)	100	26.0 ± 0.4	20.9 ± 0.3

[a] Protein was determined by kjeldahl method. [b] Expressed as weight percentage of rice bran. ND, not detected.

Ara, arabinose; Xyl, xylose; Glu, glucose; Gal, galactose; Rhamnose; Man, mannose

AX: arabinoxylan = 0.88 × (% Xyl + % Ara); Ara/Xyl: arabinose to xylose ratio. DSDPB, destarched, deproteinised rice bran; ADSDPB, autoclaved destarched, deproteinised rice bran.

4.2.2 Phenolic content

4.2.2.1 Total free and bound phenolic content

The total free phenolic and bound phenolic content of three different samples are shown in Table 4.4. Total bound phenolic content was higher than total free phenolic content for all samples. After removal of starch, free sugar and

protein by enzymatic method, the total free phenolic was reduced by 90% of the defatted rice bran. However, the enzymatic treatment increased total bound phenolic for 213% of rice bran. The total free phenolic content was increased while the total bound phenolic content was decreased after autoclaving. It indicated that the majority of free phenolic acids and some of bound phenolic acid were eliminated by this process. It could be assumed that the processes of autoclave of DSDPB at 121 ^{o}C for 4 h could affect the rice bran structure, and then bound phenolic were released. Consequently, ADSDPB contained a higher free phenolic acid.

Table 4.4 Total free and bound phenolic content of rice bran, DSDPB and ADSDPB (based on dry weight of each fraction).

Samples	**Total free phenolic (mg FAE [a] /100g)**	**Total bound phenolic (mg FAE [a] /100g)**
Rice bran	490.6 ± 40.2	830.9 ± 70.3
DSDPB	48.9 ± 0.2	2,599.2 ± 438.9
ADSDPB	282.0 ± 29.2	1,692.5 ± 239.2

[a] FAE, ferulic acid equivalent. DSDPB, destarched, deproteinised bran; ADSDPB, autoclaved destarched, deproteinised bran.

Due to the dense structure of cell wall of cellulosic biomass, a hydrothermal treatment was employed to obtain arabinoxylooligosaccharides from several biomasses; for instance, corn cobs, barley hulls, brewery spent grains, and rice hull. Beside xylooligosaccharides, the water or steam treatments also released other compounds including monosaccharides, acetic acid, acid-soluble lignin and furfural

from pentose dehydration, soluble inorganic components, and protein-derived products (Moure *et al*., 2006). Total solids of 32.6-36.3% were released from wheat bran upon hydrothermal extraction of arabinoxylooligosaccharides at temperature of 110-130^{o}C (Rose and Inglett, 2010). Production of xylooligosaccharides from almond shell by autohydrolysis could obtain nonvolatile products of 29.4%, consisting of xylooligosaccharides, xylose, arabinose, glucose, hydroxymethylfurfural, Klason-type lignin, ash, and non-identified products (Nabarlatz, Farriol, and Montane, 2005).

Thai rice bran was very high in total phenolic amounts compared to previously reported values. For example, rice bran from Thai rice variety of Khao DawkMali 105 contained 297 mg FAE/100g (Sunan Butsat and Sirithon Siriamornpun, 2010). Jung, Kim, Hwang and Ha (2007) reported that total phenolic acids of defatted rice bran were 27.86 mg of FAE/100 g. Goffman and Bergman (2004) found that light-brown rice bran exhibited lower total phenolic content than red rice bran for around 10 times. Similarly, Zhang, Zhang, Zhang, and Liu (2010) reported that average values of free, bound, and total phenolic contents of black rice bran were higher than those of white rice bran. Iqbal, Bhanger and Anwar (2005) reported that a significant difference was observed for total phenolic content among the varieties of bran extract which was in the range of 251-359 mg/100 g.

4.2.2.2 Free and bound phenolic acids content

The free-and bound phenolic acid composition of rice bran, DSDPB and ADSDPB are shown in Table 4.5 and 4.6, respectively. They showed that rice bran, DSDPB, and ADSDPB contained hydroxycinnamic acids: caffeic, ferulic and coumaric acids, and contained hydroxybenzoic acids: gallic, vanilic, protocatechuic and 4-hydroxybenzoic acids. The bound form of phenolic acids was

higher than the free form for all samples (Table 4.5, 4.6). *p*-Coumaric acid and ferulic acid were the majorities of free phenolic acid (Table 4.5), while caffeic acid, vanilic acid, and 4-OH-benzoic acid were found in small contents. This result is concomitant to the finding of Sunan Butsat and Sirithon Siriamornpun (2010) and Jung, Kim, Hwang and Ha (2007). When compared between rice bran and DSDPB, free phenolic acids were diminished from rice bran after destarch and deprotein process. Autoclaved bran had higher free phenolic acid content than that of DSDPB.

For bound phenolic acids, ferulic acids were found in predominant amount followed by *p*-coumaric acid and others phenolic acids were found in trace. However, the majority of rice bran-bound phenolic acid was ferulic acids. After autoclave treatment, the quantities of bound ferulic acid and *p*-coumaric acid in DSDPB were increased from 24,020 to 44,816μg/g and from 16,052 to 19,733 μg/g, respectively (Table 4.6). Regard to this result, hydrothermal treatment could probably break down rice bran hemicellulose as suggested by the higher phenolic acid content in both bound and free form of ADSDPB. It should be recognized that rice bran had substantially high ferulic acids (9,139 μg/g, Table 4.6). Zhao and Moghadasian (2008) reported that rice endosperm cell wall and whole brown rice had the ferulic acid contents of 9,100 μg/g and 420 μg/g, respectively.

Table 4.5 Free phenolic acid content of defatted rice bran, DSDPB and ADSDPB.

Free phenolic acids (μg/g)[a]	**Rice bran**	**DSDPB**	**ADSDPB**
Gallic acid	ND	ND	37.7 ± 2.9
Protocatechuic acid	ND	ND	2.7 ± 0.3
4-OH-Benzoic acid	58.7 ± 7.8	0.4 ± 0.0	13.2 ± 2.1
Vanilic acid	66.0 ± 1.0	ND	3.7 ± 0.6
Caffeic acid	89.4 ± 9.0	ND	34.1 ± 2.7
p-Coumaric acid	275.7 ± 2.1	81.1 ± 2.0	879.1 ± 56.6
o-Coumaric acid	ND	ND	ND
Ferulic acid	225.1 ± 8.7	38.0 ± 3.7	650.1 ± 6.1

Data are expressed as mean ± SD, n = 3. [a] based on dry weight each fraction ND, not detected; DSDPB, destarched, deproteinised bran; ADSDPB, autoclaved destarched, deproteinised bran.

Table 4.6 Bound phenolic acid content of defatted rice bran, DSDPB and ADSDPB

Bound phenolic acids (μg/g)	**Rice bran**	**DSDPB**	**ADSDPB**
Gallic acid	ND	5.4 ± 1.8	14.2 ± 4.9
4-OH-Benzoic acid	69.0 ± 11.4	213.2 ± 19.0	218.7 ± 23.1
Vanilic acid	131.4 ± 0.8	492.5 ± 43.7	589.2 ± 21.2
Caffeic acid	24.7 ± 3.3	197.2 ± 53.9	151.5 ± 22.2
p-Coumaric acid	3,418 ± 28.8	16,052 ± 2,244	19,733 ± 813.3
o-Coumaric acid	ND	ND	ND
Ferulic acid	9,139 ± 380.2	24,020 ± 3054.5	44,816 ± 1,865

Data are expressed as mean ± SD, n = 3. ND, not detected; DSDPB, destarched, deproteinised bran; ADSDPB, autoclaved destarched, deproteinised bran.

4.2.2.3 Arabinoxylan structure

Structural information on arabinoxylans of rice bran, DSDPB and ADSDPB was obtained by methylation analysis (Table 4.7). Their molar ratios are given in Table 4.7. They were based on their retention time and mass spectral patterns. The results revealed that xylan backbone of rice bran WUAX composed of substituted xylose rather than unsubstituted xylose. The present of the arabinofuranosyl residues were highly monosubstituted at the xylan backbone through position C-(*O*)-3 (referred as 3, 4- linked Xyl), followed by C-(*O*)-2 monosubstitution (referred as 2, 4- linked Xyl), and disubstituted (referred as 2,3,4- linked Xyl in Table 4.7) on the xylopyranosyl unit, respectively.

Table 4.7 shows that the predominant arabinose residues of rice bran (37.78%) were α-1, 3-linked to monosubstituted xylos. The arabinose residues of 14.10% occurred as a monosubstituted α-1, 2-linkage and those of 2.94% on doubly substituted xylose. ADSDPB was also composed of a predominant α-1, 3-linked arabinose residues (44.54%), but followed by disubstituted xylose (9.03%) and α-1, 2-linked arabinose residues (5.77%).

Since a high amount of non-reducing ends of arabinose (referred as T- linked Ara) was observed, it indicated that arabinose (19.90-66.64% molar ratio) was the mainly branched sugar. This result is similar to those reported in native and malted rice (Rao and Muralikrishna, 2007). Shibuya and Iwasaki (1985) inferred from the methylation analysis of rice bran hemicellulose that most of the side chains was composed of single arabinofuranosyl unit. The terminal arabinose residues was assumed as short side-chains on the xylan backbone and provided as a site for covalent adjunct of ferulic acid (Izydorczyk and Biliaderis, 1995). This observation

corresponded to Izydorczyk and Biliaderis (1995) who described that terminal xylosyl, galactosyl, and glucosyl were present in minor amounts.

Table 4.7 Methylation analysis of rice bran, DSDPB and ADSDPB rice bran.

Linkages [a] (% molar ratio)	Rice bran	DSDPB	ADSDPB
T- linked Xyl	2.77 ± 0.24	6.55 ± 0.7	5.89 ± 0.2
T- linked Ara	38.21 ± 0.16	66.64 ± 3.43	19.90 ± 1.63
4-linked Xyl	4.21 ± 0.02	ND	14.87 ± 1.2
2,4- linked Xyl	14.10 ± 0.78	14.44 ± 0.73	5.77 ± 0.64
3,4- linked Xyl	37.78 ± 2.14	ND	44.54 ± 2.64
2,3,4- linked Xyl	2.94 ± 0.03	12.36 ± 0.1	9.03 ± 0.02

[a] Data represents the substitution patterns of the xylan backbone as molar ratios(%) T- linked Xyl, terminal nonreducing end xylose; T- linked Ara , terminal nonreducing end arabinose. ND, not detected. DSDPB, destarched, deproteinised bran; ADSDPB, autoclaved destarched, deproteinised bran.

4.3 Antioxidant properties

4.3.1 Quantification and identification of phenolic compounds by high performance liquid chromatography

4.3.1.1 Total phenolic and phenolic compounds of rice bran arabinoxylans from graded ethanol precipitation

Rice bran WUAX was extracted by endoxylanase enzyme and was fractionated into three fractions by precipitation with three different levels of ethanol,

0-60% (F60), between 60% and 90% (F6090), and above 90% (F90). The total bound and free phenolic content of those three fractions are shown in Table 4.8. From overall result, the whole, the total free and bound phenolic content of fractionated rice bran WUAX ranged from 726.7-4,636.7, 716.7-3,253.4 mg FAE/100g and 9.98-3,299.4 mg FAE/100g, respectively. F6090 fraction contained the highest total phenolic amounts and then followed by F60 and F90 fractions, respectively. F60 and F90 fractions had comparable free phenolic content; however, F90 fraction had a very low bound phenolic content. The bound phenolic contents were of 82%, 30% and 1% of total phenolic for F60, F6090 and F90 fractions, respectively and free phenolic acids, the rest of content, were 8%, 70% and 99% in the corresponding fractions. Rao and Muralikrishna (2004) reported that water unextractable non-starch polysaccharide contained higher bound phenolic acids than water extractable non-starch polysaccharide. These results indicated that fractionation by graded ethanol precipitation could fractionate the enzyme-digested into unique portions.

Table 4.8 Total free phenolic and total bound phenolic contents of three fraction of rice bran WUAX from graded ethanol precipitation.

Ethanol fractionated WUAX	Free phenolic (mg FAE/100g)	Bound phenolic (mg FAE/100g)	Total [a]
F60	727.1 ± 63.2^{a}	$3,299.4 \pm 435.0^{c}$	4,026.5
F6090	$3,253.4 \pm 174.9^{b}$	$1,383.3 \pm 317.1^{b}$	4,636.7
F90	716.7 ± 21.7^{a}	9.98 ± 2.6^{a}	726.7

Data are expressed as mean ±SD (n = 3); FAE, ferulic acid equivalent. [a] Total phenolic content was calculated from free phenolic and bound phenolic. Values in the same column with different alphabets are significantly different at $p < 0.05$.

Table 4.9 Free Phenolic acids content of three fraction of rice bran WUAX from graded ethanol precipitation.

Compounds (μg/g)	**F60**	**F6090**	**F90**
Gallic acid	ND	ND	ND
Protocatechuic acid	ND	ND	47.3 ± 1.2
4-OH-Benzoic acid	12.7 ± 0.5	ND	39.8 ± 1.0
Vanilic acid	27.4 ± 2.1	ND	106.6 ± 9.5
Caffeic acid	8.8 ± 1.0	ND	217.4 ± 27.5
p-Coumaric acid	203.7 ± 21.9	32.4 ± 4.9	858.3 ± 18.5
o-Coumaric acid	7,492.2 ± 417.8	59,247.3 ± 14.3	ND
Ferulic acid	643.8 ± 36.8^{b}	199.5 ± 9.9^{a}	737.2 ± 9.6^{c}
Total	8,388.6	59,479.2	2,006.6

Data are expressed as mean ± SD (n = 3), ND, not detected. Values in the same column with different alphabets are significantly different at $p < 0.05$.

Table 4.10 Bound phenolic acids of rice bran WUAX from graded ethanol precipitation.

Compounds (μg/g)	**F60**	**F6090**	**F90**
Gallic acid	65.1 ± 19.9	20.0 ± 2.7	ND
Protocatechuic acid	ND	ND	ND
4-OH-Benzoic acid	88.1 ± 11.8	62.1 ± 6.35	5.6 ± 0.86
Vanilic acid	365.4 ± 27.4	305.8 ± 2.8	ND
Caffeic acid	78.5 ± 12.0	156.6 ± 20.4	ND
p-Coumaric acid	3,424.0 ± 583.6	1,411.6 ± 104.1	9.9 ± 0.69
o-Coumaric acid	ND	ND	ND
Ferulic acid	57,276.5 ± 932.5^{b}	65,670.0 ± 235.4^{c}	107.8 ± 10.88^{a}
Total	61,297.6	67,626.1	123.3

Data are expressed as mean ± SD (n = 3); ND, not detected; Values in the same column with different alphabets are significantly different at $p < 0.05$.

The free and bound phenolic acid contents of three precipitated fractions of rice bran WUAX are shown in Table 4.9 and 4.10. The ethanol fractionated rice bran WUAX contained predominantly two groups of phenolics, namely hydroxycinnamic acids (caffeic, ferulic and coumaric acids) and hydroxybenzoic acids (gallic, vanilic, protocatechuic and 4-hydroxybensoic acids). F6090 fraction contained the highest amounts of phenolic acid followed by F60, while F90 had the least phenolic acid amounts. Ferulic acid was a majority of bound phenolic acids followed by *p*-coumaric acid (Table 4.10). Ferulic acid existed in the bound form more than the free form for F60 and F6090 fractions. The *p*-coumaric acid was found in both free and bound form while *o*-coumaric acid was found only in the free form.

Ferulic acid of F60 and F6090 mostly existed in the bound form of phenolics. Their content of the bound form were 89 and 329 fold higher than that of the free form (Table 4.10). The results were consistent with the finding that ferulic acid of rice non-starch polysaccharide existed in the bound form rather than the free form (Rao and Muralikrishna, 2004). In contrast to the fractions of F60 and F6090, the F90 fraction contained only 13% bound ferulic acids and the rest of 87% ferulic acids were in the free form.

Similar to ferulic acid, high content of *p*-coumaric acid was also existed in the bound form rather than the free form in both F60 and F6090 fractions, but the finding was opposite in F90 fraction. This results is concomitant with the investigation on the water unextractable nonstarch polysaccharide from rice grain that coumaric acid was the second most abundance after ferulic acid and was around 4-fold lower than ferulic acids (Rao and Muralikrishna, 2004).

There was no free gallic acid found in all fractions but only small amount of bound form was found in F60 and F6090 fractions. Free protocatechuic acid was only found in the trace in F90 fraction. The F6090 fraction contained the highest free form of coumaric acid. However, there were no free forms of gallic acid, protocatechuic acid, 4-OH-Benzoic acid, vanilic and caffeic acid for the F6090 fractions.

In summary, the three major phenolic acids found in ethanol fractionated rice bran WUAX were ferulic, *p*-coumaric and *o*-coumaric acids. Ferulic acid and *p*-coumaric were predominantly presented in the bound form, whereas *o*-coumaric acid was mostly found in the free form. Among the three fractions, F6090 fraction contained the highest amount of bound ferulic acid with the concentration of 65,670 μg/g. Distribution of phenolic acids was similar for both F60 and 6090 fractions; however, some free phenolic acids (gallic, protocatechuic, 4-OH-benzoic acid, vanilic and caffeic acids) were not detected in the F6090 fraction. The F90 fraction contained free phenolic acid more than the bound form. This study showed that ferulic acid was the most abundant phenolic acid in the rice bran WUAX, which agreed with the fact that ferulic acid, is linked to arabinoxylans in the cell wall of aleurone layers (Shibuya and Iwasaki, 1985; Izydorczyk and Biliaderis, 1995).

4.3.1.2 Molecular characteristics of three fractions of rice bran arabinoxylans from graded ethanol precipitation

Molecular characteristics of three ethanol fractionated rice bran WUAX fractions are shown in Table 4.11. The molecular weight was systematically decreased with increasing the level of ethanol. The average molecular weight of F60 (5.786×10^4 g/mol) was 1.5 and 40 times higher than that of F6090 (4.137×10^4 g /mol)

and F90 (1.525×10^3 g/mol), respectively. It indicated the difference of molecular size among the fractions.

Table 4.11 An average molecular weight (Mw), number-average molecular weight (Mn), Z-value molecular weight (Mz) and polydispersity index (Mw/Mn, Mz/Mn) of three fraction of rice bran WUAX.

	Mw (g/mol)	Mn (g/mol)	Mz (g/mol)	Mw/Mn	Mz/Mn	DP [a]
F60	5.786×10^4	3.169×10^4	9.501×10^4	1.826	2.998	438
F6090	4.137×10^4	1.894×10^4	1.078×10^5	2.185	5.693	313
F90	1.525×10^3	2.932×10^2	1.446×10^4	5.199	49.307	12

[a] Degree of polymerization (DP) was calculated from MW/ molar mass of xylose; 132 g/mol.

The molecular weight data were consistent with the alkali degraded rice bran arabinoxylans with two peaks of 40 and 6 kDa from size exclusion chromatography analysis (Cherukuri and Cheruvanky, 2007). With regard to the distribution of molecular size and the degree of polymerization, these ethanol fractionated rice bran WUAX were different to the previous study which reported that arabinoxylans from enzymatic extraction were relatively uniform and exhibited a low degree of polymerization (Zhou *et al*., 2010). The largest molecular weight of these rice bran ethanol fractionated WUAX was 5.786×10^4 g/mol, which was much lower than that of heteropolysaccharides obtained from hot water extraction (defatted rice bran), which has an average molecular weight of 90,000 Da (Wang, *et al*., 2008).

The polydispersity of molecule was estimated by the values of Mw/Mn and Mz/Mn. The polydispersity index of all fractions was greater than one, suggesting that they were polydisperse or non-uniform molecule. The F90 fraction exhibited more polydisperse than that of F60 and F6090 fractions which may relate to its lower precipitation behavior. The result suggested the higher polydispersity with increasing ethanol level. With regard to the graded ethanol precipitation, a hydrophobic system was increased with increasing ethanol level, and then the rice bran WUAX fraction which was more hydrophilic, was early precipitated. However, besides the molecular size and hydrophilic and hydrophobic properties of rice bran WUAX, the polydispersity also affected its precipitation behavior.

The degree of polymerization, which was determined by dividing the peak molecular weight of each fraction by molar mass of xylose, 132 g/mol, was proportional decreased from 438 to 12 with increasing ethanol concentrations from 60% to > 90%. The F90 fraction contained smaller molecular weight with degree of polymerization corresponding to oligosaccharide.

The ethanol fractionated rice bran WUAX showed a precipitation behavior similar to enzymically degraded wheat bran WUAX in which the degree of polymerization was lower with higher ethanol concentration (Swennen *et al*., 2006). It was explained that the precipitation behavior of large molecules composed of high degree of substitute was less susceptible to enzymatic degradation; thus, higher molecular mass was obtained and precipitated first.

In summary, the molecular weight characteristics of three ethanol fractionated rice bran WUAX were in the range of 5.786×10^{4} to 1.525×10^{3} g/mol. F60 fraction had the highest molecular weight, while F90 fraction had the

lowest molecular weight. The molecular weight of F6090 fraction was not different from that of F60 fraction.

4.3.1.3 Monosaccharide compositions, protein content and structural characterization of rice bran arabinoxylans from graded ethanol precipitation

The sugar composition of rice bran WUAX of three different fractions from graded ethanol precipitation was listed in Table 4.12. The major monosaccharides in both F60 and F6090 fractions were xylose (560.22 and 576.99 mg/g, respectively) and arabinose (164.44 and 170.20 mg/g, respectively) which was counted for approximately 640 mg/g of arabinoxylans. Thus, it was reasonable to indicate that these two fractions were arabinoxylans. A considerable amount of galactose, glucose, and rhamnose and a small amount of mannose was also observed. It is an inherent feature of rice bran arabinoxylans (Shibuya and Iwasaki, 1985; Izydorczyk and Biliaderis, 1995) and water-soluble arabinoxylans from rice grain (Rao and Muralikrishna, 2007). Monosaccharide composition of F60 fraction was similar to that of F6090 fraction, but an amount of galactose was higher (72.35 and 43.51 mg/g respectively) but it contained no mannose. Both F60 and F6090 fractions contained similar monosaccharide composition to rice bran polysaccharides treated with *Aspergillus Oryzae* and *Lentinus edodes* enzymes. They were composed of arabinose, xylose, galactose, glucose, mannose, and others (Ghoneum and Maeda, 1996).

Table 4.12 Compositions of rice bran WUAX from graded ethanol precipitation.

Components [a] **(mg/g)**	**F60**	**F6090**	**F90**
Ara	164.44± 10.02	170.20 ± 1.81	11.09 ± 1.32
Xyl	560.22 ± 21.88	567.99 ± 16.83	20.45 ± 1.61
Glu	37.05 ± 2.79	52.07 ± 2.06	49.04 ± 2.11
Gal	72.35 ± 4.03	43.51 ± 4.68	ND
Rham	15.65 ± 3.12	19.75 ± 2.93	ND
Man	ND	5.46 ± 0.73	ND
Glucuronic acid	ND	ND	ND
Galacturonic acid	3.7 ± 0.52	1.18 ± 0.07	ND
Protein[b]	76.9 ± 0.26	26.1 ± 0.02	6.2 ±0.03
AX	637.70	649.60	27.75
Ara/Xyl	0.29	0.30	0.54

[a] Expressed as mg/g of each fraction. [b] Protein was determined by Bradford assay. ND, not detected. Ara, arabinose; Xyl, xylose; Glu, glucose; Gal, galactose; Rhamnose; Man, mannose; AX: arabinoxylan = 0.88 × (Xyl + Ara); Ara/Xyl: arabinose to xylose ratio.

Galacturonic acid was only uronic acid that was present in small amounts. Its content in F60 fraction (3.7 mg/g) was higher than that of F6090 fraction (1.18 mg/g). However, Wang *et al*., (2008) found that uronic acid content of rice bran water extractable arabinoxylan was about 23 mg/g. Uronic acids were found in rice bran arabinoxylans as terminal residues by linking to xylose residues at their *O*-2 positions (Shibuya and Iwasaki, 1985). Arabinoxylans of native and malted rice grain contained uronic acid around 80-130 mg/g (Rao and Muralikrishna, 2007).

Arabinose to xylose ratio (1:3) was rather low and comparable for F60 and F6090 fractions. The F90 fraction had higher degree of substituted arabinose than the other fractions; however, it contained a very low xylose and arabinose contents (20.45 and 11.09 mg/g respectively). F90 fraction was also different in sugar composition and its dominant monosaccharide was glucose (49.04 mg/g).

Methylation analysis of glucose-containing polysaccharide fraction from rice bran studied by Shibuya and Iwasaki (1985) showed that approximately 40% of α-(1-4)-linked glucan backbone was branched at *O*-6 positions. According to Shibuya and Iwasaki (1985), the rice bran hemicelluloses were also mainly consisted of xyloglucan besides arabinoxylan. Therefore, it was very likely that xyloglucan could be the major polymer in F90 fraction. The ethanol fractionated rice bran WUAX fractions contained protein with different amounts. The F60 fraction had higher protein content than that of F6090 and F90 fractions (76.9%, 26.1% and 6.2 mg/g, respectively).

The interglycosidic linkages between monosaccharide residues of arabinoxylans in each ethanol fractionated WUAX was determined by methylation analysis. Arabinose and xylose were the only sugars to be considered. Their molar ratios are given in Table 4.13. They were based on their retention time and mass spectral patterns.

Table 4.13 Methylation analysis of rice bran WUAX from graded ethanol precipitation.

Linkages[a] (% molar ratio)	**F60**	**F6090**	**F90**
T- linked Xyl	3.51 ± 0.01	3.64 ± 0.2	9.94 ± 0.07
T- linked Ara	22.85 ± 0.83	23.32 ± 1.47	21.60 ± 0.05
4-linked Xyl	25.44 ± 1.04	28.40 ± 0.56	27.11 ± 1.52
2,4- linked Xyl	13.27 ± 0.92	12.67 ± 0.83	6.96 ± 0.5
3,4- linked Xyl	31.88 ± 4.13	28.83 ± 1.16	32.72 ± 2.16
2,3,4- linked Xyl	3.05 ± 0.77	3.15 ± 0.24	1.67 ± 0.13

[a] Data represents the substitution patterns of the xylan backbone as molar ratios (%). T- linked Xyl, terminal nonreducing end xylose; T- linked Ara , terminal nonreducing end arabinose.

Arabinose was a terminal linked sugar (referred as T-linked Ara in Table 4.13) in all of fractionated WUAX. Its content was 21.60-23.32 % molar ratio of all linkages. The large majority of terminal-linked arabinose was mainly side chain sugar. All of rice bran WUAX fractions were highly branched by C-(*O*)-3 monosubstituted xylose residues (referred as 3, 4- linked Xyl) of 28.83-32.72% molar ratio. However, C-(*O*)-2 monosubstituted xylose residues (referred as 2, 4- linked Xyl) and a small amount of di-substituted xylose (2, 3, 4-linked Xyl) residues were also detected. This result is concomitant with those reported in rice bran hemicellulose which mainly consisted of highly branched arabinoxylan in which xylose was substituted at C-(*O*)-2 and C-(*O*)-3 as 24.6-31.3% and di-substituted xylose as 6.7% (Shibuyu and Iwasaki, 1985). Most of arabinofuranosyl residues of

cereal arabinoxylans were found as monomer substitutes, but only small proportion of side chains consisted of two or more arabinose residues (Izydorcztk and Biliaderis, 1995).

Non-reducing end arabinose (referred as T-linked Ara, Table 4.13) and non reducing end xylose (referred as T-linked Xyl, Table 4.13) is present or located at the end of the chain. With a high amount of non-reducing ends of terminal-linked arabinose (21.60–23.32% molar ratio) and the presence of mono-, di-substituted xylose, it indicates that the polymer is not totally linear. It is a branched xylose unit where arabinose is the main branch sugar as compared to the xylose one. It is similar to those reported in native and malted rice (Rao and Muralikrishna, 2007). Shibuya and Iwasaki (1985) inferred from the methylation analysis of rice bran hemicellulose that the most of the side chains were composed of single arabino-furanosyl units. Beside the terminal linked arabinose, there was also a little xylose side chain. This observation corresponded to Izydorczyk and Biliaderis (1995) who described that terminal xylosyl, galactosyl, and glucosyl were presented in minor amounts.

According to many literatures (Rao and Muralikrishna, 2007; Izydorczyk and Biliaderis, 1995; Shibuya and Iwasaki, 1985), the terminal-linked arabinose residues were assumed to be short side-chains on the xylan backbone. In addition, they provided a site for covalent adjunct of ferulic acid. The C-(*O*)-3 mono-substituted xylose residues were predominated in all fractions. In F90 fraction, it was nearly 5-fold higher than C-(*O*)-2 mono-substituted xylose. It was reported that ferulic acid covalently linked via an ester linkage to C (*O*)-5 of the arabinose residue (Smith and Hartley, 1983, quoted in Izydorczyk and Biliaderis, 1995) which were linked to

C-(*O*)-3 of xylose units (Mueller-Harvey and Hartley, 1986, quoted in Izydorczyk and Biliaderis, 1995).

The F6090 and F60 had a comparable amount of doubly branched xylose. The proportion of doubly branched xylose residues was far lower than monosubstituted xylose. This was in accordance with Shibuya and Iwasaki (1985). The doubly branched xylose of arabinoxylans was also reported in the arabinoxylan from wheat bran (Beaugard *et al*., 2004), dehusked barley (Han, 2000), native rice and malted rice (Rao and Muralikrishna, 2007). An unsubstituted xylose which referred as 4-linked Xyl was much lower in all fractions as compared to the substituted xylose.

The above results, suggested that the rice bran arabinoxylan, which was extracted by endoxylanase and then followed with a gradient ethanol precipitation, had a branched structure with C-(*O*)-3 monosubstituted xylose as the predominant residues.

4.4 Antioxidant activity of rice bran arabinoxylans from graded ethanol precipitation

4.4.1 Antioxidant activity as evaluated by FRAP and DPPH assays

The radical scavenging capacity of rice bran arabinoxylans from graded ethanol precipitation evaluated by DPPH assay is shown in Table 4.14. The concentration of antioxidant needed to scavenge 50% of the initial DPPH radical (EC_{50}) is used to measure the antioxidant power. The lower EC_{50} means the higher antioxidant power. The EC_{50} of three fractions of rice bran WUAX was determined along with the three standard antioxidants: trolox, ascorbic acid and ferulic acid. The

radical scavenging capacity of F60, F6090 and F90 expressed as EC_{50} values were 556.6, 389.6 and 1,314.7 μg/mL, respectively. The F6090 fraction exhibited the highest antioxidant capacity and followed by F60 and F90 fractions. The DPPH scavenging activity of F60 (EC_{50}=556.6 μg/mL) was comparable to the reported value of wheat bran feruloylated oligosaccharide (EC_{50}=520 μg/mL), treated with xylanase from *Bacillus subtilis* (Yuan, Wang, Yao, and Chen, 2005).

FRAP assay assesses antioxidant power of phytochemicals through the reduction of ferric to ferrous ion. The reducing capacity was calculated by the formation of ferrous-tripyridyltriazine purple color complex when fitting with the calibration curve of ferrous sulfate. The reducing power capacity is related to the amount of ferrous-tripyridyltriazine produced. The reducing capacity of the three fractions of ethanol fractionated WUAX was determined along with the three standard antioxidants. The results of FRAP values in term of ferrous (Fe^{2+}), trolox, ascorbic acid and ferulic acid equivalents are shown in Table 4.14. The reducing power of F6090 fraction was 896.06 μmol Fe^{+2}/g and was not significantly different from the F60 fraction (786.18 μmol Fe^{+2}/g). Among these fractions, F90 had the lowest reducing power (106.21 μmol Fe^{+2}/g).

Table 4.14 Antioxidant activity the three fractions of rice bran WUAX determined by FRAP and DPPH assay.

	DPPH	FRAP value			
	EC_{50} (μg/mL)	μmol Fe^{+2}/g	mg TRE/mg	mg AAE/mg	mg FAE/mg
F60	556.6 ± 31.3[c]	786.2 ± 2.6[b]	0.56± 0.02	0.37 ±0.01	0.41 ± 0.01
F6090	389.5± 11.0[b]	896.1 ± 16.7[b]	0.65 ± 0.04	0.43 ± 0.02	0.48 ± 0.02
F90	1,314.7 ± 17.0[d]	106.2 ± 0.31[a]	0.07 ± 0.01	0.05 ± 0.00	0.05 ± 0.00
Trolox	2.98 ± 0.0[a]	14,065 ± 142[c]			
Ascorbic acid	2.85± 0.03[a]	21,670± 420[d]			
Ferulic acid	5.28 ± 0.07[a]	21,347 ± 866[d]			

Data are expressed as mean ± SD, (n = 3). TRE, trolox equivalent; AAE, ascorbic acid equivalent; FAE, ferulic acid equivalent. Values in the same column with different alphabets are significantly different at $p < 0.05$.

The results from *in vitro* reagent-based antioxidant activity demonstrated that F60 and F6090 fractions had comparable total reducing power (FRAP value) while F90 had the least power. For DPPH scavenging activity, the activity could be ranked in the following decreasing order of F6090 > F60 > F90. However, none of the fractions displayed higher antioxidant activity than all three standard antioxidants, assessed by DPPH and FRAP assays. Results from the present study were agreed with the antioxidant activity of bran and husk fractions of a Thai rice variety (Khao Dawk Mali 105), which showed high values of antioxidant activity based on the DPPH and FRAP assays (Sunan Butsat and Sirithon Siriamornpun, 2010).

Regard to antioxidant activity and phenolic compositions, F60 and F6090 fractions had high phenolic acids and exhibited high antioxidant activity than that of F90 which had lower content of phenolic acids. These results agreed with Rao and Muralikrishna (2006) who reported that the water soluble feruloylated arabinoxylans from rice containing bound ferulic acid had strong antioxidant property assessed by DPPH assay. Similarly, Chatchawan Chotimarkorn *et al*. (2008) reported the EC_{50} values of DPPH scavenging capacity of rice bran phenolic-rich extracts obtained from Thai long grain variety were between 0.38-0.74 mg/mL; however, differences in sample preparations could result in varied extractable phytochemicals resulting in different antioxidant activity. It had been reported that bound phenolic in barley extract certainly contributed to antioxidant activity over free phenolic. The extract contained soluble to insoluble phenolic ratio ranging from 1:27 to 1:35, as determined by trolox antioxidant capacity (TEAC) assay, DPPH and oxygen radical scavenging capacity (ORAC) (Madhujith and Shahidi, 2009).

Considering antioxidant activity and molecular weight, the higher antioxidant activity was related to the higher average molecular weight of ethanol fractionated rice WUAX. An important of the molecular weight of arabinoxylans to their biological activity was reported. Arabinoxylans from wheat bran containing molecular weight of 3.252×10^4 and 3.517×10^5 Da had potent stimulating effects on innate and acquired immune responses of mice, and also had effect on macrophage phagocytosis (Zhou *et al*., 2010). Non-starchy rice bran polysaccharides which had molecular weight between 4 to 7 KDa and 35 to 45 KDa had been claimed to have benefits for human health (Cherukuri and Cheruvanky, 2007).

As the major components of ethanol fractionated rice bran WUAX were xylose, arabinose, and glucose, the antioxidant activity of a combining sugar with phenolic was supported by several constituents such as sugar and α-tocopherol (Faraji and Lindsay, 2004), chitosan-arabinose and chitosan-galactose mixtures (Mahae, Chalat,and Muhamud, 2011). Antioxidant activity of fructose, sucrose, raffinose, sorbitol and mannitol was confirmed in fish oil emulsions (Farali and Lindsay, 2004). The effect of oligosaccharides on DPPH was reported in that agarohexaose showed a high scavenging DPPH capability (Chen and Yan, 2005).

4.4.2 Relationships of phenolic compounds and antioxidant activities based on FRAP and DPPH assays

The correlations between total free phenolics (TFP), total bound phenolics (TBP), total phenolics (TP), total ferulic acid (TFA), free ferulic acid (FFA), bound ferulic acid (BFA), free *p*-coumaric acid (FCA), bound *p*-coumaric acid (BCA), MW and antioxidant capacities of ethanol fractionated rice bran WUAX were observed (Table 4.15). A substantially high correlation between TP and FRAP

value and also DPPH was observed (r = 1.000 and -1.000, respectively). A high correlation has been found between TFA and FRAP values and also DPPH (r = 1.000 and -0.998, respectively) (Table 4.15). The study showed that BFA was extremely correlated with FRAP value and EC_{50} of DPPH (r = 1.000 and -0.999, respectively while BCA was moderately correlated with FRAP value and EC_{50} of DPPH (r = 0.728 and -0.898, respectively). TBP was highly correlated with the molecular weight (r = 0.946). Moreover, the high molecular weight ethanol fractionated WUAX was highly correlated with high FRAP values (r = 0.914) and also were well correlated with low EC_{50} in DPPH scavenging activity (r = -0.897). There were inverse, however, a correlation between FFA and FCA and their FRAP value (r = -0.728 and -0.944, respectively) and the EC_{50} of DPPH (r = 0.756 and 0.919, respectively). These indicate that the major contribution of antioxidant capacities are total phenolic, bound phenolic and bound ferulic acid of ethanol fractionated rice bran WUAX. A well correlation was observed between TBP and their FRAP value (r = 0.733) and their DPPH EC_{50} (r = -705). The total free phenolic was also correlated with FRAP value and EC_{50} of DPPH even though on a moderate effect (r = 0.610 and -0.642, respectively). Similar observation was reported by Adom and Liu (2002) in that a highly correlation of bound ferulic acid, bound phenolic acid and total phenolics of whole brown rice extract with total antioxidant activity (r^2 = 0.999, 0.991 and 0.983, respectively).

Table 4.15 Correlation between the contents of phenolic compounds and antioxidant capacity for ethanol fractionated rice bran WUAX.

	TFP	**TBP**	**MW**	**TP**	**TFA**	**FFA**	**BFA**	**FCA**	**BCA**
TFP	1	-0.91	0.236	0.623	ND	ND	ND	ND	ND
TBP		1	0.946	0.722	ND	ND	ND	ND	ND
FRAP	0.610	0.733	0.914	1.000	0.745	-0.829	0.747	-0.944	0.728
DPPH	-0.642	-0.705	-0.897	-1.000	-0.717	0.628	-0.910	0.919	-0.898

TFP, total free phenolics; TBP, total bound phenolics; TP, total phenolics (TFP + TBP);
FFA, free ferulic acid; BFA, bound ferulic acid; TFA, total ferulic acid (FFA+ BFA);
FCA, free coumaric acid; BCA, bound coumaric acid; MW, molecular weight; ND, not determined.

The results indicated that bound ferulic acid and bound *p*-coumaric acid, the main hydroxycinnamic acid of ethanol fractionated rice bran WUAX were the major contributors to antioxidant capacities. However, other hydroxycinnamic acids even presented in small amounts in the ethanol fractionated rice bran WUAX could also contribute to some antioxidant power. Caffeic acid had a higher antioxidant power than that of ferulic and *p*-coumaric acid due to the present of its second phenolic hydroxyl group (Nardini *et al*., 1995; Soobrattee, Neergheen, Luximon-Ramma, Aruoma and Bahorun, 2005). The antioxidant activity of the monomeric hydroxycinnamates was ranked in the following decreasing order of: caffeic acid > sinapic acid > ferulic acid > *p*-coumaric acid (Andreasen, Landbo, Christensen, Hansen and Meyer, 2001). Besides hydroxycinnamic acid, the ethanol fractionated rice bran WUAX was also composed of hydroxybenzoic acid. Gallic acid was reported as the most potent antioxidants among simple hydroxybenzoic acid (Soobrattee *et al*., 2005).

In general, total phenolic content was a main factor responsible for the observed of cereal rich-phenolic extract. The correlation analysis revealed a significant correlation between total phenolic content of ethanol fractionated rice bran WUAX and FRAP value and also EC_{50} of DPPH (r = 1.000 and - 1.000, respectively). This correlation had also been demonstrated for other methanolic extract of cereal products (R^2 = 0.905; $p < 0.001$) (Velioglu, Mazza, Gao, and Oomah**,** 1998) and rye extract (Andreasen *et al*., 2001).

In summary, the total phenolics particularly bound ferulic acid were the major contributors to the reducing power and antioxidant activity of the ethanol fractionated rice bran WUAX.

4.4.3 Antioxidant capacity as evaluated within a life cell

4.4.3.1 Effect of ethanol fractionated rice bran WUAX on HepG2 cell viability by MTT assay

The evaluation of cytotoxicity of ethanol fractionated rice bran WUAX was determined in HepG2, a differentiated human hepatocellular carcinoma cells, using the colorimetric MTT assay. The effect of ethanol fractionated WUAX on HepG2 cell viability (%) and their IC_{50} values are shown in Figure 4.3A-4.3C. F60 fraction induced slightly cytotoxicity. At 50-2,500 μg/mL, F60 fraction reduced the cell viability of HepG2 by about 12% to 35%, respectively. The IC_{50} value of F60 fractions was more than 2,500 μg/mL (Figure 4.3A). F6090 fraction at a range of concentration from 500 to 12,500 μg/mL had no inhibitory effect on HepG2. In contrast, this ethanol fractionated rice bran WUAX could slightly stimulate growth of liver cells at 5,000-10,000 μg/mL concentration. The IC_{50} of F6090 was more than 12,500 μg/mL (Figure 4.3B).

F90 fraction at the concentration ranging from 50-1,000 μg/mL did not alter the cell viability (Figure 4.3C). However, at a range of concentration from 5,000-11,500 μg/mL, F90 fraction significantly inhibited growth of HepG2 cells in a dose related manner. The cell viability of HepG2 was reduced by almost 20% to 80% at 5,000-11,500μg/mL, respectively. The IC_{50} of F90 was 8,132 ± 296.4 μg/mL. Moreover, higher concentrations of F90 fraction were more toxic to HepG2 than F6090 fraction.

In conclusion, the cytotoxicity against HepG2 of various ethanol fractionated from rice bran WUAX are varied. The toxicity ranged in the order of F60 > F90 > F6090. From this range finding test, the concentration range of each fraction

that was not toxic to HepG2 was chosen to evaluate the intracellular antioxidant activity in the subsequent DCFH DA assay.

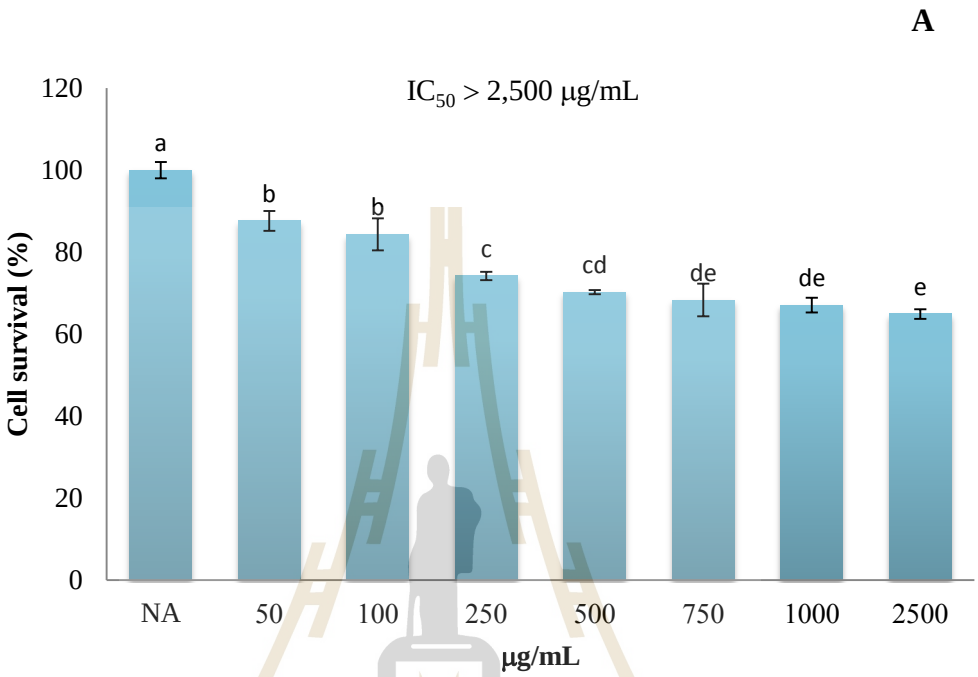

Figure 4.3 Effect of ethanol fractionated rice bran WUAX on cell viability of HepG2 by MTT assay; F60 (A), F6090 (B), and F90 (C). NA, Naïve; the results were shown as mean ± S.D. (n=3). Statistical analysis was performed by SPSS program, ANOVA ($p < 0.05$) and compared control by DMRT.

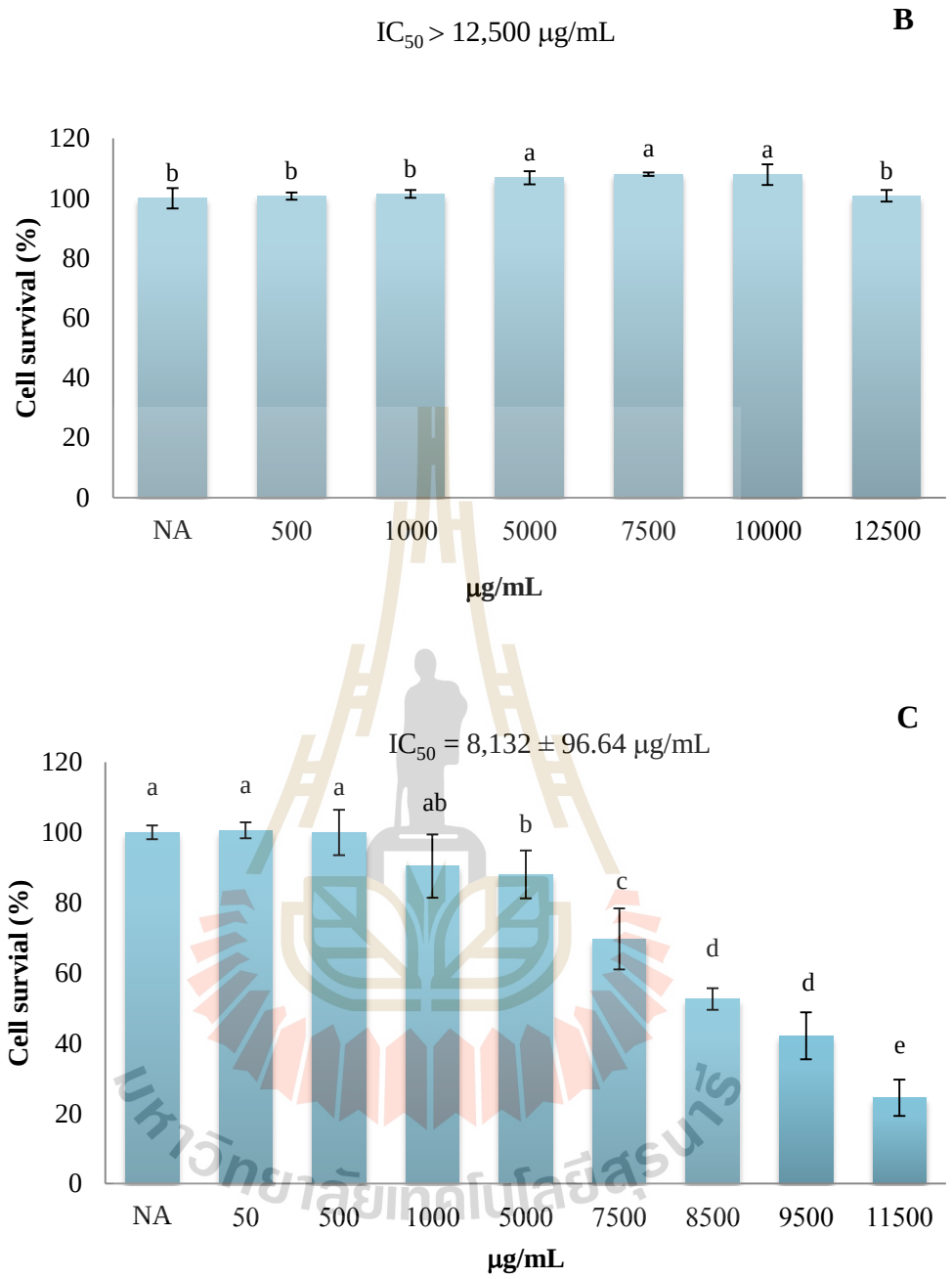

Figure 4.3 Effect of ethanol fractionated rice bran WUAX on cell viability of HepG2 by MTT assay; F60 (A), F6090 (B), and F90 (C). NA, Naïve; The results were shown as mean ± S.D. (n=3). Statistical analysis was performed by SPSS program, ANOVA (p < 0.05) and compared control by DMRT (Continuted).

4.4.3.2 Intracellular ROS scavenging activity

The intracellular ROS scavenging activity of three ethanol fractionated rice bran WUAX was investigated in human hepatocarcinoma HepG2 cells using quercetin as a standard antioxidant. HepG2 cells were directly exposed to *tert*-butyl hydroperoxide (*t*-BuOOH) to induce intracellular ROS level. The levels of ROS were measured using DCFH-DA probe. DCFH-DA enters the cells and is deacetylated by esterases to DCFH, which, because of the negative charge, are trapped within the cells. When the DCFH is oxidized by ROS, it is transformed into the highly fluorescent DCF (Wolfe and Lui, 2007). Therefore, an increase in cellular fluorescence reveals the elevated levels of ROS.

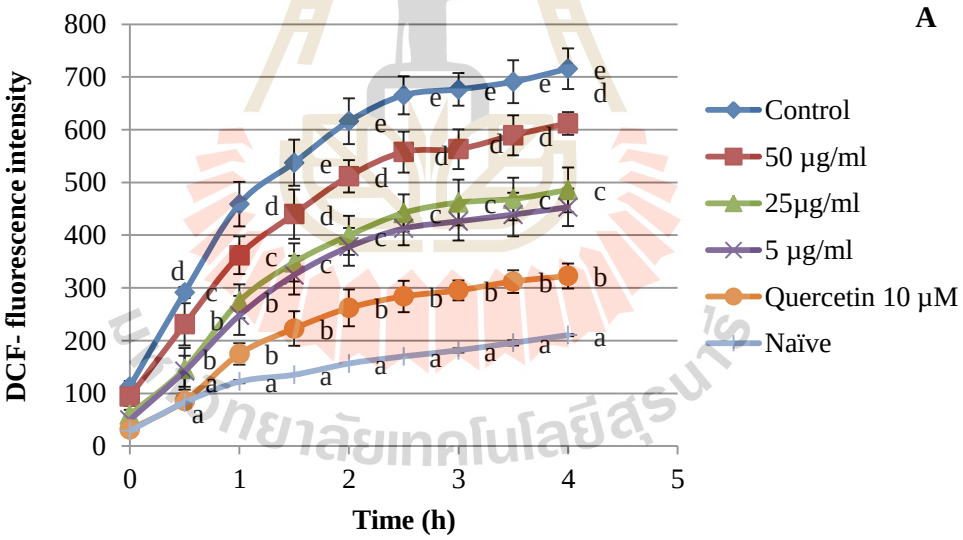

Figure 4.4 Effect of ethanol fractionated rice bran WUAX on *t*-BuOOH induced intracellular ROS level in HepG2 cells; F60 (A), F6090 (B), and F90 (C). Statistical analysis was performed by SPSS program, ANOVA and compared to the induced control at the same time point by DMRT. Data in the same time point with different alphabets are significantly different ($p < 0.05$).

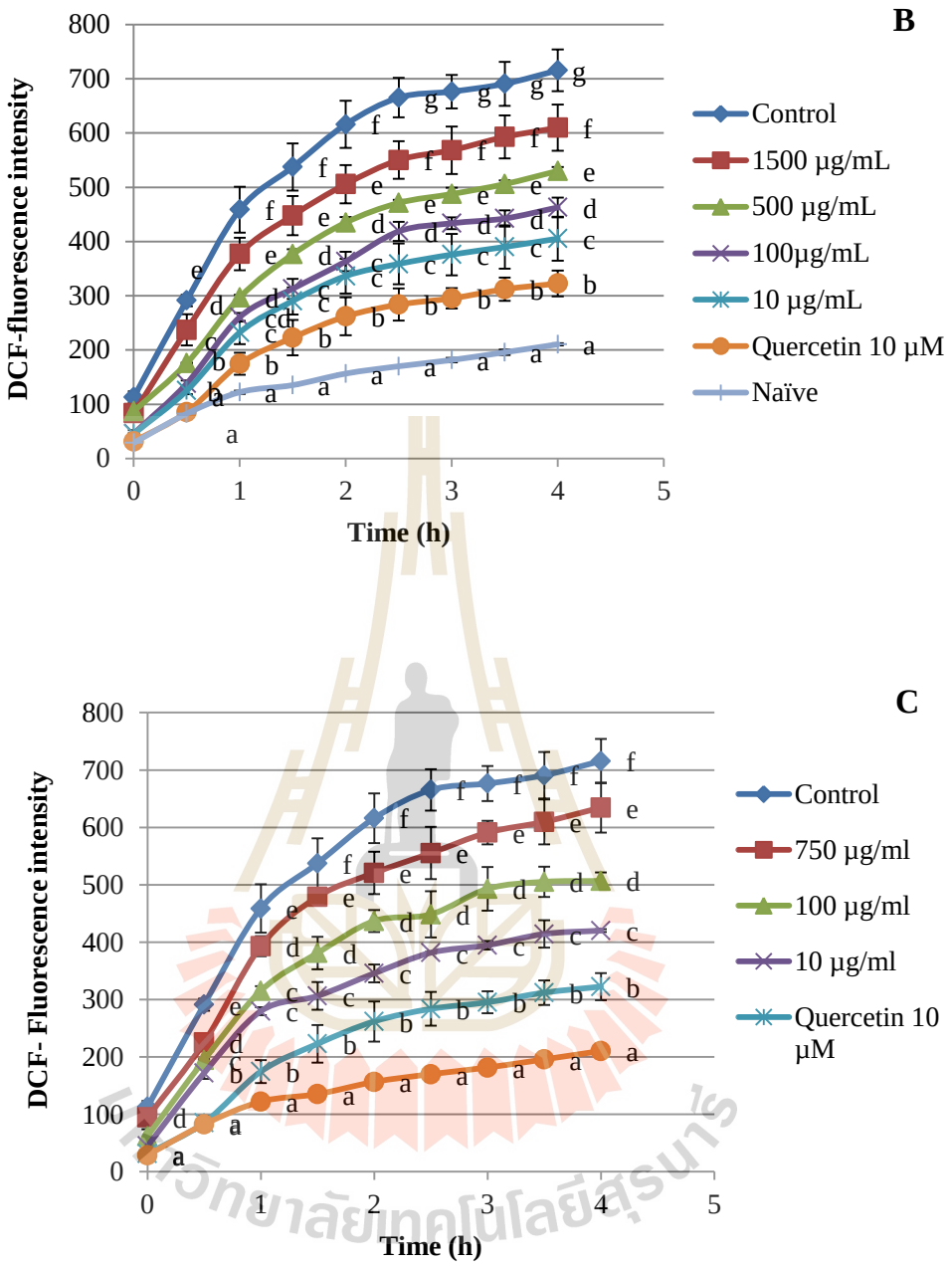

Figure 4.4 Effect of ethanol fractionated rice bran WUAX on *t*-BuOOH induced intracellular ROS level in HepG2 cells; F60 (A), F6090 (B), and F90 (C). Statistical analysis was performed by SPSS program, ANOVA and compared to the induced control at the same time point by DMRT. Data in the same time point with different alphabets are significantly different ($p < 0.05$) (Continuted).

The kinetics of DCFH oxidation in HepG2 cells by intracellular radicals generating from *t*-BuOOH in the presence and absence of ethanol fractionated rice bran WUAX is shown in Figure 4.4. The production of DCF fluorescence of the vehicle-treated control in HepG2 increased in a time dependent manner and reach a plateau to about 715.61 intensity level at 4 h. Pre-incubation with rice bran WUAX of all fractions significantly reduced the increased fluorescence at all studied time points from 0.5 up till 4 h (Figure 4.4A-4.4C). Notably, the lowest concentration of all fractions induced the strongest intracellular ROS scavenging activity, and the activity was significantly decreased with increased concentration.

The most effective concentration of F60, F6090 and F90 were 5 μg/mL, 10 μg/mL and 10 μg/mL, respectively (Figure 4.4A-4.4C). Though all fractions display strong intracellular ROS scavenging activity, the activity of the most effective concentration of all fractions were slightly less effective than 10 μM quercetin.

Different concentrations of substances can induce distinct biological response. This could be explained by hormesis; the dose response phenomenon of substance characterized by low dose stimulation and high dose inhibition. Examples of hormesis can be observed in some parameters of good health such as growth rate, fecundity, and longevity, or the potent antioxidant (Nielsen, Ostergaard, and Larsen, 2008). The present study demonstrated that all fractions of ethanol fractionated rice bran WUAX showed the highest efficiency of ROS scavenging at the lowest dose. The activity was not improved but gradually decreased, with increasing concentration. Furthermore, it has been reported that caffeic acid also enhances the ROS level on HT-1080 human fibrosarcoma cell line as

evidenced by the increased DCF fluorescence (Prasad, Karthikeyan, Karthikeyan, and Reddy, 2011). Similarly, the hermetic nature of dietary phytochemicals have been reviewd (Son, Camandola, and Mattson, 2008). The toxic properties of phytochemicals against the plant's pests can activate adaptive cellular stress response pathway, and in turn, provide the protective health benefits when they are consumed by humans in the appropriate dose.

The present study demonstrated that rice bran arabinoxylans from ethanol fractional precipitation are potent intracellular ROS scavenger. The cytoprotective effects of other cereal arabinoxylans also have been reported. Wang, Sun, Cao, and Tian, (2009) revealed that wheat bran feruloyl oligosaccharides efficiently protected normal human erythrocytes against oxidative stress by suppressing the depletion of reduced glutathione (GSH), lipid peroxidation, and methaemoglobin and protein carbonyl group formation. The used concentrations (10-500 μmol/L) showed no cytotoxicity and genotoxicity to normal human lymphocytes. In addition, DNA damage induced by H_2O_2 in human lymphocytes was also inhibited by 91.1% at the concentration of 500 μmol/L (Wang, Sun, Cao, Song, and Tian, 2008). Ghoneum and Maeda, (1996) reported that rice bran polysaccharide produced by enzymatic extraction, which contained xylose and arabinose as main components, exhibited immunomodulatory properties by enhancing the NK activity of patients.

There are two opportunities for compounds to exert their antioxidant effects in CAA assay. They can act at the cell membrane and break peroxyl radical chain reactions at the cell surface, or they can be taken up by the cell and react with ROS intracellularly. Therefore, the efficiency of cellular uptake and/or membrane binding combined with the radical scavenging activity likely dictates the

efficacy of the tested compound (Yokomizo and Moriwaki, 2006). Earlier presented data showed that F6090 fraction had the highest free and bound phenolic acids contents at 59,479.2 and 67,626.1 µg/g, respectively (Table 4.9 and 4.10). It had the highest FRAP values of 896.1µmol Fe^{+2}/g and the lowest EC_{50} values of DPPH (389.5 µg/mL, Table 4.14). The CAA assay indicates that phenolic compounds of the F6090 fraction could efficiently enter and/or absorbed by life cell and consequently act as antioxidant within a biological system.

Besides those two opportunities for compounds to exert their antioxidant effects in CAA assay, other properties of phytochemicals also contribute to their effectiveness as antioxidants in cell culture. Structural components of phenolic compound play an important role for protecting cells against oxidative stress, the 3′,4′-hydroxyl groups in the B ring and a 2,3-double bond conjugated with a 4-oxo group in the C ring of quercetin provided its strong antioxidant property of protecting cells against H_2O_2-induced oxidative stress (Wang and Joseph, 1999). Hydroxybenzoic acid and hydroxycinnamic acid have lesser antioxidant property than quercetin, they contain only one phenyl ring. Their antioxidant property is consistent with the electron withdrawing potential of the single carboxyl functional group attached to the aromatic ring, dampening the H-donation capacity of the hydroxyl group (Soobrattee, Neergheen, Luximon-Ramma, Aruoma, and Bahorun, 2005).

The CAA assay results showed the same inhibitory effect on ROS of all 3 ethanol fractionated rice bran WUAX fractions, even though they had different structure and chemical components. Zhou *et al*. (2010) studied immunological activity of arabinoxylans from wheat bran which was extracted by alkaline-and enzyme-based methods; they reported the alkaline-extracted AX (AXA)

contained a lower amount of protein (4.10%) than enzyme-extracted AX (AXE, 9.85%) and had no ferulic acid. An arabinose to xylose ratio for AXA and AXE were 0.83 and 0.56 and their molecular weight was 3.517×10^5 Da and 3.252×10^4 Da, respectively. Between them, there were no significant differences in enhancing lymphocyte proliferation. Similarly, Cherkuri and Cheruvanky (2007) found non-starchy polysaccharides, producing by alkali extraction from rice bran, had immune enhancing, anti-carcinogenic, and anti-viral effects. These polysaccharides contained glucose (92.4 mole %) as the main sugar and the rest are arabinose and xylose (5.2 and 1.9 mole%, respectively). Frenzel, Richter and Eschrich, (2002) also reported the scavenging ROS property of sugar; fructose inhibited apoptosis induced by reoxygenation in rat hepatocytes by decreasing ROS via stabilization of the glutathione pool. According to Bland, Keshavarz, and Bucke (2004) oligosaccharides with a degree of polymerization greater than 6 had a tendency to form helical structures which are most effective in influencing the immune system.

From the CAA assay results of ethanol fractionated rice bran WUAX and above reports, the sugar structure seems to affect their functional properties. Oku *et al.* (2003) investigated the mechanism of antioxidant function of trehalose by NMR and quantum chemical. They reported one trehalose molecule stoichiometrically interacts with one *cis*-olefin double bond of unsaturated fatted fatty acid, computer modeling study indicated that trehalose forms a stable complex with an olefin double bond through OH•••π and CH•••O types of hydrogen bonding. Furthermore, a significant increase in the activation energy is found for hydrogen abstraction reaction from the methylene group located between the double bonds that are both interacting with the trehalose molecules. Therefore, trehalose has a

significant depression effect on the oxidation of UFA through the weak interaction with the double bonds.

Based on those Zhou *et al*. (2010); Bland *et al*. (2004) and Oku *et al*. (2003), the structural feature and conformation of sugar affect their functional properties. Dervilly-Pinel, Thibault, and Saulnier (2001) studied conformational characteristics of wheat flour water-soluble arabinoxylans, deferuloylated, fractionated and purified by HPSEC resulting in 48 subfractions with low polydispersity index. The revealed conformational characteristics (persistence length *q*, hydrodynamic parameter n and Mark–Houwink exponent *a*) were similar among all subfractions and fitted with a semi-flexible conformation, no matter what their structural characteristics are. Substitution degree of the xylan backbone by arabinose residues has no influence on the conformation of arabinoxylans.

Polarity of the compounds is other important properties, evidences from *in vivo* study show that the net transfer of flavonoids across the brush border of rat small intestine was found to be related to their lipophilicity, rather than their spatial conformation (Crespy *et al*., 2003). Hydrophobic flavonoids may become deeply embedded in membranes, where they can influence membrane fluidity and break oxidative chain reactions. More polar compounds interact with membrane surfaces via hydrogen bonding, where they are able to protect membranes from external and internal oxidative stresses. This physical property of flavonoids was also presumably for other classes of phytochemicals and determines their interactions with the cell membrane (Oteizai, Erlejman, Verstraeten, Keen and Fraga, 2005). This may be a reason why F90 fraction was so effective as an antioxidant in this CAA

experiment. This fraction contains lesser phenolic compounds than other ethanol fractionated rice bran WUAX fractions while having more hydrophobicity.

Wolfe and Lui (2007) revealed that, among of CAA protocol when performed by washing or without washing with PBS could affect the antioxidant activity. For example gallic acid, ascorbic acid, and caffeic acid displayed dramatically lower antioxidant activity when had PBS wash. On the other hand, quercetin showed little, if any, difference in antioxidant efficacy whether or not a PBS wash was done. In our study we conducted the experiment using the PBS wash protocol, this may somehow affected especially to F90 fraction which contained more free form of caffeic acid.

There was a strong positive correlation between molecular weight of ethanol fractionated rice bran WUAX and FRAP (r = 0.914) and EC_{50} of DPPH (r = -0.897, Table 4.15), Moreover CAA assay results revealed that the most effective fraction in inhibition of ROS production was F60 fraction, the highest molecular weight ethanol fractionated rice bran WUAX. These implied that molecular weight may be responsible for the detected radical scavenging ability. In fact, several reports revealed an effect of molecular weight and molecular size of cereal arabinoxylans and other oligosaccharides on antioxidant activity and immune therapy properties. Zhou *et al.* (2007) reported that wheat bran arabinoxylans from enzymatic hydrolysis having molecular weight (3.252×10^4 Da) 10 times lower than that from alkali hydrolysis, showed more macrophage phagocytosis and delayed hypersensitivity reaction.

Several oligosaccharides which were different in degree of polymerization (DP) were tested on ROS production. The levels of antioxidant

activities related to degree of polymerization of agaro-oligosaccharides were investigated. The study revealed that their scavenging ROS capability was associated with the improvement of the cell viability. In these oligosaccharides, agaro-hexaose possessed the highest scavenging capability more than biose tetraose, and decaose (Chen and Yan, 2005). Bland, Keshavarz, and Bucke (2004) reported manno-oligosaccharides (DP 5-7), arabino-oligosaccharides (DP 6-8) showed greater inhibitory tendency towards ROS production as the DP increases. The results indicated that oligosaccharides with a degree of polymerization greater than 6 and with a tendency to form helical structures are most effective in influencing the immune system**.** Similar finding was also observed in manno-oligosaccharides derived from locust bean gum and arabino-oligosaccharides (Bland, 2002). Sargassum (seaweed) polysaccharide inhibited oxidative stress by increasing the amount of reduced glutathione, promoting the activities of superoxide dimutase and glutathione peroxidase and reducing the level of ROS in chicken bursal lymphocytes. Their molecular weights were more than 50,000 Da and contain furanopolysaccharide which is composed of galaetose, xylose, arabinose, glucose, rhamnose, and fructose. The infrared spectrum of sargassum polysaccharide has shown to contain β-glycosidic bonds in its molecule structure and has an absorbance peak characteristic of α-D-galactopyranosyl (Zhanga, Hua, Liua and Shuai, 2011).

CHAPTER V

CONCLUSIONS

The current study was to provide systematic information on potential health-beneficial properties of rice bran water unextractable arabinoxylans (WUAX). Extraction of feruloylated AX from rice bran by enzymatic endoxylanase hydrolysis provided a little yield. Hydrothermal heat treatment with autoclave of destarched, deproteinised rice bran for 4 h before extraction with 5 Unit endoxylanase for 4 h displayed the higher WUAX yield of 9.14%. Rice bran comprised predominantly bound phenolic acids which were abundant of ferulic acid and *p*-coumaric acid. Enzymatic hydrolysis of rice bran WUAX followed by graded ethanol precipitation could fractionate the rice bran into 3 major fractions, F60, F6090 and F90 with appropriate yield. F60 and F6090 fractions comprised predominantly phenolic acids: ferulic acid, *p*-coumaric acid, gallic acid, 4-OH-benzoic acid, vanilic acid, chlorogenic acid, and caffeic acid. The main compound was ferulic acid and *p*-coumaric acid. Enzymatic hydrolysis of rice bran by endoxylanase released rice bran arabinoxylans with molecular weight varying from 1.525×10^{3} to 5.786×10^{4} g/mole. The major monosaccharide compositions of F60 and F6090 fractions were xylose (66.89% and 64.54%, respectively) and arabinose (21.10% and 19.34%, respectively). F90 fraction contained glucose as the major monosaccharide. Rice bran evaluated in this research represents superior sources of feruloylated arabinoxylans. Structural information was

obtained by methylation analysis; all fractions of rice bran WUAX from graded ethanol precipitation are not totally linear. They are predominant with the glycosidic linkage of C-(*O*)-3 monosubstituted xylose residue and small amounts of C-(*O*)-2 of monosubstituted xylose and disubstituted xylose. The large majority of terminal end was arabinose, suggesting that arabinose was the main side chain sugar. The study of quantity of unsubstituted xylose and mono- and di-substituted xylose showed that F60 and F6090 had comparable higher degree of substituted (DS) than that F90. These could be assumed that F60 and F6090 fractions were hydrolyzed from the highly DS region of hemicellulose molecule. In contrast, F90 may be released from the lesser DS region.

F6090 and F60 fractions had a good reducing capacity (FRAP assay), while F6090 fraction showed an effective antioxidant activity by DPPH assay. F90 had the lowest antioxidant activity. The cytotoxicity against HepG2 of various ethanol fractionated from rice bran WUAX was performed. F60 fraction induced slightly cytotoxicity at the concentration 50-2,500 μg/mL. Their IC_{50} was more than 2,500 μg/mL. F6090 fraction with a range of concentration from 500 to 12,500 μg/mL had no inhibitory effect on HepG2 cells, and had slightly stimulated growth of liver cells at concentration 5,000-10,000 μg/mL. The IC_{50} of F6090 was more than 12,500 μg/mL. At a range of concentration from 5,000-11,500 μg/mL, F90 fraction significantly inhibited growth of HepG2 cells with the IC_{50} of 8,132 μg/mL. F60, F6090 and F90 fractions exhibited similar ROS inhibition with dose-dependent manner. The most effective concentration of F60, F6090 and F90 were 5 μg/mL, 10 μg/mL and 10 μg/mL, respectively. Regard to this study, the activity of the most

effective concentration of all fractions was slightly less effective than 10 μM quercetin.

The study shows that F60 and F6090 were similar in antioxidant activity in both reagent-based assay and cell-based assay. These fractions contained high amount of bound phenolic acids and had high molecular weight. In addition, they contained high amount of AX. Therefore, it could be stated that bound phenolic content, MW and AX content were the major contributor on antioxidant activity of F60 and F6090. For F90, this fraction contains lesser phenolic compounds and MW than that of F60 and F6090 while having more hydrophobicity. The hydrophobic property and small molecule were important for embedding in cell membrane. Therefore, it could be the reason for the effectiveness of F90 on antioxidant in this DCFH DA experiment.

Future research directions

According to its intracellular antioxidant in cell culture–based assay, the present study has provided valuable systematic information which can be further extended towards understanding of other biologic activities of the fractionate rice bran WUAX. Several epidemiological studies have revealed correlation between health-beneficial effects and an intake and bioavailability of polyphenols and phenols. Therefore, the future study could be extended to the concept of bioavailability as interaction between phenols and food matrix is definitely complex, resulting in affecting their absorption and metabolism. To understand these interactions, extensive future studies are required, in particular on metabolites as well as the initial material.

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