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Functionality of egg yolk lecithin and protein and functionality enhancement of protein by controlled enzymatic hydrolysis

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Functionality of egg yolk lecithin and protein and functionality enhancement of protein by controlled enzymatic hydrolysis

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CHAPTER 1. GENERAL INTRODUCTION

LITERATURE REVIEW

Egg yolk is a good source of choline, omega-3 fatty acids, and natural antioxidant for humans, as well as a high quality protein. Egg yolk contains 31.8-35.5% lipids, 15.7-16.6% proteins as illustrated in table 1, almost all vitamins (A, B, D, E, K) except for vitamin C, and multiple minerals [1, 2].

Yolk lipids are almost all associated with proteins among which phospholipids (PLs) comprise about 30% of total lipids [1]. Egg yolk PLs, also known as egg yolk lecithin (EYL), are mainly composed of phosphatidylcholine (lecithin, PC, 66-76%) and phosphatidylethanolamine (cephalin, PE, 15-24%) [3]. EYL has been extensively used in foods, pharmaceuticals, and cosmetics because it is a good emulsifier, and it is usually believed to be well-tolerated and non-toxic in the native form.

Native yolk proteins mainly exist as lipoproteins that are separated into a plasma fraction (soluble materials upon centrifugation) and a granular fraction (precipitants upon centrifugation). Egg yolk protein (EYP) is almost denatured after total lipid extraction where ethanol and hexanes are applied [4]. It is critically important to determine and improve its functional properties in order to make the further processing more economically feasible for industry.

This review will place emphasis on EYL and lipid-free EYP, which has been subjected to ethanol and hexanes extraction. Three aspects of EYL and EYP will be covered: 1)

emulsification properties of EYL; 2) oxidation stability of EYL; 3) controlled enzymatic hydrolysis of protein and its application on lipid-free EYP.

Table 1 Composition of egg yolk [1, 2, 3]

Water	Protein	Lipid	Carbohydrate	Ash
48-52%	15.7-16.6%	31.8-35.5%	0.2-1.0%	1.1%
	Plasma 78%	TAG 66%		
	Granular 22%	PLs 28%		
	Phosvitin 16%	Cholesterol 3%		
	Lipovitellins 70%			
	LDL 12%			

LDL: low density lipoprotein; TAG: triacyglycerol; PLs: phospholipids

Emulsification properties of phospholipids

An emulsion comprises two immiscible liquids, with one of the liquids finely dispersed as small droplets in the other. The liquid making up the droplets in an emulsion is known as the dispersed phase, whereas the other liquid surrounding the dispersed phase is defined as the continuous phase. Oil and water are commonly used liquids in making emulsion. Emulsion can be conventionally categorized into two classes, e.g., oil in water (o/w) and water in oil (w/o) systems according to the relative spatial distribution of the oil and aqueous phase [5]. Most food-based emulsions are o/w formation where the oil is the dispersed phase and the water is the continuous phase, such as beverage, mayonnaises, and salad dressings. Margarine is an example of w/o emulsion.

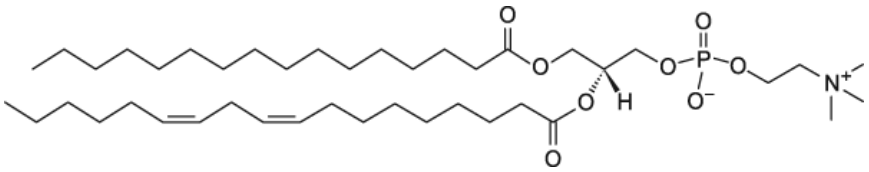
Because most oils in a food matrix contain medium- and long-chain fatty acids and are highly hydrophobic (water-hating), the surface tension is quite high if the two liquids are directly emulsified without any emulsifiers. Typically, separation into two distinct layers happens immediately after homogenization. Surface active materials, known as emulsifiers or

surfactants, such as fatty acids, PLs, protein, polysaccharides, are usually used as amphiphilic materials, and they could reduce surface tension, thereby kinetically stabilize emulsions for a relatively long shelf-life.

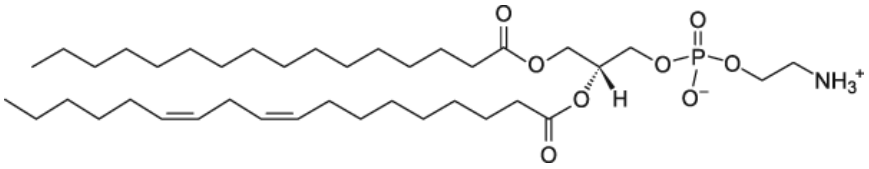
As emulsifiers, PLs function by coating on oil droplets and reducing surface tension between immiscible phases. Similar to other emulsifiers, various interactions are involved in stabilizing PL-stabilized o/w emulsions, such as Van der Waals force, steric interaction, hydration interaction, electrostatic force, and hydrophobic interaction [5]. Emulsification properties of purified individual PL originating from both soybean and egg yolk have been investigated under various conditions [6, 7]. However, EYL contain several types of PL and the interaction among PL class composition should be taken into account when it is applied into food as a whole.

As mentioned earlier, EYL is mainly composed of PC and PE, which are at 66~76% and 15~24%, respectively. Lysophospholipid (LPC, 3-6%), sphingomyelin (SM, 3-6%), and phosphatidylinositol (PI, 0.6%) are also found in EYL as minor components [8]. EYL have different PL class compositions from soy lecithin (SL) (Chapter 2, Table 1). SL contains less proportion of PC (33%) and PE (14.1%) but more PI (16.8%) and phosphatidic acid (PA, 6.4%). Among these phospholipids, PC and PE are zwitterions, whereas PI and PA are anions as illustrated below:

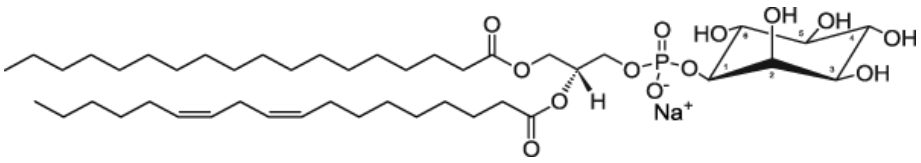
Phosphatidylcholine (PC)



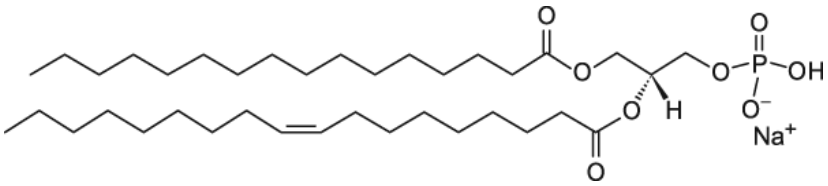
Phosphatidylethanolamine (PE)



Phosphatidylinositol (PI), sodium salt



Phosphatidic acid (PA), sodium salt



The electrostatic repulsion is usually the dominant force in the emulsion stabilized by charged PLs, such as PI and PA in SL. It was also reported that zwitterionic PLs stabilize emulsion mainly through hydration interaction due to the electronic characteristics of zero net charge, though electrostatic interaction was also observed [9, 10]. Hydration repulsion of PC and PE becomes predominant as the distance between the droplets gets closer [11, 12].

Van der Meeren [9] studied the emulsion stability with a mixture of the anionic PA in the zwitterionic PC and found that the emulsion stability was improved by partly replacing PC with PA when the amount of PC used was not high enough to form a stable emulsion. This study indicates that anionic PA and PI are good at stabilizing emulsions compared with zwitterionic PC and PE at lower concentrations.

These emulsions stabilized mainly through electrostatic repulsion, such as PI and PA stabilized ones, are susceptible to change of ionic strength, pH, surface charge density, and

surface potential. For those stabilized through hydration interaction, such as PC and PE, they are usually sensitive to temperature change [5].

Lecithin oxidation in bulk systems and emulsions

Lipid oxidation has long been recognized as a major cause of deterioration of fatty foods. Several mechanisms have been proposed and widely accepted to explain lipid oxidation by autoxidation, photooxidation, and enzyme-catalyzed oxidation. Lipid oxidation results in off-flavor (rancidity) and changes in texture and other sensory characteristics of fatty foods. It also affects physical properties, such as smoke point and viscosity. The oxidation products, hydroperoxides and their secondary decomposites, such as short-chain volatile compounds, ketodienes, and epoxy compounds, are potentially toxic compounds and may cause chronic disease, such as atherosclerosis when consumed [13].

Three steps involved in lipid oxidation are initiation, propagation, and termination. The abstraction of hydrogen atom from allylic or bisallylic position has been found to be the rate-determining step in the oxidation process [14]. Because the bond dissociation energy for monoallylic C-H is ~10 kcal/mol higher than that for bisallylic C-H, which is ~85 kcal/mol [14], oxidation rate is largely determined by the amount of di- and poly-unsaturated double bonds present in the lipid. Research has demonstrated that fatty acids with two conjugated double bonds oxidize 10 times faster than do mono-unsaturated fatty acids; a further increase in each activated methylene group causes an increase of oxidation of 2-3 times [15].

Though its fatty acid composition is influenced by feed composition, generally EYL contains much less polyunsaturated (PUFA) than does plant source SL, especially for linoleic

acid and linoleinic acid (Chapter 2, Table 2). It is obvious to predict that EYL oxidizes at a slower rate than SL.

However, quite a few factors are involved in PL oxidation besides fatty acid composition. One is PL class proportion. Oxidative and antioxidative properties of individual pure PE and PC extracted from soy and egg have been studied for decades [16, 17, 18], and it has been elucidated that egg PE oxidizes at a faster rate than egg PC because of its higher content of arachidonic acid (13.6% in PE fraction) in comparison with that in egg PC (3.6% in PC fraction). This is the fatty acid composition effect. Lea [19] observed that the free amino group of PE that disappeared during oxidation was approximately proportional to the oxygen absorption. Corliss and Dugan [17] reported the Maillard-type discoloration during oxidation of individual egg PE and egg PC and verified that PUFA content was not the only reason that caused greater reactivity of egg PE The nitrogen moiety of PE also disappeared during oxidation. Hidalgo and others [18] proposed that the secondary oxidative products, such as short-chain ketones and aldehydes, may continue to react with the primary amino group in PE proceeding as the Maillard reaction.

Though oxidative properties of individual egg PLs have been extensively studied, the practical PL directly extracted from egg yolk is a mixture of PE, PC, and minor components, and the factors influencing its oxidative stability very complex. Therefore, it is important to test the natural mixture of PLs for its stability.

A concise review by McClements and Decker [20] comprehensively postulated lipid oxidation in o/w emulsion, which suggested that the interactions between hydroperoxide located on the droplet surface and the transition metals in the continuous phase are the most common causes for lipid oxidative instability [20]. In the case of EYL application in food

emulsions, it is valuable to compare EYL with SL regarding their oxidative behavior in emulsions because of their different PL class proportions.

When the amphiphilic PLs stabilize the emulsion, they prefer to coat the surface of the oil droplet with the hydrophobic tails buried into the oil phase and with the hydrophilic polar head projected toward aqueous phase. PI and PA have negatively charged polar heads, and thus they make the overall surface charge negative for SL-stabilized emulsion. The surface charge density would be significant because of a relatively large proportion of PI and PA. Driven by strong electrostatic attraction force, aqueous soluble transition metal ions, such as cupric ion, will migrate and accumulate on the surface of droplet where they can access and catalyze formation and breakdown of hydroperoxides and, therefore, accelerate SL oxidation. Because of this chemical structure of PI and PA, SL is expected to have poorer oxidative stability in an emulsion in the presence of transition metals.

It recently was found that the interaction between hydroperoxide and transition metal was affected by another PL fraction in EYL, e.g., plasmalogen. Plasmalogen PL contains an enol ether structure as shown in Figure 9, Chapter 2. Palacios and Wang [21] estimated the content of plasmalogen as about 3.6% of total yolk PLs. Engelmann reported that one enol ether double bond prevented the oxidation of the four double bonds contained within arachidonic acid as evidenced in micelles using ^{1}H-NMR spectroscopy, though the detailed pathway was not found [22]. The role of plasmalogen PE in cupric-induced lipid oxidation was evaluated *in vitro*, and it is believed that the formation of a complex between cupric ions and the plasmalogens (on enol moiety, 1:1) accounts partly for their inhibition of cupric-induced lipid oxidation [23].

Application of controlled enzymatic hydrolysis in enhancing protein functionality

The protein content in raw egg yolk is about 15.7-16.6% on wet weight basis. The major portion of yolk protein exists as lipoproteins except for phosvitin and livetins. In the process of delipidation by using ethanol and hexanes under mild conditions, lipid-free egg yolk protein (EYP) is produced, and this delipidated yolk protein is considered as a co-product of the lipid and protein separation. Because the protein is exposed to polar solvent, its secondary or tertiary structure may have been altered and it may have poor functional properties.

Sakanaka and others [24] reported that the lipid-free EYP had an amino acid score of 100. Digestibility *in vitro* test showed that EYP experienced faster digestion than milk casein when treated with pepsin and panceatin. In the test of protein efficiency ratio (PER), the criteria to determine the nutritional value of proteins, EYP showed significantly higher value than that of milk casein.

However, applying lipid-free EYP as functional ingredient in foods could be challenging because of its expected poor functionalities as a result of solvent denaturation. When ethanol is applied to the egg yolk dispersion, it partly replaces the hydration shell of water [25]. This replacement causes surface hydrophilic side chains twist inwards and hydrophobic side chains become exposed to the outside where they interact favorably with the organic solvent. The net effect in the process is causing drastically conformational changes, and, in many cases, this conformational changes cause further aggregation and precipitation of proteins [26].

Application of enzymatic hydrolysis of protein and large protein aggregates has been utilized for a long time to modify and enhance functionalities of various proteins including soy proteins and whey proteins [27, 28]. Limited enzymatic hydrolysis produces smaller

pieces of peptides and changes surface hydrophobicity of target proteins. Hydrolyzed proteins always have increased solubility in comparison with the original ones. Functionalities, especially foaming and emulsification properties of hydrolyzed proteins, usually are dependant on degree of hydrolysis (DH) and also are protein-dependent. Excess hydrolysis may decrease either foaming or emulsification or even both properties. Another main concern during protein hydrolysis is the bitterness, which may become outstanding when the hydrolysates are designed to use as main food ingredients. Hydrolysis of soy protein has been studied extensively since World War II because of its potentially wide applications. However, a systematic study of EYP hydrolysis has not been found until recently, especially its application as food ingredient. Sakanaka and others [29] reported that EYP hydrolysates from complete hydrolysis showed antioxidant activity in a linoleic acid oxidation system. However, there is little information on the effect of controlled enzyme hydrolysis of EYP on its foaming and emulsification properties.

In this study, two food-grade proteases were investigated to hydrolyze ethanol-denatured egg yolk protein at two different DHs. Protein solubility, emulsification properties, and foaming properties of the corresponding hydrolysates were studied to examine the effectiveness of such treatments.

THESIS ORGANIZATION

This thesis consists of a general introduction, followed by two manuscripts of research papers and a general conclusion section. The papers are in the required corresponding journal formats.

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CHAPTER 2. EMULSIFICATION PROPERTIES AND OXIDATIVE STABILITY OF EGG YOLK LECITHIN

A manuscript to be submitted to *the Journal of the American Oil Chemists' Society*

Guang Wang[1] and Tong Wang[1,2]

ABSTRACT

Egg yolk lecithin (EYL) is a good source of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and it is different from soy lecithin (SL) in both fatty acid and phospholipid class composition. These factors may lead to different behavior in oxidative stability and emulsification properties in food systems. Therefore, these characteristics were investigated in this study. Emulsification properties were evaluated at two oil-to-water ratios, two emulsifier concentrations, two pHs, and with the addition of xanthan gum. The results showed that low concentration of EYL (2.5% in oil) gave poorer emulsion stability than did 5.0%, whereas emulsions with oil-to-water ratio of 50:50 were more stable than with a 20:80 ratio. Under neutral pH, EYL gave poorer emulsion stability than SL at both oil-to-water ratios and emulsifier concentrations. However, under acidic condition, EYL created a more stable emulsion than did SL. Adding xanthan (0.05%) increased stability of EYL emulsions and minimized stability differences caused by lecithin concentrations. Oxidative stability of egg yolk was determined in bulk and in emulsion. EYL showed better oxidative stability in both bulk and emulsion systems than did SL. Cupric ion did not accelerate oxidation of EYL in an emulsion system, but it did accelerate oxidation of SL.

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Keywords: Egg yolk, emulsification properties, lecithin, oxidative stability, soy lecithin.

INTRODUCTION

Lipid content of egg yolk is about one-third of that in whole yolk on dry weight basis, and phospholipids (PLs) make up 21~31% of the total lipid [1]. Egg yolk lecithin (EYL) is mainly composed of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which are 60~73% and 15~26% of total PLs, respectively [1]. Lyso-phosphatidylcholine (LPC), sphingomyelin (SM), and phosphatidylinositol (PI) are also found in EYL as minor components [2]. Among various known functionalities of lecithin, emulsification is the most important and has been studied most extensively for decades [3, 4]. EYL has been used as food emulsifier and stabilizer for mayonnaises, infant formula, hand and body lotions, as well as margarines. Recently, there are more applications of using EYL as emulsifier in cosmetics [5].

An emulsion is a mixture of two immiscible liquids, one finely dispersed in the other with the help of certain surfactants. Most food emulsions are thermodynamically unstable, though current interests are producing a thermodynamically stable system, which is referred to as microemulsion [6]. Various physicochemical factors can affect emulsion stability, such as Van der Waals force, steric interaction, hydration interaction, electrostatic force, and hydrophobic interaction in emulsion system. Homogenization process produces high energy input and breaks down oil phase into much smaller droplets, therefore minimizing settling velocity caused by gravitational force according to Stokes's law. Theoretically, a minimal amount of emulsifier should be used to fully cover the surface of emulsion droplet in order to achieve good stability. Too much or too little emulsifier may not achieve stabilization of

specific emulsion system [7]. Oil phase concentration also influences emulsion stability. In oil-in-water (o/w) emulsion, it is not necessary to lower oil concentration for making stable emulsion. Mayonnaise and salad dressing are classic applications, where oil phase could be as high as 80% and 50%, respectively [8].

In addition to the factors already discussed, we know that, for mayonnaise and salad dressing, high acidic condition is often preferred for product quality.

It is well known that lecithin is composed of both zwitterionic (PC, PE) and anionic (PI, PA as phosphatidic acid) PLs. EYL differs from soy lecithin (SL) in PL class composition in that SL contains more than 19% of PI, whereas only trace amount (0.6%) of PI is found in EYL [1]. Change in pH will alter PL net charge and therefore may affect its effectiveness in creating stable emulsion. Also in food applications, emulsifier usually could not achieve good emulsion stability without addition of other stabilizers, among which xanthan gum is commonly used as a thickener. It is important to know how these additives affect the stability of EYL-created emulsions.

Another interesting research area of lecithin is its antioxidative or synergistic antioxidative properties [9, 10]. However, the oxidative stability of lecithin itself has not been well studied. PLs are rich in di- and poly- unsaturated fatty acids, which are prone to autoxidation. PL oxidation products, such as hydroperoxides and other volatile compounds like ketodienes and epoxy compounds, could not only lead to food rancidity but also cause chronic disease including atherosclerosis [11, 12]. The oxidative stability of EYL is predicted to be better than does SL in bulk system because EYL contains more saturated fatty acids than does SL. In emulsion system, PI and PA coating the oil droplet may attract metal ions more effectively than do PC and PE. So EYL is expected to have better oxidative stability

than SL in emulsions when prooxidant metal ions are present because EYL contains less proportion of anionic PI and PA than does SL.

The objectives of the present study were to first investigate the factors influencing emulsion stability (ES) of EYL emulsion, such as emulsifier concentration, oil phase concentration, pH, thickener addition, and secondly, to study the EYL oxidative stability in bulk system and in emulsion system, and with the addition of prooxidant cupric ion.

EXPERIMENTAL PROCEDURES

Materials

Fresh eggs (Boomsma's Inc., Alden, IA) used in lecithin extraction were purchased from a local grocery store. Egg yolk was separated from egg white carefully by hand. Methods of Palacios and Wang's study [22] was used for lecithin extraction from fresh yolk, and HPLC analysis followed to quantify PL purity and individual PL class compositions. Briefly, EYL was extracted with ethanol and hexanes and acetone wash was used to remove any acetone-soluble material. Final EYL had purity as high as 95% as measured by high performance liquid chromatography with evaporative light scattering detectors (ELSD). Thin-layer chromatography (TLC) was used to separate individual PE and PC fraction in EYL, and gas chromatography (GC) was followed for analysis of fatty acid compositions as stated by Palacious and Wang [22]. Soy lecithin (99%, Fisher Scientific, Pittsburg, PA) was used as control throughout this study and when proper functionalities were tested. It was acetone-washed by the same method as for EYL.

Preparation of EYL Emulsion

To make oil-in-water (o/w) emulsion, soybean oil with dispersed lecithin was heated in a 50°C water bath for 15 min with gentle agitation. Then, a few drops of FAT RED 7B (0.1%, w/v in vegetable oil) were added into above oil solution followed by adding water. The lecithin concentrations were all based on the quantity of oil (w/v, %). A Polytron PT3100 homogenizer with PT-DA 3012/2T generator was set at 24,000 rpm for 60 s. After homogenization, emulsion was transferred into a graduated cylinder (50 ml) with mark interval of 1 ml. Cylinder was sealed by using Parafilm® and allowed to stand at room temperature for visualizing phase separation.

Effect of Emulsifier Concentration and Preparation Method on Emulsion Stability

Four emulsions with lecithin concentration of 0.5, 1.0, 2.5, and 5.0% were created by using a Hamilton Beach blender (Model 51101, Southern Pines, NC) for 3 min to compare the effect of lecithin concentration on emulsion stability (ES). Oil separation was recorded at 30 min after initial preparation. Oil-to-water ratio was 20:80 for this comparison. SL-stabilized emulsions were used as controls, and all treatments were duplicated. The same experiment was repeated by using a high speed homogenization method to compare efficiency of the two methods in producing a stable emulsion. Phase separation was recorded either in the form of cream separation or water separation (%, based on the total volume of corresponding oil and water phase). Cream separation was recorded where no obvious coalescence of oil droplets was observed with short standing time (less than 2 hrs), and water phase separation was recorded where obvious water layer formed during the prolonged

observation time. Oil phase separation was recorded when oil droplet coalescence readily occured.

Effect of Oil-to-Water Ratio, pH, and Thickener on Emulsion Stability

Four treatments of the two factors (oil-to-water ratio, pH)-two levels combination were prepared with lecithin concentration of 5% by the homogenization method described above. For the acidification treatments, hydrochloric acid (1 N) was added to deionized (D.I.) water before emulsion was made. All treatments were duplicated.

To better understand the effect of thickener on PL-stabilized food matrix, addition of xanthan gum (0.05%, in water), a commonly applied emulsion stabilizer, was tested under neutral pH. Xanthan was dissolved in D.I. water and emulsion preparation was the same as homogenization method. Preliminary test showed that this concentration of xanthan could not give long term stability but short-time stability (4 hrs) was observed at oil-to-water ratio of 20:80 with no lecithin added. Soy lecithin was used as control.

Characterization of phase separation was achieved by visualizing dye boundary after prolonged standing at room temperature. The upper layer increased in both volume and darkness and looked creamy while the lower layer looked more watery with time. In this study, both creamy phase and watery phase separation (%, based on total volume of corresponding phase) were used to characterize emulsion stability according to specific observation. Oil separation % was given when obvious oil coalescence and aggregation occurred.

Oxidative Stability of EYL in Bulk System and in Emulsion, and with Addition of Cupric ion

A reported method designed to measure oxidative stability of EYL in bulk system was followed [13]. Briefly, in this method, 6mg stock PL was coated on multiple glass beans and oxidized in a conventional oven at 55˚C for 16 days with sampling every 48 hrs. At the end of the storage, peroxide values (PV) of PLs were quantified by using a ferric thiocyanate methods [13]. Iodine analysis method was used to measure PV formed in emulsion, and the method was stated as follows: Lecithin (10 g) dispersed in 100 ml water were put into heated mineral oil (100 ml, at 50°C) and magnetically stirred for 15 min (note: mineral oil is composed mainly of alkanes and paraffin, which are not oxidizable). For the samples with cupric ion incorporation, 1 ppm (based on total volume, 200 ml) cupric sulfate pentahydrate was added into mixture. Then the mixture was homogenized at 24,000 rpm for 60 s with Polytron® PT3100 homogenizer. The final emulsions were o/w type with 10% emulsifier relative to oil phase volume.

All treatments were put into a conventional oven at 55°C for 10 days. During storage, re-homogenization, under the same condition as initial homogenization, was done two to three times every day depending on the extent of emulsion breakdown. Every 24 hrs, 10 ml of emulsions was sampled immediately after homogenization. During storage under elevated temperature, water was lost faster than that under room temperature. Oil contents were re-calculated each day as based on the moisture loss (by weighing moisture loss between sampling interval).

All samples were stored in freezer before peroxide value (PV) test. For PV quantification, samples were directly transferred into 30 ml acetic acid-chloroform solution (3:2, v/v). PV

test was conducted according to AOCS Official Method Cd-8-53 [14]. All treatments were duplicated.

Data Analysis

All treatments were duplicated except for the treatments with the addition of xanthan. SAS program (version 9.1, SAS Institute Inc., Cary, NC) was used for one-way Analysis of Variance (ANOVA) and General Linear Model (GLM) analysis. Least Significant Differences (LSD) were calculated at $P<0.05$.

RESULTS AND DISCUSSION

Effect of Emulsifier Concentration on Emulsion Stability

In a previous study where a conventional blender was utilized to create emulsion [13], it was found that at higher emulsifier concentration, 10%, the emulsion was not as stable as that at lower concentration, 5%, when oil-to-water ratio was 20:80. Figure 1a shows emulsion stability of EYL-stabilized emulsion at concentration of 0.5, 1, 2.5, and 5% when the same method of emulsion preparation was applied. The result indicates that emulsion with lower concentration showed poorer stability, especially when lecithin concentration was less than 1%. For the three lower concentration treatments, apparent oil aggregation and eventually oil layer appeared shortly after emulsion preparation. No emulsification ability was found at lecithin concentration of 0.5%. EYL concentration for making a relatively stable o/w type emulsion should be about 5% if we combine our result and Palacios and Wang's result under

the same given condition [13]. This figure also shows SL created a more stable emulsion than did EYL.

The samples with homogenization treatments showed much better emulsification and emulsion stability (Figure 1b). No visible oil phase separation was observed within 4 hrs, and only creamy phase slowly appeared with time. The treatment with 5% EYL had lower cream layer separation than the other three treatments did at early stage of the storage. All the SL treatments showed similar pattern of stability as the function of lecithin concentration, though SL gave better stability at each concentration than did EYL (Figure 1a and 1b).

Effect of Oil-to-Water Ratio on Emulsion Stability

As shown in Figure 1b, the diagram of phase separation versus time is presented to describe the emulsion stability. Also, two indexes, which are the maximal phase separation (Vmax, %), and the time to reach the maximal phase separation (Tmax, hrs), are used to quantitatively describe this stability. The means of all measurements are listed in Table 1. Stabilities of emulsions with oil-to-water ratio of 20:80 and 50:50 were compared as shown in Figure 2. Under both conditions, we observed that EYL-stabilized emulsions had poorer stability than did the SL-stabilized ones. Again, concentration of lecithin affected the stability of emulsion. Most importantly, we found that the system with oil-to-water ratio at 50:50 performed much better than that at 20:80 ($P<0.0001$ for both Vmax and Tmax). The Vmax of EYL-stabilized emulsion decreased from 92.5% to 66.5%, whereas separation of SL-stabilized emulsion decreased from 81.3% to 0% for emulsion with 5% emulsifier after standing for 20 hrs.

Because all treatments received the same homogenization, they are expected to have the same energy input. However, energy density per mass of droplet varies when oil content changes, e.g., higher energy density is received for low-oil-content (20:80) emulsion and lower energy density for high-oil-content (50:50) emulsion. Higher energy density created smaller oil droplets [15]. According to Stokes's law, which states that settling velocity of droplet is proportional the square of the radius of the droplet, emulsion with smaller oil-to-water ratio should be more stable against gravitational separation. Stokes's law is useful in explaining single droplet movement in emulsion system. However, factors influencing emulsion stability involves not only properties of single droplet but also interaction of droplets. When oil-to-water ratio increases, the droplet concentration increases though single droplet radius increases. Under this condition, the settling velocity can be retarded because droplets are so highly packed together and are restricted from moving at sufficiently high dispersed phase volume fraction (oil phase volume/total volume), as shown in Figure 3 [6]. This could partially explain why the concentrated emulsion was more stable under our experimental conditions.

Effect of pH and Thickener on Emulsion Stability

Experiments performed under neutral pH as described above showed that both concentrated dispersed phase (50:50 o/w) and concentrated emulsifier (5%, lecithin in oil) improved emulsion stability (P<0.0001 for Vmax), and SL gave better emulsion stability than did EYL (P<0.0001 for both Vmax and Tmax). When this experiment was performed under acidic condition (pH=2.3), Tmax for SL decreased, whereas it increased for EYL

though Vmax had no significant change in the case of oil-to-water ratio of 20:80, as shown in Fig. 4 and Table 1.

When oil-to-water ratio increased to 50:50, SL systems had both oil on the top layer and water separation on the bottom within a few hours after formation of emulsion, as shown in Figure 5, whereas EYL system had relatively more stable emulsion at both lecithin concentrations. pH decrease did not significantly decrease Vmax, but it significantly increased Tmax at each lecithin concentration for each lecithin (***P***<.0002). This phenomenon may be due to the different phospholipid compositions between EYL and SL. SL contains large proportion of phosphatidylinositol (PI, 17%), whereas only trace PI was found in EYL (Table 2). PI is anionic and has only one negatively charged phosphate group under neutral pH, which stabilizes the emulsion by strong electrostatic repulsion. Under acidic condition, hydrogen ions in the water phase will shield its negatively charged phosphate group and its strong polarity is reduced, which weakens its efficiency as an emulsifier. Both PC and PE are zwitterionic PLs and stabilize emulsion through hydration repulsion under neutral condition. Under acidic condition, they are positively charged because the negatively charged phosphate group is neutralized by the hydrogen ions. These positively charged molecules surround the oil droplets, preventing them from coalescencing with each other. Therefore, egg lecithin is more favorable at stabilizing o/w type emulsion under acidic conditions than did under neutral condition because of its high percentage of PC and PE (Table 2).

In the food industry, emulsifiers are usually used with other thickeners, such as xanthan gum, to give better emulsion stability. Xanthan was used to investigate the effect of thickeners or viscosity on lecithin-stabilized emulsion. As we know that xanthan gum is an anionic polysaccharide, low pH may reduce its function as an emulsion stabilizer. Our

preliminary test verified that, at highly acidic condition (pH 2.3), emulsion stabilized by xanthan gum showed much poorer stability in comparison with the ones at neutral condition (data not shown). The following experiment was carried out under neutral pH and oil-to-water ratio of 50:50, and results are shown in Figure 6. In comparison with the treatments without addition of xanthan (Figure 2, 50:50 o/w), the initial breakdown time for SL (5%) was extended from 20 hrs to 120 hrs and for EYL (2.5%, 5%) was extended from 0 hrs to 40 hrs. Figure 6 also indicated that all treatments had better emulsion stability than the control, which did not have any lecithin but only 0.05% xanthan.

Another observation from Figure 6 was that difference in emulsion stability due to emulsifier concentration as shown in Figure 2 was reduced after adding a low concentration of xanthan. Xanthan gum contains high molecular weight (~10^3 kDa) polysaccharide, and its stabilization effect is possibly due to its high viscosity and electrostatic repulsion [6]. This indicates that by combination of emulsifier and suitable stabilizer, EYL could be utilized in a more efficient way.

Oxidative Stability of EYL in Bulk System

Figure 7 shows lipid hydroperoxide generation and degradation with time. For both EYL and SL, the starting peroxide values (PV) were relatively high (between 15 and 30 meq/kg). This indicates that oxidation had occurred during fractionation and/or storage. EYL was oxidized to a maximal value of 28.9 meq/kg at 48 hrs, whereas the peak value of soybean lecithin was 52.2 at 192 hrs. After reaching peak value, PV declined because of peroxide decomposition and termination of the free radical chain reaction.

Soybean oil oxidized for 8 days (192 hrs) under the same conditions had PV about 120 meq/kg. And its maximal value was reported as 400 meq/kg at 290 hrs under the same storage condition [13]. This experiment shows that both EYL and SL were much more stable towards oxidation than soybean oil. However, the reason for peroxide breakdown or termination of reaction at such a low PV is unknown.

EYL was more stable than SL (LSD = 2.0 meq/kg) as shown in Figure 7, possibly because of its high content of saturated and mono-unsaturated fatty acids (Table 3), even though antioxidants such as tocopherols may not have been completely removed by acetone wash of SL. In comparison with oxidation curve obtained by Palacios and Wang where soybean lecithin was washed with acetone, induction stage of SL was longer in this experiment possibly because of antioxidant effect [13]. This experiment indicates convincingly that EYL has much better oxidative stability than soybean lecithin.

Oxidative Stability of EYL in Emulsion, and with Addition of Cupric Ion

Figure 8 shows oxidation of EYL and SL alone and in the presence of cupric ion (1 ppm) in emulsion. Both EYL and SL alone showed similar trend of hydroperoxide formation during first 7 days of storage. But EYL appeared to have stable PV from day 8 till the end of the storage period, whereas SL showed continued uptrend. Cupric ion is a strong prooxidant, even in emulsion system [16]. However, cupric-containing EYL system showed similar oxidation trend as EYL alone in emulsion as shown in Figure 8. This study indicates the prooxidant effect of cupric ion was retarded in EYL emulsion, and EYL with cupric ion incorporated seemed to be slightly more resistant to oxidation.

As expected, Figure 8 shows that cupric-containing SL emulsion sample had much faster oxidation rate than did non-cupric ion SL emulsion (LSD =5.4 meq/kg). Its PV increased from 9.4 to 134.0 meq/kg, whereas the PV of non-cupric emulsion increased from 10.4 to 53.0 meq/kg. This increase in oxidation rate indicates cupric ion functioned as pro-oxidant for SL oxidation in emulsion.

A few factors may account for the fact that cupric ion behaved differently in EYL and SL stabilized emulsion systems: the PL components of EYL and SL are different, especially the presence of plasmalogen phospholipids in EYL. Plasmalogen phospholipid contains an enol ether structure as shown in Figure 9 [17]. Palacios and Wang estimated the content of plasmalogen at about 3.6% of total yolk PLs [13]. Engelmann reported that one enol ether double bond prevented the oxidation of the four double bonds contained within arachidonic acid molecule [18]. The role of plasmalogen PL for cupric-induced lipid oxidation was evaluated *in vitro*, and it is believed the formation of a complex between cupric ions and the plasmalogens (on enol moiety) accounts partly for their inhibition of cupric-induced lipid oxidation [19]. Besides the contribution of plasmalogen, SL contains higher content of anionic PI and PA. And the negatively charged PLs will attract cupric ion and make it concentrate on the interface of the lipid/phospholipid droplet, thereby, cupric ion accelerates oxidation of SL more easily than of EYL. Both factors may account for the oxidative stability difference observed between EYL and SL in emulsion.

ACKNOWLEDGEMENTS

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Table 1 The effect of oil phase proportion, pH, and lecithin type on emulsion stability of lecithin-created oil-in-water emulsion.

pH	Oil,%	Lecithin	Lecithin Concentration			
			2.5%		5.0%	
			Vmax, %	Tmax, hrs	Vmax, %	Tmax, hrs
7.0	20	Soy	88.5±4.9 b,c	18.3±1.1 b	81.3±8.8 b,c	22.0±1.4 b
7.0	20	Yolk	96.0±5.7 b,c	0.8±0.3 e	92.5±3.5 b	1.0±0.0 e
7.0	50	Soy	30.0±14.1 e	34.0±1.4 a	10.5±2.1 d	33.5±4.9 a
7.0	50	Yolk	76.5±2.1 c,d	8.5±0.7 c	66.5±2.1 c	11.3±1.8 c
2.3	20	Soy	99.0±1.4 b	0.5±0.0 e	97.3±0.4 a,b	2.1±0.8 d,e
2.3	20	Yolk	120.0±14.1 a	1.8±0.3 d,e	115.0±21.2 a	6.3±0.4 d
2.3	50	Soy	60.0±11.3 d	6.5±4.9 c,d	16.0±5.7 d	5.5±2.1 d,e
2.3	50	Yolk	61.0±1.4 d	22.5±3.5 b	17.5±3.5 d	21.0±1.4 b
$LSD_{0.05}$			19.9	5.2	19.9	5.0

Different letters in the same column denote significant difference at $P<0.05$. LSD: least significant difference. Vmax: Maximal phase separation, %. Tmax: the time to reach maximal phase separation, hrs.

Table 2 Class composition (mole, %) of phospholipids in crude soy and egg yolk lecithin [20]

	PC	PE	PS	PI	PA	LPC	LPE	SPM
Soy lecithin	33	14.1	0.4	16.8	6.4	0.9	0.2	n.a.
Egg lecithin	66-76	15-24	1	n.a.	n.a.	3-6	3-6	3-6

n.a. : not available because of low content. PC: phosphatidylcholine; PE: phosphatidylethanolamine; PS: phosphatidylserine; PI: phosphatidylinositol; PA: phosphatidic acid; LPC: lyso-phosphatidylcholine; LPE: lyso-phosphatidylethanolamine; SPM: sphingomyelin.

Table 3 Fatty acid composition (mole, %) of the main PL classes of egg yolk and soybean lecithin

Source	PL	16:0	18:0	18:1	18:2	18:3	20:4	20:5	22:6
Soybean*	PE	16.0	8.3	6.8	57.3	11.7	--	--	--
	PI	22.2	19.3	6.1	43.4	9.3	--	--	--
	PC	11.2	11.9	8.6	58.6	9.9	--	--	--
Egg Yolk	PE	16.6	29.6	22.2	13.6	--	13.0	2.0	3.0
	PC	31.1	14.2	31.4	18.3	--	3.6	0.6	0.8

*Soybean data from Hammond et al. [21].

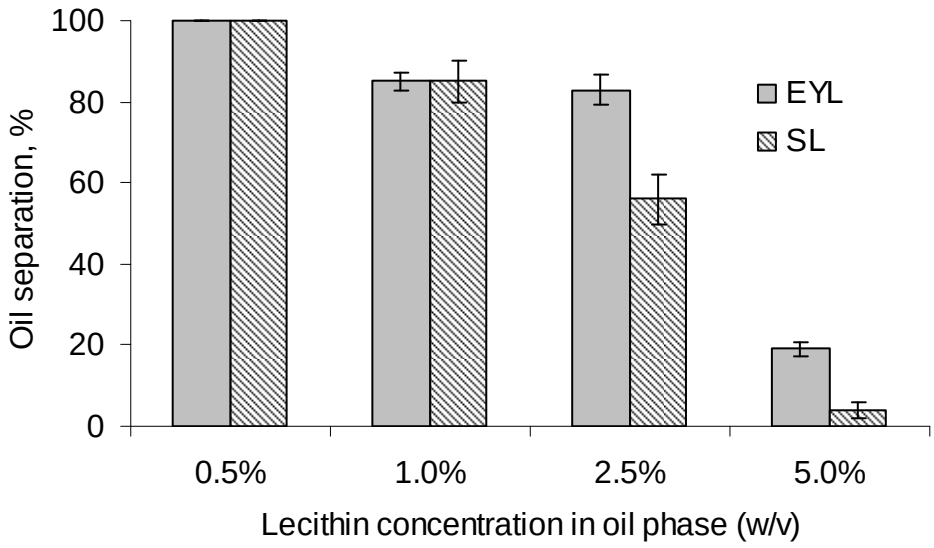


Fig. 1a Effect of EYL concentration on emulsion stability of o/w system (20:80) with conventional blending method. Oil separation was observed 30 min after emulsion preparation.

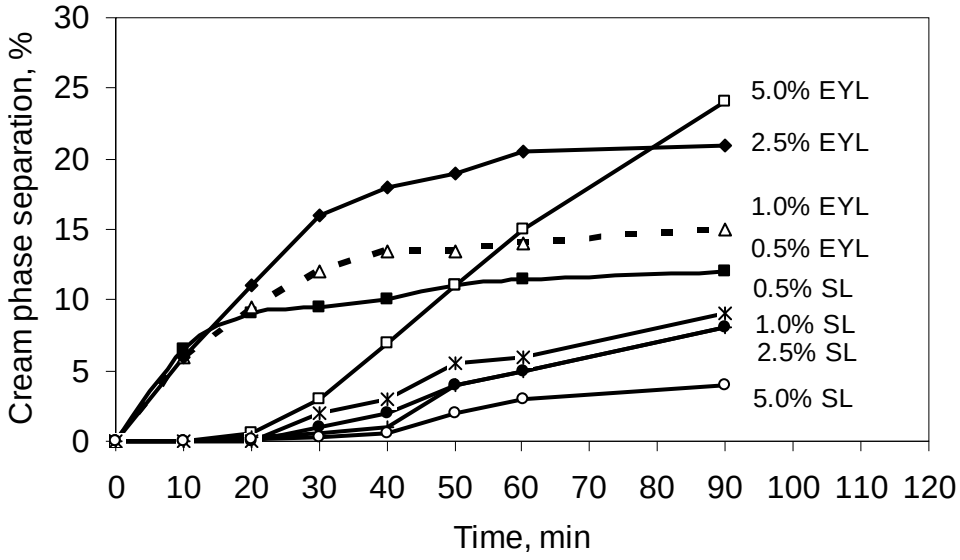


Fig. 1b Effect of EYL concentration on emulsion stability of o/w system (20:80) with homogegnization method.

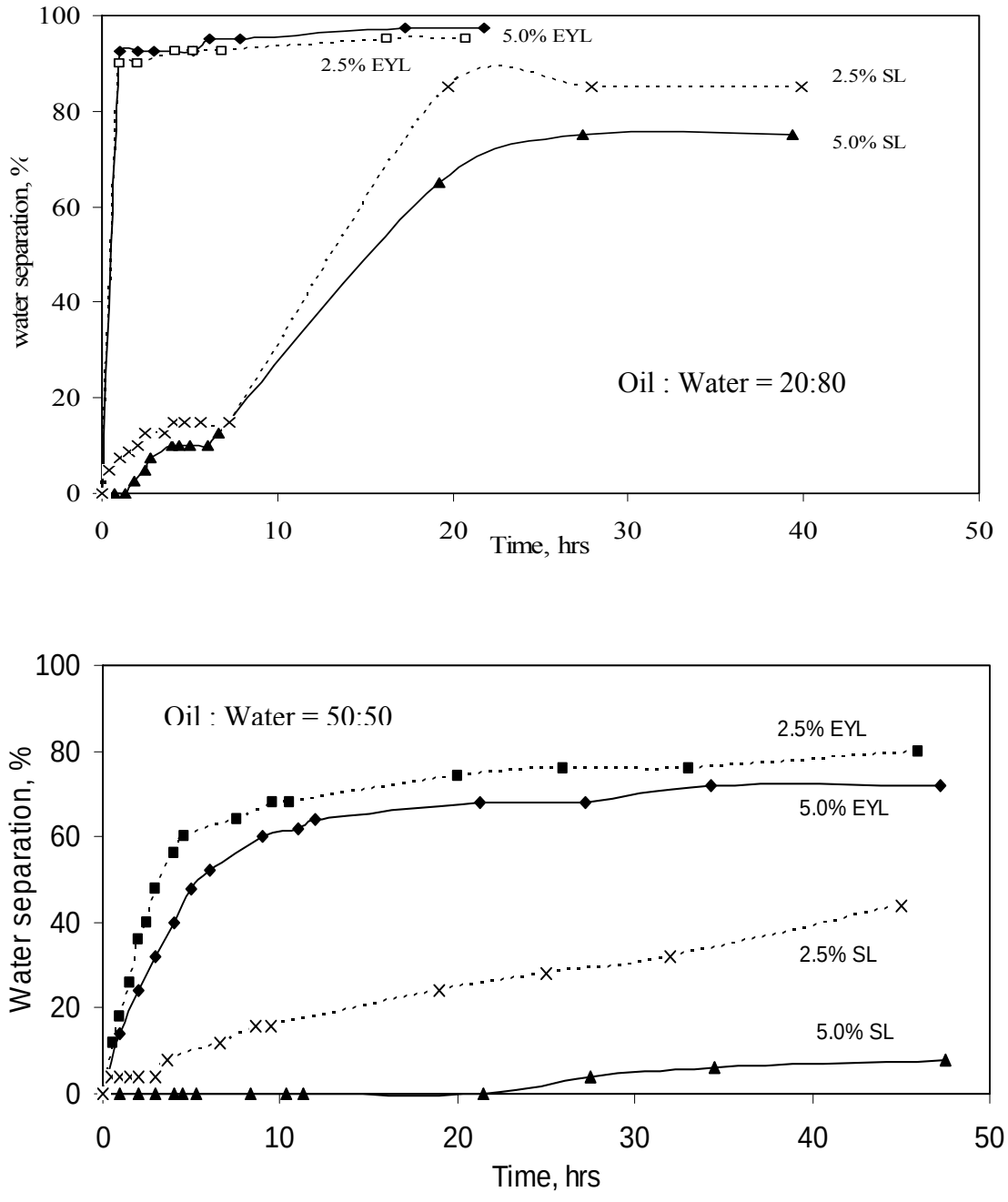
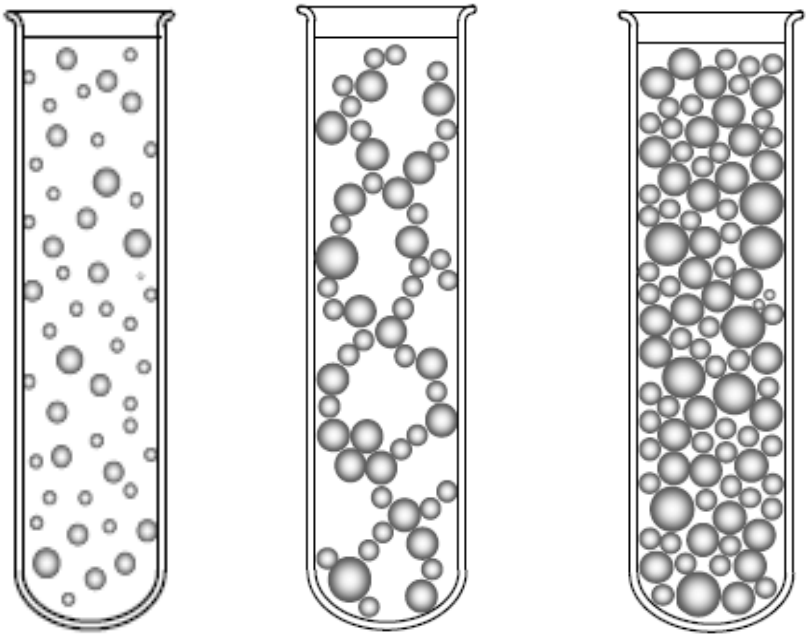


Fig. 2 Effect of oil-to-water ratio on emulsion stability of EYL- and SL-stabilized system.



No packing Open packing Close packing

Fig.3 Gravitational separation is prevented when dispersed phase volume fraction get higer (Adapted from McClements[13]).

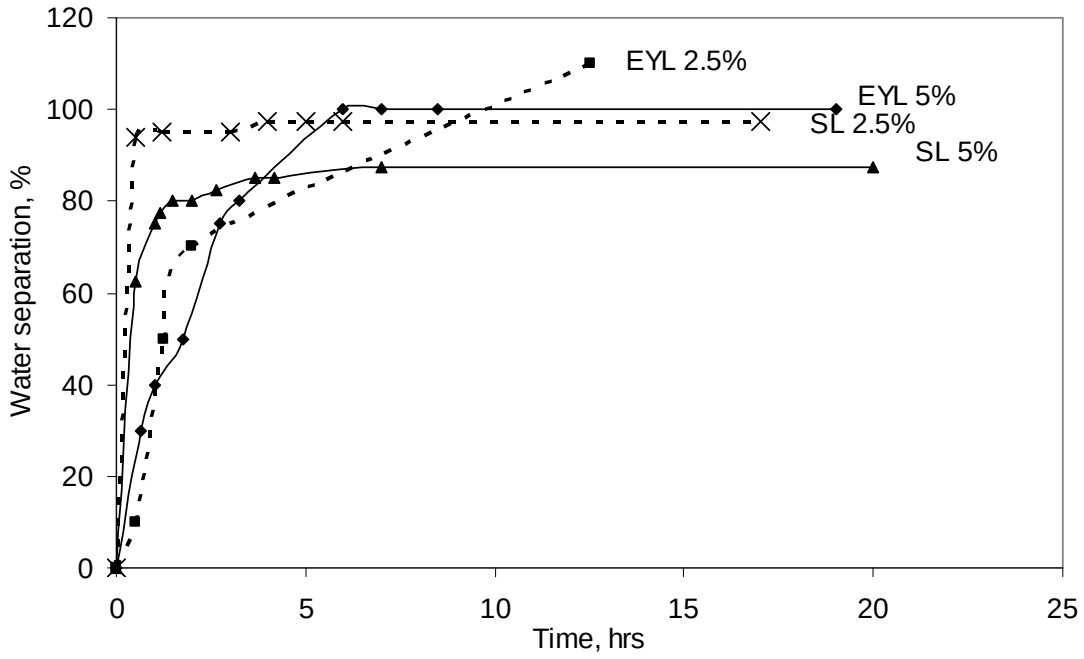


Fig. 4 Emulsion stability of lecithin under acidic condition (pH 2.3) when oil-to-water ratio was 20:80.

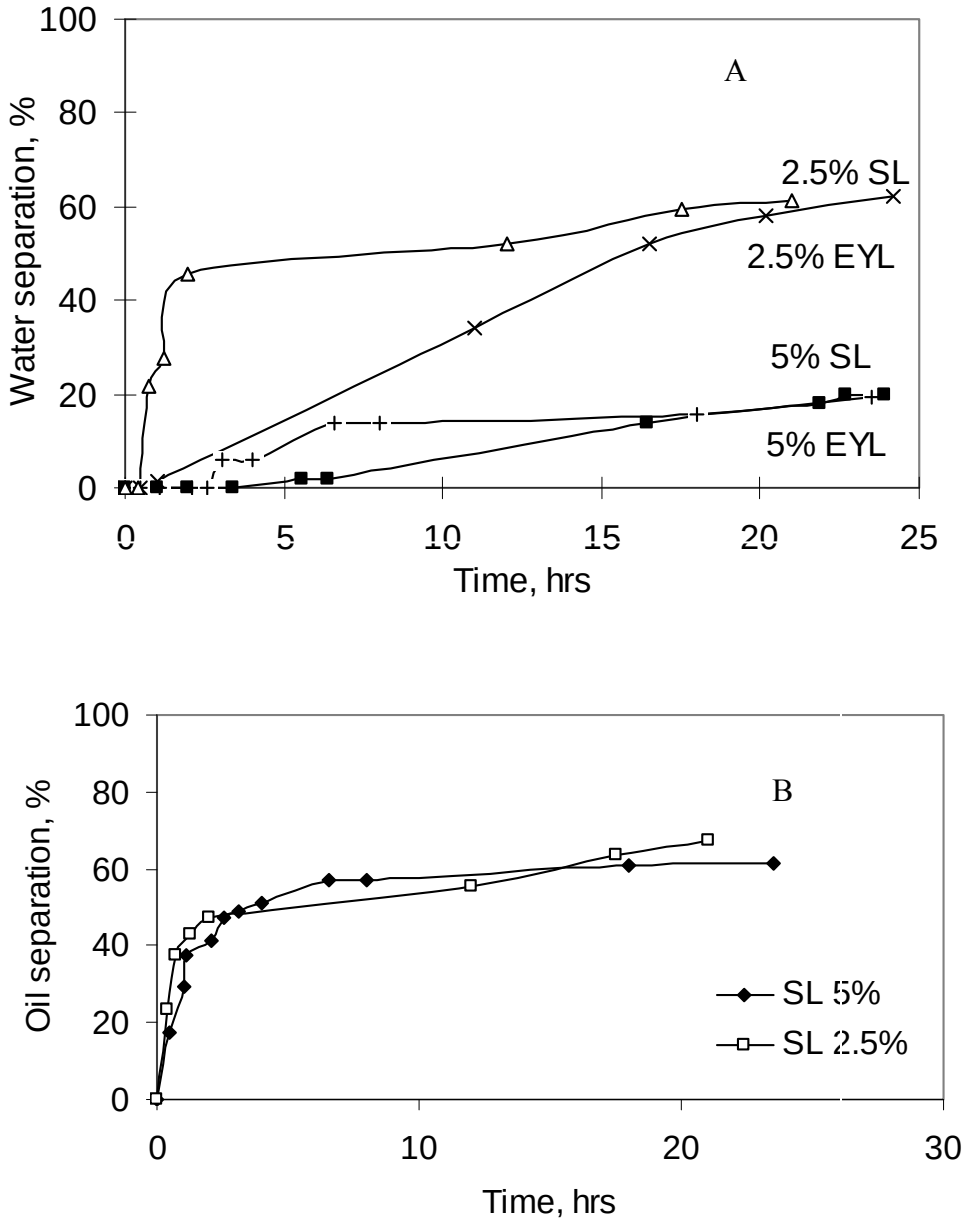


Fig. 5 Emulsion stability of EYL and SL emulsions under acidic condition (pH2.3), when oil-to-water ratio was 50:50. A: expressed as water phase separation, %; B: expressed as oil phase separation, %. Note: no oil separation was observed for EYL stabilized emulsion in B.

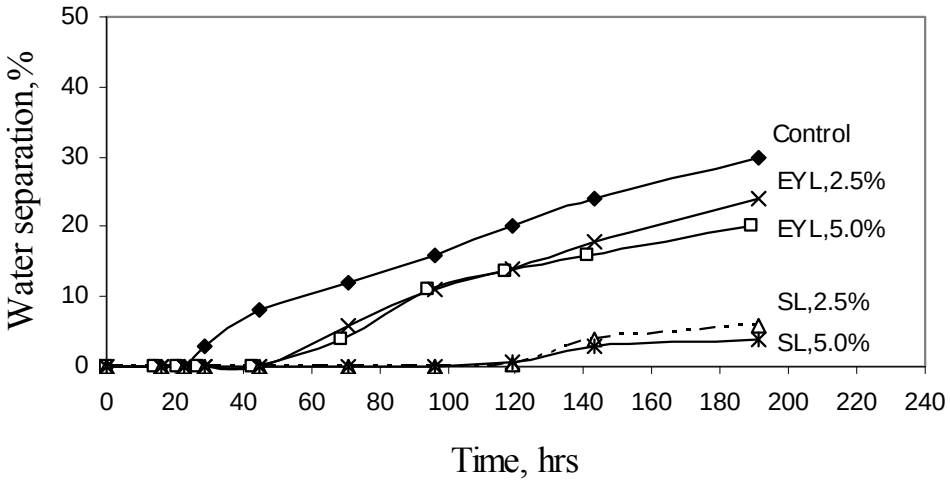


Fig. 6 Emulsion stability when xanthan (0.05%) was used as thickener (neutral pH, oil-to-water = 50:50).

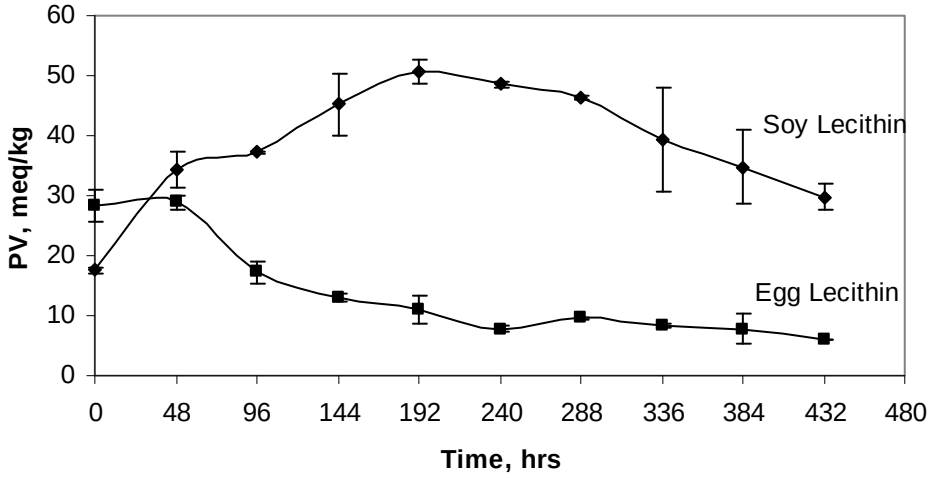


Fig. 7 Peroxide value change of egg lecithin and soy lecithin vs. heating time at 55˚C in bulk system.

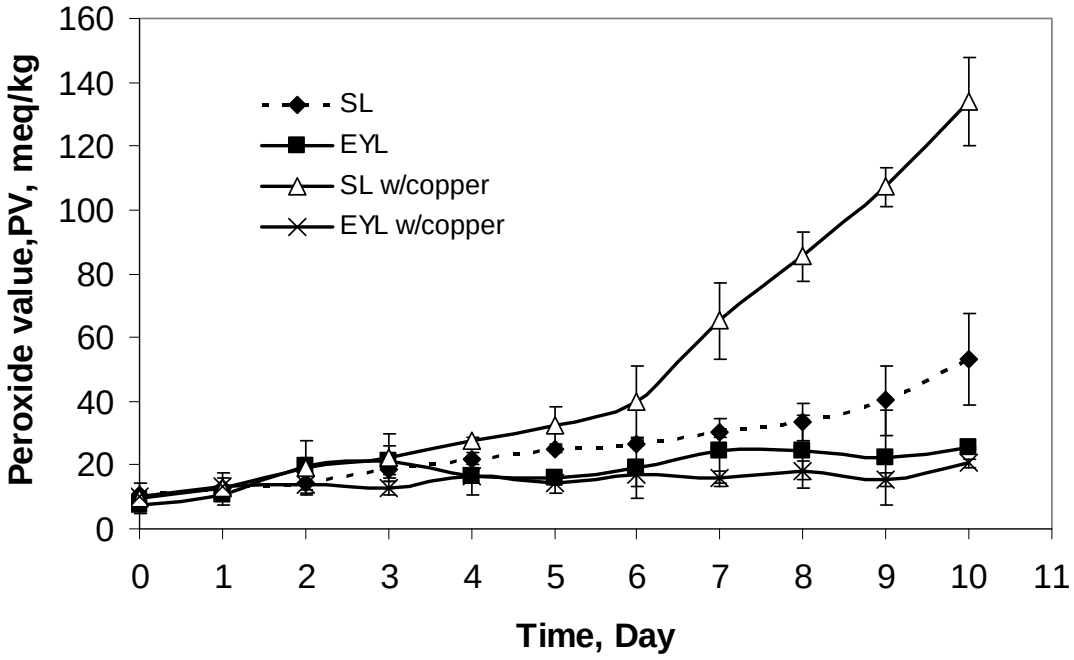


Fig. 8 Peroxide value change of egg lecithin and soy lecithin with heating time at 55℃ in o/w (50:50) emulsion and with addition of cupric ion (1ppm).

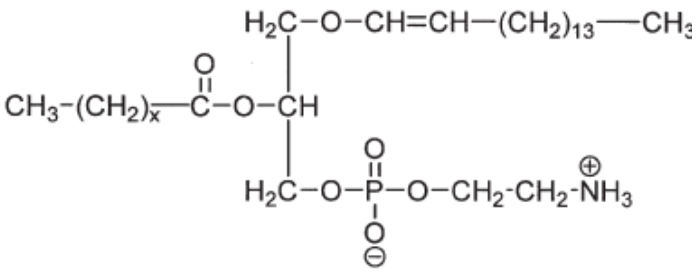


Fig. 9 Chemical structure of a PE plasmalogen.

CHAPTER 3. EFFECT OF CONTROLLED ENZYMATIC HYDROLYSIS ON FUNCTIONALITIES OF EGG YOLK PROTEIN

A paper to be submitted to *the Journal of Food Science*

G. Wang[1] and T. Wang[1,2]

ABSTRACT

Delipidated egg yolk protein (EYP) is produced as a co-product of egg yolk lecithin extraction. This EYP showed poor functionality possibly because of protein denaturation caused by ethanol treatment during lecithin extraction. Two food grade endo-proteases were used to produce EYP hydrolysates (EYPh) with two degrees of hydrolysis (DH), 3% and 6%. Protein solubility improved as DH increased, and both solubility profiles for EYP and EYPh were relatively less pH-dependent compared to soy protein. Except for foaming capacity, EYPh showed good improvement in foam stability, the speed of liquid being incorporated into foam, and maximal foam volume. Emulsion stability was improved for all EYPh treatments. Treatments at DH of 6% showed significant increase in emulsion capacity, but low DH of 3% gave reduced emulsion capacity in comparison with EYP and soy protein isolate. Overall, controlled enzymatic hydrolysis could be applied to ethanol-treated lipid-free EYP to increase the solubility, and improve the foaming properties and emulsification properties of EYP.

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Food Chemistry and Toxicology

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Keywords: delipidation, egg yolk protein, emulsification, enzymatic hydrolysis, foaming, solubility

INTRODUCTION

Egg yolk is a good source of phospholipids, especially phosphatidylcholine and phosphatidylethanolamine. We have developed a method for total lipid extraction and phospholipid fractionation from fresh egg yolk (Palacious and Wang 2005). In the process of delipidation by using ethanol and hexanes under mild condition, lipid-free egg yolk protein (EYP) is produced, and this delipidated yolk protein is considered as a co-product. It is critically important to determine and improve its functional properties and find its application to make the egg operating process more economically feasible for industry.

Sakanaka and others (2000) reported that the lipid-free EYP had amino acid score of 100. *In vitro* test for digestibility showed that EYP experienced faster digestion than milk casein when treated with pepsin and panceatin. In the test of protein efficiency ratio (PER), the criteria to determine the nutritional value of proteins, EYP showed significantly higher value than that of milk casein.

However, applying this lipid-free EYP as functional ingredient in food could be challenging because of its expected poor protein functionalities as a result of its solvent denaturation. Organic solvents can lead to drastic conformational changes, and, in many cases, these changes cause further aggregation and precipitation of proteins (Lapanje 1978). Plant proteins such as soy protein subjected to hexanes extraction still show good solubility and functionalities as shown in commercial soy protein concentrate and isolate. However, the ethanol washed soy protein concentrate has significantly reduced functionalities. In our lipid

extraction process, both ethanol and hexanes were used and this protein denaturation can lead to lowered water solubility and functionalities, e.g., foaming properties and emulsification properties. The reason why this difference arises may be because ethanol is much more polar than hexanes, though it is less polar than water. Large amount of ethanol may access to the hydrated protein and displace part of the bound water, which hydrates protein; therefore, denature the protein. On the contrary, non-polar hexanes are water immiscible and are difficult to reach hydration layer of protein. Therefore, hexanes can not displace the bound water, which is believed to protect protein from conformational change (Khmelnitsky 1991). However, hexanes alone will not extract all lipids from moist material.

It has been shown that controlled enzyme hydrolysis is an effective method in modifying functionalities of food protein, and this method has been used in various soy proteins (Lamsal and others 2006; Jung and others 2005) and whey proteins (Severin and Xia 2006). For egg yolk protein, Sakanaka and others (2004) reported that EYP hydrolysates from complete hydrolysis showed antioxidant activity in a linoleic acid oxidation system. However, there is little information on the effect of controlled enzyme hydrolysis of egg yolk protein on its foaming and emulsification properties. Enzymatic hydrolysis of protein causes breakdown of protein molecules and increase in solubility. Solubility of protein is essential for most proteins to give functionalities, such as foaming and emulsification properties. We hypotheses that controlled enzymatic hydrolysis could improve the foaming and emulsification properties of ethanol-treated lipid-free EYP.

In this study, two food grade proteases were investigated to hydrolyze ethanol-denatured egg yolk protein at two different degrees of hydrolysis. Protein solubility, emulsification

property, and foaming property of the corresponding hydrolysates were studied to examine the effectiveness of such treatments.

MATERIALS AND METHODS

Materials and preparation of egg yolk protein

Ethanol-treated egg yolk protein was obtained as the co-product of egg lecithin extraction. Fresh eggs purchased from a local store were broken and egg white was carefully removed. Fresh egg yolk protein, 400 g, was mixed with 400 ml of 100% ethanol for dehydration and lipid extraction. The mixture was centrifuged for 15 min at 4895xg and 20°C. Liquid phase was removed, and this process was repeated another two times. The ethanol-insoluble fraction was mixed with 270 ml hexanes, for three separate times, and centrifuged at the same condition as for ethanol extraction. The hexanes-insoluble fraction after air drying was extracted by adding another 140 ml of 100% ethanol followed by centrifugation to ensure complete extraction of polar lipids. The insoluble fraction was lipid-free EYP. The resulting EYP was pre-dried overnight followed by vacuum oven drying for 24 hrs. All protein samples were ground by using a lab mill (Arthur H. Thomas Co., Philadelphia, PA) with a 40 mesh filter and sealed with polyethylene zip bag and stored in refrigerator (4˚C) until use.

Total residual lipid was extracted with 10 times (volume to weight) of chloroform:methanol (2:1, v/v) and solvent was evaporated after filtration. Lipid was put in vacuum oven overnight to remove any residual solvent, and lipid content was presented in

percentage. The residual oil content was measured as 4.76%, and it was similar to the oil content in soy protein isolate (SPI) as listed on the product report.

Two food-grade enzymes used in this study were liquid Protex 7L and solid Protamex 1.5. Protex 7L (Genencor International, Rochester, N.Y., USA) is a bacterial neutral endo-protease originated from *Bacillus amyloliquefaciens* and its effective component is protease neutral (E.C. 3.4.24.28). Enzyme Protamex 1.5 (Novozymes North America, Inc., Franklinton, NC, USA) is also an endo-protease orignated from *Bacillus amyloliquefaciens* and *Bacillus licheniformis*. Both enzymes are non-specific proteases. Physical information about these enzymes is presented in Table 1.

Soy protein isolate (SPI) (Profam-974®) is generously donated by ADM Company (Decatur, IL, U.S.A).

Enzymatic hydrolysis

The pH-stat method was used to determine degree of hydrolysis (DH). According to this established method (Alder-Nissen 1986), the amount of base consumed in mole is proportional to the amino groups liberated in mole during the hydrolysis process. DH was calculated using Alder-Nissen's equation: $DH\%=100*V_B*N_B/(\alpha*Mp*h_{tot})$, where α is degree of dissociation of α –amino group, Mp is the mass of protein (g), h_{tot} is the total number of peptide bonds in the protein (meq/g protein), and V_B and N_B are volume and concentration of alkaline added. At fixed temperature and constant pH environment, α and h_{tot} are constants for a specific protein. We used pH and temperature of 7.0 and 50°C. The values of α and h_{tot} for soy protein were used because they are not available for EYP yet. Therefore, the resulting DH% values shown in this study were rough estimates rather than

exact DH, and such relative measurement should not interfere with the question the experiment was designed to answer.

The hydrolysis reaction was operated in a 250 ml jacketed glassware with a pH-STAT automatic titration (718 Model, Brinkmann, Switzerland) connected with an iso-thermal water bath. EYP slurry 10% (w/w) was prepared and stirred for 30 min to fully disperse the protein in suspension. Protein suspension was preheated to 50˚C and pH was adjusted to the neutral by using 2 N sodium hydroxide. Based on above equation, fixed amount of enzymes (Table 2) for achieving DH of 0, 3, and 6% were added to the suspension. To determine the optimal dose of enzyme, EYPs were previously treated with a series of enzyme doses for hydrolysis, and DH change versus reaction time was plotted. When the slope of diagram became flat, we then chose this what as optimal dose. Regarding reaction time at each level of DH%, we wanted the two enzymatic treatments to have similar time to avoid deviation from heating time. Reaction was carried out until the set DH value was reached. Inactivation of the enzymes was done by acidifying hydrolysates to pH 4.0-4.2 immediately after titrations were finished (Adler-Nissen 1986). Egg yolk protein hydrolysates (EYPh) were then put into freezer (-20°C) until functionality tests. Being one of the most common methods of enzyme inactivation, heat inactivation was also examined by heating EYPh for 15min in water bath at about 90˚C for Protex 7L-treated EYPh.

All EYPhs were neutralized to pH 7 by using 1 N hydrochloric acid before functional properties were determined.

Water solubility profile

Water solubility curve was determined in the pH range of 2 to 10 with 1 unit interval. Freshly prepared 10% EYPh dispersion was diluted to 2% with deionized water and gently agitated for one hour. pH was adjusted at 30 and 60 min, respectively, using 1 N sodium hydroxide or 1N hydrochloric acid. All treated samples were centrifuged at 10,000xg for 10 min at 20˚C (Jung and others 2005). Protein content in the supernatant was determined by using the Biuret protein assay (Gornall and Bardawill 1949). Duplicate preparations were done for each treatment. Bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used to establish standard curve.

Foaming property

Freshly prepared 10% EYPh dispersion was diluted to 100 ml at 0.5% concentration at pH 7. The dispersion was loaded into a 400 ml glass column fitted with a medium size fritted glass disk at the bottom. Nitrogen gas was purged at 100 ml/min to make a final volume of 300 ml from 100 ml of 0.5 % protein solution. Three measurements were made: time to reach final volume of 300 ml, t_f in seconds; volume of liquid sample consumed at the end of foaming, V_{max} in ml; and time used for half of the incorporated liquid to drain back, $t_{1/2}$ in seconds. Three foaming property parameters were calculated as based on these measurements (Wang and others 2004):

1) Foaming capacity denotes ml of foam formed per ml of N_2 purged, and it is calculated as:

$$F_c = \frac{300mL}{100mL/\min \times t_f \times 1\min/60\sec}, \text{ in mL/mL unit}$$

2) K value, the specific rate constant for liquid drainage, which is used to describe foam stability, is calculated as:

$$K = \frac{1}{V_{max} \times t_{/2}}, \text{ in } mL^{-1}s^{-1}$$

3) V_i, which is the speed of liquid being incorporated into foams:

$$V_i = \frac{V_{max}}{t_f}, \text{ in mL } s^{-1}$$

Emulsification properties

EYPh (25 g, 2%) prepared from fresh 10% of stock hydrolysate dispersion was mixed with dyed (Sudan Red 2B, about 4 ppm) soybean oil at constant addition rate of the oil (5 ml/min). A Bamix brand hand-held blender (Switzerland) was used to continuously mix oil and EYPh dispersion at "Low" speed until obvious phase inversion was observed, at which point the oil-in-water emulsion system lost viscosity. The amount of oil added till phase inversion was measured and calculated as emulsification capacity (g oil/g protein).

Emulsion stability was measured by following method: dyed vegetable oil, 8 ml, was added into 32 ml (2%) EYPh and mixed for 1 minute by using the same hand-held blender at "High" speed setting. From the resulting emulsion, 10 ml was transferred into 15ml plastic centrifuge tube that has a marker interval of 0.5 ml. Emulsion stability (EC, %) was calculated by dividing non-separated volume by total volume after 1 day standing at ambient temperature. The formula is shown as followings:

$$EC\ (\%) = \frac{\text{Volume,non-separated, mL}}{\text{Volume,total, mL}} \times 100$$

Statistical analysis

All treatments were duplicated, and data analyses were done with SAS program (version 9.1, SAS Institute Inc., Cary, NC). One-way Analysis of Variance (ANOVA) was used and Least significant differences was calculated at $P<0.05$.

RESULTS AND DISCUSSION

Enzymatic hydrolysis

Because the two enzymes used showed different catalytic activities, different amount of enzyme needed to be used in order to obtain the same degree of hydrolysis. The least amount of enzyme used in each treatment was ensured that at the end point of reaction, hydrolysis speed (represented as slope of DH% versus time), was low as indicated by flat curve (Figure 1). Two enzymes did not show significantly different reaction time in producing EYPhs with DH of 3%, whereas Protamex-treated EYP had significantly longer reaction time than Protex at DH 6% (Table 2). All four treatments showed good hydrolysis reproducibility as shown in Figure 1.

Water solubility profile

Bovine serum albumin (BSA) was used as the standard to establish standard equation of solubility, which is Absorbance at 540 nm= 0.0206 (mg/ml protein) + 0.0025, R^2=0.9999. The solubility was measured in the pH range from 2 to10, and solubility curves of both EYP and corresponding EYPhs are shown in Figure 2. Solubility curve of soy protein isolate was determined and shown in Figure 2 as a comparison. EYP and its hydrolysates showed flat

curves throughout pH range, and there were no obvious isoelectric points. On the contrary, SPI showed expected U-shape profile as pH increased from 2 to 10, which is consistent with other reports (Jung and others 2005; Lamsal and others 2006). Kong and others (2007) mentioned similar pH-independent solubility curve of wheat gluten hydrolysates, though the non-hydrolyzed wheat gluten was pH-dependent. The low solubility of untreated EYP could be the effect of protein denaturation in the process of ethanol dehydration and extraction of lipid. Ethanol is less polar than water, which caused exposure of hydrophobic core of native EYP, e.g., causing protein unfolding, and hydrophobic interaction and aggregation. Thus, the water solubility was lowered. Enzymatic hydrolysis was expected to break protein molecules or aggregates into smaller pieces, thus increasing solubility. The evidence of high solubility at DH value of 6% and low solubility at DH value of 3% for both enzyme treatments supports this hypothesis. Solubility profile at even higher DH value such as 10% was also measured in our study, and it showed maximal solubility great than 60% (data not shown). However, its foaming properties were obviously impaired. This decrease in foaming properties indicates that DH of 10% may have been excessively hydrolyzed for optimal protein functionality.

Various factors may influence protein solubility, e.g., the extent of protein denaturation, amino acid composition, hydrogen bonding, ionic strength, aromatic/aliphatic ratio, and hydrophobicity. These factors usually are interactive and influence each other. Among these factors, the effect of amino acid net charge and protein hydrophobicity on protein solubility attracted most attention (Dill 1990; Hayakawa and Naika 1985; Shaw and others 2001). Tanford (1961) pointed out that the solubility of a protein is proportional to the square of the net charge on the protein. To evaluate the general net charge information on soy and yolk

proteins, we used amino acid composition of soy protein isolate (SPI) and egg yolk (dried) from USDA protein database as shown in Table 3. The net charge ratio is expressed as the negative charge (sum of glutamic acid and aspartic acid) divided by positive charge (sum of arginine and lysine). SPI has a net charge ratio of 2.9, whereas EYP has only 1.5. This difference in net charge may in part explain why solubility profile of EYP was less sensitive to pH change.

Foaming properties

As shown in Table 4, no significant difference was observed for foaming capacity (Fc) among all EYPhs and the two controls. For K value, which denotes the rate of liquid drainage and is a measure of foam stability, the three hydrolyzed treatments (EaDH3, EbDH3, and EbDH6) showed significantly lower values than that of EYP control except for EaDH6. The lower value of K means that foams created from these treatments were more stable. Meanwhile, all hydrolysis treatments showed significantly higher Vi value than EYP and SPI. This higher Vi indicates that the formation of foam by the hydrolysates was much faster in comparison with the EYP control. The possible reason for this improvement is that small peptide chains are more mobile to migrate onto the surface and change structural conformation to form protein film enclosing air. Parameter Vmax represents the maximal liquid volume consumed to create 300 ml foam during purging process and indicates the density of the foam. All treatments created higher density of foam than EYP and SPI controls. This means all EYPhs produced smaller bubbles and finer foam than did the controls. Therefore, we can generally conclude that controlled hydrolysis of EYP improved the foaming properties of ethanol-treated delipidated EYP.

Emulsification properties

Whole egg yolks are good natural emulsifiers that have been used in mayonnaise-making for a long time because of their large emulsion capacity and high stability. Emulsification capacity (EC) is the amount of oil that can be emulsified under specific condition per gram of protein. And emulsion stability (ES) describes the ability of protein to keep an emulsion without phase separation over time at a given temperature and gravitational field (Panyan and Kiara 1996). Mean EC and ES for EYP and its hydrolysates are presented in Table 5. The two treatments (EaDH6, EbDH6) with DH of 6% showed significantly higher EC than EYP and SPI. But EaDH3 and EbDH3, which had DH of 3%, had significant lower EC values than did EYP, and this indicates that higher DH (6%) is good for increasing emulsification capacity. Literature review by Lamsal and others (2006) suggested that soy protein hydrolysates had increased EC with DH up to 5%. It is not the case in our study for EYP hydrolysates. Emulsion stability of EYP was significantly improved by enzymatic hydrolysis in both DHs for both enzyme treatments. Again, higher DH gave better emulsion stability.

Another phenomenon we found in this study is that heating inactivation of the enzymes had marked detrimental effect on EC of EYP hydrolysates. After the enzymatic hydrolysis to achieve DH of 6%, we tried to terminate the hydrolysis reaction by heating hydrolysates at over 90°C for 15 min. The EC change was shown in Table 6. Heated hydrolysate had decreased EC by 3 fold compared with the one that was not heated and also lower than EYP control. The EC difference caused by heating and freezing inactivation indicates that EYP and EYPhs are heat sensitive, as the majority of other proteins are. This result is consistent

with other studies done on emulsion properties of whole egg yolks (Guilmineau and Kulozik 2006).

Effect of DH% and enzyme type on functional properties of EYP hydrolysates

In this experiment, we found that two enzymes showed similar hydrolysis trend with time. But generally the hydrolysates created by using Protex showed higher solubility curve at the same DH value as shown in Figure 2. This difference in solubility indicates the peptide bonds they attacked during hydrolysis maybe a little bit different, though they are both non-specific proteases. DH values positively relate to solubility profile for both type of enzyme hydrolysates. Treatments with lower DH showed better foaming performance, but they had poor emulsification properties. Also at DH of 6%, the treatment of Protex had poorer foaming properties while it had best emulsification capacity and stability.

Conclusion

This study illustrates that application of controlled enzymatic hydrolysis can significantly improve functionalities, such as solubility, foaming properties and emulsification properties of ethanol-treated EYP. Further work should be conducted on sensory evaluation to study how controlled enzymatic hydrolysis affects its bitterness and other sensory attributes.

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Table 1 Properties of proteases Protex (Genencor) and Protamex (Novozymes)

	Protex 7L (a)	Protamex (b)
Form	Liquid	Granular
Activity	Min. 1600 azocasein/g	1.5 AU
Concentration	1-5%	1-10%
Optimal pH	7	7
Optimal temperature , °C	25-65	24-60

Optimal pH and temperature range was cited from BRENDA's comprehensive enzyme information http://www.brenda.uni-koeln.de/index.php4

Table 2 Parameters used in enzymatic hydrolysis of egg yolk protein

Treatment	EaDH3	EaDH6	EbDH3	EbDH6
Enzyme	Protex	Protex	Protemax	Protemax
Desired DH, %	3	6	3	6
EYP, g	10	10	10	10
Water, g	90	90	90	90
E/S, %	0.05	0.15	0.075	0.20
Enzyme amount	166.7 ul	499.5 ul	150 mg	400 mg
NaOH consumed, 1 N,mL	0.515	1.03	0.515	1.03
Reaction time, min	32.4±0.6^{c}	80.4±5.0^{b}	41.0±6.0^{c}	94.0±8.5^{a}

E/S, %: ratio of enzyme to substrate (g/g). Concentration of Protex was estimated as 3% and Protemax was estimated as 5% to calculate E/S, %. EaDH3: Protex, DH = 3%; EaDH6: Protex, DH = 6%; EbDH3: Protemax, DH = 3%; EbDH6: Protemax, DH = 6%. DH: degree of hydrolysis. Different letters in reaction time represent significant difference at 95% confidence level.

Table 3 Charged amino acid content in egg yolk protein and soy protein isolate

	Charge	g/100g SPI	Relative Molar ratio	g/100g Egg yolk,dried	Relative Molar ratio
Aspartic acid	(-)	10.232	0.09	3.348	0.03
Glutamic acid	(-)	17.452	0.14	4.34	0.03
Arginine	(+)	6.67	0.04	2.444	0.02
Lysine	(+)	5.32	0.04	2.718	0.02

Net charge ratio of SPI : 2.9

Net charge ratio of EYP: 1.5

Data obtained from USDA national nutrient database for standard reference

Relative molar ratio for each amino acid is calculated by dividing amino acid content by its molecular weight. Histidine is not included here because its pI (6.3) is close to neutral. Net charge ratio for each protein is calculated by dividing sum of negative molar ratio by sum of positive molar ratio.

Table 4 Foaming properties of egg yolk protein hydrolysates and controls

	Fc, ml/ml	K x10^3, 1/(mL*s)		Vi x10^3,mL/s		Vmax, mL	
SPI	1.429±0.016	8.247±0.742	a	5.3±0.3	d	40.0±2.8	e
EYP (DH0)	1.446±0.008	5.727±0.060	b	7.0±0.0	c	52.0±0.0	d
EaDH3	1.417±0.000	2.339±0.012	d	12.5±0.0	a	95.0±0.0	b
EaDH6	1.319±0.007	8.035±0.378	a	9.0±0.1	b	74.0±1.4	c
EbDH3	1.412±0.008	2.397±0.019	d	12.4±0.1	a	95.0±0.0	b
EbDH6	1.369±0.022	2.549±0.069	c	12.7±0.2	a	100.0±0.0	a
LSD	0.250	0.800		0.4		3.0	

Fc: foaming capacity; K: specific rate constant for liquid drainage; Vi: speed of liquid being incorporated into foams; Vmax: maximal foam volume. EYP: Egg Yolk Protein; SPI: Soy Protein Isolate; EaDH3: Protex, DH = 3%; EaDH6: Protex, DH = 6%; EbDH3: Protamex, DH = 3%; EbDH6: Protamex, DH = 6%. DH: degree of hydrolysis. LSD: Least Significant Difference. Different letters in the same column indicate significant difference at 95% confidence level.

Table 5 Emulsification properties of egg yolk protein hydrolysates

	Emulsification Capacity, mL/mL	Emulsion Stability,%
SPI	551.0±1.3 d	30±0 b
EYP (DH0)	661.0±7.3 c	25±1 c
EaDH3	495.0±9.8 d	31±1 b
EaDH6	935.0±48.4 a	38±3 a
EbDH3	396.0±6.5 e	32±1 b
EbDH6	739.0±23.0 b	36±1 a
LSD	69.0	3

EYP: Egg Yolk Protein; SPI: Soy Protein Isolate; EaDH3: Protex, DH = 3%; EaDH6: Protex, DH = 6%; EbDH3: Protamex, DH = 3%; EbDH6: Protamex, DH = 6%. DH: degree of hydrolysis. LSD: Least Significant Difference. Different letters in the same column indicate significant difference at 95% confidence level.

Table 6 Emulsion capacity (EC, mL/mL) as affected by heat inactivation of the protease Protex 7L.

Treatment	EYP (DH0)	EYPh, DH6%, non-heating	EYPh, DH6%, heating
EC, g oil/ g protein	509 ± 25.6 b	918 ± 67.2 a	300 ± 3.5 c

EYP: Egg yolk protein control, not hydrolysis treatment; EYPh, 6%, Non-heating: Protex hydrolyzed EYP with DH of 6%, freeze-dried; EYPh, 6%, heating: Protex hydrolyzed EYP with DH of 6%, inactivation by heating for 15 min in 90˚C water bath. DH: degree of hydrolysis.

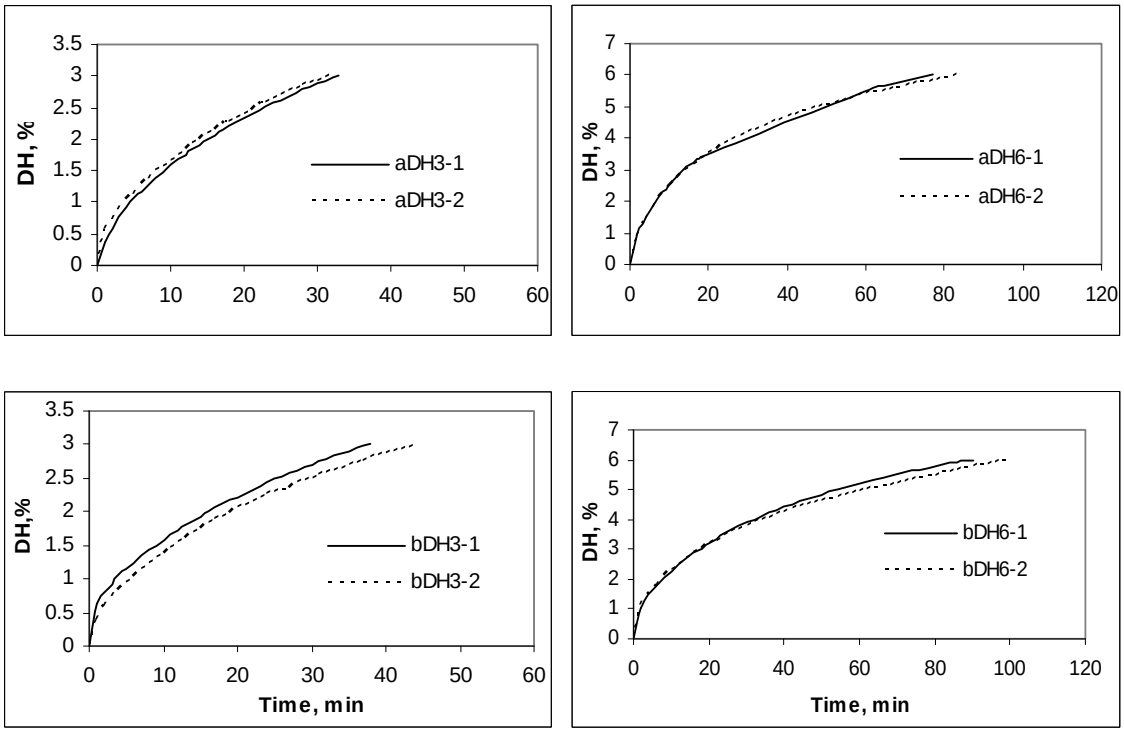

Fig. 1 Duplicated hydrolysis of egg yolk protein at pH 7 and 50˚C to achieve 3 and 6% hydrolysis by using two proteases as measured by pH-STAT. EaDH3: Protex, DH = 3%; EaDH6: Protex, DH = 6%; EbDH3: Protamex, DH = 3%; EbDH6: Protamex, DH = 6%.

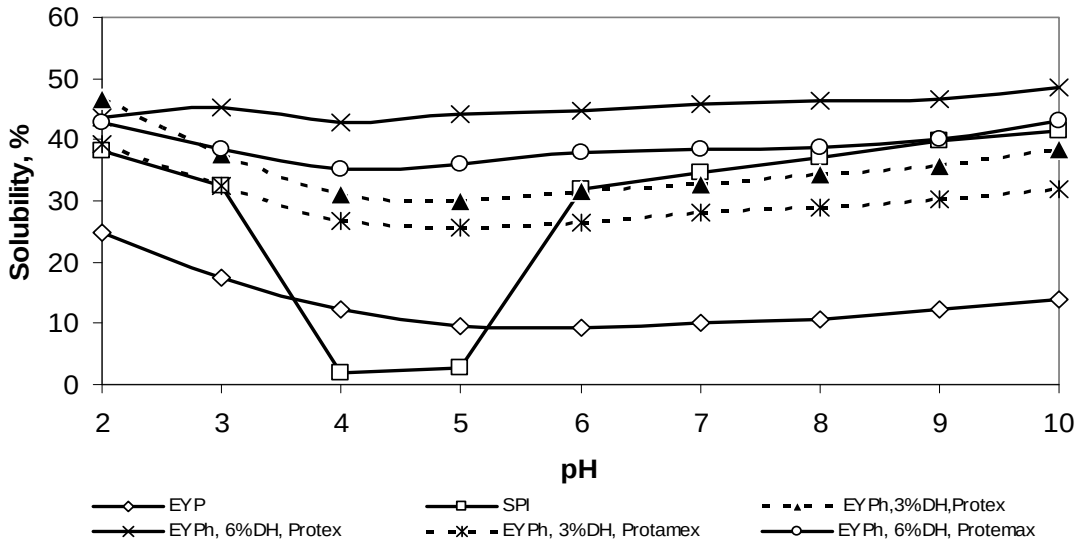

Fig. 2 Water solubility profile of egg yolk protein hydrolysates (EYPh), egg yolk protein (EYP) control, and soy protein isolate (SPI). DH: degree of hydrolysis.

CHAPTER 4. GENERAL CONCLUSIONS

Egg yolk lecithin (EYL) has unique fatty acid composition and phospholipid (PL) classes compared with other sources of lecithin, such as soybean lecithin (SL), which is the primary emulsifier and nutritional supplement. EYL contains more phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and less phosphatidylinositol (PI) and phosphatidic acid (PA) in comparison with SL. This difference in PL classes made the emulsion created by EYL have certain similarities in stabilizing emulsion as SL; meanwhile, they showed some difference in specific emulsion environment. Both EYL and SL stabilized emulsion of oil-in-water much better at higher oil and PLs concentration because of possible steric effect. The minimal concentration of EYL needed is higher than SL to obtain the stable emulsion under neutral pH. In acidic condition, EYL stabilized oil-in-water emulsion much better than what it did in neutral pH and better than SL did also. PL classes also affect the oxidative stability of EYL in the emulsion system. EYL showed better oxidative stability compared with SL in both bulk oil and emulsion systems, especially in the presence of prooxidant ion, such as cupric ion. Charged PLs, such as PI and PA in SL, could effectively attract transition metal, such as cupric ion, onto the surface of lipid droplets. This accelerates the oxidation of SL surrounding the droplet when it is used as emulsifiers. Plasmalogen found in EYL may contribute to this stability also. The amount of the total polyunsaturated fatty acid (PUFA) content mainly determined the oxidation rate, where EYL contains less PUFA, especially linoleic and linoleinic acid than did SL.

Delipidated egg yolk protein (EYP) is produced as a co-product of egg lecithin extraction. This EYP showed poor functionality possibly because of protein denaturation

caused by ethanol treatment during lecithin extraction. Two food-grade proteases were found to be effective at improving functionalities of delipidated EYP. Controlled enzymatic hydrolysis was conducted at two degrees of hydrolysis (DH), which were 3 and 6%. Solubility profile of treated EYP positively related to the DH. EYP with lower DH showed better foaming properties. Emulsion stability was improved for both enzyme treated hydrplysates at both DH. Low DH impaired emulsification capacity, whereas higher DH improved emulsification capacity of EYP. Further work should be conducted on sensory evaluation to study how controlled enzymatic hydrolysis affects its bitterness and other sensory attributes.

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