รายงานวิจัยฉบับสมบูรณ์

ผลของโปรตีนไฮโดรไลเสตจากรำข้าวและสารสกัดฟีนอลิกจากแกลบ ต่อความเสถียรของอิมัลชันและความคงตัวต่อปฏิกิริยาออกซิเดชัน

Effects of rice bran protein hydrolysates and rice hull phenolic extract on emulsion and oxidative stability

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ชื่อโครงการ: Effects of rice bran protein hydrolysates and rice hull phenolic extract on emulsion and oxidative stability

ผลของโปรตีนไฮโดรไลเสตจากรำข้าวและสารสกัดฟีนอลิกจากแกลบต่อความเสถียรของอิมัลชันและความคงตัวต่อปฏิกิริยาออกซิเดชัน

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บทคัดย่อ

อิมัลชันแบบน้ำมันในน้ำเป็นระบบที่พบเป็นองค์ประกอบในผลิตภัณฑ์อาหารหลากหลายชนิด ความเสถียรต่อการเกิดการแยกชั้นและปฏิกิริยาลิพิดออกซิเดชันมีความสำคัญต่อการยอมรับของผู้บริโภคในผลิตภัณฑ์ ปัจจุบันมีความสนใจเกี่ยวกับการใช้วัตถุเจือปนอาหารจากธรรมชาติเพิ่มขึ้นอย่างต่อเนื่อง วัตถุประสงค์ของการศึกษาครั้งนี้คือเพื่อผลิตวัตถุเจือปนอาหารจากธรรมชาติเพื่อใช้ส่งเสริมเสถียรภาพทางเคมีกายภาพของระบบอิมัลชันแบบน้ำมันในน้ำ โดยทำการผลิตโปรตีนไฮโดรไลเซตจากรำข้าว (Rice bran protein hydrolysate, RBPH) ที่มีสมบัติอิมัลซิไฟเออร์ที่ดีได้ผ่านทางกระบวนการไฮโดรไลซิสด้วยเอนไซม์โปรติเอส RBPH ที่ได้ยังสามารถลดการเกิดออกซิเดชันในระบบอิมัลชันได้อีกด้วยซึ่งคาดว่าเป็นผลมาจากกรดอะมิโนองค์ประกอบที่มีสมบัติต้านออกซิเดชันได้นั่นเอง จากนั้นผลิตสารสกัดฟีนอลิกจากแกลบ (Rice hull phenolic extract, RHPE) ที่มีสมบัติต้านออกซิเดชันที่ดีได้จากกระบวนการสกัดด้วยเมทานอล จากการศึกษาพบความสัมพันธ์ในระดับสูงระหว่างปริมาณสารฟีนอลิกและประสิทธิภาพในการต้านออกซิเดชันของสารสกัด บ่งชี้ถึงบทบาทสำคัญของฟีนอลิกต่อสมบัติต้านออกซิเดชันของ RHPE การเติม RHPE ลงในอิมัลชันส่งผลช่วยพัฒนาความคงตัวต่อการเกิดออกซิเดชันของระบบได้ โดยเฉพาะที่ระดับความเข้มข้นของ RHPE เพิ่มขึ้น และยังพบการเสริมฤทธิ์กันระหว่าง RBPH และ RHPE ในการพัฒนาความคงตัวทางเคมีกายภาพของตัวอย่างอิมัลชัน จากนั้นศึกษาผลการทำงานร่วมกันของ RBPH และ RHPE ต่อเสถียรภาพของตัวอย่างอิมัลชันที่เตรียมขึ้นจากน้ำมันชนิดต่างๆ ได้แก่ น้ำมันถั่วเหลือง (Soybean oil, SBO) น้ำมันรำข้าว (Rice bran oil, RBO) และน้ำมันปาล์มโอเลอิน (Palm olein, PO) พบว่าความ คงตัวทางเคมีกายภาพของอิมัลชันนั้นขึ้นอยู่กับชนิดของน้ำมัน โดย RHPE สามารถช่วยพัฒนาความคงตัวทางกายภาพของตัวอย่างอิมัลชันที่เตรียมจาก SBO และ RBO ได้ แต่อิมัลชันจาก SBO มีระดับการเกิดลิพิดออกซิเดชันสูงที่สุดเมื่อเทียบกับตัวอย่างที่เตรียมขึ้นจากน้ำมันชนิดอื่นๆ ทั้งนี้คาดว่าเป็นผลเนื่องมาจากความไม่คงตัวต่อการเกิดออกซิเดชันของกรดไขมันไม่อิ่มตัว ซึ่งพบเป็นปริมาณมากใน SBO นั่นเอง การศึกษาครั้งนี้บ่งชี้ว่า RBPH และ RHPE ซึ่งผลิตขึ้นจากวัสดุเหลือใช้ทางการเกษตร สามารถใช้เป็นวัตถุเจือปนอาหารธรรมชาติที่มีผลช่วยพัฒนาความคงตัวทางเคมีกายภาพของระบบอิมัลชันได้

Abstract

Oil-in-water (O/W) emulsion is generally found as a composition in various food products. To ensure consumer acceptability in the emulsion, stability against phase separation and lipid oxidation has to be concerned. Recently, there is continuously growing interest in using natural additives in food products. The present work aimed to produce natural additives and used to enhance physicochemical stability of O/W emulsion. Rice bran protein hydrolysate (RBPH) with a potent emulsifying ability could be prepared via Protease aided process. Moreover, RBPH could enhance oxidative stability of the emulsion attributed to its amino acid composition with antioxidative ability. Further, preparation of rice hull phenolic extract (RHPE) with effective antioxidative properties could be accomplished via methanolic extraction. High correlation between phenolic contents and antioxidative capacity of the extract was established, suggesting to the predominant role of phenolic compounds on antioxidative ability of RHPE. Incorporation of RHPE to the emulsions led to improve oxidative stability of the sample, especially at the increased RHPE concentration. A synergistic affect between RBPH and RHPE to improve physicochemical stability of the emulsion was observed. To more elucidate the effects of RBPH and RHPE on stability of emulsion, various oils, *i.e.,* soybean oil (SBO), rice bran oil (RBO), and palm olein (PO), were employed as a dispersed phase to prepare the emulsions. By using different oil types, physicochemical stability of the emulsions was affected. The greatest emulsion formability was observed for the SBO emulsion. RHPE could improve colloidal stability of the samples, when SBO and RBO were employed as a dispersed phase. Nonetheless, the most oxidative degree was observed for the SBO emulsions, supposed since a susceptibility against oxidative reaction of unsaturated fatty acids abundantly present in SBO. The present work suggested that RBPH and RHPE which were prepared from the agricultural waste could be a potent agent employed as natural additives to enhance physicochemical stability of emulsion model.

Key words: rice bran protein hydrolysate, rice hull phenolic extract, emulsion, colloidal stability, lipid oxidation, and phenolic compounds

1. Introduction

Emulsion is generally found as a composition in various products, involving pharmaceuticals, cosmetics, food products, and so on. Regarded to be its thermodynamically unstable system, phase separation tends to occur and leads to unacceptability of consumer in the emulsified products. To prevent phase separation, emulsifier, a surface active agent capable of locating at oil–water interfaces, has to be employed. Proteins are widely used as emulsifier in food products, not only because of their amphiphilic characteristic, but also nutritive value, safety and bioavailability. During emulsification process, proteins facilitate emulsion formation by lowering interfacial tension, and retard drop aggregation by forming a protective barrier around dispersed drop through "*surface denaturation*" process (McClements, 2004). Protein hydrolysates, defined as a protein fraction produced from hydrolysis reaction, have attracted much attention in recent years for their functional properties, *e.g.,* emulsifying ability, foamability, and antioxidant activity (Bandyopadhyay and Ghosh, 2002; Bandyopadhyay *et al*., 2008; Tang *et al*., 2003b; Paraman *et al*., 2007; Aewsiri *et al*., 2009, 2010, 2013). Upon hydrolysis, molecular size of protein became smaller, leading to improve solubility of the hydrolyzed peptides (Bandyopadhyay and Ghosh, 2002; Bandyopadhyay *et al*., 2008; Tang *et al*., 2003b; Paraman *et al*., 2007). It is recognized that solubility is one of the important prerequisite properties controlling emulsifying ability of proteins (Damdoran, 2005).

Lipid oxidation has a crucial role in lowering emulsion quality by rising off-flavor, reducing nutritive value of essential fatty acids and some vitamins, and also producing some health risk compounds, *e.g.* free radicals and reactive aldehydes (Halliwell *et al.,* 1995). To tackle the problem, both synthetic [*e.g*., butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate, and tert-butylhydroquinone (TBHQ)] and natural antioxidants [*e.g.,* α-tocopherol and phenolics] have been introduced. At a present, a special attention has been given to natural antioxidants because of a worldwide trend to avoid or minimize the use of synthetic food additives (González-Montelongo *et al*., 2010). Protein hydrolysates are one of the interesting candidates to be employed as a natural antioxidant, because of their safety, biodegradability, high nutritional or physiological value, and producible from a renewable source. Antioxidant properties in emulsion systems of the hydrolysates prepared from various sources of proteins, *e.g.,* potato (Cheng *et al.,* 2010a, b; Nieto *et al*., 2009; Wang and Xiong, 2005), soybean (Park *et al.,* 2012), and hemp (*Canabis sativa L.*) (Tang *et al.,* 2009)

have been reported. The mechanism of protein hydrolysates to prohibit lipid oxidation is attributed to free radical scavenging and metal chelating abilities provided by some amino acid residues exposed after the hydrolysis process (Peña-Ramos *et al.,* 2004).

Phenolic compounds could improve emulsion stability against lipid oxidation through many mechanisms including free radical scavenging, singlet oxygen quenching, peroxides and other reactive oxygen species inactivating, pro–oxidant metal ions chelating, secondary oxidation products quenching, and pro–oxidative enzyme inhibiting activities (Shahidi and Zhong, 2011). The phenolic compounds in sage extract, *i.e.,* carnosic acid, carnosol, and rosmarinic acid (Cuvelier, *et al.,* 1996), and oregano extract, *i.e*., thymol and carvacrol (Baratta *et al*., 1998), exhibited the antioxidant ability with a comparable efficacy with BHT, and resulted in the extended shelf life of salad dressing up to 6 months (Abdalla and Roozen, 2001).

Thailand is an important rice exporter with the commercial value higher than 196 billion baths in 2011, and the highest income belongs to polished rice products (Department of International Trade Promotion, Ministry of Commerce, 2011). From a polishing process, a large amount of hull and bran are generated as a by–product with low market value. Rice bran may be useful as a source of nutritive protein: Rice bran contains *ca*. 12–20 % protein content with a good essential amino acid profile (Tang *et al*., 2002, 2003a, b). Various essential amino acids, *e.g*., histidine, threonine, valine, and tryptophan, are found in rice bran with content as high as soy protein isolate and casein (Fabian and Ju, 2011). Moreover, various bioactive properties of rice bran protein, *e.g*., antioxidant, anticancer, and anti–inflammatory activities, have been reported (Chrastil, 1992; Fabian and Ju, 2011). Generally, a price of rice bran is *ca*. 4–5 baht/kg, whereas a price of rice bran oil is *ca.* 90–100 baht/liter. Using rice bran as a source to produce protein as food additives however, can increase a value of rice bran up to 1700 baht/kg (Jiamyangyuen *et al.,* 2005). On the other hand, hull of rice is found as an excellent source of antioxidant agents (Ramarathnam *et al.,* 1989; Butsat and Siriamornpun, 2010; Jeon *et al*., 2006; Lee *et al.*, 2003; Shih and Daigle, 2003). Major phenolics with the effective *in vitro* radical scavenging capability present in the indigenous Thai rice were ferulic, vanillic, and *p*–coumaric acids (Butsat and Siriamornpun, 2010), whereas isovitexin was the predominant phenolic compound in the hull of Katakutara (indica) rice (Ramarathnam *et al.,* 1989). Shih and Daigle (2003) extracted the phenolics from rice hull which were able to effectively retard lipid oxidation in ground beef model.

To innovate natural food additive, therefore, the use of rice bran protein hydrolysates (RBPH) as emulsifier and application of rice hull phenolic extract (RHPE) as antioxidant agent might be a promising approach to enhance both colloidal and oxidative stability of emulsion system. As consequence, rice processing by–products can be better utilized and natural food additives for processing aids can be obtained for further application.

2. Objectives

- To produce rice bran protein hydrolysates (RBPH) with effective emulsifying activity and ability to retard lipid oxidation in emulsion system

- To prepare phenolic extracts from rice hull and observe their effects on colloidal and oxidative stability of emulsion system

- To investigate the influences of RBPH and rice hull phenolic extract (RHPE) on the stability of emulsion system prepared from different oil types

3. Literature review

3.1 Protein isolate and hydrolysates and emulsifying properties

Proteins are widely employed as emulsifier in food products due to their amphiphilic characteristic. Rice bran, a by-product from polished rice processing, is one of the interesting sources providing proteins with effective functional and physiological properties, because of its availability, biodegradability, and high nutritive value of some essential amino acids. To isolate the protein from rice bran, alkaline extraction is always conducted which might lead to some inferior functional properties, involving lowering solubility, foaming, and emulsifying properties (Shin and Daigle, 1997; Were *et al*., 1997; Paraman *et al.,* 2006), and destroying a nutritive value (Ansharullah, 1997) of the derived protein. To improve extractability and functional properties of the isolated proteins, enzymatic hydrolysis has been implemented. Hydrolysis, in general, markedly increases protein solubility and offers a feasible means to improve the physicochemical characteristics and bioactivities that are not found in the original proteins, *e.g.,* antioxidant activity, water holding capacity, emulsifying, and foaming properties, (Cheng *et al.,* 2010a, b; Cumby *et al.,* 2008; Park *et al.,* 2012; Tang *et al.,* 2009). Upon hydrolysis, solubility which is one of crucial prerequisite properties

determining the effective emulsifying activity of proteins could be improved (Bandyopadhyay *et al*., 2002, 2008; Were *et al*., 1997; Wu *et al*., 1998). Furthermore, hydrolysis leads to an exposure of some buried hydrophobic amino acids to molecular surface, resulting in increase hydrophobicity of the proteins. Hydrophobicity of protein enzymatically extracted from rice endosperm was markedly higher than the products derived from alkaline extraction (Paramen *et al.,* 2007). The developed hydrophobicity was correlated with the improved emulsifying ability of the proteins (Paramen *et al.,* 2007; Horax *et al*., 2011). To prepare protein hydrolysates with efficient emulsifying activity, degree of hydrolysis (DH) has to be considered. Higher DH always provided better solubility, but led to aninferior emulsifying ability since the hydrolyzed protein was lacking of secondary and tertiary structure to provide a strong interfacial film covering around dispersed drops (Paramen *et al.,* 2007; Tang *et al.,* 2003b).

3.2 Oxidative stability

Lipid oxidation is one of the most important factors deteriorating quality and reducing shelf–life of food products (Estévez *et al.,* 2004, 2005). In emulsion, lipid oxidation progresses rapidly due to a largely present interfacial areas that facilitates the attack of oxygen available in the non–polar phase and water soluble pro–oxidants, *e.g*., metal ions (McClements and Decker, 2000; Waraho *et al*., 2011). The oil–water interface, therefore, has a great impact on the progress of lipid oxidation (McClements and Decker, 2000; Waraho *et al*., 2011). It has been suggested that the interfacial films effectively preventing the incorporation between water soluble pro–oxidants and lipid soluble oxygen could markedly reduced oxidation rate (Berton *et al*., 2011, 2012). The stability against lipid oxidation depends on intrinsic factors, *e.g.,* physical characteristics of the emulsion itself (*e.g*., size, size distribution pattern, and electrical charge of the dispersed drops), molecular characteristics of the chemical compounds present in the system (*e.g*., employed oil and emulsifier types), and interaction between those compositions at the interfacial area (McClements and Decker, 2000; Waraho *et al*., 2011). On the other hand, the extrinsic factors, such as pH, temperature, a presence of pro–oxidant or antioxidant agents, and a pressure that the emulsified system in exposed, also affect the oxidation rate (McClements and Decker, 2000; Waraho *et al*., 2011). According to the polar paradox theory, it has been suggested that the surfactant with lower hydrophilic–lipophilic balance (HLB) might be

active than the higher counterpart to retard oxidation in emulsion system (Berton *et al*., 2012; Shahidi and Zhong, 2011). Furthermore, a greater oxidative stability was given by the interfacial films that can effectively prevent the access of pro–oxidants from aqueous to lipid phase through generating of electrostatic (Cheng *et al.,* 2010a, b; Tong *et al.,* 2000) or steric force by a big hydrophilic head group (Berton *et al*., 2012) to repel the pro–oxidants out off the interfacial areas.

3.3 Antioxidative activities of protein hydrolysates

Antioxidant activity of the protein hydrolysates prepared from either plant (Park *et al*., 2012; Cheng *et al*., 2010a, b; Nieto *et al.,* 2009; Wang and Xiong, 2005; Tang *et al.,* 2003b; Megias *et al*., 2008; Moure *et a*l., 2006; Pownall *et al.*, 2010) or animal (Hattori *et al.*, 1998; Aewsiri *et at.,* 2009, 2010, 2011; Bougatef *et al*., 2010) sources has been reported. Many mechanisms, *e.g*., free-radical scavenging, metal ion chelating, oxygen quenching or hydrogen donating, and protective barrier forming against a penetration of lipid oxidation initiator to the oil phase (Moure *et a*l., 2006; Elias *et al.,* 2006; Cheng *et al.,* 2010a, b; Megias *et al.,* 2008; Peña-Ramos *et al.,* 2004) have been proposed for protein hydrolysates. After hydrolysis, exposure of some buried amino acids, *e.g.,* tyrosine, methionine, tryptophan, and proline, to the molecular surface provokes the antioxidant activities of the peptides (Megias *et al*., 2008). The antioxidant activity of the hydrolysates depended on many factors, *e.g*., size of peptides (Kitts and Weiler, 2003; Peng *et al.,* 2009; Cheng *et al*., 2010a, b), hydrophobicity (Tang *et al.,* 2009; Cheng *et al*., 2010a, b; Pownall *et al.*, 2010), and preponderance of hydrophobic amino acids and their sequence (Peña-Ramos *et al.,* 2004; Mendis *et al.,* 2005).

The molecular size of the peptides played the important role in determining antioxidant activity of protein hydrolysates. The effective antioxidative ability tended to be found in a small peptide fraction (Peña-Ramos *et al.,* 2004; Cheng *et al.,* 2010a, b; Kitts and Weiler 2003). The MW of the antioxidant active peptides of the potato protein was in a range of 0.5–0.9 kDa corresponding to 5–7 amino acid residues (Cheng *et al*., 2010b). The most effective peptide derived from Alcalase hydrolyzed chickpea (*Cicer arietinum L.*) protein to inhibit the oxidation of linoleic acid was reported in the fraction with the MW of *ca.* 0.2–3 kDa (Li *et al*., 2008). The alfafa leaf protein hydrolysate showed a strong superoxide scavenging activity, when the MW was lower than 3 kDa (Xie *et al*., 2008). From the study of Peña-Ramos *et al.*, (2004), however, the most effective antioxidant activity of whey

protein hydrolysates was found for the large size peptide: The order of antioxidant efficacy of whey protein fractions was in the order of the large (MW≈66 kDa) > small (MW≈2.5 kDa) > intermediate peptides (MW≈12.4 kDa). The ability to suppress lipid oxidation provided by the large fractions was also illustrated in the whey protein fraction after isolation through 3500 MW cut–off dialysis tube.

3.4 Antioxidative activity of phenolics

Phenolics have been reported to exhibit interfacial and antioxidative activities in emulsion systems. The ability of gallic acid, catechin, and quercetin (Di Mattia *et al*., 2010) and oleuropein (Di Mattia *et al*., 2011) to reduce the oil–water interfacial tension was reported due to its ability to be accumulated at the interfacial border. Addition of anthocyanin from a berry juice extract could enhance stabilization of whey protein based emulsion, especially at increased anthocyanin concentration increased (Viljanen *et al.,* 2005a, b). It has been previously reported that phenolic compounds could improve oxidative stability of protein stabilized emulsions (Yuji *et al*., 2007; Oda *et al.,* 1998; Almajano *et al.,* 2004, 2007). Phenolic compounds tended to irreversibly associate with proteins via non–covalent and covalent interactions, resulting in the increased accumulation of phenolics at the oil–water interface, and hence, successfully improved their antioxidant capability (Bonoli-Carbognin *et al.,* 2008; Oda *et al.,* 1998; Almajano *et al.,* 2004, 2007; Aewsiri *et al*., 2009, 2010, 2011). Heinonen *et al.,* (1998) reported that BSA bound with ferluric acids successfully improved the prohibition of hexanal formation in lecithin–liposome system. This tendency was also observed in the sunflower O/W and W/O emulsions: The oxidation rate was significantly decreased by the synergistic effect of BSA–caffeic acid (Conde *et al*., 2011), ovalbumin–catechin, and BSA–catechin adducts (Almajano *et al.,* 2007). Covalently linking of oxidized phenolic compounds with cuttlefish skin gelatin could improve antioxidant ability of the gelatin (Aewsiri *et al.,* 2009, 2011). Hydrophobicity of the protein molecules after modification with phenolic compounds played important role in stability of the emulsion system (Aewsiri *et al*., 2011).

To prepare the plant phenolic extracts, various solvents have been used. González-Montelongo *et al.,* (2010) reported that the banana peel extracts derived by using a mixture of acetone and water (1:1) showed the highest yield and most effective antioxidant activity compared to those treated by solely acetone or methanol. However, methanol was reported to be the most selective solvent for

grape pomace extract (Pinelo *et al.,* 2005). Goli *et al*., (2005) extracted the phenolics from pistachio hull and suggested that the extractability could be promoted by increasing a solvent polarity. By using a dissimilar extracting solvents, different phenolic compounds were liberated, and hence, vaying antioxidative capacity of the extracts. The main phenolics present in rice hull extracts were cinnamic acid and benzoic derivatives, when using MeOH:H_2O (75:25) as the solvent (Asamari *et al*., 1996), whereas *p*-coumaric acid was predominant when 100% MeOH was used (Nam *et al*., 2004). Wu *et al*., (1994) reported that the metanolic and ethanolic rice hull extracts exhibited better ability to inhibit oxidation in ground beef and lard models than the ethyl acetate rice hull extract. Generally, increase temperature affected to enhance phenolic liberation because high temperature could promote diffusion rate and solubility of the analytes in solvent, thereby better antiradical activity of the extracts could be expected (Pinelo *et al.,* 2005; González-Montelongo *et al.,* 2010). Extraction using a severe condition, however, might cause the degradation of the phytochemical compounds (Larrauri *et al.,* 1997). The elevated temperature often induces the interaction between the components of plant materials, thus lowering the extractability (González-Montelongo *et al*., 2010).

4. Research Methodology

4.1. *Study on the emulsifying and antioxidative properties of rice bran protein hydrolysates (RBPH)*

4.1.1 Preparation of rice bran

The defatted bran of rice (*Oryza sativa* L., cultivar Thai Hom Mali) was received from Thai Edible oil CO., LTD. (Bangkok, Thailand). The bran was stored in a polyethylene bag at 4°C for less than 3 months before use. The approximate analysis (protein, ash, fat, moisture, and carbohydrate) of the defatted rice bran was performed (AOAC, 2000).

4.1.2 Preparation and characterization of RBPH

RBPH was prepared using Viscozyme and Protease following the modified methods of Paraman *et al.,* (2007) and Bandyopadhyay *et al.,* (2008) as shown in Fig. 1.

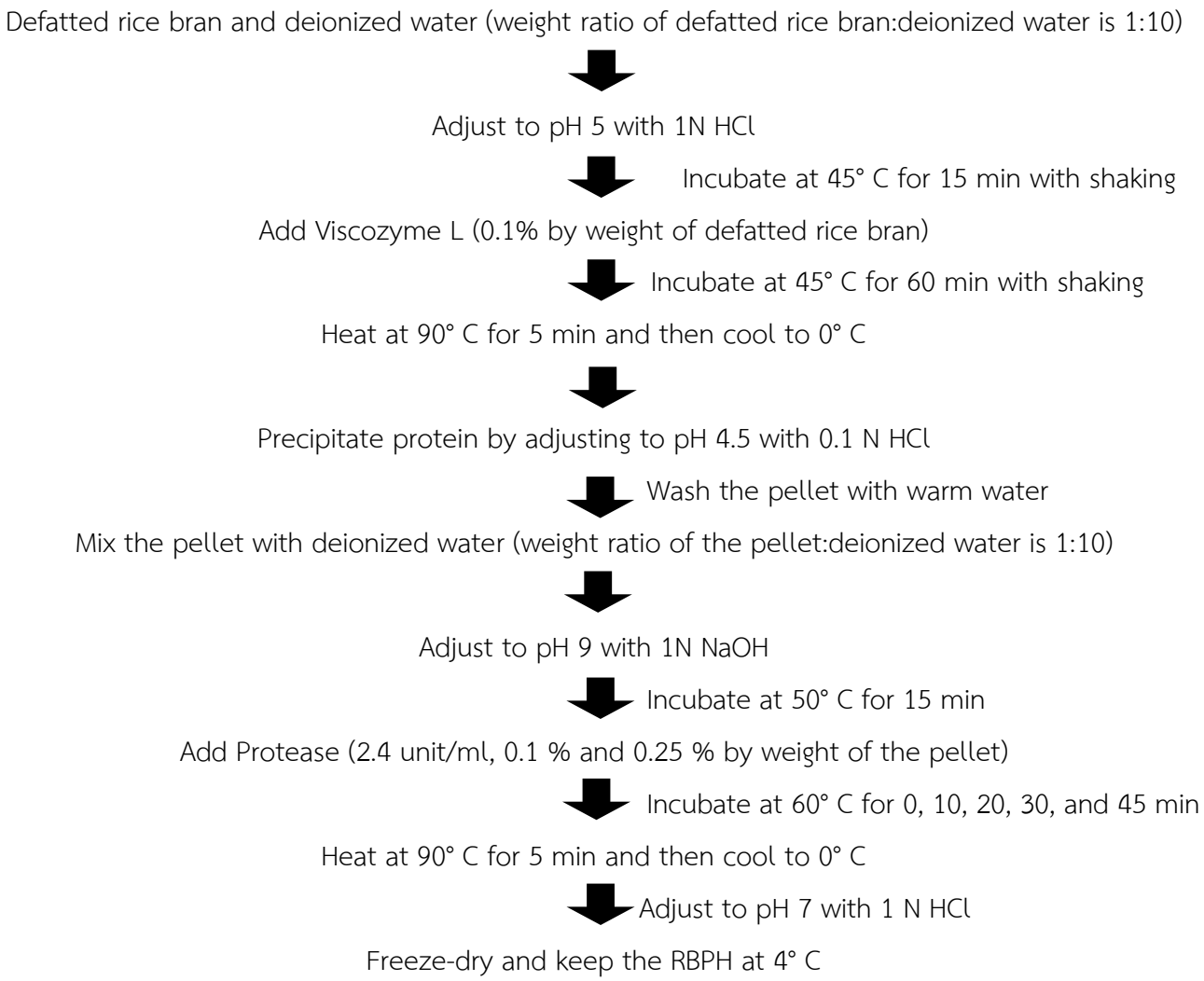

Fig. 1 Preparation of RBPH via the Viscozyme and Protease aided extraction

All RBPH samples will be subjected to analyses:

- Degree of hydrolysis (DH) using 2,4,6–trinitrobenzene acid (TNBS) assay (Alder-Nissen, 1979)
- Molecular size using tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970)
- Surface hydrophobicity using 1–anilinonaphthalene–8–sulfonic acid (ANS) assay (Hayakawa and Nakai, 1985)
- Soluble protein content by Bradford assay (Bradford, 1976)

4.1.3 Study on emulsifying property and antioxidative ability of RBPH

4.1.3.1 Emulsifying property

RBPH was dissolved in 10 mM phosphate buffer pH 7 at various concentrations (0.5, 0.75 and 1 % w/v) with the presence of 0.02 % NaN_3. The RBPH solution was then homogenized with soybean oil

(10 % v/v) at 20000 rpm for 5 min. The emulsion sample was kept at room temperature and the colloidal stability indices were determined at different storage times.

- Oil droplet size using optical microscope or laser particle size analyzer
- Emulsifying ability index (EAI) and turbidity estimation according the methods of Pearce and Kinsella (1978) and Rangsansarid and Fukada (2007), respectively

4.1.3.2 Antioxidative ability

The RBPH based O/W emulsions was prepared employing stripped soy bean oil as a dispersed phase. The indigenous tocopherol and impurity present in the oil was firstly removed via the solvent–free method, as per method of Maldonado-Valderrama *et al*., (2008). Emulsion contains RBPH at different levels was stored in a screw capped bottle at 37°C in the dark. Progressive of lipid oxidation was monitored along a period of 2 weeks, by measuring.

- Peroxide value (PV) according to the method of Sakanaka *et al*., 2004
- Thiobarbituric acid reactive substances (TBARS) by the procedure described by Tong *et al.,* (2000)

4.2 Study on effect of rice hull phenolic extract on the physical and oxidative stability of RBPH based O/W emulsion

4.2.1 Preparation of rice hull phenolic extracts (RHPE)

The hull of (*Oryza sativa* L., cultivar Sangyod) received from rice milling community enterprise (Khuan-Khanun, Phatthalung) was pulverized and passed through a 40–mesh sieve before keeping in a plastic container at -20° C until use. Mixtures of methanol–water at different mixing ratios (3:1, 2:1, and 1:1) were used as extracting media. The extraction was carried out at 30 or 50°C for various times. The RHPE preparation procedure is depicted in Fig. 2.

Ground rice hull and solvents (ratio 1:5, w/v)

↓ Homogenize at 12,000 *g* for 1 min

Incubate in water bath at 30° C or 50° C for 0, 30, 60, 120, 180, and 300 min

↓

Filter through Whatman No. 42 filter paper

↓

Freeze–dry and store at -20° C for less than 1 month

Fig. 2 RHPE preparation (modified from Goli *et al.,* 2005)

The extracts were then subjected to analyses.

- Total phenolic content (TPC) by Folin–Ciocalteu assay, according to the method of Javanmardi *et al*., (2003). TPC was expressed as gallic acid equivalent per gram dry weight of sample.
- Phenolic profile analysis using HPLC as per method of Arslan *et al*., (2013)
- The DPPH (2,2–diphenyl–1–picrylhydrazyl) radical scavenging activity (Aewsiri *et al*., 2009)
- The ABTS (2,2–Azinobis (3–ethylbenzothiazoline–6–sulfonic acid) diammonium salt) assay by the method of Aewsiri *et al*., (2009)
- Metal chelating activity on Fe^{2+} (Decker and Welch, 1990)

4.2.2 Study on the effects of RHPE on the stability of the RBPH based O/W emulsion

RHPE prepared by the selected condition was incorporated into the RBPH stabilized emulsion through different stepwises: Before and after emulsification process. The emulsion preparation is depicted in Fig. 3.

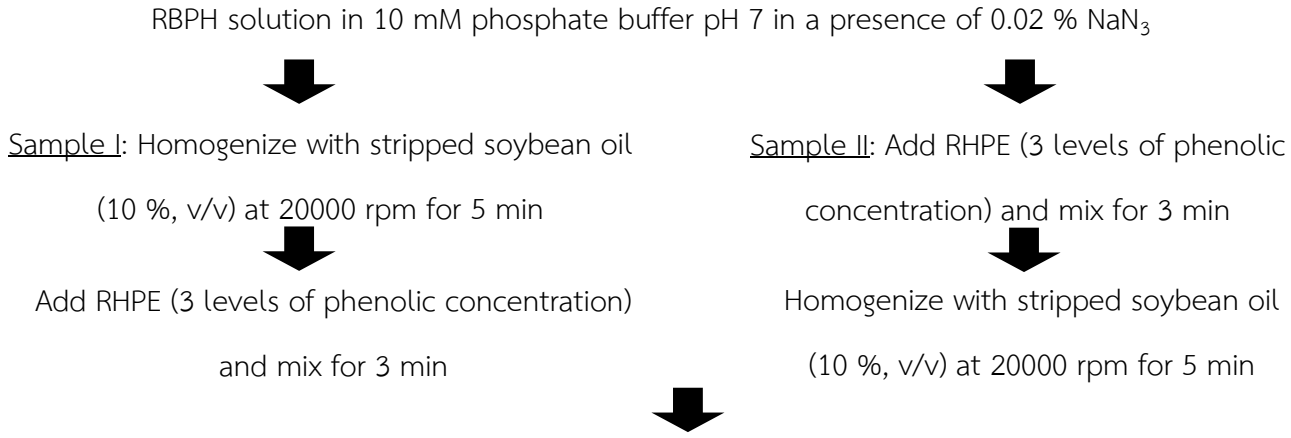

Fig.3 Emulsion preparation with different RHPE incorporating steps (modified from Cheng *et al.,* 2010a)

Colloidal stability of the prepared emulsion was investigated by measuring oil droplet size, EAI, ζ-potential, and turbidity loss rate as the aforementioned methods. Moreover, degree of lipid oxidation occurring in the samples was evaluated measuring PV and TBARS content.

4.3 Study on the effects of RBPH and RHPE on physical and oxidative stability of O/W emulsions with different oil components

Influence of RBPH and RHPE on the physicochemical stability of the emulsion model was further elucidated, by varying the types of oils. Different oil types, *i.e.,* soybean oil (SBO), rice bran oil (RBO), and palm olein (PO) were employed as a dispersed phase. Dispersibility and oxidative stability of the emulsions were then monitored as per the measurements mentioned earlier.

4.4 Statistical analysis

The experiments were run in triplicate, and the data were reported as means ± standard deviations. Two–way analysis of variance (ANOVA) was used and means comparison was performed using Duncan multiple range test by the SPSS statistic program (Version 10.0; SPSS Inc., Chicago, IL, USA).

5. Results and Discussion

5.1. Study on the emulsifying and antioxidative activities of rice bran protein hydrolysates

5.1.1 Preparation of protein hydrolysates

Chemical composition of the bran of rice (*Oryza Sativa*, L.) was firstly quantified as shown in Table 1. The bran contained a trace amount of fat, *ca*. 1 %. The protein content was *ca.* 15 % which was in agreement with the previous results, reporting as 12–20 % (Tang *et al*., 2002) and 15.4 % (Hamada, 2000). This result suggested that the defatted rice bran which is a by–product from rice bran oil processing might be a promising source of protein.

Table 1 Chemical composition of the defatted rice bran

compositions	content (%, wet basis)
protein	15.38±0.06
lipid	1.11±0.0.07
moisture	9.24±0.11
ash	9.12±0.11
carbohydrate	*ca*. 65.15

Means ± standard deviations (*n*=3) were shown.

To isolate protein from rice bran, a dual carbohydrase aided extraction was carried out, according to the procedure of Cheetangdee (2014). The bran was treated with Viscozyme–L (0.1 % by weight of bran) at 45°C pH 5 for 1 h, and then subsequently treated with α–amylase (0.1 % by weight of bran) at 45°C pH 6.2 for 1 h. During the extraction, a mechanical force was incorporated by sonicating at 40 mHz, in order to enhance protein liberation from the bran (Cheetangdee, 2014). By using this method, protein could be isolated from the bran with the extraction yield of *ca.* 50 %. The rice bran protein isolate was then hydrolyzed using Protease (0.75 % or 1 % by weight of the protein isolate) at various hydrolytic times to prepare rice bran protein hydrolysates. Fig. 4 illustrates degree of hydrolysis (DH) of the hydrolysates prepared at different Protease concentrations and hydrolytic times. Increasing of DH with hydrolytic time was observed, and higher DH was found when Protease was applied at higher concentration: After reacting for 45 and 60 min, the DH of 0.75 % (1 %) Protease treated RBPH was *ca.* 7.5 and 10 % (9.5 and 11 %), respectively.

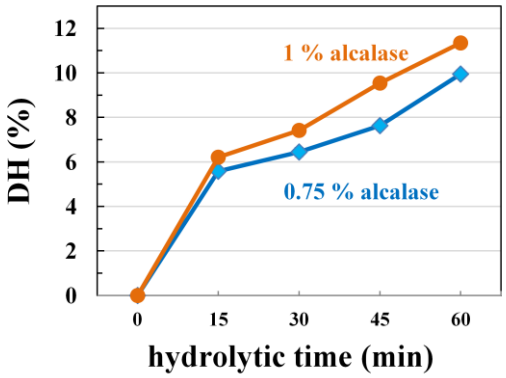

Fig. 4 Degree of hydrolysis (DH) of the bran protein hydrolysates prepared by using 0.75 % (◆) and 1 % (●) Protease at various hydrolytic times. Means ± standard deviation (*n*=3) were shown.

The electrophoretograms of the hydrolysates prepared at different Protease concentrations and hydrolytic times were depicted in Fig. 5. Decrease in molecular size of the proteins with DH increasing was reported. Upon hydrolysis, the peptides tended to decrease their size: The predominant bands of the rice bran protein isolate (hydrolytic time 0 h) was *ca.* 40 kDa (see lanes 2 and 8), whereas the bands at the MW of *ca.* 29 kDa was gradually appeared with increasing hydrolytic time. At the same hydrolytic time, the band at the MW of *ca.* 29 kDa became more distinct when Protease was applied

at increase concentration, suggesting more pronounced decreasing of peptide size. Extending a hydrolysis reaction, the band at *ca*. 15 kDa expected to be rice prolamin became more distinct. Barber *et al*., (1998) suggested that rice prolamin consisted of three polypeptide subunits with the MW of 10, 13, and 16 kDa. Tang *et al*., (2003b) preparing RBPH via Protease-*P* aiding found the peptides with MW in the range of 6.5–66.2 kDa with the dense bands at 6.5 and 14.4 kDa. The alcalase treated RBPH with the DH of 7.5 % consisted of medium (10–90 kDa) and small (1–3 kDa) sized peptides with the contents of *ca*. 85.3 and 5.3 %, respectively (Hamada, 2000). Dominant difference between the SDS-PAGE patterns of the RBPH in non-reducing and reducing conditions was not found in this study.

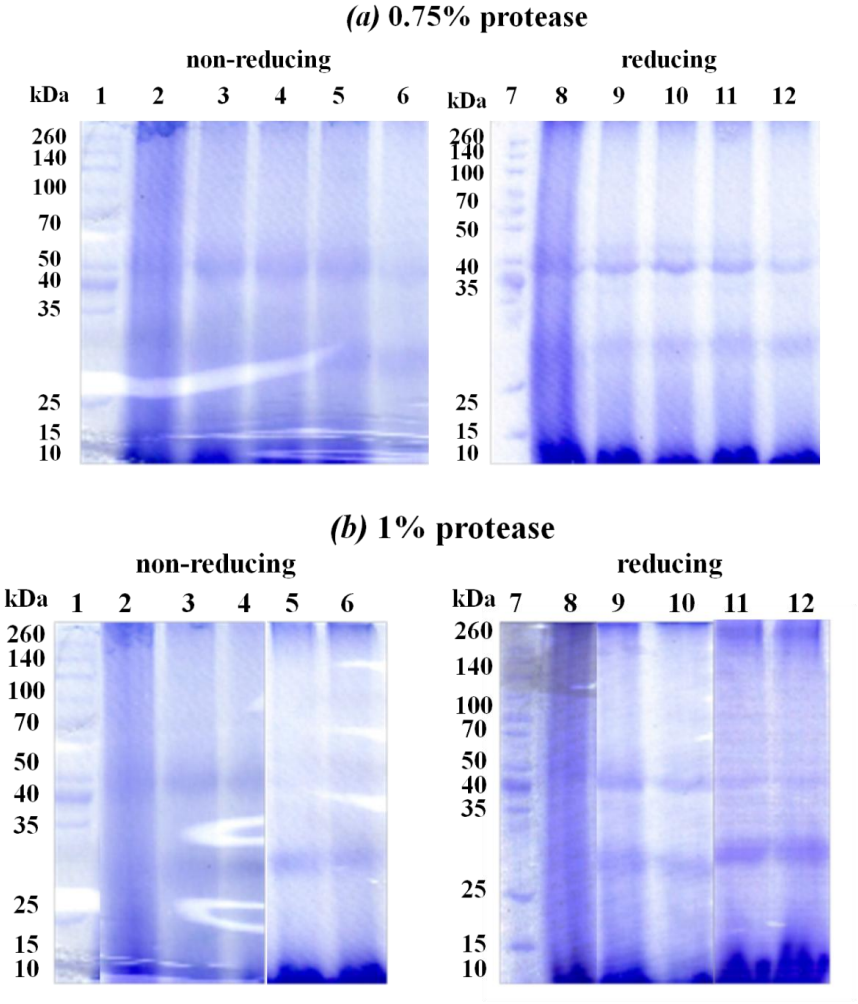

Fig. 5 Electrophoretograms of the rice bran protein hydrolysates prepared by (***a***) 0.75 % and (***b***) 1 % Protease at various hydrolytic times: *lane 1* and *7*, molecular weight marker; *lane 2, 3, 4, 5, 6* (*8, 9, 10, 11*, 12) RBPH prepared at hydrolytic times of 0, 15, 30, 45, and 60 min in non-reducing (reducing) conditions, respectively.

Hydrophobicity of the hydrolysates prepared at various hydrolytic times was determined as depicted in Fig. 6. S_0 is one of the important factors governing emulsifying activity of proteins (Phillips *et al*., 1994). S_0 was gradually increased with hydrolytic time, and higher S_0 was observed when the peptides were treated with Protease at higher concentration. Increasing of S_0 was expected since an exposure of some originally buried hydrophobic amino acids due to a partial denaturation of the hydrolyzed proteins (Qi *et al*., 1997; Paraman *et al*., 2006). Development of S_0 with increasing DH was also observed in hydrolyzed rice bran (Tang *et al*., 2003b), rice endosperm (Paraman *et al*., 2006) and soy (Qi *et al*., 1997) proteins. Higher S_0 might effect to promote surface activity of proteins to interact with non–polar phase, and thereby effecting to enhance emulsifying activity of proteins (Qi *et al*., 1997).

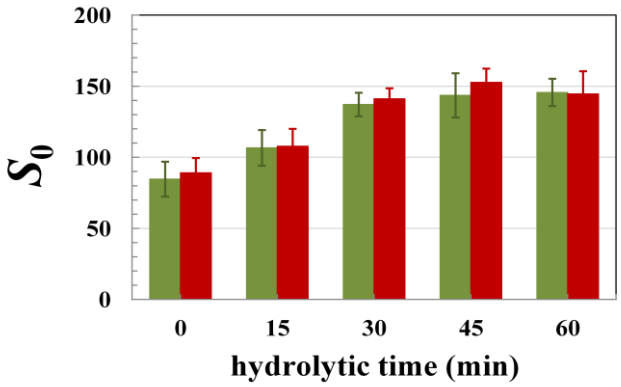

Fig. 6 Surface hydrophobicity (S_0) of the bran protein hydrolysates prepared by using 0.75% (■) and 1% (■) Protease at various hydrolytic times. Means (n=3) ± standard deviation were shown.

5.1.2 Emulsifying activities of the protein hydrolysates

The rice bran protein hydrolysates prepared by using Protease at different concentrations and hydrolytic times were employed as emulsifier to prepare soybean oil-in-water (O/W) emulsions at different concentrations (0.5, 0.75, and 1% w/v). Colloidal stability of the emulsions was then observed. Fig. 7 shows emulsifying ability index (EAI) of the emulsions stabilized by the hydrolysates at different concentrations. Higher EAI was observed for the emulsions stabilized by the hydrolysates prepared by 1 % Protease compared to the counterpart prepared using 0.75 % Protease. This tendency implied better capacity of the former peptides to facilitate emulsion formation, which might be postulated since their higher S_0 (see Fig. 6). Hydrophobic amino acids exposed due to hydrolysis

process might effect to enhance interfacial activity, resulting in improved emulsion formability of the proteins (Qi *et al*., 1997). Better emulsifying activity, moreover, might cause from improved solubility, since decreasing in the size, of peptides upon hydrolysis process (Qi *et al*., 1997; Bandyopadhyay and Ghosh 2002; Bandyopadhyay *et al*., 2008; Paraman *et al*., 2007; Tang *et al*., 2003b). Higher solubility affected to facilitate diffusion and spreading of proteins at the oil–water interfacial areas, so more protein content localizing at the interfaces during emulsification process could be expected (Qi *et al*., 1997). Improved solubility of proteins by hydrolysis reaction was previously confirmed (Qi *et al.*, 1997). In the present study, the hydrolysates with higher DH could develop EAI of the peptides. Interestingly, the highest EAI was observed for the emulsions stabilized by the hydrolysates prepared by 1 % Protease for 45 min, irrespective of the hydrolysate concentrations. Longer hydrolytic time, or in turn higher DH, than this level led to lower EAI. DH played a crucial role on functional properties of protein hydrolysates. Qi *et al.,* (1997) reported that EAI of soy protein hydrolysates was increased with DH, till the maximum value was reached at the DH of 15 %. Nonetheless, the peptides with DH higher than 15 % led to lowered EAI. Casein hydrolysates exhibited the improved emulsifying activity with DH level up to 8 %, whereas more pronounced DH than this resulted in inferior functional properties of the hydrolysates (Hu *et al*., 2003).

Considering on the concentration of protein hydrolysates, decreased EAI with increasing peptide concentration was evident at the same hydrolytic time: The EAI values of the emulsions stabilized by the hydrolysates prepared using 0.75 % (1 %) Protease for 45 min were *ca*. 0.7, 0.6, and 0.5 (0.95, 0.7, and 0.6) m^2/g, respectively.

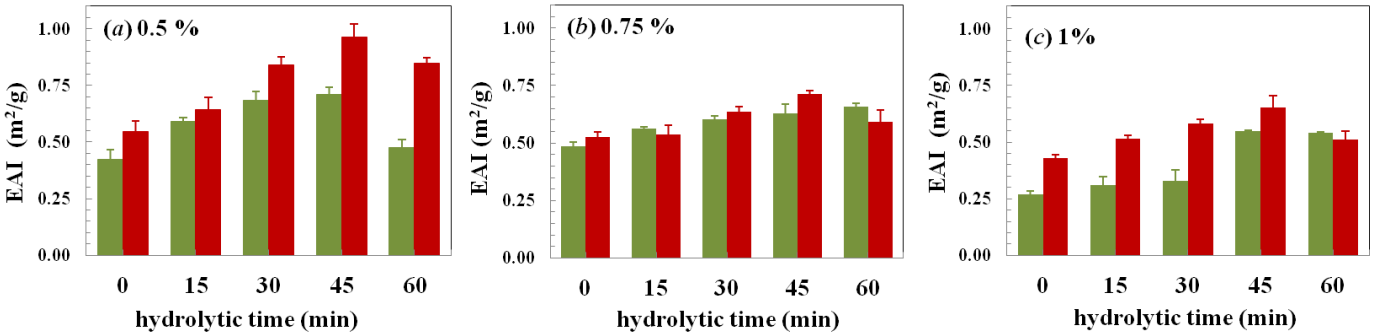

Fig. 7 Emulsion stability index (EAI) of soybean O/W emulsions stabilized by the hydrolysates prepared by using 0.75 % (■) and 1 % (■) Protease at various hydrolytic times (0-60 min). The concentrations of the hydrolysates were (***a***) 0.5 %, (***b***) 0.75 % and **(*c*)** 1 % and oil volume of the emulsion was 10 %. Mean ± standard deviation (*n*=3) were shown.

Turbidity loss rate, the indicator suggesting emulsion dispersibility under accelerated condition (dilute form) (Labuza *et al.,* 1991), was estimated. The emulsion samples were diluted to the oil volume of 0.25 %, before reading the absorbance at 500 nm. Note that, less turbidity loss rate (lower in absolute value) indicates to a slower change in emulsion turbidity, thus implying higher emulsion stability than the larger ones.

Fig. 8 depicts the transmission loss rate (ΔT) of the emulsions. The ΔT generally tended to decrease with increasing concentration of the hydrolysates, suggesting to the improved emulsion dispersibility: ΔT of the emulsions stabilized by the hydrolysates prepared by 0.75 % (1 %) Protease for 15 min was *ca.* -0.1, -0.08, and -0.06 (-0.07, -0.06, -0.05), when the hydrolysates concentrations were 0.5 %, 0.75 %, and 1 %, respectively. At high DH level (*ca.*≥10 %), however, ΔT became larger, when the hydrolysates concentration was increased: ΔT of the emulsions stabilized by the hydrolysates prepared by 0.75 % (1 %) Protease for 60 min was *ca.* -0.066, -0.044, and -0.051 (-0.042, -0.041, -0.064), at the hydrolysates concentrations of 0.5 %, 0.75 %, and 1 %, respectively.

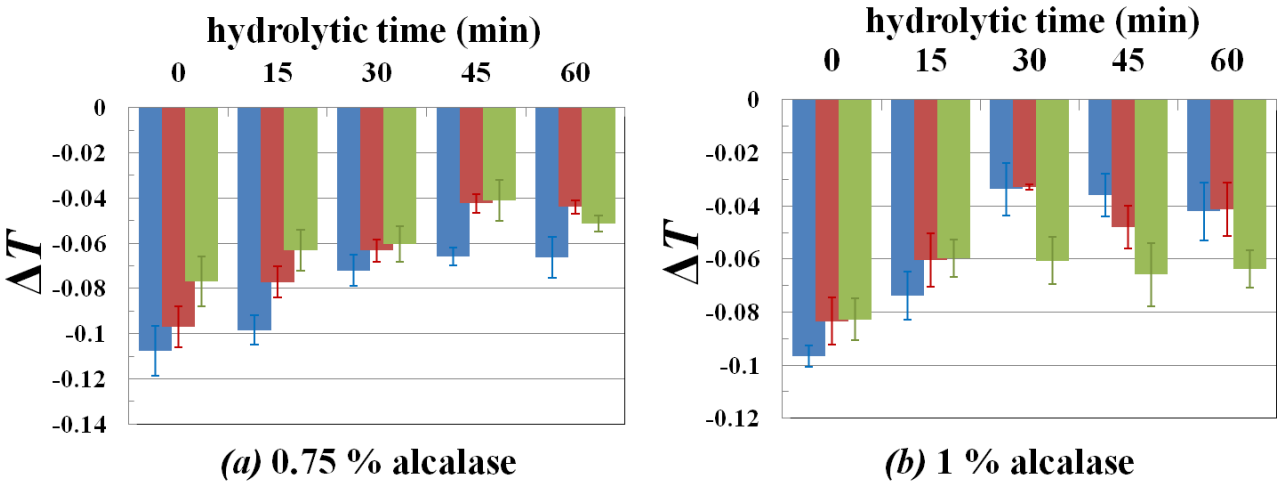

Fig. 8 Transmission loss rate (ΔT) of the emulsions stabilized by the hydrolysates prepared using ***(a)*** 0.75 % and ***(b)*** 1 % Protease at various hydrolytic times (0–60 min). The concentrations of the hydrolysates used to stabilize emulsions were 0.5 % (■), 0.75 % (■), and 1 % (■). Means ± standard deviations (n=3) were shown.

Fig. 9.1 and 9.2 illustrate mean diameter of the emulsions stabilized by the hydrolysates prepared using 0.75 % and 1 % Protease at various hydrolytic times, respectively. Smaller oil droplets could be observed when the emulsions were stabilized by the hydrolyzed peptides, compared to the unhydrolyzed counterpart (the hydrolytic time of 0 h). This result indicated improved emulsifying activity of the peptides upon hydrolysis process. Interestingly, the hydrolysates with DH of *ca*. 8 % (the ones prepared by using 0.75 % and 1 % Protease for 45 and 30 min, respectively) providing the smallest d_{32} compared to the others, when RBPH was applied at the concentration of 0.75 %. This is in well accordance with the tendency in turbidity loss rate expressed in Fig. 8.

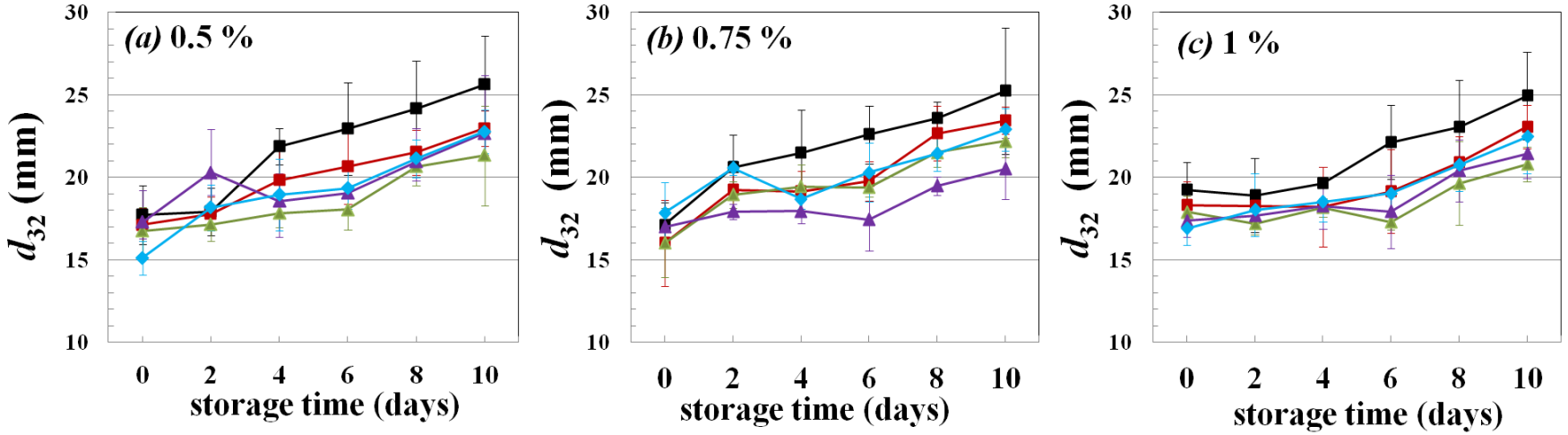

Fig. 9.1 Mean diameter (d_{32}) of the emulsions stabilized by the hydrolysates at different concentrations: ***(a)*** 0.5 %, ***(b)*** 0.75 %, and ***(c)*** 1 % as a function of storage times. The hydrolysates were prepared by using 0.75 % Protease at various hydrolytic times: 0 (■), 15 (■), 30 (▲), 45 (▲), and 60 (▲) min. Means ± standard deviations (n=3) were shown.

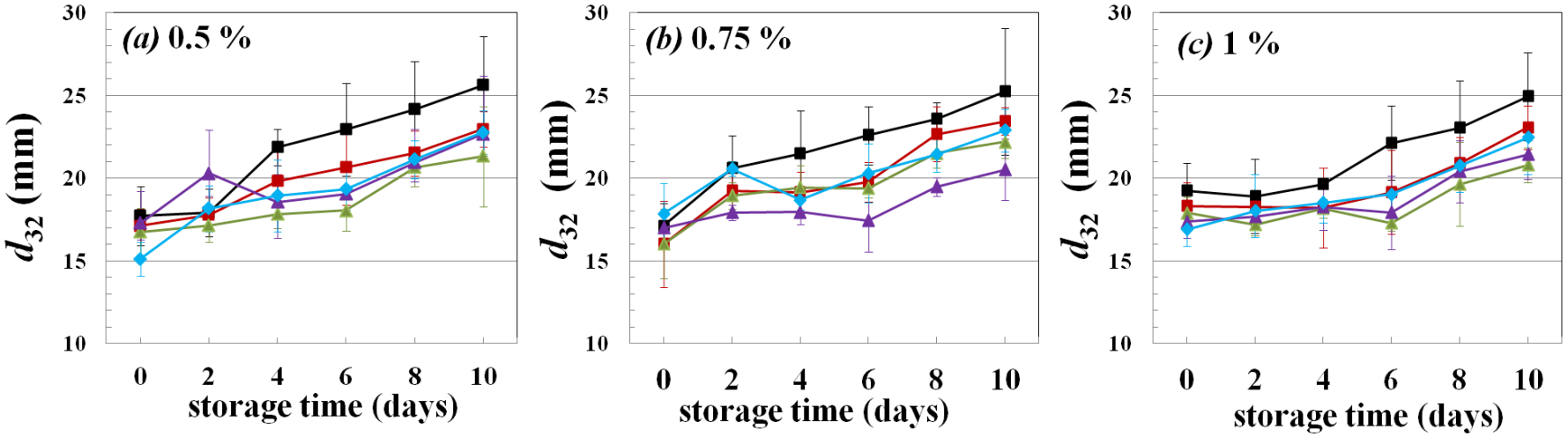

Fig. 9.2 Mean diameter (d_{32}) of the emulsions stabilized by the hydrolysates at different concentrations: ***(a)*** 0.5 %, ***(b)*** 0.75 %, and ***(c)*** 1 % as a function of storage times. The hydrolysates were prepared by using 1 % Protease at various hydrolytic times as remarked in Fig 9.1. Means ± standard deviations (n=3) were shown.

5.1.3 Antioxidant properties of the protein hydrolysates

To observe antioxidant activities of the hydrolysates, soybean O/W emulsions were prepared employing Tween 20 (0.5 %) as an emulsifier to ensure emulsion dispersibility. The hydrolysates were added to the emulsions at different concentrations (0.5, 0.75, and 1 %, w/v), and progressive of lipid oxidation was monitored measuring peroxide value (PV) and thiobarbituric reactive substances (TBARS) formation during a storage period of 2 weeks.

Fig. 10.1 and 10.2 reveal PV development of the emulsion samples incorporated with the hydrolysates prepared using 0.75 % and 1 % Protease at various hydrolytic times, respectively. From Fig. 10.1, a lag phase of 2 days was observed for all emulsions, and PV was then increased in different manners, depending on the added hydrolysates. For the control sample, PV gained drastically to reach the maximum at *ca*. 7 mg hydroperoxide eq/L, after keeping for 8 days, before turning to decline. A longer lag phase of 10 days was observed for the emulsions containing the hydrolysates prepared by 1 % Protease (Fig. 10.2). The decreased PV could be expected, because hydroperoxides were unstable product that could degrade and/or interact with other species through oxidative process (Cheng *et al*., 2010a). The hydrolysates prepared by both of 0.75 and 1 % Protease at the hydrolytic times of 0 and 15 min showed the least ability to prohibit PV formation, as suggested by higher PV than the others. For the hydrolysates prepared at the hydrolytic times of 30–60 min, ability to suppress PV formation depended on the applied concentration. Lower PV was observed with increasing hydrolysates concentration. When the hydrolysates prepared using 1 % Protease for 30 and 45 min were added, the PV of *ca.* 2.5, 2, and 1.7 mg hydroperoxide eq/L was observed for the emulsions containing the hydrolysates at the concentrations of 0.5, 0.75, and 1 % (see Fig. 10.2). For the emulsions incorporated with the hydrolysates treated by 1 % Protease for 30–60 min, the PV continuously increased through 2 weeks when the hydrolysates concentrations were 0.75 % and 1 %, (see Fig. 10.2d). These results suggested better ability of the smaller peptides to retard primary product formation of lipid oxidation than did the bigger ones.

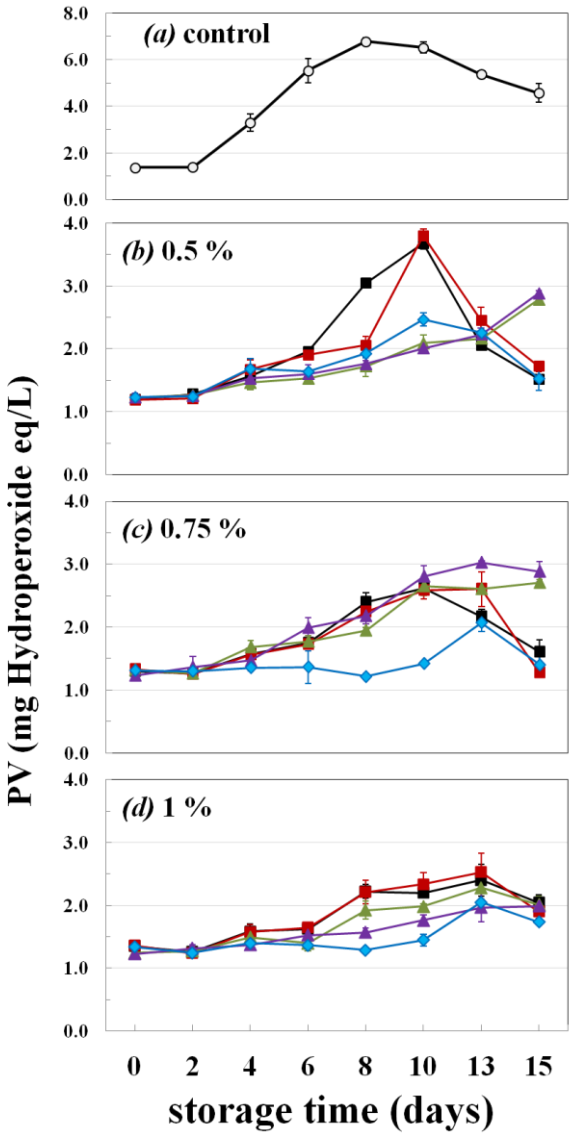

Fig. 10.1 Peroxide value (PV) of the emulsions incorporated with the hydrolysates prepared by using 0.75 % Protease at various hydrolytic times as remarked in Fig 9.1. The emulsions contained the hydrolysates at different concentrations: ***(a)*** control emulsion (emulsion without RBPH adding), **(*b*)** 0.5 %, ***(c)*** 0.75 %, and ***(d)*** 1 %. Means ± standard deviations (*n*=3) were shown.

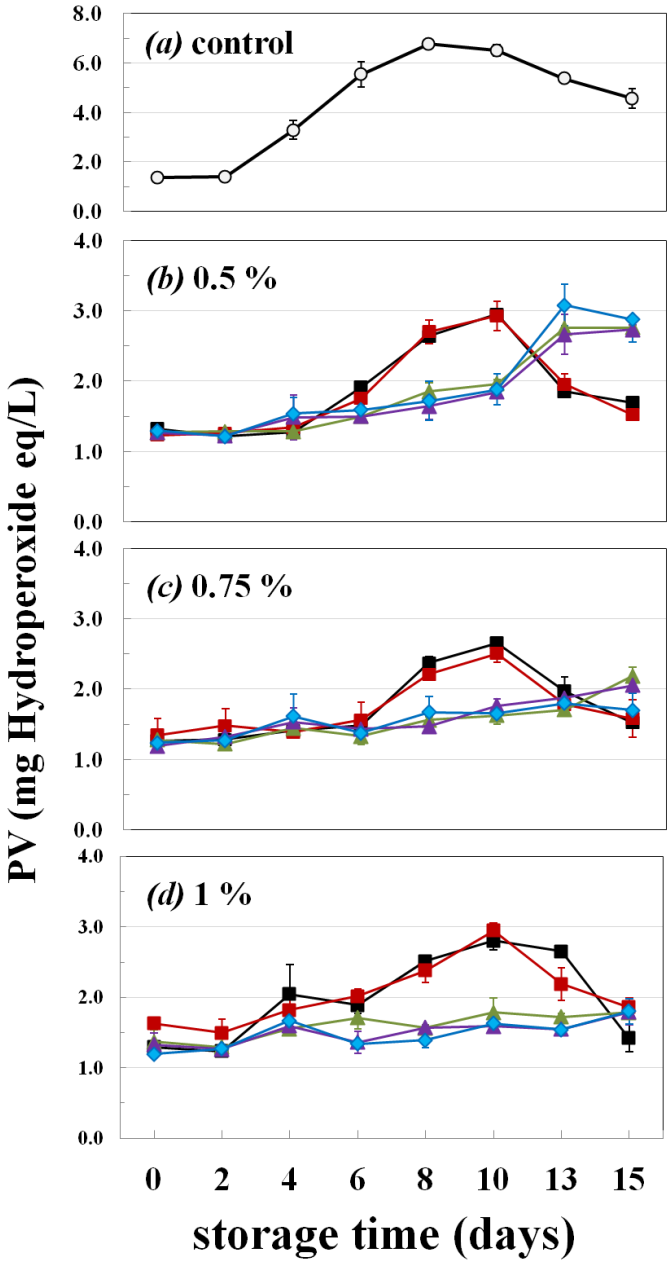

Fig. 10.2 Peroxide value (PV) of the emulsions incorporated with the hydrolysates prepared by using 1 % Protease at various hydrolytic times as remarked in Fig 9.1. The emulsions contained the hydrolysates at different concentrations: ***(a)*** control emulsion (emulsion without RBPH adding), **(*b*)** 0.5 %, ***(c)*** 0.75 %, and ***(d)*** 1 %. Means ± standard deviations (*n*=3) were shown.

Fig. 11.1 and Fig. 11.2 illustrate TBARS content present in the emulsions incorporated with the hydrolysates prepared using 0.75 % and 1 % Protease at various hydrolytic times. Higher TBARS was observed for the control sample, compared to the emulsions added with the hydrolysates. TBARS of the control was continuously increased and got the highest value at *ca.* 1.8 mg malondialdehyde (MDA) eq/L after 10 days of storage. For the emulsions containing the hydrolysates hydrolyzed for 0 and 15 min, there was a slower increasing of TBARS through 2 weeks and the highest value of *ca*. 1.1 mg MDA eq/L was observed at the hydrolysates concentration of 1 %. For the emulsions incorporated with the hydrolysates hydrolyzed for 30–60 min, there was almost no change in TBARS, excepted when the hydrolysates were added at 0.5 % (see Fig. 11.1a and 11.2a).

According to the present results, the hydrolysates from rice bran proteins could improve oxidative stability of the model O/W emulsions in different manners, depending on DH and applied concentration. Ability of potato protein hydrolysates to retard lipid oxidation in Tween 20 based emulsion was reported and supposed since improved hydrophobicity of the peptides after hydrolysis process (Cheng *et al*., 2010a). In emulsion system, hydrophobicity played a crucial role to govern antioxidative capacity of peptides: Higher hydrophobicity of the peptides, better solubility in non–polar phase, and hence, better interfacial activity. This effected to facilitate an adsorption of peptides to the interfacial areas to form a strong interfacial film that act as a physical barrier to prevent pro–oxidants approaching to lipid phase (Cheng *et al*., 2010a, b; Rajapakse *et al.,* 2005; Saiga *et al*., 2003). Development of antioxidant activity of the peptides with increasing of hydrophobicity has been confirmed (Tang *et al*., 2009; Rajapakse *et al*., 2005; Saiga *et al*., 2003; Xie *et al.,* 2008; Cheng *et al*., 2010a, b).

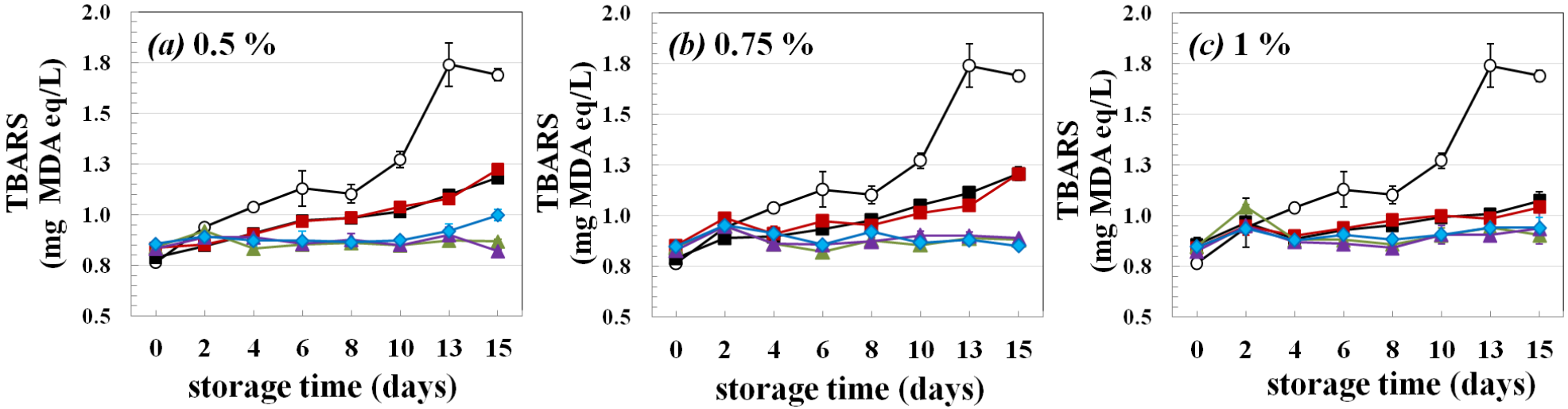

Fig. 11.1 TBARS of the emulsions added with the hydrolysates at different concentrations *(a)* 0.5 %, *(b)* 0.75 %, and *(c)* 1 % at various storage times. The different symbols indicated the control (emulsion without RBPH adding, ○), emulsions with the hydrolysates prepared by using 0.75 % Protease at 0 (■), 15 (■), 30 (▲), 45 (▲), and 60 (▲) min. Means ± standard deviations (*n*=3) were shown.

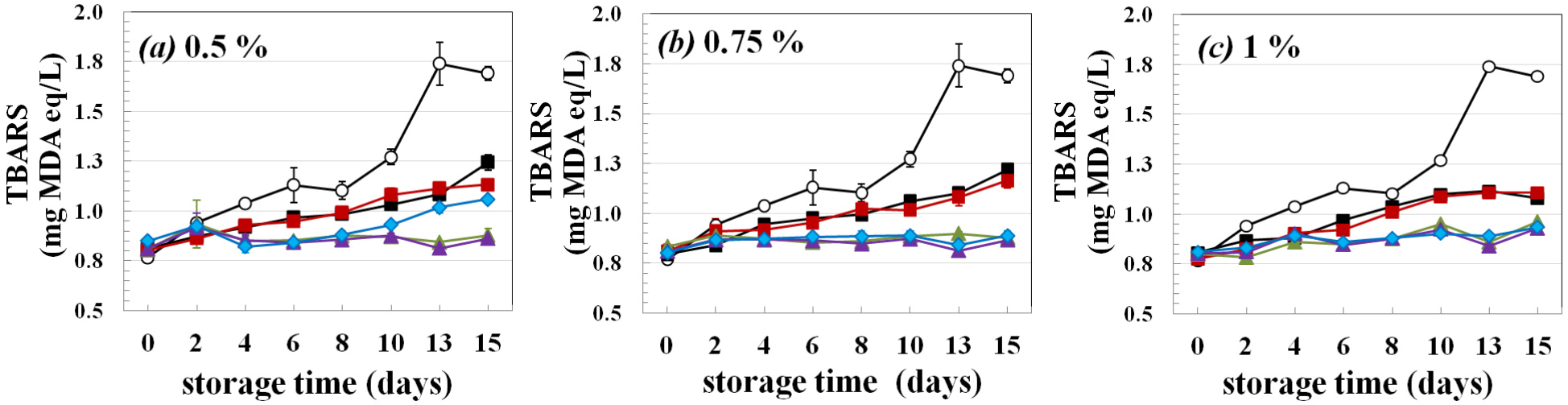

Fig. 11.2 TBARS of the emulsions added with the hydrolysates at different concentrations ***(a)*** 0.5 %, ***(b)*** 0.75 %, and ***(c)*** 1 % at various storage times. The different symbols indicated the control (emulsion without RBPH adding, ○), emulsions with the hydrolysates prepared by using 1 % Protease at 0 (■), 15 (■), 30 (▲), 45 (▲), and 60 (▲) min. Means ± standard deviations (*n*=3) were shown.

Moreover, the antioxidant activity of the hydrolyzed peptides might be attributed to a decreasing in molecular size (Moure *et al*., 2006; Li *et al*., 2008; Peña-Ramos *et al.,* 2004; Wang *et al.,* 2007; Suetsuna *et al*., 2000). Potent antioxidant activity tended to be observed in low MW peptides: The effective antioxidant activity of chickpea protein hydrolysate, potato protein hydrolysate, and alfafa leaf protein hydrolysate was found for the fractions with MW ranging from 200–3000 Da (Li *et al*., 2008), 500–900 Da (Cheng *et al*., 2010a), and < 3kDa (Xie *et al.,* 2008), respectively. Improved free radical scavenging activity of the peptides could be observed when their MW was decreased (Li *et al.,* 2008; Cheng *et al*. 2010a; Xie *et al.,* 2008). Antioxidant activity of the hydrolysates found in the present work, therefore, might be supposed since some exposed hydrophobic amino acid residues as indicated by increased S_0, and also decreasing in peptide size after hydrolysis.

Hydrolyzed peptides could exhibit antioxidative properties in various ways such as ferrous iron chelating ability (Wang and Xiong, 2005; Pownall *et al*., 2010) and radical scavenging effect (Cheng *et al.,* 2010a; Jao and Ko, 2002; Li *et al*., 2008; Pownall *et al*., 2010; Park *et al*., 2012). Antioxidant activity of hydrolyzed peptides could be supposed due to a presence of some amino acid residues, *e.g.,* Tyr (Wang and Xiong, 2005; Xie *et al*., 2008), Phe (Kudo *et al*., 2009; Pownall *et al*., 2010), Trp, Leu, Val (Pownall *et al.,* 2010), Cys, Met, and His (Xie *et al.,* 2008). It has been suggested that the predominant amino acids in rice bran protein isolate and protease treated rice bran proteins were sulfur–containing amino acids, *i.e*., Cys and Met (Hamada, 2000). These amino acids played important role to protect cell membrane from oxidative stress by subjecting to be precursors of important natural antioxidants, *i.e*., taurine and glutathione (Horton, 2003). According to their effective antioxidative properties, the hydrolysates prepared using 1 % Protease for 0 and 60 min were selected to determine composited amino acids, and the result was shown in Table 2 (Cheetangdee and Benjakul, 2015). The selected RBPH was rich in Glu/Gly, Arg, Lys, Leu, Pro, and Val, which constituted 34.32, 10.86, 7.06, 5.89, 5.85, and 5.41%, respectively. Acidic amino acids, *e.g*., Glu could effectively quench metal ions through charge interactions (Saiga *et al.,* 2003). Potent free radical scavenging ability of Pro was reported since it was able to donate proton to maintain its stability through resonance mechanism (Rajapakse *et al*., 2005 Yen *et al.,* 2002). Leu and Val were also suggested to be a potent free radical scavenger (Duh *et al*., 1999). Aliphatic amino acids, *e.g*., Arg and Glu, powerfully acted as a ligand to reduce free metal availability (Cheng *et al.*, 2010b). Thus, antioxidant activities of the RBPH were more likely generated

by its amino acid composition. Regarding to the increased hydrophobicity, moreover, the affinity between peptides and the used emulsifier might be enhanced, thereby facilitating a partitioning of the peptides at the interfaces. Therefore, peptides could exhibit their antioxidative properties effectively.

Table 2 Amino acid composition (% of total amino acid) of RBPH prepared using 1 % Protease for 0 and 60 min

Amino acids	0 min	60 min
Alanine (Ala)	3.43	4.76
Arginine (Arg)	9.81	10.86
Aspartic acid+Asparagine (Asp+Asn)	23.11	4.40
Glutamic acid+Glycine (Glu+Gly)	25.30	34.32
Histidine (His)	2.62	4.76
Isoleusine (Ile)	2.65	2.30
Leucine (Leu)	5.14	5.89
Lysine (Lys)	6.89	7.06
Phenylalanine (Phe)	3.41	3.79
Proline (Pro)	3.46	5.85
Serine (Ser)	2.19	2.78
Threonine (Thr)	2.30	2.14
Tryptophan (Trp)	0.24	1.57
Tyrosine (Tyr)	4.32	4.07
Valine (Val)	3.89	5.41
Total hydrophobic amino acids[a]	50.14	68.65

[a] Total hydrophobic amino acids: determined from the overall content of Glu+Gly, Ala, Val, Leu, Pro, His, Phe, Trp and Ile (Zhu *et al.,* 2006).

From all points of views, the bran protein hydrolysate prepared by using 1 % Protease for 30 min exhibited the highest emulsifying and antioxidative activities, especially when it was used at the concentration of 0.75 %. This selected hydrolysates was referred as rice bran protein hydrolysates (RBPH) to be used in a further study.

5.2 Study on effect of rice hull phenolic extract on the colloidal and oxidative stability of RBPH based O/W emulsion

5.2.1 Preparation of rice hull extracts containing phenolic compounds

The hull of rice (*Oryza sativa* L, cultivar Sangyod) was used to prepare phenolic extracts via methanolic extraction method. The pulverized hull was mixed with the mixtures of methanol–water (the mixing ratios were 1:1, 2:1, and 3:1, v/v) at the ratio of 1:10. The extraction was conducted at different temperatures (30 or 50°C) for various times (0–300 min). Total phenolic content (TPC) and antioxidant activities of the extracts were then observed.

Table 3 shows TPC of the hull extracts prepared at various conditions. Increasing of extraction time and temperature effected to increase TPC of the extracts ($P<0.05$). The highest TPC of *ca*. 3.8 mg gallic acid eq (GAE)/g rice hull (dry weight) was provided by the extraction using a mixture of methanol–water at the ratio of 3:1 at 50°C for 180 min ($P<0.05$).

Table 3 Total phenolic content (TPC) of the hull extracts prepared at various extraction times, methanol contents, and temperatures

methanol:water	Temperature (°C)	Extraction time (min)					
		0	30	60	120	180	300
1:1	30	2.64±0.03Cd	3.01±0.019CcY	3.16±0.04CbY	2.95±0.05CbY	3.12±00.07CaY	3.05±0.01CaY
	50		3.16±0.05CcX	3.36±0.03CbX	3.30±0.02CbX	3.41±0.04CaX	3.45±0.07CaX
2:1	30	2.78±0.04Bd	2.94±0.07BcY	3.27±0.07BbY	3.19±0. 05BbY	3.38±0.08BaY	3.39±0.06BaY
	50		3.27±0.02BcX	3.45±0.10BbX	3.49±0.11BbX	3.64±0.08BaX	3.75±0.10BaX
3:1	30	2.83±0.06Ad	3.20±0.11AcY	3.20±0.11AbY	3.22±0.05AbY	3.48±0.06AaY	3.77±0.10AaY
	50		3.41±0.05AcX	3.51±0.01AbX	3.45±0.05AbX	3.80±0.08AaX	3.34±0.02AaX

Table 4 DPPH radical inhibition effect (%) of the hull extracts prepared at various extraction times, methanol contents, and temperatures

methanol:water	Temperature (°C)	Extraction time (min)					
		0	30	60	120	180	300
1:1	30	44.03±1.58Ce	60.07±0.89CdY	64.11±0.67CcY	59.77±1.61CbY	60.84±0.82CaY	57.52±0.72CaY
	50		62.92±0.18CdX	66.84±0.82CcX	65.12±0.91CbX	66.25±0.37CaX	68.09±1.08CaX
2:1	30	55.32±0.91Be	57.93±1.86BdY	70.71±2.05BcY	68.39±0.80BbY	73.14±2.24BaY	74.99±0.21BaY
	50		74.39±1.66BdX	72.73±0.31BcX	73.74±1.04BbX	72.13±0.57BaX	77.84±1.07BaX
3:1	30	56.21±0.90Ae	69.76±1.07AdY	69.64±1.38AcY	73.26±1.63AbY	77.72±0.99AaY	79.32±0.36AaY
	50		73.92±2.76AdX	77.24±1.35AcX	73.56±0.54AbX	81.28±0.00AaX	74.63±2.50AaX

Table 5 ABTS radical inhibition effect (%) of the hull extracts prepared at various extraction times, methanol contents, and temperatures

methanol:water	Temperature (°C)	Extraction time (min)					
		0	30	60	120	180	300
1:1	30	24.90±0.92Bd	43.25±2.50BcY	46.01±0.70BbY	42.60±0.70BbY	51.89±1.74BaY	45.08±3.35BaY
	50		50.03±1.74BcX	53.96±1.39BbX	55.48±2.66BbX	59.19±2.74BaX	62.02±1.76BaX
2:1	30	36.16±0.92Ad	43.39±1.59AcY	57.44±3.69AbY	54.06±3.11AbY	58.68±5.21AaY	58.13±3.93AaY
	50		55.75±0.87AcX	62.22±3.12AbX	64.15±1.15AbX	68.04±3.42AaX	72.14±2.63AaX
3:1	30	39.43±2.48Ad	49.38±2.38AcY	51.89±3.18AbY	52.55±3.05AbY	60.02±2.94AaY	71.56±4.00AaY
	50		58.06±0.92AcX	61.16±1.49AbX	59.40±3.58AbX	71.76±2.75AaX	55.41±0.84AaX

Table 6 Reducing power (Abs_{700}) of the hull extracts prepared at various extraction times, methanol contents, and temperatures

methanol:water	Temperature (°C)	Extraction time (min)					
		0	30	60	120	180	300
50	30	0.621±0.007Cd	1.018±0.013CcY	1.098±0.021CbY	0.958±0.024CbY	1.115±0.020CaY	1.056±0.021CaY
	50		1.173±0.018CcX	1.289±0.052CbX	1.230±0.051CbX	1.396±0.061CaX	1.414±0.048CaX
65	30	0.755±0.025Bd	1.019±0.008BcY	1.356±0.015BbY	1.350±0.019BbY	1.338±0.054BaY	1.395±0.035BaY
	50		1.309±0.032BcX	1.453±0.056BbX	1.475±0.024BbX	1.620±0.067BaX	1.581±0.055BaX
75	30	0.847±0.011Ad	1.315±0.021AcY	1.596±0.028AbY	1.527±0.049AbY	1.530±0.019AaY	1.833±0.019AaY
	50		1.498±0.032AcX	1.441±0.019AbX	1.587±0.036AbX	1.866±0.028AaX	1.522±0.004AaX

Table 7 Ferrous chelating effect (%) of the hull extracts prepared at various extraction times, methanol contents, and temperatures

methanol:water	Temperature (°C)	Extraction time (min)					
		0	30	60	120	180	300
50	30	40.81±3.26Ad	50.45±1.69AbX	49.18±3.69AaX	56.35±2.99AaX	56.43±1.70AcX	53.96±1.35AbX
	50		51.94±2.03AbX	55.83±1.62AaX	60.09±1.25AaX	62.86±2.69AcX	67.56±4.55AbX
65	30	28.33±3.48Bd	41.26±1.95BbX	39.61±4.51BaX	49.03±2.03BaX	35.05±3.58BcX	38.34±3.14BbX
	50		35.65±1.25BbX	41.55±2.30BaX	36.55±2.81BaX	27.65±2.70BbX	26.76±4.57BbX
75	30	30.79±3.42Cd	38.49±2.62CbX	38.79±4.26CaX	38.19±2.25CaX	32.36±2.12CbX	30.94±3.26CbX
	50		31.17±0.98CbX	42.23±2.84CaX	32.59±3.24CaX	31.39±0.39CbX	30.04±1.75CbX

For Table 3-7

Means ± standard deviations (*n*=3) were represented. The statistical analysis was performed by factorial design using SPSS program at a confidential level of 95 %.

The different capital letter in the same column indicates significant difference between means.

The different small letter in the same row indicates significant difference between means.

X and Y in the same column indicate significant difference between means under the same methanol concentration.

Antioxidant activities of the hull extracts were investigated through DPPH radical scavenging activity (Table 4), ABTS radical scavenging effect (Table 5), reducing power (Table 6), and ferrous chelating ability (Table 7). The hull extracts with higher DPPH radical inhibition ability could be prepared by increasing methanol content, temperature, and extraction time up to 180 min (P<0.05). Extraction at the time longer than 180 min had no further effect on the improvement of the activity (P>0.05). This tendency was in accordance with a reducing power of the extracts. The highest DPPH radical scavenging activity (*ca.* 75 % DPPH inhibition effect) and reducing power were observed for the hull extracts prepared by using 3:1 methanol–water at 50 °C for 180 min (P<0.05). ABTS radical scavenging ability of the extracts was increased by increasing temperature up to 180 min (P<0.05). The activity was significantly improved by increase methanol content to the mixing ratio of 2:1 (P<0.05), whereas using 3:1 methanol–water had no further effect on the improvement of the activity (P>0.05). Extraction using 2:1 methanol–water at 50 °C for 180 min could provide the extracts with the highest ABTS radical inhibition activity of *ca*. 70% (P<0.05). Table 4–6 imply antioxidant activities of the extracts through primary mechanism. By donating protons/electrons, the extracts might able to stabilize free radicals, and thereby inhibiting oxidative chain reaction (Duh *et al.,* 1999; Klompong *et al.,* 2007). Ability of the extracts to quench ferrous ion was investigated as shown in Table 7. Transition metal ions, *e.g.*, Fe^{2+}, Cu^{2+}, and Co^{2+}, could enhance lipid oxidation through Fenton reaction, so lipid oxidation might be retarded by limiting free metal ion availability (Gordon *et al*., 2001). The extracts exhibiting the highest metal ion chelating ability were found when the 1:1 methanol–water was used as an extracting media for 60–120 min (P<0.05), whereas the inferior ability was observed when the extraction time was extended to ≥ 180 min (P<0.05). Increasing in methanol content affected to impair ferrous ion chelating ability of the extracts (P<0.05). Increasing extraction temperature had no effect on the improvement of ferrous ion chelating ability of the extracts (P>0.05). This tendency differed from other antioxidant activities, *i.e.,* DPPH and ABTS radical scavenging abilities and reducing power. Hinneburg *et al*., (2006) preparing the extracts from various culinary herbs and spices also observed a similar contradiction behavior between metal chelating ability and other antioxidant activities (*i.e*., DPPH radical scavenging, reducing power, and inhibition of linoleic acid peroxidation). This was postulated due to the difference in active compounds with dissimilar antioxidative modes of action of the extracts derived by using various extracting media

(Škerget *et al*., 2005). Chemical structures of phenolic compounds strongly related to their antioxidant activities (Chen *et al.,* 1999; Burda and Oleszek 2001; Škerget *et al.,* 2005).

In the present work, the hull extracts exhibited antioxidant activities through both primary and secondary mechanisms in different manners, depending on extraction condition. Extraction condition greatly influenced on antioxidant properties of plant extracts (Exarchou *et al*., 2002; Miliauskas *et al*., 2004; Djeridane *et al.,* 2006; Dudonné *et al.,* 2009). The correlations between TPC and antioxidant activities of the extracts were plotted in Fig. 12. Positive correlation between TPC and DPPH, ABTS radical scavenging activities and reducing power was evident, as indicated by the high correlation factors of 0.8511, 0.9643, and 0.8793 for DPPH, ABTS, and reducing assays, respectively. This tendency suggested important role of phenolic compounds on antioxidant capacities of the extracts. This behavior was in agreement with the previous reports (Exarchou *et al*., 2002; Lee *et al*., 2003; Miliauskas *et al.,* 2004; Djeridane *et al.,* 2006; Dudonné *et al.,* 2009; Butsat and Siriamornpun, 2010). With their structure containing aromatic rings and hydroxyl groups, phenolics could exhibit antioxidant activities via a redox property, by forming resonance–stabilized phenoxyl radicals to make them as a reducing agent, hydrogen–donor, metal chelator, and oxygen quencher (Bors and Michel, 2002).

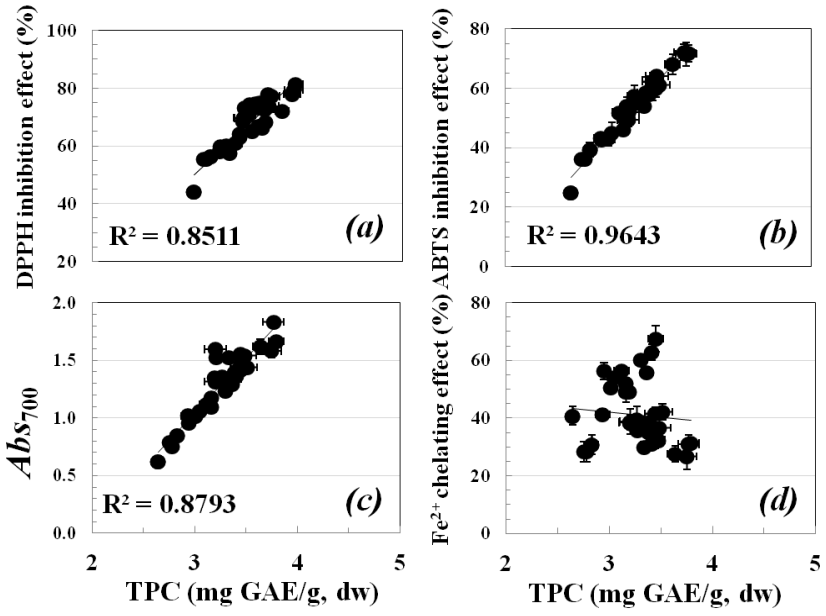

Fig. 12 Correlation between TPC and antioxidant activities of rice hull extracts: ***(a)*** DPPH radical inhibition effect, ***(b)*** ABTS radical inhibition effect, ***(c)*** reducing power, and ***(d)*** ferrous chelating ability.

From the results, the hull extracts prepared using the mixture of 3:1 methanol–water at 50°C for 180 min successfully provided the extract with the highest TPC and antioxidant activities, as suggested by DPPH and ABTS scavenging abilities and reducing power (P<0.05). When, this selected rice hull phenolic extracts (RHPE) was identified, HPLC chromatogram was depicted in Fig. 13. The prevalent phenolics in RHPE were the derivatives of cinnamic (*i.e., p*–coumaric and ferulic acids) and benzoic (*i.e*., vanillic acids) acids. Benzoic and cinnamic derivatives were normally found as phenolic compositions in various cereals (Kim *et al*., 2006). Various phenolic compositions were reported in rice hull extracts depending on rice varieties, *e.g*., vanillic and *p*–coumaric acids for Khao Dawk Mali 105 rice (Butsat and Siriamornpun, 2010), *N*–indolyl acetate, *p*–coumaric, and *o*–methoxycinnamic acid for Japonica rice (Lee *et al*., 2003). Phenolic composition in plants varied depending on species, growth environment, as well as extraction condition, *e.g*., solvent type, concentration, and extraction time (Lee *et al*., 2003; Butsat and Siriamornpun, 2010).

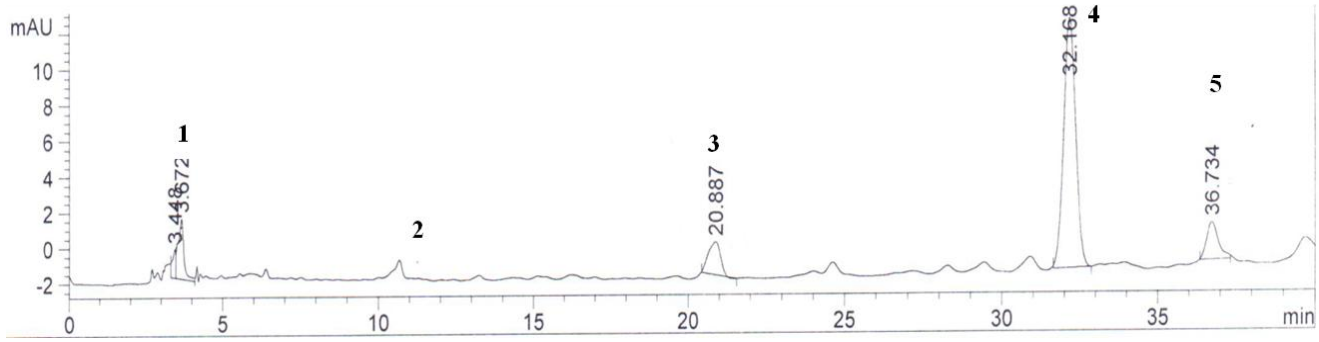

Fig. 13 HPLC profiles of RHPE prepared using the mixture of methanol–water at the ratio of 3:1 at 50 °C for 180 min, where: 1, solvent; 2, unknown compound; 3, vanillic acid; 4, *p*–coumaric acid; and 5, ferulic acid.

Table 8 shows the contents of phenolic compounds, mainly present in the extracts prepared using methanol at different contents and extraction times. Increasing methanol content extracted more *p*–coumaric acid: The *p*–coumaric contents of the extracts prepared using the methanol–water at the mixing ratios 1:1, 2:1, and 3:1 for 60 min were 8.72, 9.57, and 9.65 mg/g sample, respectively. At the extraction time of 180 min, the corresponding extracts had *p*–coumaric acid for 10.33, 13.47, and 15.90 mg/g sample, respectively. For vanillic acid, however, it was recovered more efficiently when

the methanol at lower concentration was used. At the extraction time of 60 min, the liberated vanillic contents were 3.62 and 1.83 mg/g sample, for the extraction using 1:1 and 2:1 methanol–water, whereas it was undetectable with the extract prepared by 3:1 methanol–water. Ferulic acid was present in the extracts at low concentration. Alcohol with higher concentration could promote releasing of lipophilic phenolics (Durling *et al.,* 2007). Therefore, the presence of vanillic (*p*–coumaric) acid in the extracts prepared by using the methanol–water 1:1 (3:1) as the extracting media could be expected. With different molecular structures, phenolics exhibited antioxidants in varying features. A presence of ethylenic group of *p*–coumaric acid could enhance electron donatability (White and Xing, 1997), thereby leading to its potent radical scavenging capacity (Andreasen *et al.,* 2001; McDonald *et al.,* 2001). Reducing capacity of phenolic compounds could be promoted by *para* orientation of phenolic hydroxyl group (Frankel *et al.,* 1995). The presence of *p*–coumaric acid of hull extract was coincident with reducing power and radical scavenging activity. Presence of *ortho* methoxy group was able to improve phenoxy radical stabilization, resulting in potent antioxidant activity of vanillic acid (McDonald *et al.,* 2001).

Table 8 Contents of vanillic, *p*–coumaric, and ferulic acids (mg/g sample) present in the rice hull extracts prepared using mixtures of methanol–water at various ratios (1:1, 2:1, and 3:1) for different extraction times.

Phenolic compounds	1:1		2:1		3:1	
	60 min	180 min	60 min	180 min	60 min	180 min
vanillic	3.62±0.16^{a}	7.25±0.16^{a}	1.83±0.40^{b}	5.61±0.53^{b}	nd^{c}	4.03±0.21^{c}
p–coumaric	8.72±0.09^{b}	10.33±0.04^{c}	9.57±0.10^{a}	13.47±0.08^{b}	9.65±0.01^{a}	15. 90±0.12^{a}
ferulic	0.78±0.05^{a}	1.35±0.08^{a}	0.78±0.21^{a}	1.39±0.11^{a}	nd^{b}	1.43±0.11^{a}

Means ± standard deviations (n=2) were present.

nd: not detectable

The different superscript letters within the same extraction time indicate significant differences between the means (P<0.05).

The RHPE prepared using the selected condition (3:1 metahnol–water at 50°C for 180 min) was prepared and lyophilized. The powder was kept at 4 °C for less than 2 months and used for a next study.

5.2.2 Study on the effects of RHPE on the stability of O/W emulsions stabilized by RBPH

5.2.2.1 Optimization of RHPE incorporation to the emulsion model

The O/W emulsion model was prepared using soybean oil as a dispersed phase. The emulsions were stabilized by RBPH (0.75 %, w/v) and incorporated with RHPE at various concentrations (0, 1, 2, and 3 %, w/v). The RHPE was introduced to the emulsions in different steps, *i.e.*, before and after emulsification process. Physicochemical stabilities of the emulsions were investigated.

5.2.2.1.1 Colloidal stability

Physical stability of the emulsions was evaluated, measuring emulsion ability index (EAI), mean diameter of oil droplets (d_{32}), transmission loss rate (ΔT), and ζ-potential value, as shown in Table 9. Emulsion formability of the RBPH based emulsions could be improved, when RHPE was incorporated at the concentration levels of 1 and 2 % (P<0.05). This result implied that emulsifying formability of the RBPH could be enhanced by interacting with phenolic compounds present in the RHPE. Due to their amphiphilic characteristic, some phenolics, *e.g.,* gallic acid, catechin, quercetin and oleuropein could be accumulated at the oil–water interfacial areas, where they enhanced emulsion formation by reducing tension at the interfaces (Di Mattia *et al*., 2010, 2011). Smaller sized oil droplets was obviously seen for the emulsions with RHPE adding compared to the control sample (P<0.05). This might be explained by the increased negative charge on oil drop surfaces since RHPE adding, as suggested by ζ-potential measurement. By increasing surface charge, dispersion of oil drops could be enhanced through electrostatic repelling force, so improved drop dispersibility could be expected. Enhancement of emulsion dispesibility as affected by RHPE adding was also implied by the lowered ΔT of the RHPE added emulsions compared to the control sample (P<0.05). Considering on RHPE introducing method, RHPE adding before emulsification provided the emulsions with lower ΔT than the counterparts incorporated with RHPE after emulsification (P<0.05). This might be expected since more pronounced protein modification induced by RHPE adding before emulsification process. During

emulsification process, proteins underwent surface denaturation to form interfacial film around oil drops through a formation of inter– and intrainteraction between adsorbed protein molecules (Cheetangdee *et al.,* 2011). Adding RHPE after emulsification might have less effect to modify protein structure, or in turn less influence on interfacial film formation, thereby effecting to different drop dispersibility.

Phenolic compounds could interact with proteins mainly via covalent interactions, and led to alter proteins' functionalities (Aewsiri *et al.,* 2009). Interfacial activity of β-lactoglobulin could be developed by adding syringic acid, tyrosol, and oleuropein (Di Mattia *et al*., 2010). Catechin successfully improved dispersibility of olive oil emulsion (Di Mattia *et al.,* 2009). In this work, surface charge of oil drops could be increased by RHPE adding, suggesting that phenolic compounds present in the extracts might alter RBPH structure by enhancing exposure of negatively charge amino acids of peptides, and led to improve dispersibility of the emulsions.

Table 9 Physical characteristics of the RBPH based emulsions containing RHPE at different concentrations (0-3 %). The RHPE was incorporated to the emulsions by different steps (before and after emulsification process).

RHPE concentration (%)	EAI (m^2/g)		d_{32} (μm)		ΔT		ζ-potential (mV)	
	before	after	before	after	before	after	before	after
0	0.636±0.017B		16.53±0.96A		0.091±0.004A		-40.68±1.52C	
1	0.700±0.002Aa	0.654±0.026Aa	12.26±0.79Bb	2.33±0.99Bb	0.083±0.004Bb	0.085±0.005Ba	-43.61±1.15Aa	-42.41±0.89Aa
2	0.739±0.022ABa	0.651±0.021ABa	11.74±0.91Bb	1.91±1.11Bb	0.064±0.007Cb	0.072±0.005Ca	-43.19±1.30Ba	-42.65±1.62Ba
3	0.639±0.057Ba	0.641±0.037Ba	12.14±0.63Bb	1.02±0.76Bb	0.070±0.007Db	0.076±0.007Da	-44.91±1.14ABa	-43.35±1.73Ba

The statistical analysis was performed by factorial design using Duncan multiple range test at a confidential level of 95%.

The different capital letters within the same column indicates significant difference between means* under the same parameter tested

The different small letter within the same row indicates significant difference between means* under the same parameter tested

**n*=3 for EAI and ΔT, whereas *n*=10 for d_{32} and ζ–potential measurements.

5.2.2.1.2 Oxidative stability

The RBPH stabilized emulsions containing RHPE at various concentrations were stored in a screw–cap bottle at 37°C in the dark, and progressive of lipid oxidation was evaluated by measuring PV and TBARS content along a period of 2 weeks. Fig. 14 depicts PV and TBARS of the emulsions at various storage times.

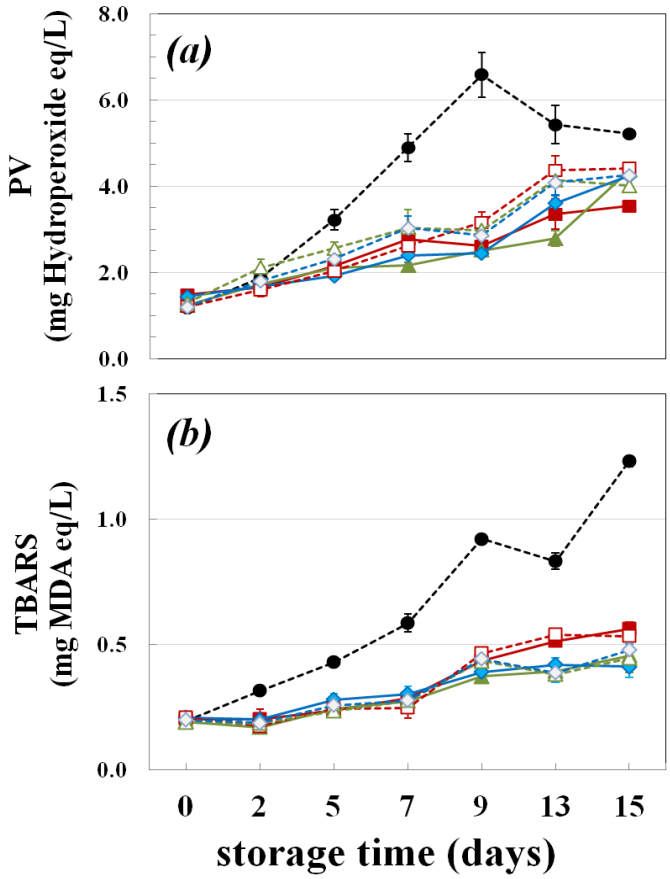

Fig. 14 ***(a)*** PV and ***(b)*** TBARS of the RHPE added emulsions measured as a function of storage time. The emulsions contained RHPE at various concentrations: ● control (no RHPE adding), ■ 1%, ▲ 2%, and ◆ 3 %, w/v. The RHPE was incorporated into the emulsions before (closed symbols) and after (open symbols) emulsification process. Means ± standard deviations (*n*=3) were shown.

RHPE successfully improved oxidative stability of the emulsions as indicated by lower PV and TBARS for the RHPE added emulsions, compared to those observed for the control sample. Enhancement of lipid oxidation in emulsion model could be accomplished using various plant phenolic extracts (Yuji *et al*., 2007; Almajano and Gordon, 2004; Almajano *et al.,* 2007). Anchoring at

the interfaces of phenolic compounds could be promoted by interacting with proteins (Almajano and Gordon, 2004; Yuji *et al*., 2007). In emulsion system, lipid oxidation always takes place at the interfaces where oil drops are exposed to aqueous–soluble pro-oxidants (Waraho *et al.,* 2011). With greater accumulated amount at the interfaces, phenolics might exhibit antioxidant activity effectively, thereby promoting oxidative stability of the emulsions (Almajano and Gordon, 2004; Almajano *et al.,* 2007; Bonoli-Carbognin *et al.,* 2008; Aewsiri *et al*., 2009). Heinonen *et al.,* (1998) reported that BSA bound with ferluric acids successfully prohibited hexanal formation in lecithin–liposome system. This tendency was also observed in the sunflower O/W and water–in–oil (W/O) emulsions: Oxidation rate was significantly decreased when the emulsions were incorporated with BSA–caffeic acid (Conde *et al*., 2011), ovalbumin–catechin, and BSA–catechin adducts (Almajano *et al.,* 2007). Antioxidant activity of cuttlefish skin gelatin could be enhanced by covalent linking with the oxidized phenolic compounds, and the modified gelatin could effectively lower rate of lipid oxidation during 10 days of storage (Aewsiri *et al.,* 2009).

Considering RHPE introducing method, there was no remarkably different PV and TBARS between the emulsions added with RHPE before and after homogenization process. Increase RHPE concentration slightly effected to decrease TBARS of the emulsions: At the end of the storage, higher TBARS was observed for 1 % RHPE added emulsions than those observed for the samples containing RHPE at 2 and 3 %. This could be expected since better antioxidative properties of the RHPE with increased concentration. Addition of anthocyanin from a berry juice extract, especially at higher concentration, could enhance oxidative stability of whey protein based emulsion (Viljanen *et al.,* 2005a, b).

From the present results, one can see that the RHPE could successfully improve physicochemical stability of RBPH based emulsions. Because of the better colloidal stability provided by RHPE adding before emulsification at 3 %, this emulsification condition was selected for a further study.

5.3 Study on the effects of RHPE on the physicochemical stability of RBPH based emulsions made from different oil types

The O/W emulsions made from different oils, *i.e.*, soybean oil (SBO), rice bran oil (RBO), and palm oil (PO) were prepared and incorporated with RHPE at the level of 3 %. The physicochemical stability of the emulsion samples was then investigated.

5.3.1 Physical characteristics of the emulsions

The physical stability indices involving emulsion ability index (EAI), initial oil droplet size, turbidity loss rate (ΔT), and ζ-potential value c of the emulsions was investigated as shown in Table 10.

Table 10 EAI, initial oil droplet size, turbidity loss rate (ΔT), and ζ-potential value of the RBPH stabilized emulsions made from different types of oil without or with RHPE adding

parameters	dispersed phase	Control (without RHPE adding)	RHPE adding (3 %, w/v)
EAI (m^2/g)	SBO	0.716±0.003Aa	0.599±0.030Ab
	RBO	0.557±0.007Ba	0.447±0.006Bb
	PO	0.463±0.006Ba	0.452±0.011Ba
Initial d_{43} (nm)	SBO	2.77±0.43Aa	2.41±0.41Aa
	RBO	2.86±0.39Aa	2.89±0.52Aa
	PO	2.86±0.44Aa	2.42±0.69Aa
ζ–potential (mV)	SBO	-39.74±3.17Aa	-47.95±2.00Ab
	RBO	-38.30±1.07Aa	-48.08±2.06Ab
	PO	-39.21±1.60Aa	-47.44±2.68Ab
ΔT	SBO	0.917±0.032Aa	0.820±0.021Ab
	RBO	0.917±0.016Aa	0.832±Ab
	PO	0.956±0.022Aa	0.824±0.027Ab

Means ± standard deviation (n=3, except for initial d_{43} measurement in which n=10) were shown.
In an each tested parameter, the different capital (small) letter indicates difference between means within a same column (row).

The highest EAI, observed for SBO emulsions compared to the samples made from RBO and PO (P<0.05), implied that emulsifying formability of RBPH could be affected by employing different oil types. Interactions between the components in a system taking place at different steps, *e.g.,* during

homogenization and/or aging processes, were complex phenomena that could affect emulsifier adsorption (Davies *et al*., 2001; Granger *et al.,* 2005). These interactions influenced to protein organization at the interfaces, competitive adsorption of surface active compounds, and affinity of the emulsifiers to hydrophobic residues of the emulsion system, thereby leading to difference initial droplet size and long term stability of emulsion (Granger *et al.,* 2005). In the present work, significant differences on initial d_{43}, ΔT, and ζ–potential for the emulsions made from dissimilar kinds of oil were not observed (P>0.05). By adding RHPE, the dispersibiltiy of all emulsions could be improved as implied by a reduction of ΔT (P<0.05), which might be ascribed to the increased ζ–potential of the emulsions when RHPE was incorporated (P<0.05).

Table 11 reveals diameter of oil drops dispersed in the emulsions produced from different kinds of oils at various storage times. For the emulsions made from SBO and RBO, RHPE adding could successfully retard the increment of emulsion size with increase storage time: For the SBO and RBO emulsions, d_{43} significantly increased after 1 week when RHPE was not added (P<0.05), whereas the increase in emulsion size was negligible over 2 weeks (P>0.05) when RHPE was incorporated to the emulsions. Nonetheless, this behavior was not observed for the PO emulsions. This might be ascribed to a dissimilar chemical composition present in different kinds of oils. A part of the emulsifiers initially adsorbed at the interfaces could be displaced by some surface active agents, resulting in inferior dispersibility of oil drops since a partial coalescence phenomenon (Bolliger *et al*., 2000; Relkin *et al.,* 2003). Displacement of proteins from oil drop surfaces by sodium dodecyl sulfate was more intense for functional dairy proteins (FDP) than in the case of skim milk powder (SMP), with the aggregation factors of 1.3 and 19.3 for FDP and SMP based emulsions, respectively (Granger *et al.,* 2005). Desorption of adsorbed proteins to aqueous phase as induced by a presence of low molecular weight surface active compounds resulted in a depletion flocculation of the emulsified drops (Holstborg *et al.,* 1999).

Table 11 Mean diameter of oil droplets (nm) dispersed in the RBPH stabilized emulsions made from different kinds of oil without (-) and with (+) RHPE adding at various storage times.

Dispersed phase	RHPE	Storage time (weeks)		
		0	1	2
SBO	-	2.77±0043b	3.77±0.79a	4.27±0.51a
	+	2.41±0.41a	2.78±0.60a	2.63±0.69a
RBO	-	2.86±0.39b	4.35±0.38a	4.19±0.74a
	+	2.89±0.52a	2.94±0.39a	2.80±0.52a
PO	-	2.86±0.44b	3.84±0.60a	3.78±0.48a
	+	2.42±0.69b	3.61±0.90a	3.78±0.39a

Means ± standard deviation (*n*=8) were shown.

The different letter indicates difference between means within a same row.

Types of oils employed as a dispersed phase could affect to physicochemical stability of emulsion system (Nor Hayati *et al.,* 2007; Protonotariou *et al.,* 2013). The composition and physicochemical properties of the oils influenced the size of dispersed drops produced during emulsification process: Variation in oil types altered viscosity ratio between dispersed and continuous phases, which further determined a minimum size of oil drops produced under a steady state (McClements, 1999). Protonotariou *et al*., (2013) observed a lower volume separation, as well as smaller drop diameter, for the olive oil dressing compared to the ones made from sesame oil, which was postulated due to a greater consistency of pseudoplastic behavior of olive than did sesame oil (Akhtar *et al*., 2009). Dissimilar rheological properties of oils affected to emulsification ability, *i.e.,* drop breakdown, consequently droplet size and stability of the emulsion (Protonotariou *et al*., 2013). Regarding composited fatty acids, it has been suggested that medium chain triacylglecerides (TAGs) provided emulsion with a greater stability than did long–chain TAGs, *e.g*., soybean oil (Driscoll *et al*., 2001). Better stability of the emulsions made from more saturated oils was reported (Granger *et al*., 2005). Nor Hayati *et al.,* (2007) replacing palm kernel oil with a high saturated fatty acids (*ca*. 71 % of total fatty acids) to SBO for salad dressing preparation, found that emulsions containing 10–30 % palm kernel oil replacement could retard drop aggregation during 1 month of storage. By decreasing

unsaturation degree of a dispersed phase, oil drops tended to form a stronger network that effected to reduce drop mobilization in the emulsified matrix, and hence, lower degree of drop aggregation could be supposed. Significant content of C6:0–C12:0 in palm kernel oil, moreover, was believed to partly contribute to a structural rearrangement, thus allowed better miscibility between dispersed and continuous phases during emulsification process (Nor Hayati *et al.,* 2007). In the present work when RHPE was not incorporated to the emulsions, the smallest d_{43} after 2 weeks of storage could be observed for the emulsion made from PO compared to the counterparts produced from SBO and RBO.

5.3.2 Chemical stability of the emulsions

Oxidative stability of the emulsions was evaluated by measuring PV and TBARS content along a storage time of 2 weeks as shown in Fig. 15a and 15b, respectively.

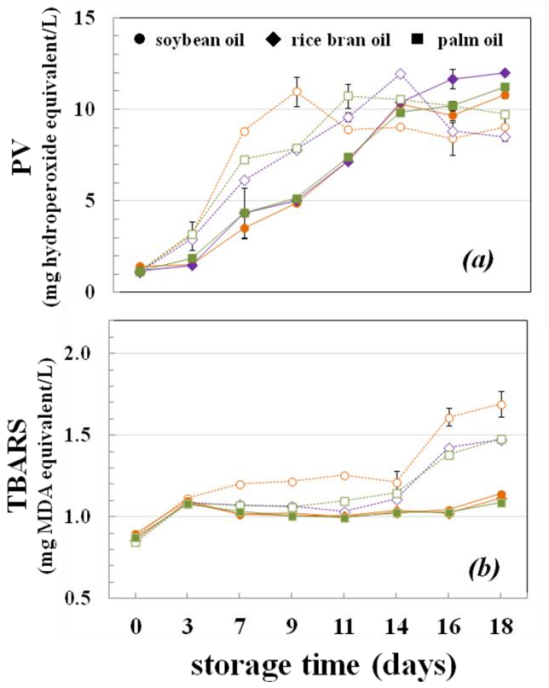

Fig. 15 ***(a)*** PV and ***(b)*** TBARS of the emulsions made from different oil types at various storage times without (open symbols) or with (closed symbols) RHPE adding.

With different fatty acid compositions of the used oils, physicochemical stability of the emulsions was affected. The most pronounced lipid oxidation degree was observed for the SBO emulsion as suggested by the highest PV development with storage time: The PV was maximized after 9 days of

storage for SBO emulsions, but the highest values were found after 11 and 14 days for the emulsions of PO and RBO, respectively. This tendency was in accordant with the result of TBARS measurement: The SBO emulsions exhibited higher TBARS than those of RBO and PO emulsions. This behavior might be explained by a different fatty acid composition and miscellaneous compounds, *e.g.,* endogenous phenolic substances present in the oils. Table 12 shows the fatty acid compositions of the employed oils. SBO is rich in C18:2, whereas C18:1 was the major fatty acid of PO and RBO.

Table 12 Fatty acid compositions and phytochemical compounds of the selected oils[a]

	SBO	RBO	PO
14:0 (g/100 g)	-	0.37	-
16:0 (g/100 g)	10.1	12.96	44.8
18:0 (g/100 g)	4.3	2.97	4.6
18:1 (g/100 g)	22.3	45.43	38.9
18:2 (g/100 g)	53.7	35.90	9.5
18:3 (g/100 g)	8.1	0.84	0.4
α-tocopherol (ppm)	116	108.4	377
α-tocotrienol (ppm)	-	34.4	52
β- tocopherol (ppm)	17	-	1
β- tocotrienol (ppm)	-	-	4
γ-tocopherol (ppm)	578	127.5	4
γ- tocotrienol (ppm)	-	11.7	132
δ- tocopherol (ppm)	263	2.92	-
δ- tocotrienol (ppm)	-	-	-
Oryzanol (ppm)	-	40	-
references	Van Niekerk and Burger, 1985; Yoshida *et al*., 1990	Most *et al*., 2005	Van Niekerk and Burger, 1985; Yoshida *et al*., 1990

[a] Fatty acid compositions were determined by GLC and are expressed as mean average weight percent composition on a fatty acid basis. Trace acids (less than 0.1 %) were excluded.

Stability of oils strongly depended on their chemical compositions, including composited fatty acids and profile of miscellaneous substances, *e.g.,* endogenous phenolic compounds. It is well recognized that unsaturated fatty acids (USFAs) are susceptible to chemical deterioration, especially oxidative reaction (Nor Hayati *et al*., 2005; Ramadan and Wahdan, 2012). Therefore, the higher PV and TBARS found for SBO emulsions than the counterparts made from RBO and PO could be supposed. Regarding RBO, moreover, a presence of several photochemical compounds, such as γ–oryzanol, the ferlulate esters of triterpene alcohol (Roger *et al*., 1993), campesterol, tocotrienols, and β–sitosterol (Itoh *et al*., 1973a, b) at a relatively high level was reported. PO was confirmed as a good source of vitamin E homologues, especially α-tocopherol (Tan, 1989). The efficiency of α-tocopherol to scavenge free radicals was reported (Kamal-Eldin, 2006). Table 13 reveals a presence of phytochemicals in some vegetable oils. These compounds, moreover, could exhibit antioxidative properties, thereby enhancing oxidative stability of the emulsions.

Table 13 Sterol and triterpene contents in some vegetable oils (mg/100 g oil)

oils	campesterol	stigmasterol	β-sitosterol	cycloartanol	cycloartenol	24-methylene-cycloartanol
Rice bran	506	271	885	106	482	494
Safflower	45	31	181	1	34	7
Corn	410	110	1180	4	8	11
sunflower	31	31	235	-	29	16
cottonseed	17	4	400	-	10	17
sesame	117	62	382	4	62	107
soybean	72	72	191	-	156	8
peanut	36	21	153	1	11	16

From: Itoh *et al.,* 1973a, b

6. Conclusion

The bran and hull of rice which are a by–product from rice milling process could be a promising candidate used to prepare functional additives to enhance stability of emulsion model. By using Protease aided–extraction, rice bran protein hydrolysates (RBPH) with emulsifying activity could be

prepared. The functional properties of the hydrolysates depended on degree of hydrolysis, (DH), in which increased with the applied Protease concentration and hydrolytic time. Upon hydrolysis, improvement of emulsifying property of the hydrolysates was supposed since the increase solubility and hydrophobicity as well as a decrease in molecular size of the peptides. The RBPH with potent functional properties could be prepared using 1 % Protease and conducting the extraction at 60°C for 30 min. The RBPH could also prohibit lipid oxidation in the model emulsions, supposed since its composited amino acids with antioxidative capacity.

Rice hull phenolic extract (RHPE) was prepared via methanolic extraction to be used as a natural antioxidant agent. The extracts with higher total phenolic content (TPC) could to be prepared by increasing methanol content and extraction time. High correlation between TPC and antioxidant abilities involving DPPH radical scavenging ability, ABTS radical trapping capacity, and reducibility was suggested. The condition providing RHPE with the highest antioxidative capacity was the extraction using methanol–water at the ratio of 3:1 as extracting medium for 180 min at 50°C. The predominant phenolic compounds present in RHPE were vanillic and *p*–coumaric acids. When RHPE was incorporated to the soybean O/W emulsions, lipid oxidation could be delayed, especially at the increased RHPE concentration. Furthermore, RHPE could enhance emulsion dispersibility, which was attributed to a synergistic effect between phenolic genres and RBPH. To be effectively improve emulsion physicochemical stability, it was found that RHPE incorporation before emulsification process at the concentration level of 3 % was effective.

Then, the physicochemical stability of the RBPH stabilized emulsions added with RHPE was investigated by using different oil types, *i.e.,* soybean oil (SBO), rice bran oil (RBO), and palm oil (PO), as a dispersed phase. Regarded to difference in fatty acid composition and profiles of microconstituents of the used oils, physicochemical stability of the emulsions was affected. The greatest emulsion formability implied by the highest EAI was found for the SBO emulsion. RHPE could improve colloidal stability of the systems, when SBO and RBO were employed as a dispersed phase as suggested by no significant droplet size increment along a storage of 2 weeks. Nonetheless, the most pronounced oxidative degree was observed for the SBO emulsions, supposed since a susceptibility against oxidative reaction of unsaturated fatty acids abundantly present in SBO. By

optimizing the types of oil employed as a dispersed phase, therefore, the emulsion with desirable physicochemical stability could be prepared.

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8. ภาคผนวก

8.1 สำเนาบทความที่ได้รับการตีพิมพ์แล้ว (Reprint)

Cheetangdee N, Benjakul S. 2015. Antioxidant activities of rice bran protein hydrolysates in bulk oil and oil-in-water emulsion. J. Sci. Food Agric. 95: 1461-1468.

8.2 ผลการวิจัยส่วนที่ยังไม่ได้ตีพิมพ์หรือตีพิมพ์ไม่ได้ แต่อยู่ในวัตถุประสงค์ของโครงการวิจัย

8.2.1 The manuscript entitled “Oxidation and colloidal stability of oil-in-water emulsion as affected by pigmented rice hull extracts” has been submitted to Journal of the American Oil Chemists’ Society, and now is in a review process.

8.2.2 The manuscript entitled “Effects of rice hull phenolic extracts on the stability of emulsions stabilized by rice bran protein hydrolysates” is now in preparation.

8.3 ข้อคิดเห็นและข้อเสนอแนะสำหรับการวิจัยต่อไป

To elucidate the synergistic effects between RBPH and RHPE on the improvement of physicochemical stability of the emulsion model, information about the interfacial phenomena and partitioning behavior of RHPE should be more elucidated. Moreover, study on the effects of RBPH and RHPE on characteristic and stability of other food models should be implemented.

8.4 บทความวิจัยที่นำเสนอที่ประชุมวิชาการ (Proceeding) (ถ้ามี)

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