# THESIS APPROVAL

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# THESIS

# EFFECT OF SOY SOLUBLE POLYSACCHARIDE ON THE STABILITY OF SOY-STABILIZED LIQUID EMULSION

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**A Thesis Submitted in Partial Fulfillment of**
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This study used soy soluble polysaccharide (SSP) and pectinase-hydrolyzed soy soluble polysaccharide (PH-SSP) from okara, a soy residue from soy milk production, as the sources of dietary fiber in oil-in-water emulsions. It was found that although pectinase hydrolysis generally shortened the molecular weight of SSP, self-association of PH-SSP occurred after hydrolysis, resulting in the formation of large particles confirmed by SDS-PAGE and Zetasizer Nano-ZS. Pectinase-hydrolyzed soy soluble polysaccharide could induce morphological changes in murine macrophage RAW 264.7. The addition of reconstituted PH-SSP (0-2%) to the liquid emulsion containing 3.75% soy protein isolate (SPI) and 3.33% refined rice bran oil resulted in excessive sedimentation at pH 2.0 ($p<0.05$). This was likely due to associative phase separation mechanism of positively charged SPI and negatively charged PH-SSP. At pH 7.0, which was above isoelectric pH of SPI, charge repulsion between negatively charged SPI and negatively charged PH-SSP reduced the formation of insoluble coacervate ($p<0.05$). Nevertheless, the formation of insoluble coacervate could be lowered by using the heat-denatured SPI. Moreover, preferential distribution of SPI in serum phase of emulsion was also regulated by pH. Adding PH-SSP up to 6% did not alter emulsion capacity during *in vitro* digestion; however, it resulted in the destabilization of o/w emulsion under peptic and tryptic digestion. Overall, this study proposed the methods in controlling oil-in-water emulsion stability and the reduction of insoluble coacervate in soy-based emulsions containing soy soluble polysaccharide and its pectinase-hydrolyzed products.

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## INTRODUCTION

Emulsion is one of the food systems found in milk products, cake batters, fruit beverages, fat reduced food, encapsulation of active ingredients or control release delivery systems for active compounds. Generally, protein and polysaccharide are used to stabilize emulsion due to their abilities to lower surface tension and viscosity-building, respectively.

Okara or soy residue is a by-product from soymilk or tofu production. It is generally underutilized as animal feed. However, it has protein and carbohydrate contents around 25% and 50%, respectively (Liu, 1997). Many researchers extracted and characterized proteins and carbohydrates from okara to maximize its utilization (Yamaguchi *et al.*, 1996; Ma *et al.*, 1997; Chan and Ma, 1999).

Liquid emulsion such as those used as enteral formulae are prone to aggregation and gelation during thermal process due to their high contents of biopolymers (Roesch and Corredig, 2002). Themally-induced gelation is a limitation to drink and beverage industry. Besides, adding polysaccharides such as pectin could further lower the emulsion stability due to the aggregation between protein and pectin (Roudsari *et al.*, 2006). Consequently, using the water soluble soy polysaccharide from okara as a source of dietary fiber, which carries pectic-like structure (Yamaguchi *et al.*, 1996; Nakamura *et al.*, 2006a), may pose difficulties in thermal process of such emulsions because of the aggregation between protein and soy soluble polysaccharide.

The hydrolysis of okara by commercial pectinase may change the characteristics of water soluble soy polysaccharide. The different characteristics of soy soluble polysaccharide from okara affect the behavior of protein-polysaccharide

aggregation that influences the stability of soy-stabilized emulsion containing soy protein isolate (SPI), rice bran oil and soy soluble polysaccharide as a source of dietary fiber, particularly during *in vitro* digestion (Na Nakornpanom *et al.*, 2010). The insights in the interactions between protein and polysaccharide may be useful in regulating the formation of insoluble coacervate in soy-stabilized emulsion containing soy polysaccharides.

# OBJECTIVES

1. To study the effect of polysaccharide hydrolysis by commercial pectinase on the characteristics of soy soluble polysaccharide from okara,

2. To understand the interactions between soy proteins and polysaccharides and their influences on the stability of soy-stabilized emulsion, and

3. To study the stability of soy-stabilized emulsion containing soy soluble polysaccharide during *in vitro* digestion.

# LITERATURE REVIEW

## 1. Emulsion

Emulsion is a colloidal system consisting of two immiscible liquids (usually oil and water), with one liquid (called dispersed phase or internal phase) dispersed as small spherical droplets in the other liquid (called continuous phase or external phase). Emulsion is classified by dispersed and continuous phase; o/w emulsion is oil droplets dispersed in aqueous phase; while w/o emulsion is aqueous droplets dispersed in the oil phase. Emulsion system is generally found in foods such as pasteurized milk, margarine, salad dressing, etc. In most foods, diameters of the droplets usually lie somewhere between 0.1 and 100 μm (McClements, 2005). Apart from normal foods, emulsion can be used to control the release of active compound. In addition to simple emulsion, double emulsion or multiple emulsion has been continuously studied in the last few decades. Multiple emulsion can be used to control the release of active compounds, reduces fat in food or in the fabrication engineer-structure of foods. There are many researchers investigated single emulsion and multiple emulsion to control the release of bioactive compounds including water-soluble vitamins, peptides or oligopeptides (such as hormones), retinol, etc. (Couvreur *et al.*, 1997; Silva-Cunha *et al.*, 1997; Morishita *et al.*, 1998; Benichou *et al.*, 2004; Hwang *et al.*, 2005; Benichou *et al.*,2007; McClements *et al.*, 2007).

Emulsion is thermodynamically unstable; and oil and water tend to separate from each other. Figure 1 shows physical mechanism of emulsion instability including creaming, sedimentation, flocculation, coalescence, and phase inversion (McClements, 2005). Gravitational separation causes creaming and sedimentation. Creaming is referred to the phenomenon that the droplets move upward due to the lower density of its droplet compared to external liquid. Sedimentation is used to describe the event that the droplets move downward due to the fact that the droplets have higher density than the external liquid. Flocculation describes the aggregation of droplets where these droplets maintain their individual integrity; whereas coalescence is the process that two or more droplets immerge together to form a larger droplet.

Phase inversion is the process that o/w emulsion becomes to w/o emulsion or vice versa.

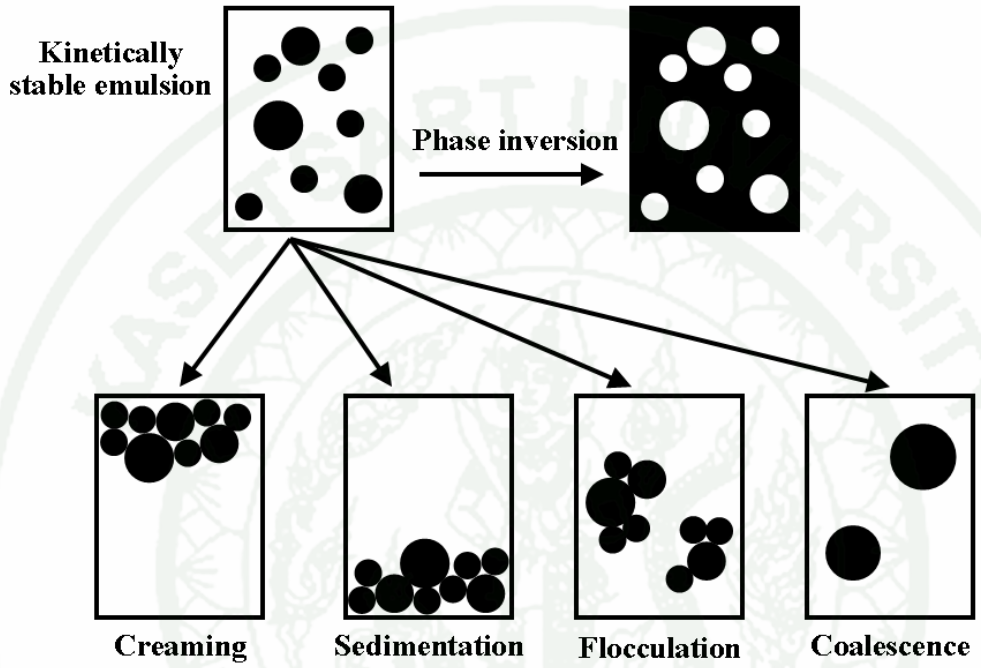


**Figure 1** Emulsion instability through a variety of physical mechanism.

**Source:** McClements (2005)

To form stable emulsion, the appropriate emulsifier and unit operation are required. Emulsifiers are surface active agent that can adsorb at the oil-water interface and form a protective membrane. This subsequently reduces the surface tension of each phase and prevents aggregation of the droplets. Most emulsifiers are amphiphilic molecules that have both hydrophilic and hydrophobic moieties in the same molecule. The most common emulsifiers used in food industry are small molecular weight emulsifiers, phospholipids and proteins (McClements, 2005).

In addition to emulsifiers, polysaccharide has been used to stabilize emulsion due to its ability to increase the viscosity of continuous phase. It is particularly used in

oil-in-water emulsion and retard droplet aggregation (McClements, 2005). Table 1 shows functional properties of polysaccharides commonly used in food industry.

**Table 1** Functional properties of polysaccharides used as viscosifier.

| Name | Structure | Solubility | Function | Aggregation Mechanism |
|---|---|---|---|---|
| Carrageenan | Linear<br>Anionic<br>200 – 400 kDa | Hot water<br>Cold water | Thickening<br>Gelling | Helix association<br>Cold-set<br>Thermoreversible |
| Alginate | Linear<br>Anionic<br>32 – 200 kDa | Hot water<br>Low $Ca^{2+}$ | Thickening<br>Gelling | $Ca^{2+}$<br>Cold-set<br>Thermoreversible |
| Pectin | | | | |
| Low methoxy | Linear<br>Anionic<br>5 – 150 kDa | Hot water<br>Cold water<br>(Low $Ca^{2+}$) | Thickening<br>Gelling | $Ca^{2+}$<br>Cold-set<br>Thermoreversible |
| High methoxy | Linear<br>Anionic<br>5 – 150 kDa | Hot water<br>Cold water<br>(Low $Ca^{2+}$) | Thickening<br>Gelling | Acid + sugar<br>Cold-set<br>Thermoirreversible |
| Starch | | | | |
| Native | Granules<br>Nonionic | Hot water | Thickening<br>Gelling | Granule swelling<br>Heat-set<br>Irreversible |
| Modified | Linear/<br>Branched<br>Nonionic | Cold water<br>Hot water | Thickening<br>Gelling | Helix association<br>Cold-set<br>Reversible |

**Source:** McClements (2005)

**2. Soy protein-stabilized oil-in-water emulsion**

Protein is an amphiphilic macromolecule. Hydrophilic region of protein molecule dissolves in the aqueous phase, while the hydrophobic region dissolves in the oil phase. Stabilization of emulsion by protein depends on 2 factors: (1) its ability to reduce the surface tension at oil-water interface, and (2) its ability to form films that provide strong repulsive forces between oil droplets via steric or electrostatic stabilization (McClements, 2004; McClements, 2005).

Protein in soybean seed can be divided by biological properties into 2 groups; metabolic proteins (e.g. structural proteins and enzymes) and storage proteins.
Most of soy proteins are globular proteins which are classified by solubility patterns into albumins and globulins. Albumins are water soluble, while globulins are soluble in salt solution. Most soy proteins, which are storage proteins, are globulin. Globulins can be further divided into two groups; i.e., legumin and vicilin (Liu, 1997). Legumins and vicilins are commonly known as glycinin and conglycinin in soybean, respectively. Moreover, soy protein can be classified by sedimentation velocity into 4 groups; 2S, 7S, 11S, and 15S. The 7S globulin fraction refers to β-conglycinin, possessing molecular weight of 180-210 kDa. β-conglycinin is a trimer which consists of 3 subunits with different physicochemical properties; α', α, and β subunits with the molecular weight of 58, 57, 42 kDa, respectively. The 11S globulin fraction is glycinin which is hexamer with molecular weight of 300-380 kDa. Glycinin consists of acidic (≈35 kDa) and basic (≈20 kDa) subunits. The quaternary structure of glycinin is stabilized by hydrophobic and electrostatic interactions. Moreover, both acidic and basic subunits of glycinin are linked by disulfide linkage, resulting in higher thermal stability than β-conglycinin (Petruccelli and Añón, 1995; Liu, 1997; Utsumi *et al.*, 1997; Fukushima, 2004).

**Table 2** Major protein fractions in soybean.

| Fraction | Total protein (%) | Components | Molecular weight (kDa) |
|---|---|---|---|
| 2S (α-conglycinin) | 22 | Trypsin inhibitors | 8, 21.5 |
| | | Cytochrome *c* | 12 |
| 7S (β- and γ- conglycinin) | 37 | Hemagglutinin | 110 |
| | | Lipoxygenase | 102 |
| | | β-Amylase | 61.7 |
| | | 7S globulin | 180-210 |
| 11S (glycinin) | 31 | 11S globulin | 350 |
| 15S | 11 | | ≈600 |

**Source:** Kilara and Harwalkar (1996)

Soy proteins have good solubility in alkaline condition (pH>8.0) and good emulsification properties. Some researchers demonstrated that soy protein has better emulsifying activity than those of some milk proteins (Utsumi *et al.*, 1997). When compared between β-conglycinin and glycinin, Rivas and Sherman (1984) found that β-conglycinin showed better emulsifying properties than that of glycinin due to the fact that β-conglycinin was more hydrophobic and had a lower molecular weight than glycinin. Therefore, it may easily adsorb at the oil-water interface. Using soy protein as emulsifier, however, has been limited under the acidic condition due to their isoelectric pH, which is around 4.5. At pH near 4.5, net charge of protein at surface is zero so the soy proteins aggregate (Liu, 1997).

Heat treatment is one of the physical modifications to improve emulsifying properties of proteins. Heat treatment causes protein denaturation. This leads to the exposure of hydrophobic patches, charged reactive groups, or sulfhydryl groups (Sorgentini *et al.*, 1995; Campbell *et al.*, 2009). Furthermore, it was found that heat treatment employed to soy protein isolate (SPI) not only induced denaturation, but also dissociation of subunits and induced protein aggregation. Keerati-u-rai and

Corredig (2009) indicated that heating SPI at 75°C for 15 min reduced droplet size of emulsion from 1 μm to 0.1 μm when compared to those stabilized by unheated SPI. This was due to the dissociation of β-conglycinin and partial denaturation of glycinin. However, heating SPI at 95°C for 15 min caused denaturation and dissociation of both β-conglycinin and glycinin, resulting in the increase of droplet size of emulsion due to the aggregation of protein subunits.

**3. Interactions between protein and polysaccharide**

Proteins and polysaccharides are macromolecules used extensively as nutrients and functional ingredients in food. The interactions between proteins and polysaccharides, such as electrostatic interactions and hydrogen bondings, influence the properties of foods. Both proteins and polysaccharides are charged molecules. When mixing proteins and polysaccharides together, either associative phase separation or segregative phase separation could occur (Figure 2), depending on their electrical surface charges (McClements, 2006). Associative phase separation occurs when protein and polysaccharide have opposite charges. They can form either soluble complex or insoluble coacervate. On the other hands, segregative phase separation occurs when protein and polysaccharide have the same charge or uncharged. It can be incompatible or cosoluble, depending on the concentrations of protein and polysaccharide (McClements, 2006).

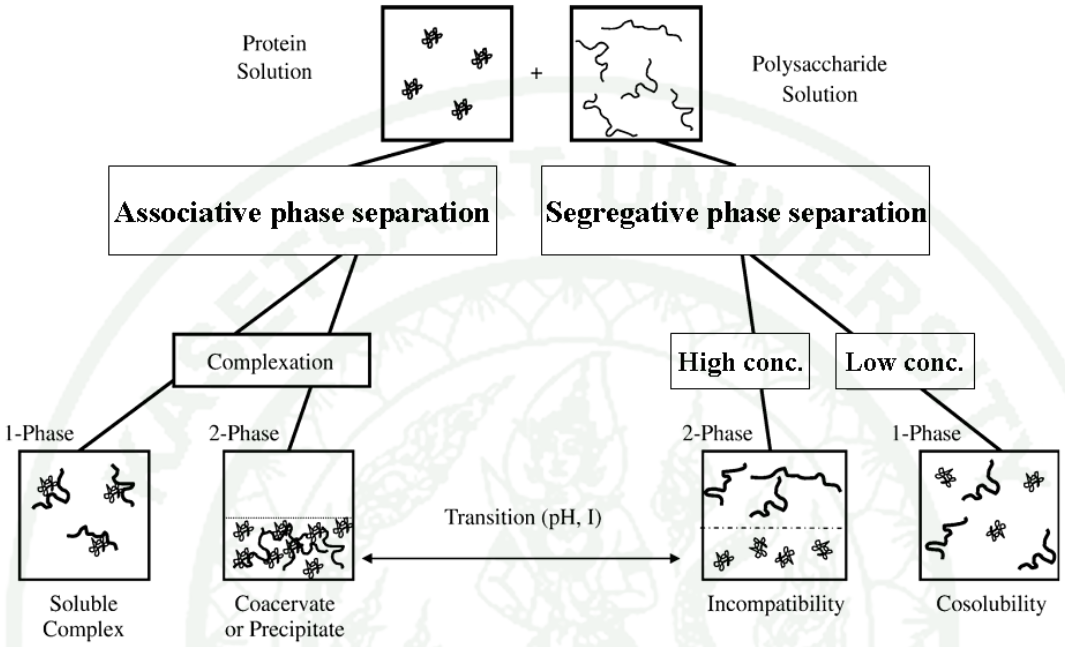


**Figure 2** The interactions between proteins and polysaccharides.

**Source:** McClements (2006)

**4. Factor affecting protein-polysaccharide coacervation**

4.1 pH

pH affects the coacervation between protein and polysaccharide because pH determines surface charge of protein and ionized groups of polysaccharide, e.g., carboxyl group of pectin or amino group of chitosan. At pH above isoelectric pH of protein, surface net charge of protein is negative. At pH below isoelectric pH of protein, net charge of protein at surface is positive. For polysaccharide such as pectin, it has a free carboxyl group in galacturonan backbone that has $pK_a$ value of ~3.5 (Thakur *et al.*, 1997). pH therefore controls the acid dissociation of carboxyl group.

At pH above $pK_a$ of pectin, carboxyl group is more ionized and pectin becomes more negative.

$$pH - pK_a = \log \frac{[COO^-]}{[COOH]}$$

Therefore, the alteration of pH will control the types and magnitudes of charges in protein and polysaccharide molecules and their interactions.

Roudsari and co-workers (2006) studied the influence of pH on stabilizing behavior of high methoxyl pectin in SPI stabilized emulsion containing 0.75% SPI, 5% soybean oil and various concentration of high methoxyl pectin. At pH 7.5, which was above isoelectric point of soy protein and $pK_a$ of carboxyl group in high methoxyl pectin, both soy proteins and high methoxyl pectin were negatively charged. Protein and pectin thus interacted by repulsive interaction so emulsion was stabilized. When adjusting the pH to acidic condition of around 3.5, protein had positively charged, while high methoxyl pectin still was negatively charged. High methoxyl pectin induced aggregation of proteins at the interface and coalescence of emulsion droplet via bridging flocculation occurred (Figure 3). Therefore, the size of emulsion droplets increased. However, when pH was below 3.5, which is below $pK_a$ of high methoxyl pectin, emulsion droplets stayed smaller than that at pH 3.5. This was due to the unionized carboxyl group in pectin at pH below 3.5, so that high methoxyl pectin became uncharged molecule, which resulted in the absence of bridging flocculation.

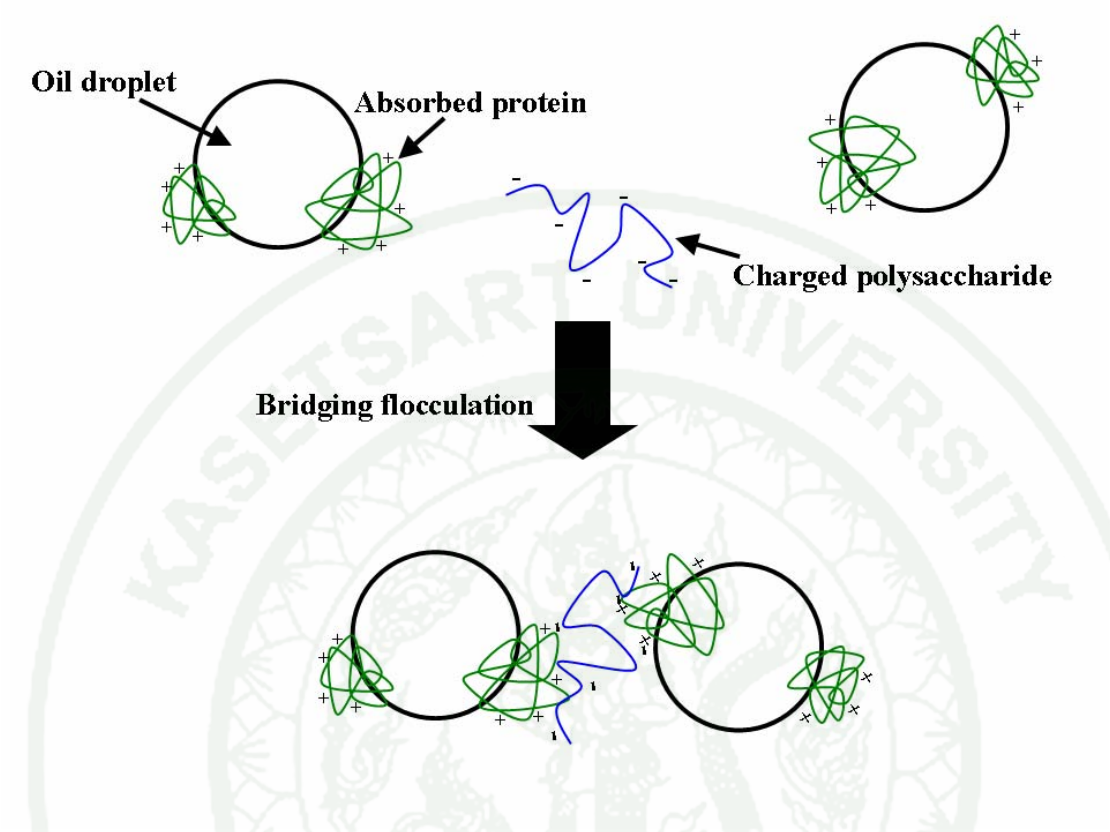


**Figure 3** Schematic diagram of bridging flocculation between interfacial protein and polysaccharide.

4.2 Charge of polysaccharide

In 2006, Surh *et al.* studied the effect of pectin types, i.e., high methoxyl pectin (59% DE; degree of esterification) and low methoxyl pectin (32% DE), which were different in the degree of esterification and charges of pectin, on the stability of o/w emulsion stabilized by sodium-caseinate. They found a little difference between pectin types, i.e., high methoxyl (59% DE) and low methoxyl (32% DE) pectin on ζ-potential and droplet aggregation of sodium-caseinate stabilized emulsion.

Nevertheless, Lam *et al.* (2007) demonstrated that charge of polysaccharide influenced soy protein aggregation at low pH (pH 3.8). They used different type of pectin (DE 32.6-71.4) to stabilize SPI dispersion at low pH. It was found that higher charged polysaccharide (lower DE value) may not effectively

stabilize protein dispersion at low concentration due to the associative phase separation between soy protein and pectin that then precipitated (Figure 2).

4.3 Size of polysaccharide

Not only has the charge of polysaccharide that affects the formation protein-polysaccharide coacervated, but also the size of polysaccharide that plays an important role in the characteristics of the coacervate. For charged polysaccharide, Liu *et al.* (2006) studied the influence of different type of pectin (i.e. low methoxyl pectin and high methoxyl pectin; DE 40.4-71.4) in casein dispersion. They demonstrated that although the charge of pectin played an important role in particle size distribution of casein dispersion at low pH, the efficiency in stabilizing the casein dispersion decreased with decreasing size of pectin from 372.7 to 174.7 kDa.

Herceg *et al.* (2007) found that adding small molecular weight (MW) saccharide, i.e., glucose and sucrose, increased emulsion stability of whey protein-stabilized emulsion because of the increase in viscosity in aqueous phase, while starch and inulin decreased the emulsion stability due to the depletion flocculation induced by those polysaccharides (Figure 4).

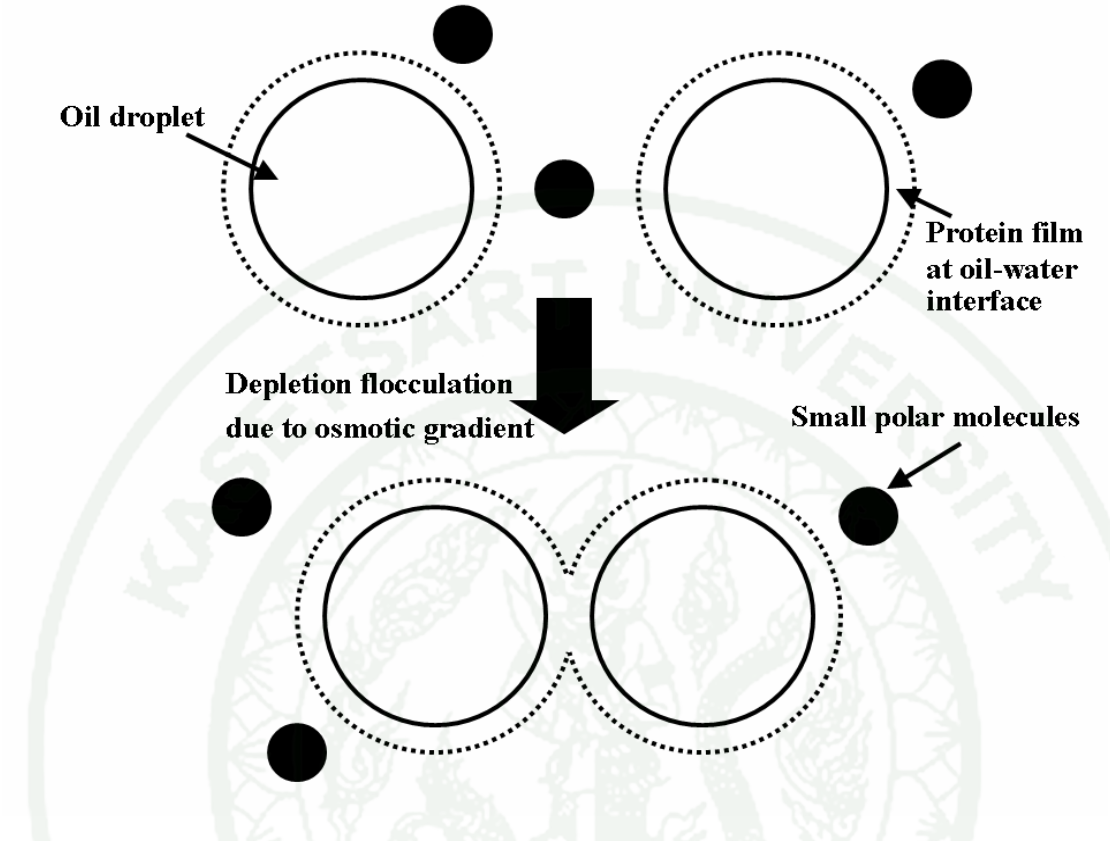


**Figure 4** Schematic diagram of depletion flocculation.

**Source:** McClements (2005)

**5. Okara**

Okara or soy residue is a by-product from soy milk or tofu processing. Every 1 kg of soybean processed for soy milk produces about 1.1 kg of fresh okara which has 76-80% moisture, 8.1-15.2% fat, and 23.6-24% protein (Liu *et al.*, 1997). However, the use of okara as human food has been limited due to the fact that okara contain high contents of moisture. Therefore, okara is easily spoiled. Moreover, okara has high water-retention capacity so it promotes highly vicous products.

5.1 Nutrition perspectives

Dried okara contain 25.4-28.4% protein, 9.3-10.9% fat, 3.8-5.3% soluble carbohydrate, 40.2-43.6% insoluble fiber and 12.6-14.6% soluble fiber (Van der Riet *et al.*, 1989). Even though okara has high protein and fiber, it has been used as animal feed only (Liu, 1997). Therefore, some researchers investigated the usage of okara as human foods such as tempeh, koji, baked products, and desserts as reviewd by O'Toole (1999). Extractions of protein and polysaccharide from okara were also investigated (Ma *et al.*, 1997; Nakamura *et al.*, 2004b; Yamaguchi et al., 1996).

Soy soluble polysaccharide is an acidic polysaccharide which has pectic-like structure containing galacturonan backbone of homogalacturonan linked by α-1,4-glycosidic linkage and rhamnogalacturonan as repeating units being comprised of α-1,2-rhamnose and α-1,4-galaturonic acid, then branched by β-1,4-galactan and α -1,3- or α -1,5- arabinan chains. Moreover, the reducing end of soy soluble polysaccharide is conjugated with protein. This protein fraction possessed the molecular weight about 10 and 50 kDa (Nakamura *et al.*, 2004a, b). Figure 5 shows possible structure of soy soluble polysaccharide proposed by Yamaguchi *et al.* (1996) and Nakamura *et al.* (2006a).

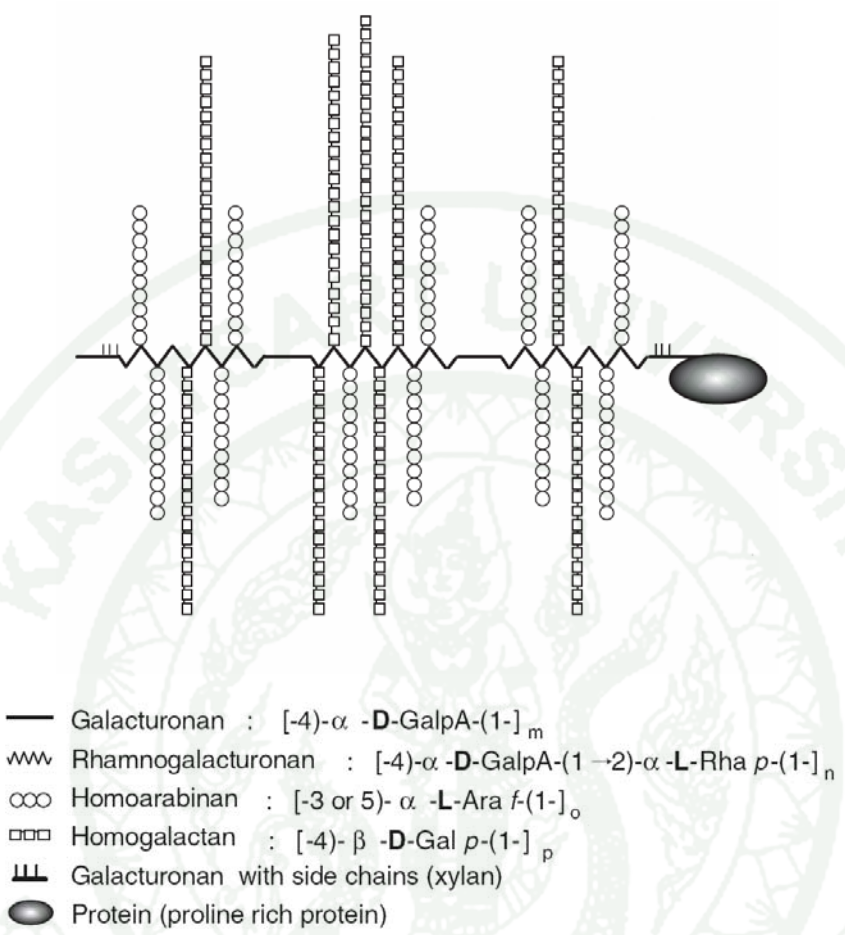


**Figure 5** Possible structure of soy soluble polysaccharide.

**Source:** Nakamura *et al.* (2006a)

Soy soluble polysaccharides were investigated for their use as ingredients in food such as emulsion and encapsulation. Soy soluble polysaccharide can be used to stabilize acidified milk beverage (Nakamura *et al.*, 2003; 2006b). Moreover, soluble polysaccharide was used as wall material in linoleic acid microencapsulation (Minemoto *et al.*, 2002). Soy soluble polysaccharide showed emulsifying properties and strong antioxidative capacity after spray drying. Protein fraction in soy soluble polysaccharide was accounted for emulsifying properties while the carbohydrate moiety acted as steric hindrance (Nakamura *et al.*, 2004a).

5.2 Immunostimulating activity by polysaccharide

Apart from functional properties of polysaccharides in food, there are many studies showed that polysaccharides from plant and fungi have immunostimulating activities on macrophage. Macrophage is a type of differentiated cells originated as blood monocytes. It has several functions such as killing pathogenic microorganism, processing and presentation of antigens to lymphocytes. Macrophage can kill microorganism by the recognition system which is mediated by toll-like receptors (TLR) that are specific for different components. TLR-2 binds peptidoglycan, TLR-4 binds bacterial lipopolysaccharide (LPS) and TLR-5 binds flagellin. As a result of recognition, phagocytic enzymes are activated such as oxidases and inducible nitric oxide synthase (iNOS), resulting in the production of bacterial reactive oxygen intermediates (ROI) and nitric oxide (NO) (Figure 6). However, large amounts of mediators can cause severe inflammatory diseases. Appropriate regulation of these immunomodulating molecules can help a host to prevent itself from pathologic attacks and cancer (Shin *et al.*, 1997; Iacomini *et al.*, 2005; Lull *et al.*, 2005; Gordon, 2006; Wichers, 2009).

Moreover, macrophage secretes protein to response microbes. The proteins called cytokines, which mediate many cellular reactions of immune systems. Cytokines are soluble proteins responsible for communications between macrophages and between macrophages and other cells. Tumor necrosis factor (TNF) and interleukin-12 (IL-12) are cytokines that macrophages produce to respond to bacterial lipopolysaccharide (LPS) (Lewis and McGee, 1992; Abbas and Lichtman, 2004).

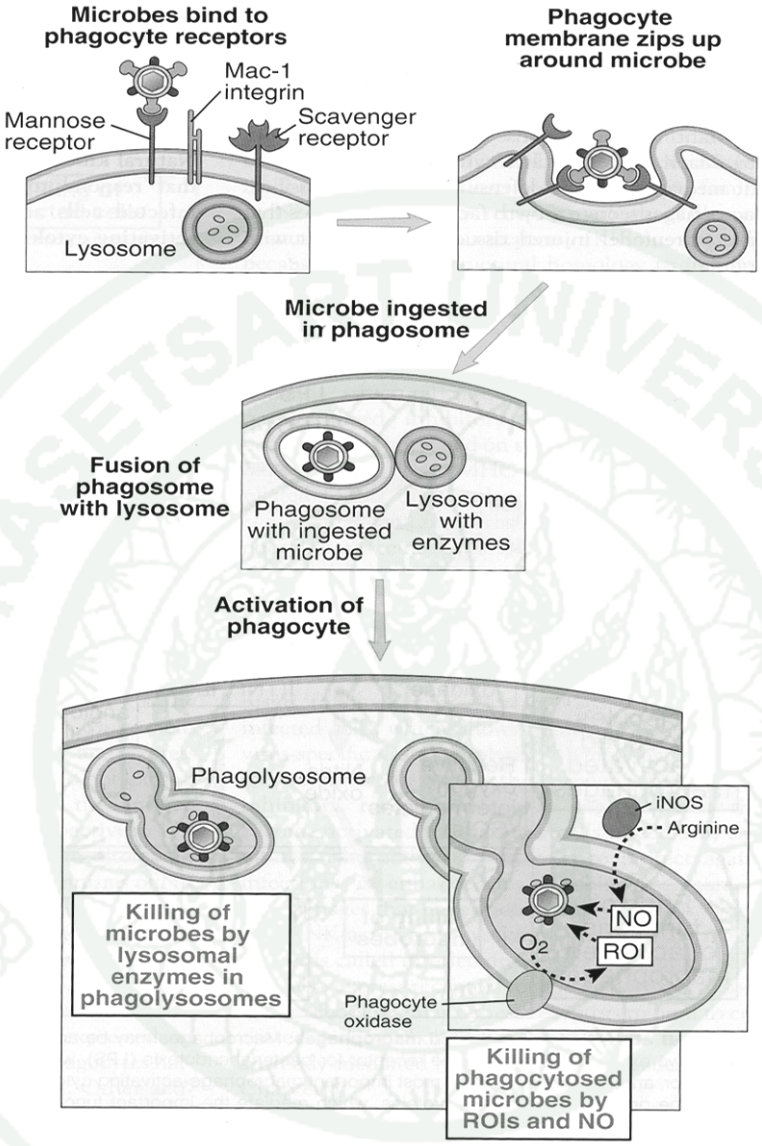


**Figure 6** The mechanism of phagocytosis by macrophage.

**Source:** Abbas and Lichtman (2004)

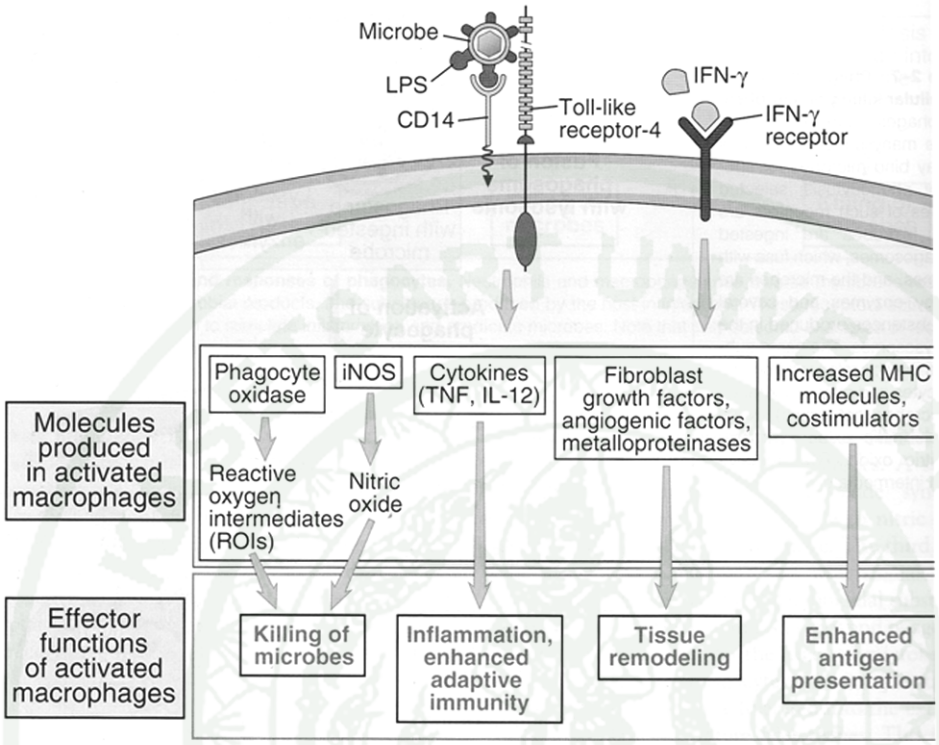


**Figure 7** Functions of activated macrophage.

**Source:** Abbas and Lichtman (2004)

The objective of this study was to characterize physiochemical and biological properties of soy soluble polysaccharides from okara and to elucidate the interactions between soy proteins and polysaccharides and their influences on the stability of liquid emulsion containing SPI, rice bran oil and different types of dietary fiber, i.e., soy soluble polysaccharide (SSP) and pectinase-hydrolyzed soy soluble polysaccharide (PH-SSP). Pre-heat treatment was also employed to SPI to alter the surface characteristics of proteins and their susceptibility to polysaccharide interactions under different pH. The understandings on protein and polysaccharide interactions may help regulating emulsion stability during processing and also *in vitro* digestion.

# MATERIALS AND METHODS

## Materials

### 1. Raw materials

1.1 Soy residue (okara) from the soy milk production was kindly provided by Lactasoy Co., Ltd. (Thailand). Fresh okara was packed in plastic bag and kept in freezer at -20°C to avoid microbial spoilage. Okara was extracted the soy soluble polysaccharide within one month after storage.

1.2 Soy protein isolate (SPI; PROFAM 974, Archer Daniels Midland, Decatur, USA). The protein content was 82.14% (determined by Kjeldahl method (AOAC, 2000) using N factor of 6.25)

1.3 Refined rice bran oil (King, Thai Edible Oil Co., Ltd, Bangkok, Thailand) was purchased from local supermarket and kept at room temperature (≈27°C)

### 2. Chemical Reagents

2.1 Reagents for hydrolysis of okara

2.1.1 Lactic acid (L-LAC FG 88; food grade, Shanxi Leda Biochemical, Taiyuan, China)

2.1.2 Commercial pectinase (EC 3.2.1.15; Pectinex® Ultra SP-L, Novozymes, Dittingen, Switzerland) was purchased from The East Asiatic (Thailand) Public Co., Ltd. Pectinase was stored at 4-8°C in refrigerator before use.

2.2 Reagents for protein analysis (Lowry's method)

2.2.1 Albumin from bovine serum (Fluka Biochemika, Fluka Chemic GmbH, Buchs, Switzerland)

2.2.2 Copper (II) sulfate ($CuSO_4 \cdot 5H_2O$; analytical grade, Fisher Chemicals, Fisher Scientific UK Limited, Loughborough, UK)

2.2.3 Folin-Ciocalteau's phenol reagent (Fluka Biochemka, Sigma-Aldrich Chemic GmbH, Buchs, Switzerland)

2.2.4 Potassium sodium (+)-tartrate ($KNaC_4H_4)6{\cdot}H_2O$; analytical grade, UNIVAR, Ajax Finechem, Seven Hills, Australia)

2.2.5 Sodium carbonate anhydrous ($Na_2CO_3$; analytical grade, UNIVAR, Ajax Finechem, Seven Hills, Australia)

2.3 Reagents for reducing sugar analysis

2.3.1 Ammonium molybdate ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2$; analytical grade, UNIVAR, Ajax Finechem, Seven Hills, Australia)

2.3.2 Benzoic acid ($C_6H_5COOH$; UNIVAR, analytical grade, Ajax Finechem, Seven Hills, Australia)

2.3.3 Copper (II) sulfate ($CuSO_4{\cdot}5H_2O$; analytical grade, Fisher Chemicals, Fisher Scientific UK Limited, Loughborough, UK)

2.3.4 D-galacturonic acid ($C_6H_{10}O_7{\cdot}H_2O$; Fluka, Sigma-Aldrich, Slovakia)

2.3.5 Di-sodium hydrogen arsenate heptahydrate ($AsHNa_2O_4{\cdot}5H_2O$; Fluka, Fluka Chemic GmbH, Spain)

2.3.6 Sodium acetate hydrated ($CH_3COONa{\cdot}3H_2O$; analyticak grade, UNIVAR, Ajax Finechem, Seven Hills, Australia)

2.3.7 Sodium chloride (NaCl; analytical grade, UNIVAR, Ajax Finechem, Seven Hills, Australia)

2.3.8 Sodium sulfate ($Na_2SO_4$; analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.3.9 Sulfuric acid ($H_2SO_4$; analytical grade, Baker Analyzed, Mallinckrodt Baker, Phillipsburg, USA)

2.4 Reagents for dietary fiber analysis

2.4.1 Acetone ($CH_3COCH_3$; analytical grade, Baker Analyzed, Mallinckrodt Baker, Phillipsburg, USA)

2.4.2 α-amylase (EC 3.2.1.1; α-amylase, heat stable, Product No. A3306, Sigma, Sigma-Aldrich, St. Louis, USA)

2.4.3 Amyloglucosidase (EC 3.2.1.3; Amyloglucosidase from *Aspergillus niger*, Product No. A9913, Sigma, Sigma-Aldrich, St. Louis, USA)

2.4.4 Boric acid ($H_3BO_3$; analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.4.5 Bromocresol green ($C_{21}H_{14}Br_4O_5S$; analytical grade, Labchem, Ajax Finechem, Auckland, New Zealand)

2.4.6 Celite®, acid-washed (Product No. C8656, SIGMA, Sigma-Aldrich, St. Louis, USA)

2.4.7 Copper (II) sulfate ($CuSO_4 \cdot 5H_2O$; analytical grade, Fisher Chemicals, Fisher Scientific UK Limited, Loughborough, UK)

2.4.8 Disodium phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$; analytical grade, MERCK, Merck, KGaA, Darmstadt, Germany)

2.4.9 Ethyl alcohol ($C_2H_5OH$; analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.4.10 Hydrochloric acid (HCl; analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.4.11 Methyl red ($C_{15}H_{15}N_3O_2$; Panreac, PANREAC QUIMICA SA, Espana, Spain)

2.4.12 Monosodium phosphate dehydrate ($NaH_2PO_4 \cdot 2H_2O$; Analytical grade, MERCK, Merck, KGaA, Darmstadt, Germany)

2.4.13 Protease (EC 3.4.21.14; Protease from *Bacillus licheniformis*, Product No. P3910, Sigma, Sigma-Aldrich, St. Louis, USA)

2.4.14 Potassium sulfate ($K_2SO_4$; analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.4.15 Sodium hydroxide (NaOH; analytical grade, Merck, MERCK KGaA, Darmstadt, Germany)

2.4.16 Sodium hydroxide (NaOH; commercial grade, Thasco chemical Co., Bangkok, Thailand)

2.4.17 Sulfuric acid ($H_2SO_4$; analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.5 Reagents for high performance liquid chromatography (HPLC)

2.5.1 Acetic acid ($CH_3COOH$; Mallinckrodt Baker, Phillipsburg, USA)

2.5.2 Hydrochloric acid (HCl; Mallinckrodt Baker, Phillipsburg, USA)

2.5.3 Sodium acetate ($CH_3COONa$; SHOWA, Showa Denko Co., Ltd. Tokyo, Japan)

2.5.4 Sodium hydroxide (NaOH; SHOWA, Showa Denko Co., Ltd. Tokyo, Japan)

2.5.5 α-L-Rhamnose ($C_6H_{12}O_5$; Sigma, Sigma-Aldrich, St. Louis, USA)

2.5.6 Glucose ($C_6H_{12}O_6$; Sigma, Sigma-Aldrich, St. Louis, USA)

2.5.7 Maltose ($C_{12}H_{22}O_{11} \cdot H_2O$; Sigma, Sigma-Aldrich, St. Louis, USA)

2.5.8 (D)-+-Raffinose ($C_{18}H_{32}O_{16} \cdot 5H_2O$; Sigma, Sigma-Aldrich, St. Louis, USA)

2.5.9 Pullulan standard (Shodex STD P5, P20, P100, SHOWA, Showa Denko Co., Ltd. Tokyo, Japan)

2.6 Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.6.1 Acrylamide PAGE ($CH_2CHCONH_2$; PlusOne, Amersham Biosciences AB, Uppsala, Sweden)

2.6.2 Ammonium persulfate (BIO-RAD, Bio-Rad Laboratories, Hercules, USA)

2.6.3 Brilliant Blue R (Coomassie Brilliant blue R-250, Usb, USB corporation, Cleveland, USA)

2.6.4 Bromophenol blue sodium salt ($C_{27}H_{28}Br_2O_5S$; Labchem, Ajax Finechem Pty Ltd., Taren Point, Australia)

2.6.5 Glacial Acetic acid ($CH_3COOH$; Baker Analyzed, Mallinckrodt Baker, Phillipsburg, USA)

2.6.6 Glycerol ($CH_2OHCHOHCH_2OH$, CARLO ERBA, Cario Erba Reagenti SpA, Rodano, Italy)

2.6.7 Glycine (BIO-RAD, Bio-Rad Laboratories, Hercules, USA)

2.6.8 Hydrochloric acid (HCl; analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.6.9 Methanol ($CH_3OH$; CARLO ERBA, Carlo Erba Reagenti SpA, Rodano, Italy)

2.6.10 N, N' Methylenebisacrylamide ($(CH_2{:}CHCONH)_2CH_2$; PlusOne, GE Healthcare Bio-science AB, Uppsala, Sweden)

2.6.11 2-Mercaptoethanol ($HSCH_2CH_2OH$; BIO-RAD, Bio-Rad Laboaratories, Hercules, USA)

2.6.12 Sodium dodecyl sulfate ($C_{12}H_{25}NaO_4S$; Usb, USB corporation, Cleveland, USA)

2.6.13 N, N, N', N'-tetramethylethylenediamine (TEMED; Usb, Amersham International, Buckinghamshire, England)

2.6.14 Tris (hydroxyl methyl) aminomethane ($NH_2C(CH_2OH)_3$; Usb, USB corporation, Cleveland, USA)

2.7 Reagent for determination of biological activities

2.7.1 Dimethyl Sulfoxide (DMSO; Sigma, Sigma-Aldrich, St. Louis, USA)

2.7.2 3-(4, 5-dimethylthi-azol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma, Sigma-Aldrich, St. Louis, USA)

2.7.3 Di-sodium hydrogen phosphate ($Na_2HPO_4$; SHOWA, Showa Denko Co., Ltd. Tokyo, Japan)

2.7.4 Dulbecco's Modified Eagle Medium (DMEM; GIBCO®, Invitrogen, Carlsbad, USA)

2.7.5 Fetal bovine serum (FBS; HyClone, Thermo Fisher Scientific, Waltham, USA)

2.7.6 Hydrochloric acid (HCl; Mallinckrodt Baker, Phillipsburg, USA)

2.7.7 Lipopolysaccharide (LPS from *E. coli*; Sigma, Sigma-Aldrich, St. Louis, USA)

2.7.8 Methanol ($CH_3OH$; Mallinckrodt Baker, Phillipsburg, USA)

2.7.9 N-(1-naphthyl) ethylene-diamine dihydrochloride (NEDD; Sigma, Sigma-Aldrich, St. Louis, USA)

2.7.10 Penicillin (GIBCO®, Invitrogen, Carlsbad, USA)

2.7.11 Potassium chloride (KCl; SHOWA, Showa Denko Co., Ltd. Tokyo, Japan)

2.7.12 Potassium di-hydrogen phosphate ($KH_2PO_4$; SHOWA, Showa Denko Co., Ltd. Tokyo, Japan)

2.7.13 Sodium chloride (NaCl; SHOWA, Showa Denko Co., Ltd. Tokyo, Japan)

2.7.14 Sodium nitrite ($NaNO_2$ Sigma, Sigma-Aldrich, St. Louis, USA)

2.7.15 Streptomycin (GIBCO®, Invitrogen, Carlsbad, USA)

2.7.16 Sulfanilamide (Sigma, Sigma-Aldrich, St. Louis, USA)

2.9 Reagent for determination emulsion stability

2.9.1 Hydrochloric acid (HCl; analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.9.2 Oil Red "O" (Product No. 75087; Fluka Chemika, St. Louis, USA)

2.9.3 Sodium bicarbonate ($NaHCO_3$; analytical grade, UNIVAR, Ajax Finechem, Seven Hills, Australia)

2.9.4 Sodium hydroxide (NaOH; analytical grade, Merck, MERCK KGaA, Darmstadt, Germany)

2.10 Reagents for determination of size distribution and ζ-potential

2.10.1 Hydrochloric acid (HCl; analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.10.2 Sodium hydroxide (NaOH; analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.11 Reagents for *in vitro* digestion

2.11.1 Bile salts (Product No. B8756, Fluka Chemika, St. Louis, USA)

2.11.2 Hydrochloric acid (HCl; analytical grade, MERCK, Merck KGaA, Darmstadt, Germany)

2.11.3 Pepsin (EC 3.4.23.1; pepsin from porcine gastric mucosa, Product No. P7000, Sigma, Sigma-Aldrich, St. Louis, USA)

2.11.4 Sodium bicarbonate ($NaHCO_3$; analytical grade, UNIVAR, Ajax Finechem, Seven Hills, Australia)

2.11.5 Sodium hydroxide (NaOH; analytical grade, Merck, MERCK KGaA, Darmstadt, Germany)

2.11.6 Trypsin (EC 3.4.21.4; trypsin from bovine pancreas, Product No. T8003, Sigma, Sigma-Aldrich, St. Louis, USA)

**3. Instruments and Apparatus**

3.1 Instruments and apparatus for sample preparation

3.1.1 2-digit weight balance (OHOUS model ARC120, Ohous Corp., Pine Brook, NJ, USA)

3.1.2 4-digit weight balance (SCALTEC model SPB31, Scaltech instruments, Germany)

3.1.3 Freeze dryer (HETO model FD 2.5, Heto Lab Equipment, Allerød, Denmark)

3.1.4 Incubator (MEMMERT model 600, Memmert GmbH&Co. KG, Germany)

3.1.5 Refrigerated centrifuge (model Sorvall RC-5C Plus, Dupont SORVALL Products, Newtown, CT, USA)

3.1.6 Test sieve (Aperture size 150 micron, Endecotts Limited, London, UK)

3.2 Instruments and apparatus for sample analysis

3.2.1 4-digit weight balance (SCALTEC model SPB31, Scaltech instruments, Germany)

3.2.2 Electrophoresis Cell (BIO-RAD model Mini-PROTEAN® 3 Cell, Bio-Rad Laboratories, Inc., Hercules, USA)

3.2.4 Hot air oven (MEMMERT model ULM400, Memmert GmbH&Co. KG, Germany)

3.2.5 Kjeldahl apparatus (BUCHI, Digestion unit K-435; Scrubber B-414; Distillation unit B-324, LabortechnikAG, Switzerland)

3.2.6 Microcentrifuge (Labnet Spectrafuge 16M, Labnet International, Inc., USA)

3.2.3 Microhaematocrit centrifuge (KHT-400, Gemmy Industrial Corp., Taipei, Taiwan)

3.2.7 Minolta Spectophotometer (model Minolta 3500D, Konica Minolta Ltd., Osaka, Japan)

3.2.8 Mix ultra-turrax® (model T25 basic, IKA®-WERKE, Staufen, Germany)

3.2.9 Muffle furnace (model Tactical 308, Gallenkamp, UK)

3.2.10 pH meter (model Orion 2-star, Thermo Fisher Scientific Inc., Waltham, USA)

3.2.11 Spectrophotometer (model Spectronic 20 plus, Spectronic Instruments, Inc., USA)

3.2.12 Vacuum pump (model VCP 8101, HARMONY, Teipei, Taiwan)

3.2.13 Vortex mixer (Vortex-Genie 2 model G-560E, Scientific Industries, Inc., USA)

3.2.14 Waterbath (MEMMERT model OB14, Memmert GmbH&Co. KG, Germany)

3.2.15 Zetasizer Nano-ZS Instruments (Zen 3600, Malvern Instrument Ltd., Worcestershire, UK)

3.2.16 Autotitrator (Multi Propose Titrator model MPT-2, Malvern Instrument Ltd., Worcestershire, UK)

3.2.17 Hydraulic press

3.2.17 ELISA microplate reader (Bio-Tek, BioTek Instruments, Inc., Vermont, USA)

3.2.18 Confocal Imager (BD$^{TM}$ CARV II, BD Biosciences, Ontario, Canada)

## Methods

### 1. Characterization of soy polysaccharide from okara

1.1 Extraction of SSP and PH-SSP

The extraction procedure used was described in Figure 8. Frozen okara was thawed at room temperature (≈27°C) and put into filtered bag, then liquid fraction was separated by hydraulic press. For pectinase hydrolysis, thawed okara was adjusted the pH to 4.5 with 85% lactic acid in order to adjust to optimum pH of pectinase and avoid microbial spoilage, then incubated with commercial pectinase (EC 3.2.1.15; Pectinex® Ultra SP-L) at the enzyme concentration of 2% (w/w of thawed okara) at 30°C for 20 h before separating the liquid fraction. Soy soluble polysaccharide (SSP) and pectinase-hydrolyzed soy soluble polysaccharide (PH-SSP) were extracted from the liquid fraction by adding 70% ethanol at the ratio between liquid fraction to ethanol as 1:3 (v/v). The precipitate was centrifuged at 10,000 rpm, 4°C for 20 min using refrigerated centrifuge (model Sorvall RC-5C Plus, rotor F-16/250, rotor code = 22, Dupont SORVALL Products, Newtown, CT, USA). SSP and PH-SSP were freeze-dried and kept at -20°C before analysis. Yield of SSP and PH-SSP was around 0.54% and 0.7% of fresh okara, respectively.

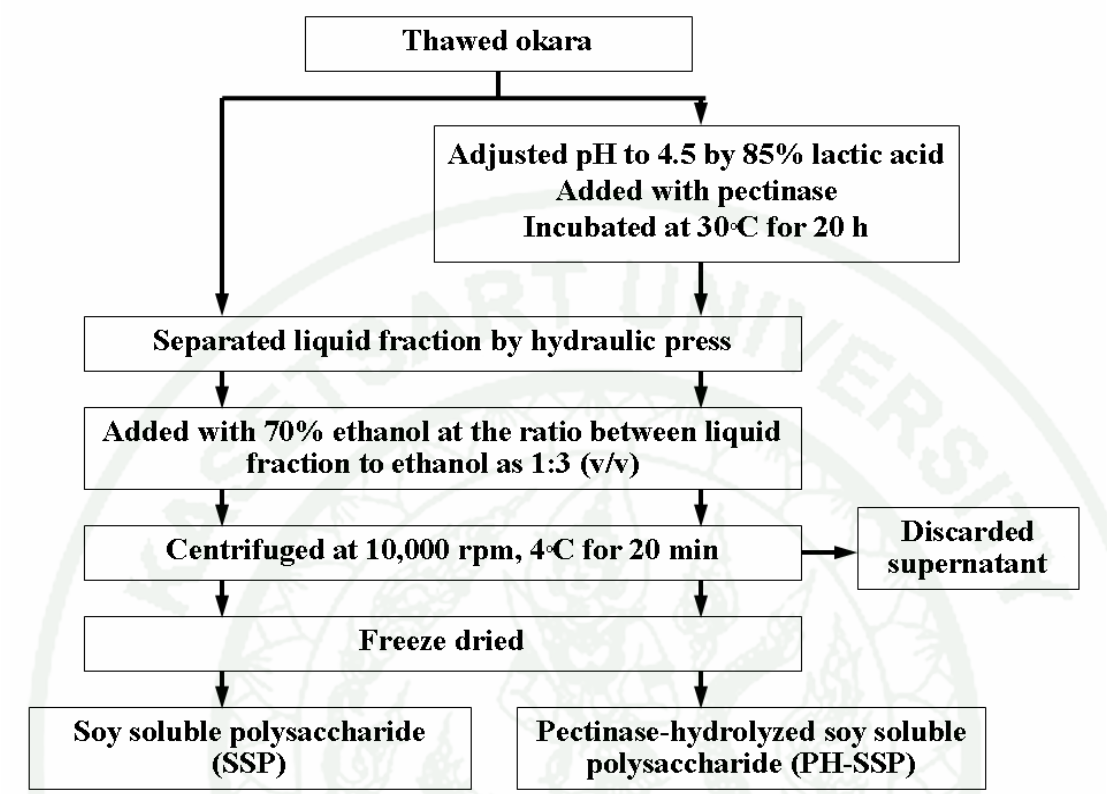


**Figure 8** Flow chart of the extraction of SSP and PH-SSP.

SSP and PH-SSP were analyzed for moisture (AOAC, 2000), protein (Lowry *et al.*, 1951), reducing sugar (Milner and Avigad, 1967), and dietary fiber contents (AOAC, 2000). CIE color values were also analyzed using spectrophotometer (model Minolta 3500D, Konica Minolta Ltd., Osaka, Japan).

1.2 High performance size exclusion chromatography (HPSEC)

High performance size exclusion chromatography was conducted at National Pintung University of Science and Technology (NPUST), Pingtung, Taiwan. Gel filtration chromatography of SSP and PH-SSP were performed using HPLC system (Hitachi L-2130 pump, Hitachi L-2490 RI detector, DEGASYS DG-1110 uniflows, and ENSHINE super CO-150 oven) (Nakamura *et al.*, 2006a) using a TSK guard column $PW_{XL}$ (6.0mm x 40 mm; Tosoh Co. Ltd., Tokyo, Japan) and TSKgel G-3000 PWXL column (7.8mm x 300 mm; exclusion limit; 100 kDa as dextran or 800

kDa as protein; Tosoh Co. Ltd., Tokyo, Japan). SSP and PH-SSP were dispersed in double deionized water to give a concentration of 1% (w/v). A 20 μL of sample solutions were injected and separation was carried out at a flow rate of 0.6 mL/min using 50 mM sodium acetate buffer pH 5.0 as a mobile phase. The molecular weight of SSP and PH-SSP was determined against standard pullulan (Showa Denko Co., Ltd. Tokyo, Japan) and sugars (Sigma-Aldrich).

1.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Molecular weight profiles of proteins in soy soluble polysaccharide were determined by SDS-PAGE (Laemmli, 1970) using Bio-Rad Mini-PROTEAN® 3 electrophoresis (Bio-Rad Laboratories, Inc, Hercules, CA, USA). A 4% acrylamide stacking gel and 15% acrylamide separating gel were used. A buffer containing 0.375 M Tris-HCl, pH 8.8 and 0.1% (w/v) SDS was used for the separating gel and 0.125 M Tris-HCl, pH 6.8 and 0.1% (w/v) SDS was used for the stacking gel. SSP and PH-SSP were dissolved in sample buffer containing 0.5 M Tris-HCl pH 6.8, 0.0125% SDS, 10% glycerol, 1% (w/v) bromophenol blue; with or without 5% β-mercaptoethanol. The addition of β-mercaptoethanol was to reduce disulfide bonds existed in soy proteins. Each sample was heated at 100°C for 3 min, cooled to room temperature and centrifuged at 5000 rpm (Labnet Spectrafuge 16M, Labnet International) for 5 min. An aliquot of sample solution containing 0.02 mg protein per well and 4 μl full-range MW markers (MW standards of 10-250 kDa; RPN800, Amersham Biosciences UK Ltd., Buckinghamshire, UK) was loaded in each well. Electrophoresis run in electrode buffer pH 8.3 containing 0.124 M Tris, 0.959 M glycine, and 0.1% (w/v) SDS using constant 150 voltage. Gel was stained in Bio-Rad Coomassie blue R-250 staining solution containing 40% methanol, 10% acetic acid and 0.1% Coomassie blue R-250 for 30 min, then destained overnight with destaining solution containing 10% methanol and 7.5% acetic acid for 2-3 times.

1.4 Size distribution of soy polysaccharide

To study the effect of pectinase hydrolysis on size distribution, both SSP and PH-SSP was reconstituted in deionized water to give 1.0% (w/v) polysaccharide suspension. Polysaccharide suspension was heated at 80°C for 30 min to fully hydrate the polysaccharides, and then cooled to room temperature. The size distribution of polysaccharide was measured by Zetasizer Nano-ZS Instruments (Zen 3600, Malvern Instrument Ltd., Worcestershire, UK).

1.5 Biological activity of soy soluble polysaccharide

Morphological changes and immonomodulationg activity of soy soluble polysaccharide on macrophage were conducted at National Pintung University of Science and Technology (NPUST), Pingtung, Taiwan. Murine macrophage cell line RAW 264.7 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C under 95% $O_2$ and 5% $CO_2$ (Chen et al., 2007; Lee *et al.*, 2008).

1.5.1 Morphological changes

Macrophage RAW 264.7 ($1\times10^5$ cells/mL) treated with PH-SSP at the concentrations of 50,100,200, and 300 μg/mL or lipopolysaccharide (LPS) as control at concentration of 1 μg/mL was performed on cover glass bottom culture dish. The treated macrophage was incubated for 8 hr and fixed on cover glass by 100% methanol for 10 min. Cover glass was put on slide and macrophage morphological changes were imaged by BD™ CARV II Confocal Imager (BD™ CARV II, BD Biosciences, Ontario, Canada) (Lee *et al.*, 2008).

1.5.2 Cell viability determination by 3-(4, 5-dimethylthi-azol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) test.

Macrophage RAW 264.7 was cultured in a 96-well plate at a concentration of $1\times10^6$ cells/mL and allowed to adhere on plate overnight. Cells were treated with SSP or PH-SSP at the concentrations of 50, 100, 200, 250, 400, 500, 750,

and 800 μg/mL and incubated at 37°C with 5% $CO_2$ for 24 h. After incubation, the suspension of media was removed and washed with 100 μL of phosphate buffer saline (PBS). A 50 μL of 0.1% MTT reagent (3-(4, 5-dimethylthi-azol-2-yl)-2, 5-diphenyl tetrazolium bromide) was added. Plate was further incubated for 3 h for the reaction to proceed, and the suspension was removed after incubation. A 50 μL of 100% DMSO was added to each well. After 30 min, the absorbance of each well was read on an ELISA microplate reader (Bio-Tek, BioTek Instruments, Inc., Vermont, USA) against a control (untreated macrophage) at wavelength of 570 nm (Chen et al., 2007; Lee *et al.*, 2008).

1.5.3 Nitric oxide determination.

Macrophage RAW 264.7 was cultured in a 24-well plate at a concentration of $5\times10^5$ cells/mL. Cells were treated with SSP and PH-SSP at the concentrations of 50, 100, 150, 200, and 300 μg/mL for 24 h and also treated with lipopolysaccharide (LPS) (1 μg/mL) as positive standard. After incubation, the amount of nitrite ion ($NO_2^-$) produced by macrophage in the extracellular media was determined by colormetric method with the reaction with Griess reagent (Sohn and Fiala, 2000; Chen et al., 2007; Lee *et al.*, 2008)). Griess reagent was prepared by mixing 200 mL of 1% sulfanilamide dissolved in 3N HCl and 200 mL of 0.1% N-(1-naphthyl) ethylene-diamine dihydrochloride (NEDD) disoloved in 3 N HCl. The amount of nitrite ion was related to the production of nitric oxide by macrophage. Then, the media was added with 80 μL of Griess reagent and incubated at room temperature for 30 min. The absorbance was read by ELISA microplate reader (Bio-Tek, BioTek Instruments, Inc., Vermont, USA) at a wavelength of 540 nm. Nitric oxide production by macrophage was calculated against the standard curve of a known concentration of $NaNO_2$.

**2. Effect of soy polysaccharide from okara on soy protein-polysaccharide interactions in soy-stabilized emulsion**

2.1 Emulsion preparation

Soy-stabilized emulsion was prepared as shown in Figure 9. SPI (0.456g) was dissolved in 7.5 mL of 20 mM carbonate buffer at specified pH (2.0, 4.5, and 7.0) to obtain 3.75% protein (w/v). Then SPI solution was homogenized with 0.333 mL of refined rice bran oil containing oil red "O" (Fluka Chemika, St. Louis, MO, USA) using an ultra-turrax® (model T25 basic, IKA®-WERKE, Staufen, Germany) at 21500 rpm for 1 min. O/W emulsion was then further homogenized again with soy soluble polysaccharide solution by ultra-turrax® (model T25 basic, IKA®-WERKE, Staufen, Germany). The SSP or PH-SSP solution was prepared by reconstituting the freeze-dried powder in 2.17 mL of 20 mM carbonate buffer at specified pH (2.0, 4.5, and 7.0). The total volume of soy-stabilized emulsion was 10 mL and the final emulsion contained 3.75% protein (unheated and heated), 3.33% rice bran oil, and 0-2% soy polysaccharide.

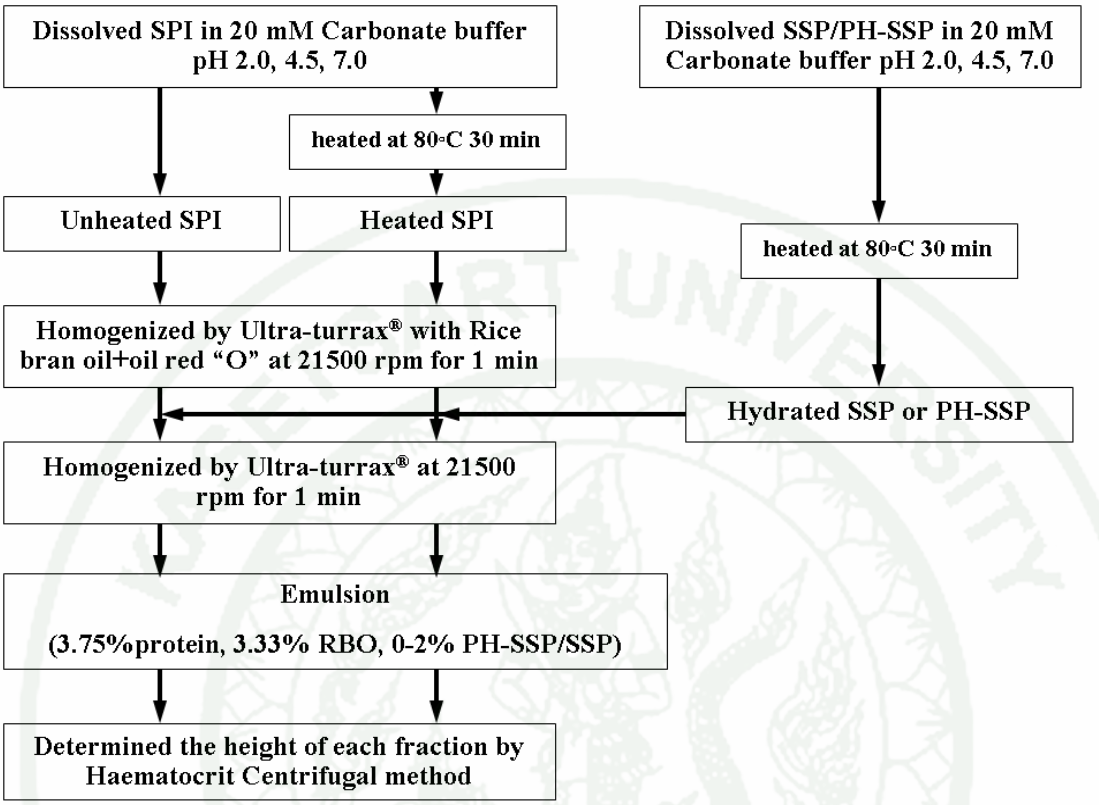


**Figure 9** Flow chart of emulsion preparation.

2.2 The stability of soy-stabilized emulsion

Soy-stabilized emulsion containing different soy soluble polysaccharides as dietary fiber was evaluated by determining the height of each fraction by haematocrit centrifugal method (McDermott *et al.*, 1981). Emulsion was centrifuged at 12000 rpm for 10 min by microhaematocrit centrifuge (KHT-400, Gemmy Industrial Corp., Taipei, Taiwan). After centrifugation, emulsion was separated into 4 layers; top red oil phase, white-pink emulsion phase, translucent serum phase, and bottom opaque sediment phases. The height of each fraction was measured by vernier caliper and reported as per cent height compared against total height. Protein content in serum and sediment phase was analyzed by Lowry's method (Lowry *et al.*, 1951) and protein profiles in both phases were determined by SDS-PAGE as described in Section 1.3.

2.3 Effect of pre-heat treatment on ζ-potential of SPI

ζ-potential of 0.02% heated (80°C, 30 min) and unheated SPI was performed by Zetasizer Nano-ZS Instruments coupled with autotitrator (Multi Propose Titrator model MPT-2, Malvern Instrument Ltd., Worcestershire, UK). The pH of SPI solution was adjusted to pH in a range of pH 2-7 by autotitrator using 1M HCl or 1M NaOH.

2.4 *In vitro* digestion

Peptic and tryptic *in vitro* digestion was determined as described by Glahn *et al.* (1998). Soy-stabilized emulsions were prepared as described in Section 2.1 to give a 3.75% protein, 3.33% rice bran oil and 0-6% PH-SSP. Emulsions were adjusted to pH 2.0 by 2M HCl and then hydrolyzed by pepsin with enzyme to protein ratio of 1:20 for 15 min at 37°C. Peptic digestion was stopped by adding 1 M $NaHCO_3$ and adjusted to pH 7.0 by 2M NaOH. Sample was further hydrolyzed by trypsin using trypsin to protein ratio of 1:100 in the presence of bile salts (0.72 μmol/mL sample). Emulsions were incubated at 37°C for 60 min. Samples were determined for emulsion stability after each step of hydrolysis at predetermined digestion time by haematocrit centrifugal method as describe in Section 2.2

**3. Statistical analysis**

The data were analyzed by Analysis of Variance (ANOVA) with significance at $p<0.05$. Significant difference among mean values was determined by Duncan's Multiple Range Test. All statistical analyses were performed using the SPSS Software Version 12.

# RESULTS AND DISCUSSION

## Part I: Characterization of soy soluble polysaccharide from okara

Freeze-dried SSP and PH-SSP contained 39% and 49% dietary fiber, respectively (Table 3). Commercial pectinase contained polygalacturonase and cellulase; therefore, the cell wall structure of okara could be hydrolyzed and became more soluble. Pectinase hydrolysis increased not only dietary fiber content but also reducing sugar content. SSP did not possess reducing sugar, while PH-SSP had an increase in reducing sugar close to 9.45% ($p<0.05$) (Table 3). This was due to the hydrolysis of polygalacturonan backbone or other side chains such as galactan or arabinan into neutral sugar, which increased the reducing power (Nakamura *et al.*, 2006a). The increase in reducing sugar of PH-SSP led to the more brownish color in PH-SSP (Table 4). Reducing sugar could involve in the Maillard reaction between the aldehyde group at reducing end and amino group in protein during freeze drying of PH-SSP observed as lower L* value and higher b* value of freeze-dried PH-SSP ($p<0.05$) (Table 4).

**Table 3** Chemical composition (wet basis) of soy soluble polysaccharide (SSP) and pectinase-hydrolyzed soy soluble polysaccharide (PH-SSP) freeze-dried powder.

| | Moisture (%) | Protein (%) | Dietary fiber (%) | Reducing sugar (%) |
|---|---|---|---|---|
| SSP | 13.42[a] | 26.39[a] | 39.61[b] | Not detected |
| PH-SSP | 10.17[b] | 27.72[a] | 49.74[a] | 9.45[a] |

Means within the same column followed by different superscript are significantly different ($p<0.05$).

**Table 4** Color values of soy soluble polysaccharide (SSP) and pectinase-hydrolyzed soy soluble polysaccharide (PH-SSP) freeze-dried powder.

| | L* | a* | b* |
|---|---|---|---|
| SSP | 88.91[a] | -0.28[b] | 8.40[b] |
| PH-SSP | 87.67[b] | 0.40[a] | 11.41[a] |

Means within the same column followed by different superscript are significantly different ($p<0.05$).

Size exclusion chromatograms showed that pectinase hydrolysis of SSP reduced its MWs (Figure 10). SSP had MWs of around 180 and 250 kDa and a small MW one of 6 kDa, as well as some sugars with MW of 0.6 kDa (as calculated by pullulan standard in shown Appendix Figure 4 and Appendix Table 2). The MWs of polysaccharide found in SSP from okara were slightly smaller than those reported by Nakamura *et al.* (2004b), who found that SSP from okara had high MW fraction processing MW of 310 kDa and low MW fraction of MW around 20 kDa. It should be noted that the okara used in this study was kindly provided by Lactasoy Co., Ltd. Of which the process of soymilk production was not revealed. However, after pectinase hydrolysis, the PH-SSP had MWs of about 150 and 20 kDa. There was an increase in the MW fractions of 0.3 – 1.2 kDa (Appendix Figure 5 and Appendix Table 3), which were possibly oligosaccharides and sugars.

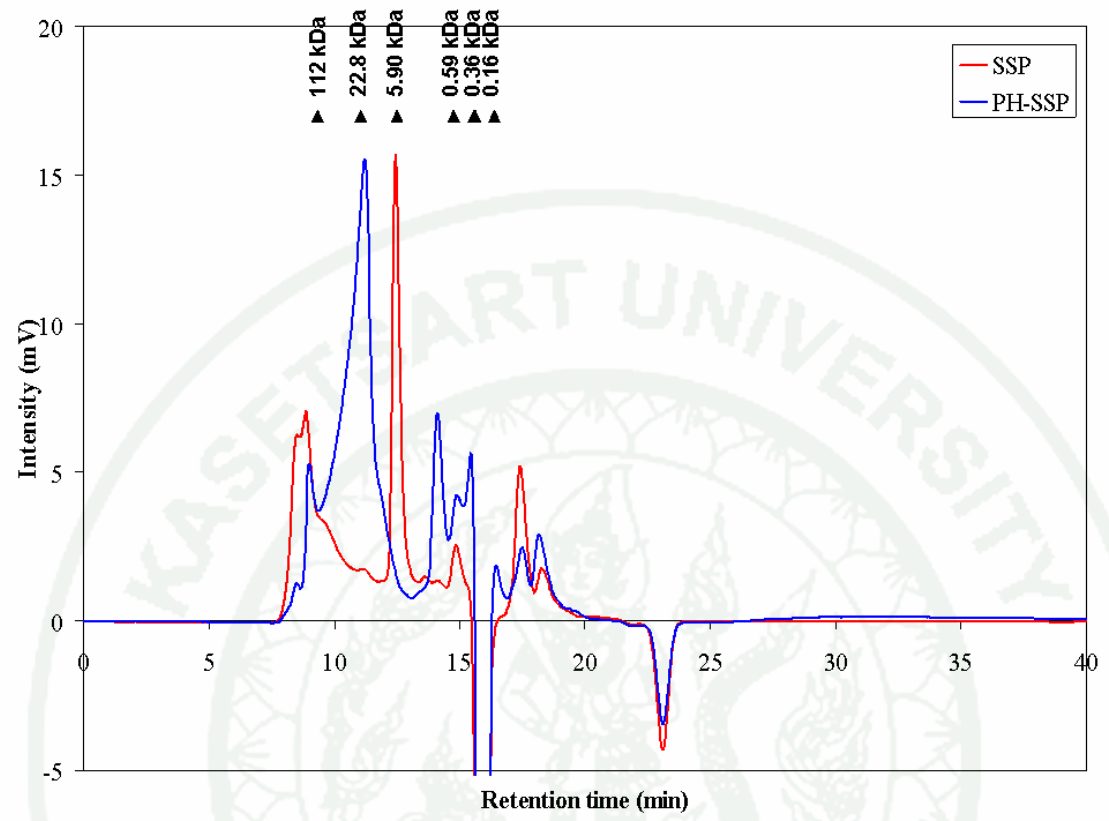


**Figure 10** Size exclusion chromatogram of SSP and PH-SSP on TSKgel G-3000 $PW_{XL}$ column. Elution was monitored by refractive index detector. MW (kDa) was calculated by using standard pullulan and sugar.

Although the protein content in SSP and PH-SSP was similar ($p \geq 0.05$) (Table 3), protein profiles in SSP and PH-SSP differed from those found in SPI at some bands (Figure 11). In the absence of β-ME, SPI, which is the most storage protein in soybean, had proteins with MW of higher than 35 kDa (Figure 11, lane 3). Moreover, most of proteins found in SSP (Figure 11, lane 2) showed faster electrophoretic mobility compared against those of SPI (Figure 11, lane 3), although they were glycated with soy polysaccharide. This suggested that the protein fraction conjugated with polysaccharides were quite small.

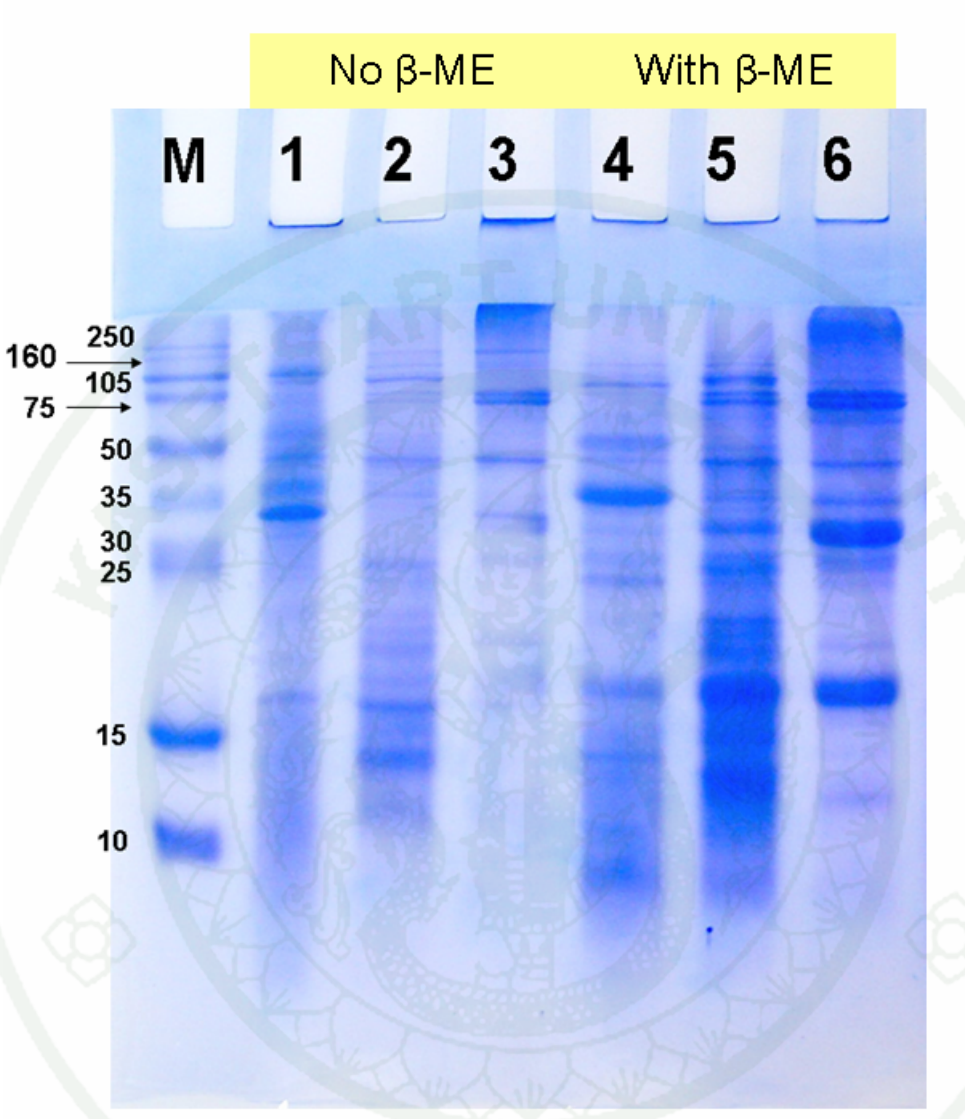


**Figure 11** MW profiles of different SSP and SPI in the absence (lane 1-3) and presence (lane 4-6) of β-mercaptoethanol by 15% separating gel; M = Standard protein marker; lane 1 and 4 = PH-SSP; lane 2 and 5 = SSP; lane 3 and 6 = SPI.

Nevertheless, PH-SSP showed the precipitation of high MW components in the well (Figure 11, lane 1) not found in SSP (Figure 11, lane 2). The aggregation of PH-SSP did not involve disulfide linkage because when β-ME was added, PH-SSP still showed precipitation in the well (Figure 11, lane 4). The hydrolysis of SSP by commercial pectinase, however, resulted in additional bands with high intensity around 30 – 50 kDa (Figure 11, lane 1), which were not present in SSP (Figure 11, lane 2). This suggested that the PH-SSP could undergo association after hydrolysis since majority of the bands were found at higher MW compared against those in SSP (Figure 11, lane 2). Some of these bands disappeared in the presence of β-mercaptoethanol with the exception of the band at 35 kDa. This suggested that these protein fractions (30-50 kDa) were associated by disulfide bond in protein; whereas, the protein fraction with MW of 35 kDa could be associated via glycation between protein and polysaccharide.

In the presence of β-ME, most of the protein bands in SSP (Figure 11, lane 5) had MWs close to those in SPI (Figure 11, lane 6), particularly those with the MW of higher than 30 kDa. This suggested that the extraction of SSP using hydraulic press also compressed residual soy proteins. Nevertheless, there were some unique bands found in SSP that not present in SPI in the presence of β-ME, i.e., 105 kDa, 25 kDa, and below. Some of these bands and typical soy proteins found in SPI were not found in PH-SSP (Figure 11, lane 4). This indicated thatalthough only polysaccharides were hydrolyzed by commercial pectinase, the MW profiles of proteins glycosylated with polysaccharides were increased. It is possible that there was an association of the PH-SSP via protein fraction in pectic-like structure.

The size distribution of biopolymers characterized by Nano-Zetasizer was shown in Figure 12. Particle size distribution of SSP and PH-SSP illustrates that the SSP displayed a broad unimodal distribution, of which average size was around 500 nm. After pectinase hydrolysis, the particle size distribution of PH-SSP was reduced to 100 nm and the large particle with average size of 2000 nm was observed. This confirmed the SDS-PAGE of proteins that there was the association of PH-SSP. Apparently hydrolysis of SSP could result in larger length-scale particle compared to

the unhydrolyzed one due to the association of biopolymers although the polysaccharide fractions were shortened by commercial pectinase.

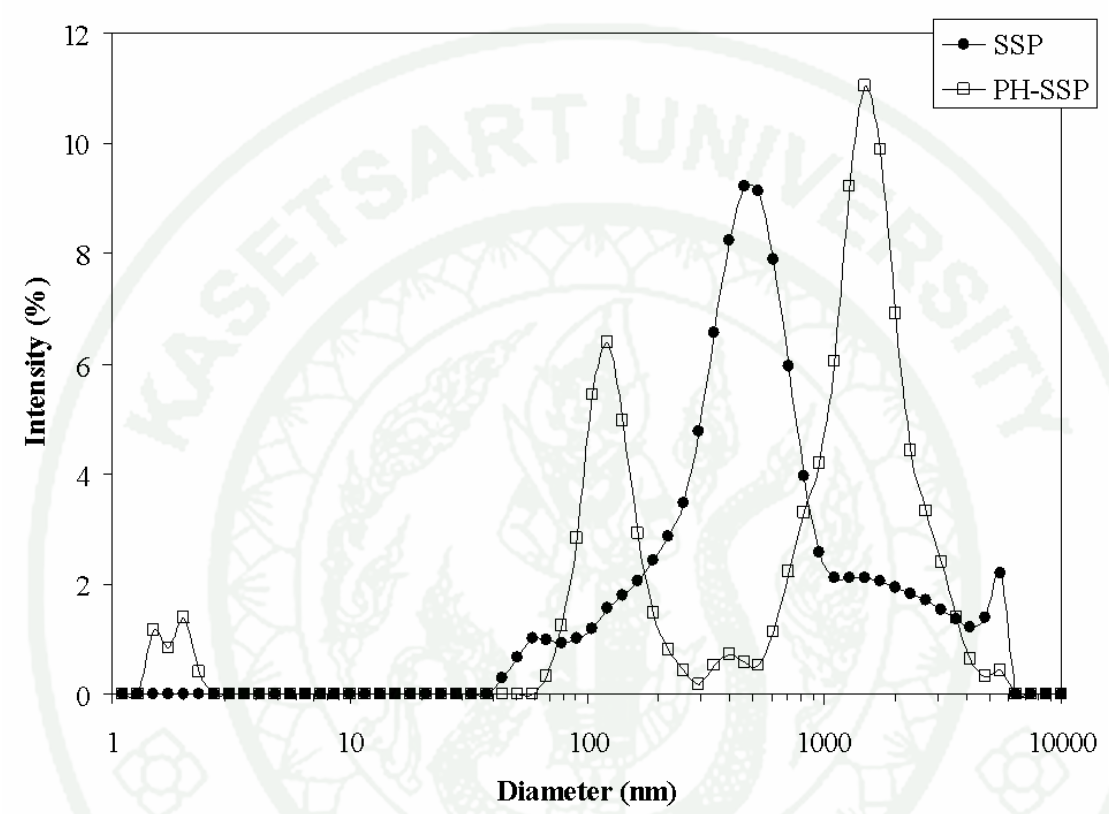


**Figure 12** Effect of pectinase hydrolysis on the particle size distribution 1% (w/v) soy soluble polysaccharide in deionized water. Bars represent standard derivation.

Both SSP were tested for biological activities of macrophage such as cytotoxicity, nitric oxide (NO) production and morphological changes. The addition of SSP or PH-SSP did not affect cell viability measured by MTT test compared to the control treatment (not added with SSP or PH-SSP) ($p \geq 0.05$) (Figure 13). This indicated that using SSP or PH-SSP were not toxic to macrophage.

Although the presence of SSP or PH-SSP did not affect cell survival, macrophage grown in medium added with SSP or PH-SSP showed different NO

production (Figure 14). At low concentration, SSP activated macrophage to produce the highest concentration of NO as did lipopolysaccharide (LPS) ($p \geq 0.05$). The increasing concentration of SSP reduced the production of NO. PH-SSP, however, did not effectively activate macrophage to produce NO (Figure 14). NO production of macrophage is one of the mechanisms involved in phagocytic uptake of microorganism by macrophage. The high amount of NO produced by macrophage indicated that immune system was stimulated.

Nevertheless, morphological changes in macrophage were observed in the presence of PH-SSP (Figure 15). The dendritric process of macrophage is one of the macrophage activation markers. The presence of PH-SSP on macrophage morphology showed similar manner of macrophage added with LPS. This suggested that PH-SSP activate macrophage to produce actin cytoskeleton. PH-SSP may activate macrophage to produce other compounds involved in immune system such as reactive oxygen species (ROS) or cytokines, e.g., tumor necrosis factor (TNF)-α, which needs further investigation.

It is apparent that pectinase hydrolysis of SSP extracted from okara induced the changes in chemical, physicochemical, and biological characteristics of PH-SSP. The molecular size of SSP was reduced from 180-250 kDa to 150 kDa after pectinase hydrolysis. However, pectinase hydrolysis induced self-association of PH-SSP to large particle confirmed by SDS-PAGE and Zetasizer Nano-ZS. Further investigation on the usage of both SSP and PH-SSP was reported in the following section.

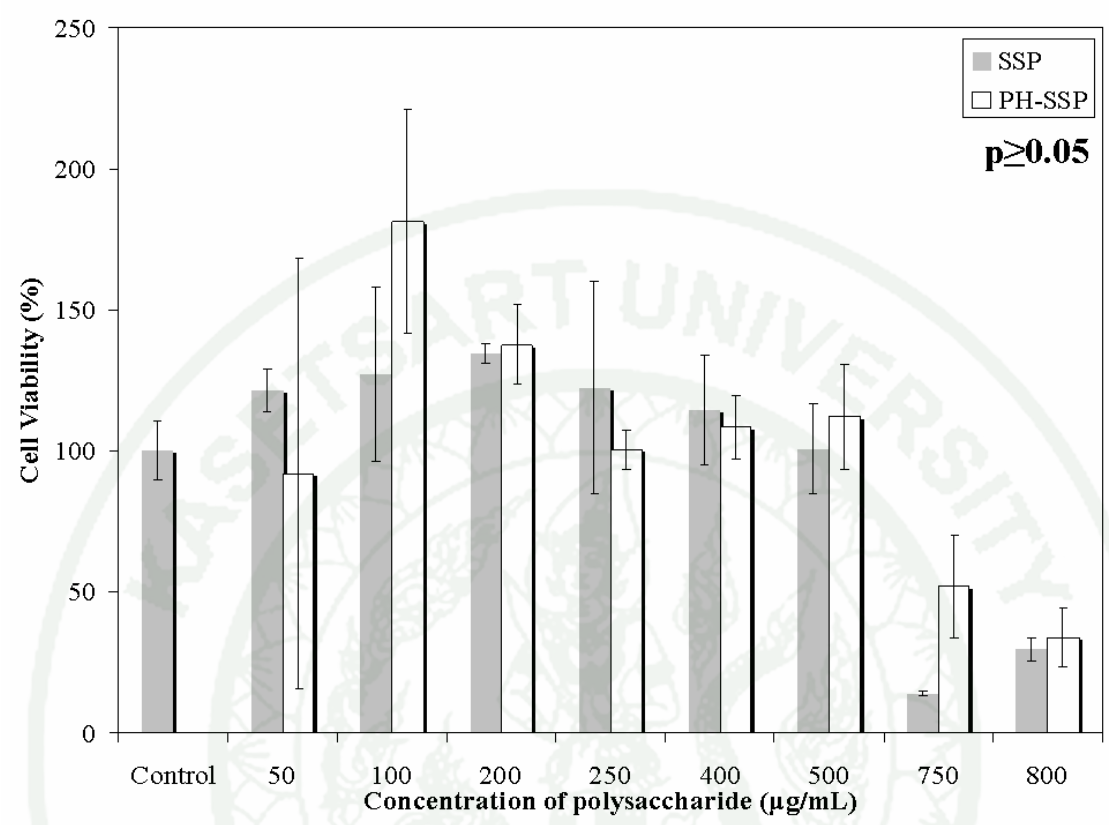


**Figure 13** Effect of soy soluble polysaccharide (SSP) and pectinase-hydrolyzed soy soluble polysaccharide (PH-SSP) in macrophage RAW 267.4 by MTT test. Bars represent standard derivation.

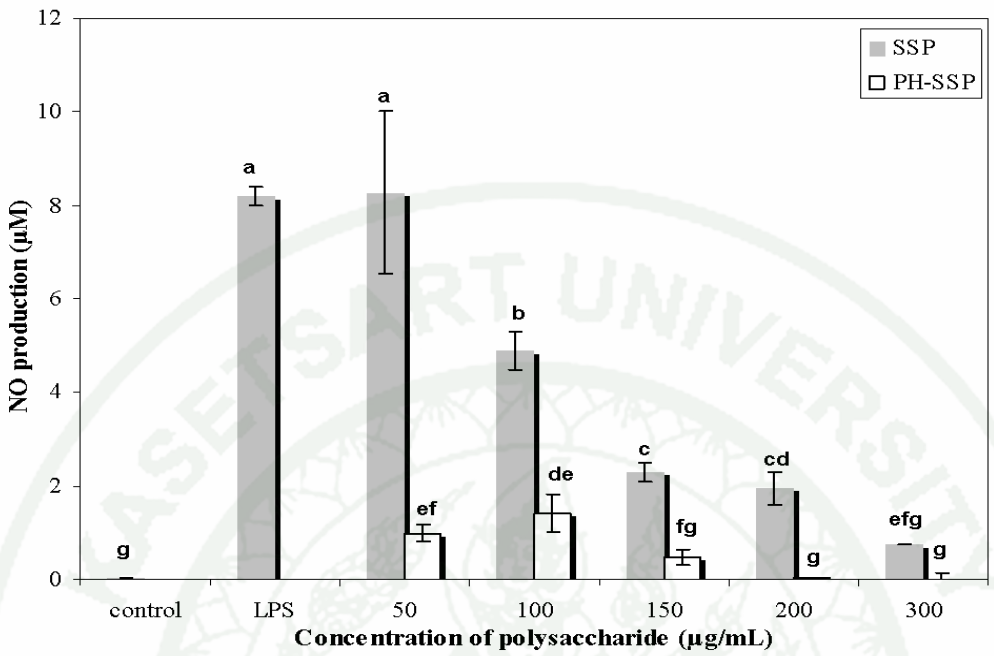


**Figure 14** Effect of soy soluble polysaccharide (SSP) and pecinase-hydrolyzed soy soluble polysaccharide (PH-SSP) concentration on NO production in macrophage RAW 267.4. Bars represent standard derivation.

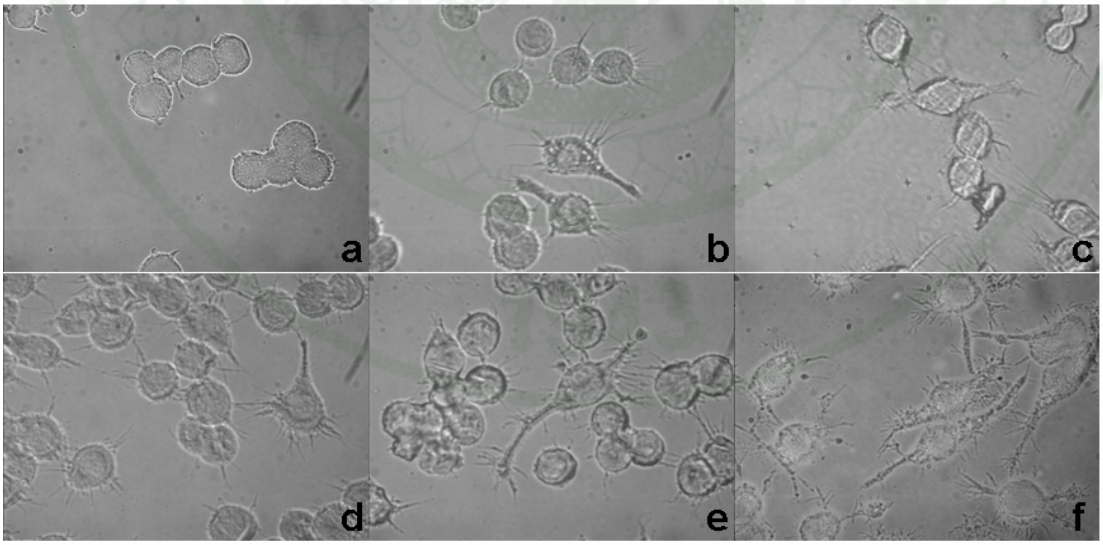


**Figure 15** Morphological change of actin cytoskeleton of macrophage RAW264.7 stimulated by PH-SSP at different concentration; 50 µg/mL(b), 100 µg/mL(c), 200 µg/mL(d), 300 µg/mL(e) and macrophage morphological changes stimulated by LPS 1.0 µg/mL(f) for 24 hr compared to normal cell(a). Magnifying power was 1000x.

## Part II: Effect of soy polysaccharide from okara on protein-polysaccharide interactions in soy-stabilized emulsion

The presence of PH-SSP in oil-in-water emulsion up to 2% did not influence oil phase and emulsion phase height ($p \geq 0.05$) (Table 5). However, at pH 2.0, which was below isoelectric pH of SPI, the sediment phase height of emulsion containing PH-SSP was higher than the others ($p < 0.05$). The sediment or insoluble coacervate formed by was likely due to the associative phase separation between positively charged SPI and negatively charged PH-SSP, a so-called bridging flocculation. On the contrary, the sediment phase height at pH 7.0 was lowered ($p < 0.05$). It was possible that electrostatic repulsion between negatively charged SPI and negatively charged PH-SSP was responsible for low degree of insoluble coacervate formation. In addition, heat treatment (80 °C, 30 min) employed to SPI prior to emulsification could reduce the formation of insoluble coacervate between SPI and PH-SSP, especially at pH 7.0 ($p < 0.05$).

**Table 5** Effect of PH-SSP concentration on emulsion stability at different pH. The emulsion was stabilized by either unheated soy protein isolate (U-SPI) or heated soy protein isolate (H-SPI).

| pH | PH-SSP concentration (%) | Oil phase height (%) | | Emulsion phase height (%) | | Sediment phase height (%) | |
|---|---|---|---|---|---|---|---|
| | | U-SPI $^{ns}$ | H-SPI $^{ns}$ | U-SPI $^{ns}$ | H-SPI $^{ns}$ | U-SPI | H-SPI |
| 2.0 | 0.00 | 0.84 | 0.65 | 3.94 | 4.97 | $18.30^{abc}$ | $17.56^{a}$ |
| | 0.75 | 0.77 | 0.79 | 3.97 | 4.05 | $14.46^{bcd}$ | $16.02^{ab}$ |
| | 1.50 | 0.79 | 0.47 | 4.88 | 4.70 | $12.17^{cd}$ | $16.84^{a}$ |
| | 2.00 | 0.96 | 0.74 | 5.06 | 4.91 | $12.41^{cd}$ | $15.74^{ab}$ |
| 4.5 | 0.00 | 0.82 | 0.78 | 4.08 | 4.89 | $19.80^{abc}$ | $2.84^{d}$ |
| | 0.75 | 0.83 | 0.74 | 4.06 | 4.50 | $19.78^{abc}$ | $8.02^{bcd}$ |
| | 1.50 | 0.82 | 0.71 | 4.18 | 4.84 | $17.51^{abcd}$ | $12.34^{abc}$ |
| | 2.00 | 1.11 | 0.72 | 4.04 | 4.76 | $15.00^{bcd}$ | $14.40^{ab}$ |
| 7.0 | 0.00 | 0.66 | 0.63 | 4.43 | 4.44 | $19.06^{abc}$ | $1.78^{d}$ |
| | 0.75 | 0.94 | 0.83 | 3.87 | 4.25 | $9.79^{d}$ | $2.97^{d}$ |
| | 1.50 | 0.70 | 0.97 | 4.14 | 4.58 | $22.07^{ab}$ | $4.60^{cd}$ |
| | 2.00 | 0.85 | 1.05 | 4.44 | 4.71 | $24.10^{a}$ | $5.37^{cd}$ |

Means within the same column followed by different superscript are significantly different ($p<0.05$).

$^{ns}$ indicates no statistical difference at 95% confident.

Pre-heat treatment at 80°C for 30 min employ to SPI prior to emulsification could reduce the formation of insoluble coacervate. It is possible that protein unfolding and the exposure of hydrophobic and/or charged reactive group were accounted for the reduction of coacervate formation. Heat treatment slightly increased the isoelectric pH of SPI from 4.26 to 4.54 (Figure 16). Besides, the charge of heated SPI at pH 7.0 was -45 mV, which was slightly higher than that of the unheated one ($p<0.05$). Because heated SPI had higher ζ-potential than did the unheated one, the formation of insoluble coacervate could be reduced at pH 7.0 (Table 5).

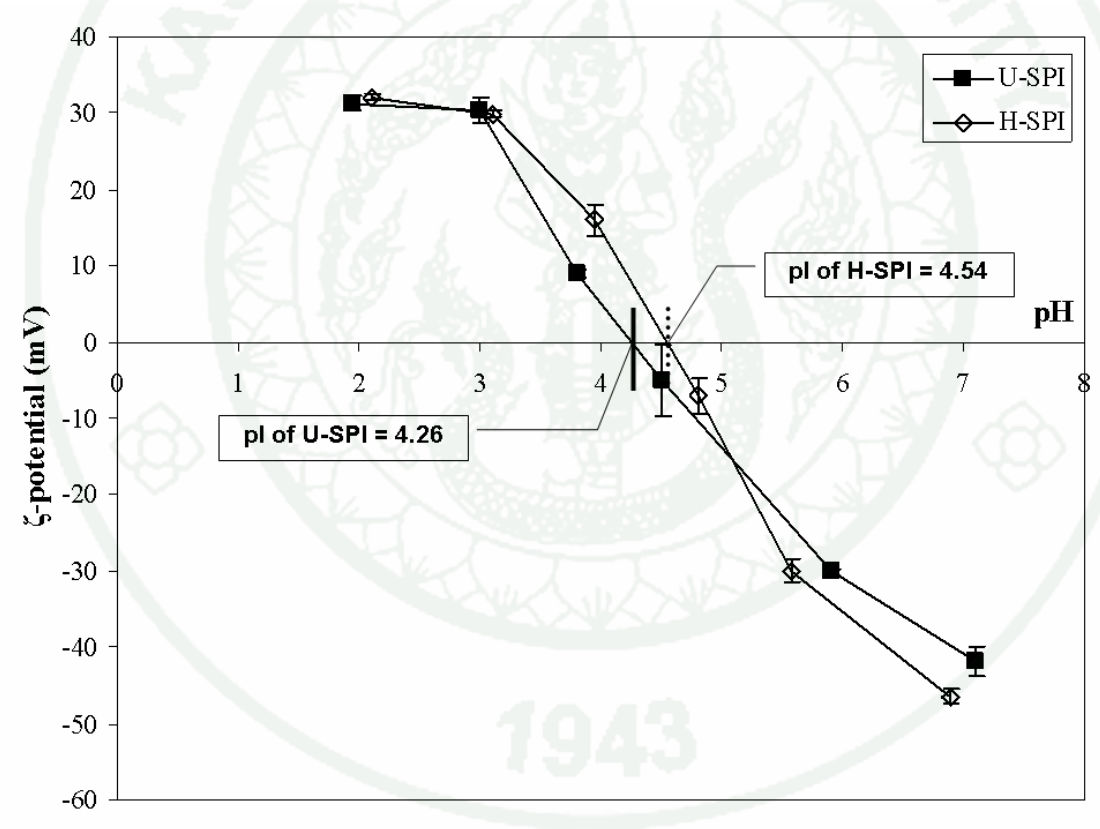


**Figure 16** Effect of heat treatment (80 °C, 30 min) on SPI on ζ-potential of 0.02% (w/v) SPI as a function of pH. Bars represent standard derivation.

Protein content in each fraction from emulsions was shown in Table 6. Protein content in the insoluble coacervate in the sediment fraction increased from 0.13% to 0.57% when 2% of PH-SSP was added in o/w emulsion at pH 7.0. This is probably due to the induction of protein-PH-SSP coacervation. Heat treatment employed to SPI reduced the formation of insoluble coacervate and the protein content in sediment fraction.

**Table 6** Protein concentration in serum and sediment phases in emulsion containing PH-SSP.

| Emulsion | Protein content (%) | | |
|---|---|---|---|
| | Serum | Sediment | Emulsion (Calculated) |
| H-SPI, 0% PH-SSP, pH 7.0 | $0.90 \pm 0.05^{a}$ | $0.13 \pm 0.02^{c}$ | $2.71 \pm 0.03^{a}$ |
| H-SPI, 2% PH-SSP, pH 2.0 | $0.49 \pm 0.14^{b}$ | $1.99 \pm 0.06^{a}$ | $1.27 \pm 0.20^{b}$ |
| H-SPI, 2% PH-SSP, pH 7.0 | $0.94 \pm 0.01^{a}$ | $0.57 \pm 0.06^{b}$ | $2.24 \pm 0.07^{a}$ |
| U-SPI, 2% PH-SSP, pH 7.0 | $0.67 \pm 0.13^{ab}$ | $2.05 \pm 0.14^{a}$ | $1.03 \pm 0.27^{b}$ |

Means within the same column followed by different superscript are significantly different ($p<0.05$).

At pH 7.0, protein in the serum phase included both 7S globulin (which has MW more than 38 kDa) and 11S globulin (which consists of basic subunit (18-20 kDa) and acidic subunit (31-38 kDa)) (Figure 17(a), lane 1, 3 and 4). Although heat treatment (80°C, 30 min) employed to SPI unfolded 7S soy protein of which the denaturation temperature was around 72°C, The MW profiles of proteins in serum phase did not alter (Figure 17(a), lane 3 and 4).

At pH 2.0, protein content in the serum phase was lower than at higher pH while the protein content in the sediment phase increased. Different protein profiles in serum phase of emulsion at pH 2.0 compared to those at pH 7.0 were also observed (Figure 17(a)). Acidic subunit of 11S soy polypeptide (31-38 kDa) and 7S soy

polypeptides (38-76 kDa) were found in serum phase of emulsion at pH 2.0 (Figure 17(a), lane2). This was similar to the study by Lam *et al.* (2008), who found that only 7S soy polypeptide was present in serum phase when adding 0.1% of high-methoxyl pectin (HMP) at acidic condition due to the bridging flocculation between SPI and HMP. The different protein species presented in both serum and sediment phases of emulsion indicated that SPI showed preferential distribution and preferential aggregation with PH-SSP.

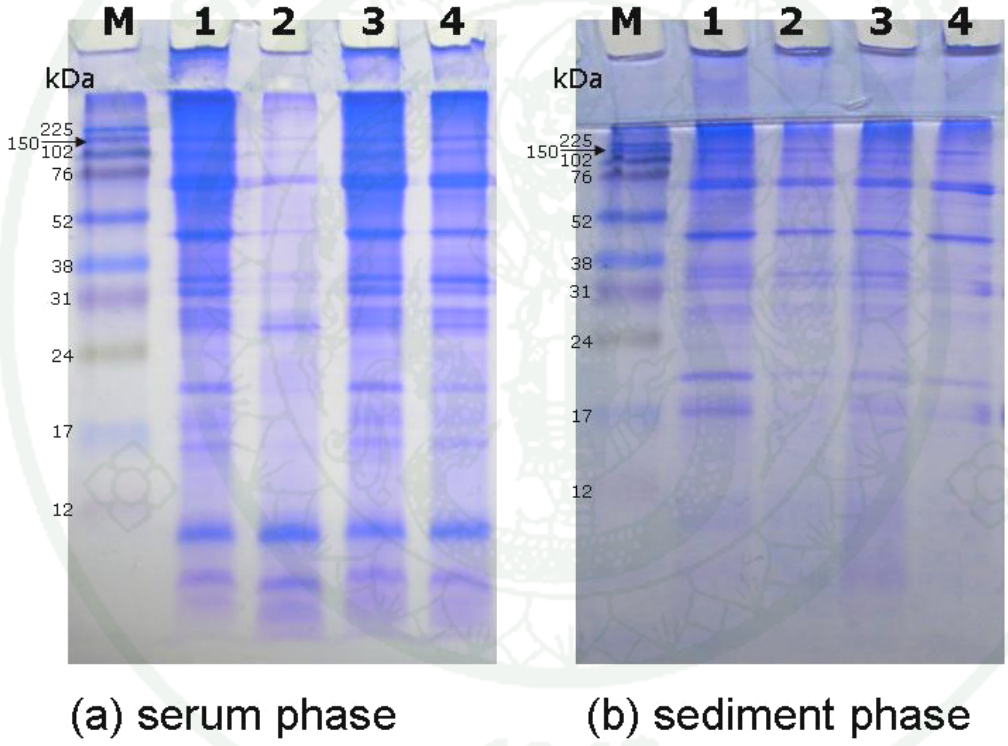


**Figure 17** Preferential distribution of proteins in serum phase (a) and sediment phase (b) in the absence of β-mercaptoethanol; M = Standard protein marker; lane 1 = H-SPI, 0% PH-SSP, pH 7.0; lane 2 = H-SPI, 2% PH-SSP, pH 2.0; lane 3 = H-SPI, 2% PH-SSP, pH 7.0; lane 4 = U-SPI, 2% PH-SSP, pH 7.0

However, adding SSP in emulsion stabilized by the heated SPI at pH 7.0 did not affect emulsion phase height, compared to that added with PH-SSP ($p \geq 0.05$). The formation of insoluble coacervate (sediment phase height) between soy proteins and polysaccharide was increased in the presence of SSP (Table 7). Adding PH-SSP reduced the formation of insoluble coacervate, not only due to the alteration of the MWs of polysaccharide, but also due to the preferential adsorption of protein species at oil-water interface. Adding PH-SSP could help 11S soy polypeptide adsorbed to the more extent at oil-water interface compared to the others so that protein content in emulsion phase of emulsion containing PH-SSP was higher than that containing SSP (Table 7).

**Table 7** Effect of soy polysaccharide type on emulsion stability by heat-denatured SPI at pH 7.0.

| Concentration of polysaccharide (%) | Oil phase height (%) | | Emulsion phase height (%) | | Sediment phase height (%) | |
|---|---|---|---|---|---|---|
| | SSP | PH-SSP | SSP | PH-SSP | SSP | PH-SSP |
| 0.00 | $0.59^{c}$ | | $3.37^{a}$ | | $2.40^{e}$ | |
| 0.75 | $0.69^{abc}$ | $0.83^{abc}$ | $4.55^{a}$ | $4.25^{a}$ | $6.54^{c}$ | $2.97^{de}$ |
| 1.50 | $0.67^{bc}$ | $0.97^{ab}$ | $4.79^{a}$ | $4.58^{a}$ | $10.35^{b}$ | $4.60^{cd}$ |
| 2.00 | $0.67^{bc}$ | $1.05^{a}$ | $4.73^{a}$ | $4.70^{a}$ | $14.10^{a}$ | $5.37^{c}$ |

Means within the same category followed by different superscript are significantly different ($p<0.05$).

The formation of insoluble coacervate was resulted mainly from the interactions between protein and polysaccharide. Therefore, emulsion containing SSP had protein content in the sediment fraction about 1.36%, which was higher than those containing PH-SSP ($p<0.05$) (Table 8). Though the formation of insoluble coacervate (sediment phase height) and the protein content were different, the protein profiles from in serum and sediment phase containing different SSP were similar (Figure 18). Both 7S (38-76 kDa) and 11S soy protein (17-38 kDa) were found in

serum phase containing either PH-SSP or SSP at pH 7.0. At pH 2.0, however, the basic subunit of 11S soy polypeptide (17-22 kDa) disappeared in emulsion adding PH-SSP (Figure 17(a), lane 2). It is suggested that the pH of emulsion regulated preferential distribution of proteins in soy-stabilized emulsion (Figure 17(a) lane 2 and 3, Figure 18(a) lane 2 and 3).

**Table 8** Protein concentration in serum and sediment phase from emulsion containing different SSP.

| Emulsion | Protein content (%) | | |
|---|---|---|---|
| | Serum | Sediment | Emulsion (calculated) |
| No polysaccharide | $0.90 \pm 0.05^{a}$ | $0.13 \pm 0.02^{c}$ | $2.71 \pm 0.03^{a}$ |
| 2% PH-SSP | $0.94 \pm 0.01^{a}$ | $0.57 \pm 0.06^{b}$ | $2.24 \pm 0.07^{b}$ |
| 2% SSP | $0.77 \pm 0.02^{b}$ | $1.36 \pm 0.13^{a}$ | $1.62 \pm 0.12^{c}$ |

Means within the same column followed by different superscript are significantly different ($p<0.05$).

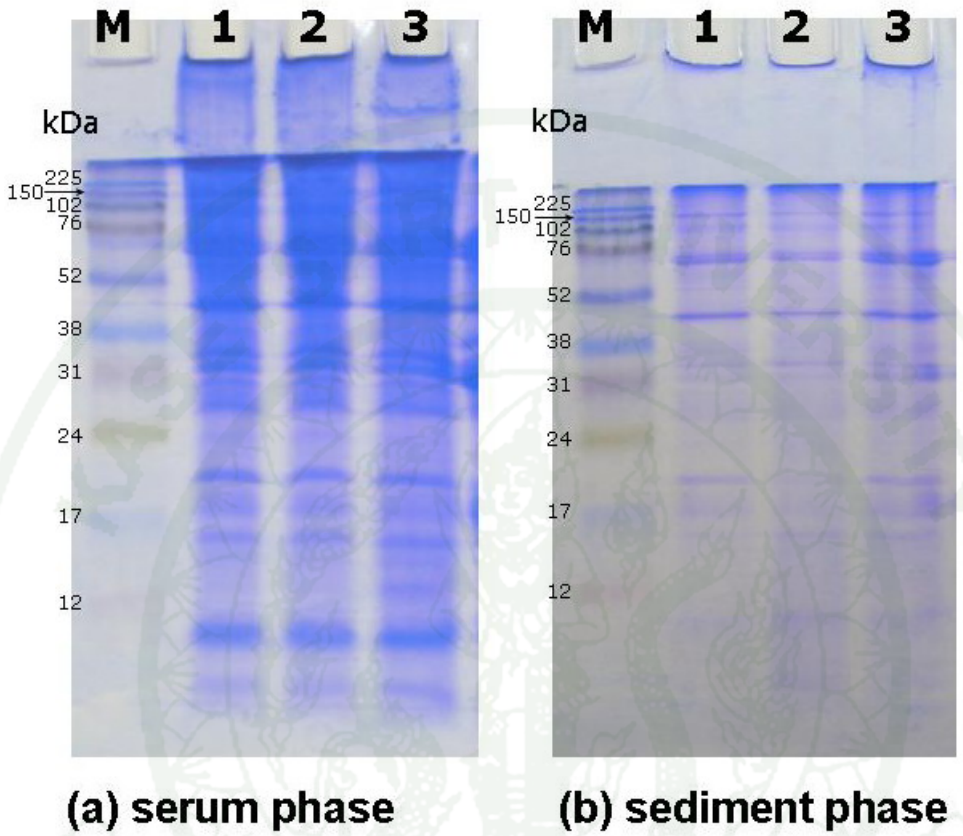


**Figure 18** Effect of soy polysaccharide types on MW of proteins in serum phase (a) and sediment phase (b) of emulsion at pH 7.0 in the absence of β-mercaptoethanol by 15% separating gel; M = Standard protein marker; lane 1 = no soy polysaccharide; lane 2 = 2% PH-SSP; lane 3 = 2% SSP.

Table 9 shows the stability of heated SPI emulsion containing PH-SSP during *in vitro* digestion. Peptic digestion at pH 2.0 referred to the digestion in stomach; while tryptic digestion referred to the digestion in small intestine. The addition of PH-SSP up to 6% did not affect emulsion phase height throughout the digestion ($p \geq 0.05$). However, oil phase height increased from 0.09% to 0.66% when 6% PH-SSP was added ($p < 0.05$). It is possible that the depletion flocculation between SPI and PH-SSP occurred. At high concentration of PH-SSP, the oil was released to the high degree. Oil was released from emulsion around 0.6% after 15 min of peptic digestion and the

addition of various concentration of PH-SSP did not alter the digestion pattern ($p \geq 0.05$). Further digestion by trypsin and bile salts for 60 min did not change the oil release after peptic digestion.

High concentration of PH-SSP increased not only oil phase height, but also the formation of insoluble coacervate. The presence of 6% PH-SSP, insoluble coacervate was increased from 1.94% to 23.38%. Under peptic digestion condition, the insoluble coacervate (sediment phase height) was increased because the acidic pH could help bridging the flocs between positively charged SPI and negatively charged PH-SSP. The insoluble coacervate was lowered after tryptic digestion due to the digestion of SPI into small MW peptides, which could not aggregate with PH-SSP.

**Table 9** Effect of PH-SSP on emulsion stability during *in vitro* digestion.

| Digestion scheme | Oil phase height (%) | | | |
|---|---|---|---|---|
| | 0% PH-SSP | 2% PH-SSP | 4% PH-SSP | 6% PH-SSP |
| undigest (pH 7.0) | $0.09^{b,B}$ | $0.24^{c,B}$ | $0.31^{b,B}$ | $0.66^{b,A}$ |
| + pepsin 15 min (pH 2.0) | $0.65^{a,A}$ | $0.58^{b,A}$ | $0.61^{ab,A}$ | $0.55^{ab,A}$ |
| + pepsin 15 min + trypsin and bile salts 60 min (pH 7.0) | $0.51^{a,B}$ | $0.70^{a,B}$ | $0.84^{a,B}$ | $1.20^{a,A}$ |
| Digestion scheme | Emulsion phase height (%) | | | |
| | 0% PH-SSP | 2% PH-SSP | 4% PH-SSP | 6% PH-SSP |
| undigest (pH 7.0) | $4.78^{a,AB}$ | $5.88^{a,A}$ | $5.91^{a,A}$ | $4.37^{a,B}$ |
| + pepsin 15 min (pH 2.0) | $4.02^{a,A}$ | $4.43^{a,A}$ | $4.83^{ab,A}$ | $5.93^{ab,A}$ |
| + pepsin 15 min + trypsin and bile salts 60 min (pH 7.0) | $3.46^{a,A}$ | $3.80^{a,A}$ | $3.32^{b,A}$ | $3.13^{b,A}$ |
| Digestion scheme | Sediment phase height (%) | | | |
| | 0% PH-SSP | 2% PH-SSP | 4% PH-SSP | 6% PH-SSP |
| undigest (pH 7.0) | $1.94^{b,B}$ | $7.73^{b,B}$ | $26.78^{a,A}$ | $23.38^{a,A}$ |
| + pepsin 15 min (pH 2.0) | $14.61^{a,C}$ | $19.42^{a,B}$ | $20.93^{a,A}$ | $19.82^{a,AB}$ |
| + pepsin 15 min + trypsin and bile salts 60 min (pH 7.0) | $0.70^{b,D}$ | $4.07^{c,C}$ | $7.48^{b,B}$ | $10.67^{b,A}$ |

Means within a same column followed by different lower case superscript are significantly different ($p<0.05$).

Means within a same row followed by different upper case superscript are significantly different ($p<0.05$).

# CONCLUSIONS AND RECOMMENDATIONS

## Conclusions

This study pointed out that the addition of soy soluble polysaccharide up to 6% as a source of fiber in soy-stabilized emulsion affected the stability of liquid emulsion containing 3.75% protein, and 3.33% rice bran oil. The formation of insoluble coacervate between SPI and SSP/PH-SSP could be regulated by pH, types of SSP and heat treatment employed to SPI. The alteration of pH from neutral pH (pH 7.0) to acidic pH (pH 2.0) led to excessive insoluble coacervate formation due to the bridging flocculation between opposite charged molecules. Although pectinase hydrolysis of okara resulted in the self-association of soy soluble polysaccharide to large particle, it could reduce the aggregation between soy proteins and polysaccharides before and after *in vitro* digestion by pepsin and trypsin in the presence of bile salts. Furthermore, the formation of insoluble coacervate could be lowered by using heated soy proteins (80°C, 30 min), especially at pH 7.0. Electrostatic interactions played a significant role in the formation of insoluble coacervate between soy proteins and polysaccharides. The understanding in interactions between soy proteins and polysaccharides could help designing the stability of liquid emulsion and the reduction of insoluble coacervate.

## Recommendations

This study suggested the possibility in the production of liquid emulsion containing high dietary fiber to have desirable properties, i.e., drinkable, low insoluble coacervate, and also the ability to control oil released. However, this study still needs further investigation on (1) the MW of polysaccharides in the SDS-PAGE to confirm the MW of protein conjugated with polysaccharide using different staining system, (2) the investigation of the macrophage activity such as phagocytic uptake and reactive oxygen species (ROS) concentration, (3) the clarification on protein at oil-water

interface of soy-stabilized emulsion influencing different characteristics of liquid emulsion, and so on.

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# APPENDIX

# Method of Analysis

## 1 Determination of moisture content (AOAC, 2000 Method 925.10)

1.1 Apparatus

1.1 Moisture can and lid

1.2 Hot air oven

1.3 Analytical balance

1.4 Desiccator

1.2 Procedure

1.2.1 Dry moisture can and lid in hot air oven at 130°C for 3 hr

1.2.2 Cool in dessicator and weigh

1.2.3 Weigh the sample about 1.0-1.5 g and place in moisture can

1.2.4 Heat the sample in moisture can at 130°C for 1 hr

1.2.5 Remove from hot air oven, cool in desiccator and then weigh

1.2.6 Repeat 1.2.4-1.2.5 until constant weight.

1.3 Calculation

$$\%\ moisture = \frac{(W_1 - W_2)}{W_1} \times 100$$

In which,

$W_1$ = weight of wet sample

$W_2$ = weight of dry sample

**2. Determination of protein content (Lowry's method, Lowry *et al.*, 1951)**

2.1 Apparatus

2.1.1 Analytical balance

2.1.2 Water bath

2.1.3 Vortex

2.1.4 Spectrophotometer

2.2 Reagents

2.2.1 Complex-forming reagent (prepare immediately before use) is prepared by mixing 100 mL of solution A with 1 mL of solution B and 1 mL of solution C.

2.2.2 Solution A is prepared by dissolving 2% (w/v) of sodium bicarbonate in distilled water.

2.2.3 Solution B is prepared by dissolving 1% (w/v) of copper (II) sulfate in distilled water.

2.2.4 Solution C is prepared by dissolving 2% (w/v) of sodium potassium tartrate in distilled water.

2.2.5 2N sodium hydroxide

2.2.6 1N Folin-Ciocalteau's phenol reagent

2.2.7 Standards bovine serum albumin stock solution is prepared by dissolving 0.01 g of bovine serum albumin in 5 mL of distilled water.

2.3 Procedure

2.3.1 A 0.3 mL of sample or standard is place in test tube and adds 0.3 mL of 2N sodium hydroxide

2.3.2 Heat at 100°C for 10 min in water bath and cool to room temperature in ice bath immediately

2.3.3 Add 3 mL of complex-forming reagent, mix thoroughly by vortex

2.3.4 Stand for 10 min at room temperature

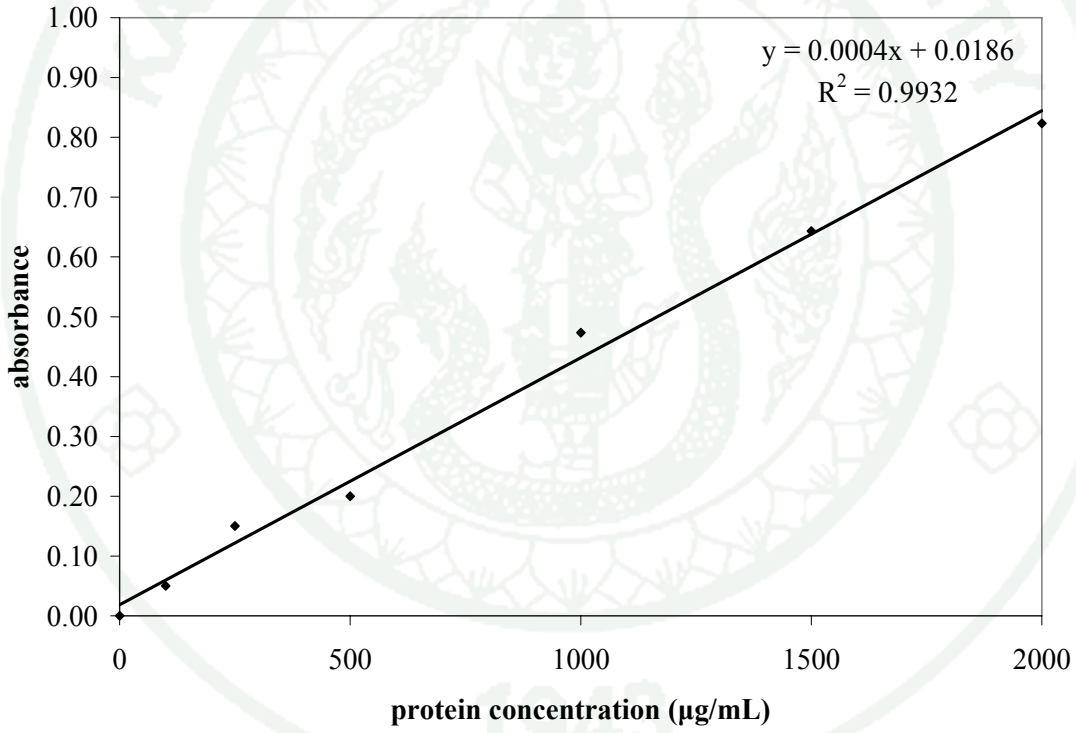
2.3.5 Add 0.3 mL of 1N Folin-Ciocalteau's phenol reagent and stand for 30 min at room temperature (but not exceed 60 min)

2.3.6 Read absorbance at 550 nm, use blank reagent to zero the spectrophotometer

2.4 Calculation

2.4.1 Plot absorbance at 550 nm against bovine serum albumin content of standard solution

2.4.2 Calculate the protein content of sample by using the equation from the standard curve of bovine serum albumin.




**Appendix Figure 1** Standard curve for protein content determination.

**3. Determination of dietary fiber content (AOAC, 2000, Method 985.29)**

3.1 Apparatus

3.1.1 Dietary fiber crucible

3.1.2 Water bath

3.1.3 Tall-form beaker

3.1.4 pH meter

3.1.5 Vacuum pump

3.1.6 Hot air oven

3.1.7 Muffle furnace

3.1.8 Desiccator

3.2 Reagents

3.2.1 98% Ethanol

3.2.2 75% Ethanol

3.2.3 Acetone

3.2.4 Di-sodium hydrogen phosphate

3.2.5 Sodium di-hydrogen phosphate

3.2.6 α-Amylase (EC 3.2.1.1; α-amylase, heat stable, Product No. A3306, Sigma, Sigma-Aldrich, St. Louis, USA)

3.2.7 Protease (EC 3.4.21.14; Protease from *Bacillus licheniformis*, Product No. P3910, Sigma, Sigma-Aldrich, St. Louis, USA)

3.2.8 Amyloglucosidase (EC 3.2.1.3; Amyloglucosidase from *Aspergillus niger*, Product No. A9913, Sigma, Sigma-Aldrich, St. Louis, USA)

3.2.9 0.275M Sodium hydroxide

3.2.10 0.325M Hydrochloric acid

3.2.11 Celite (acid washed)

3.3 Procedure

3.3.1 Weigh duplicate 1.0g of sample in tall-form beaker which each portion should not differ more than 0.020g

3.3.2 Add 50 mL of phosphate buffer pH 6.0 and pH of sample should be around 6.0 ± 0.2

3.3.3 Add 0.1 mL of α-amylase and cover beaker with aluminium foil

3.3.4 Heat in water bath at 95-100°C for 30 min and shake gently at 5 min intervals

3.3.5 Cool at room temperature and add 10 mL of 0.275M sodium hydroxide; pH should be around 7.5 ± 0.2

3.3.6 Add 0.1 mL of protease and cover beaker with aluminium foil

3.3.7 Incubation at 60°C with continuous agitation for 30 min and cool at room temperature

3.3.8 Adjust pH to 4.0-4.6 by adding 10 mL of 0.325M hydrochloric acid

3.3.9 Add 0.3 mL of amyloglucosidase and cover beaker with aluminium foil

3.3.10 Heat at 60°C with continuous agitation for 30 min and cool at room temperature

3.3.11 Add 280 mL of 95% ethanol and preheat at 60°C

3.3.12 Allow to precipitate at room temperature for 60 min

3.3.13 Weigh crucible containing Celite, then wet by 78% ethanol

3.3.14 Apply suction to draw Celite onto crucible as even mat and the transfer precipitate to crucible

3.3.15 Wash residue three times with 20 mL of 78% ethanol, two times with 10 mL of 95% ethanol, and two times with 10 mL of acetone

3.3.16 Dry crucible containing residue overnight at 105°C until constant weight

3.3.17 Subtract crucible and Celite weight to determine weight of residue

3.3.18 Analyze residue from one test of set of duplicates for protein by Kjeldahl method (AOAC, 2000, method 960.52) and another residue for ash determination by heating at 525°C until the constant weight

3.3.19 Substract crucible and Celite weight to determine ash

3.4 Calculation

$$B = \textit{weight of blank (mg)} = \textit{weight of residue} - P_B - A_B$$

In which,

*weight of residue* = average of residue weights (mg) for duplicate blank determinations

$P_B$ = weight (mg) of protein determined in first blank residue

$A_B$ = weight (mg) of ash determined in second blank residue

$$\textit{Total dietary fiber (\%)} = \frac{(\textit{weight of residue} - P - A - B)}{\textit{weight test portion}} \times 100$$

In which,

*weight of residue* = average of weights (mg) for duplicate blank determinations

$P$ = weights (mg) of protein in first test portion residues

$A$ = weights (mg) of ash in second test portion residues

*weight test portion* = average of two test portion weights (mg)

**4. Determination of protein content (Kjeldahl method; AOAC, 2000, method 960.52)**

4.1 Apparatus

4.1.1 Analytical balance

4.1.2 Digestion kjeldahl tube

4.1.3 Digestion unit (model K-435, Buchi)

4.1.4 Scrubber (model B-414, Buchi)

4.1.5 Distillation unit (model B-324)

4.1.6 Titration apparatus

4.2 Reagents

4.2.1 2% (w/v) Boric acid

4.2.2 Bromocresol green

4.2.3 Copper (II) sulfate

4.2.4 Methyl red

4.2.5 Potassium sulfate

4.2.6 40% (w/v) Sodium hydroxide

4.2.7 Sulfuric acid

4.2.8 0.1N Sulfuric acid

4.3 Procedure

4.3.1 Weigh the sample 0.5-1.0 g of solid sample or 1.0 mL of liquid sample into digestion kjeldahl tube

4.3.2 Add catalysts (10 g of potassium sulfate and 0.5 g of copper (II) sulfate) and a few of glass beeds

4.3.3 Add 20 mL of conc. sulfuric acid and then digest in digestion unit until the solution is clear

4.3.4 Cool to room temperature

4.3.5 Take digestion kjeldahl tube to distillation unit and then add 60 mL of distilled water and 60 mL of 40% sodium hydroxide

4.3.6 Distill for 3 min and using 60 mL of 2% boric acid to collect distillate

4.3.7 Titrate with 0.1N sulfuric acid. Reagent blank should be run

4.4 Calculation

$$\%Nitogen = \frac{(S-B)\times N \times f \times 1400}{W}$$

$$\% \text{ Protein} = \% \ Nitrogen \times \text{conversion factor}$$

In which,

S = Volume of using acid in sample titration (mL)

B = Volume of using acid in blank titration (mL)

N = Normality of sulfuric acid

W = Weight of sample (g)

$f$ = Factor of sulfuric acid

$$f = \frac{E}{121.14 \times N \times V}$$

In which,

$E$ = Weight of tris buffer (120 mg)

$N$ = Normality of sulfuric acid

$V$ = Volume of using acid in tris buffer titration (mL)

**5. Determination of reducing sugar content (Milner and Avigad, 1967)**

5.1 Apparatus

5.1 Analytical balance

5.2 Water bath

5.3 Vortex

5.4 Spectrophotometer

5.2 Reagents

5.2.1 Copper reagent is prepared by dissolving 28 g of sodium sulfate and 4 g of sodium chloride in 72 mL of distilled water (hard to dissolve). Then add 10 mL of 2M acetate buffer (pH 5.1) and 6.5 mL of 0.32 M copper sulfate to salt solution. Solution is heated at 60°C until complete dissolution. Final pH is adjusted to 4.8 and the volume is adjusted to 100 mL by distilled water. The reagent is kept in brown bottle at 37°C, which is stable at least 2 months.

5.2.2 Arsenomolybdate color reagent is prepared by dissolving 2.5 g of ammoniummolybdate in 45 mL of distilled water. A 2.1 mL of conc. sulfuric acid and 0.3 g of di-sodium hydrogen arsenate in 2.5 mL of distilled water are added in the solution. The reagent is kept in brown bottle, covered by aluminium foil, at 37°C for 24 to 48 hr before use.

5.2.3 Sugar stock solution is prepared by dissolving 0.001 g of galacturonic acid in 5 mL of 0.05% benzoic acid.

5.3 Procedure

5.3.1 A 0.5 mL of sample or standard solution is mixed with 1.5 mL of copper reagent

5.3.2 Heat at 100°C for 10 min in water bath

5.3.3 Cool to room temperature in ice bath without shaking

5.3.4 Add 1.0 mL of arsenomolybdate color reagent, and then mix thoroughly

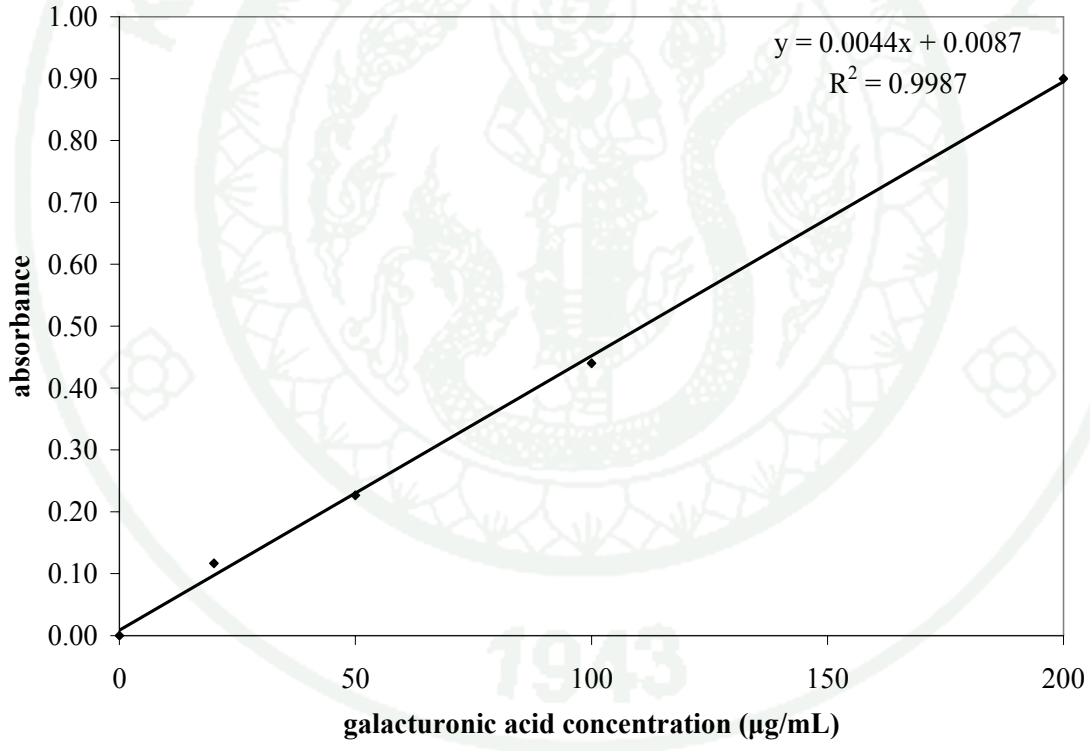
5.3.5 Add 2.0 mL of distilled and mix gently

5.3.6 Read the absorbance at 600 nm, use blank reagent to zero the spectrophotometer

5.4 Calculation

5.4.1 Plot absorbance at 600 nm against reducing sugar content of standard solution

5.4.2 Calculate the reducing sugar content of sample by using the equation from the standard curve of galacturonic acid



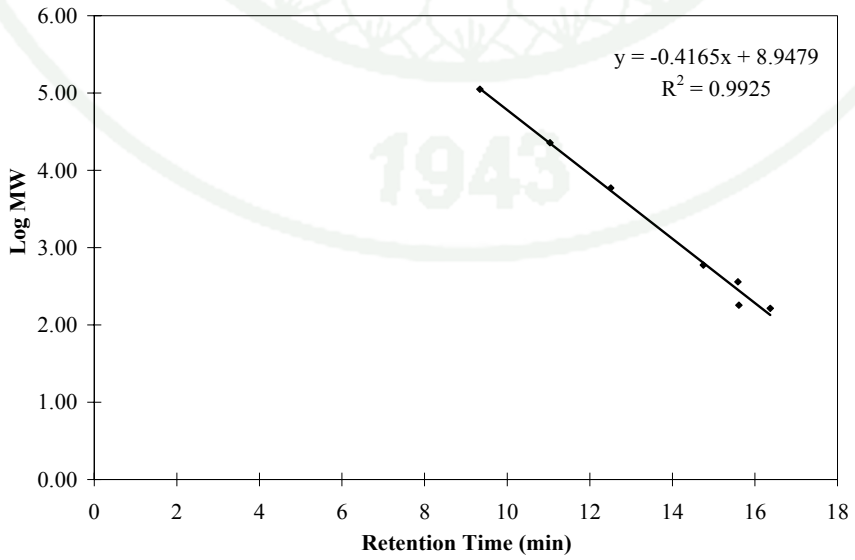

**Appendix Figure 2** Standard curve for reducing sugar content determination.

**6. Molecular weight analysis of polysaccharide by HPLC**

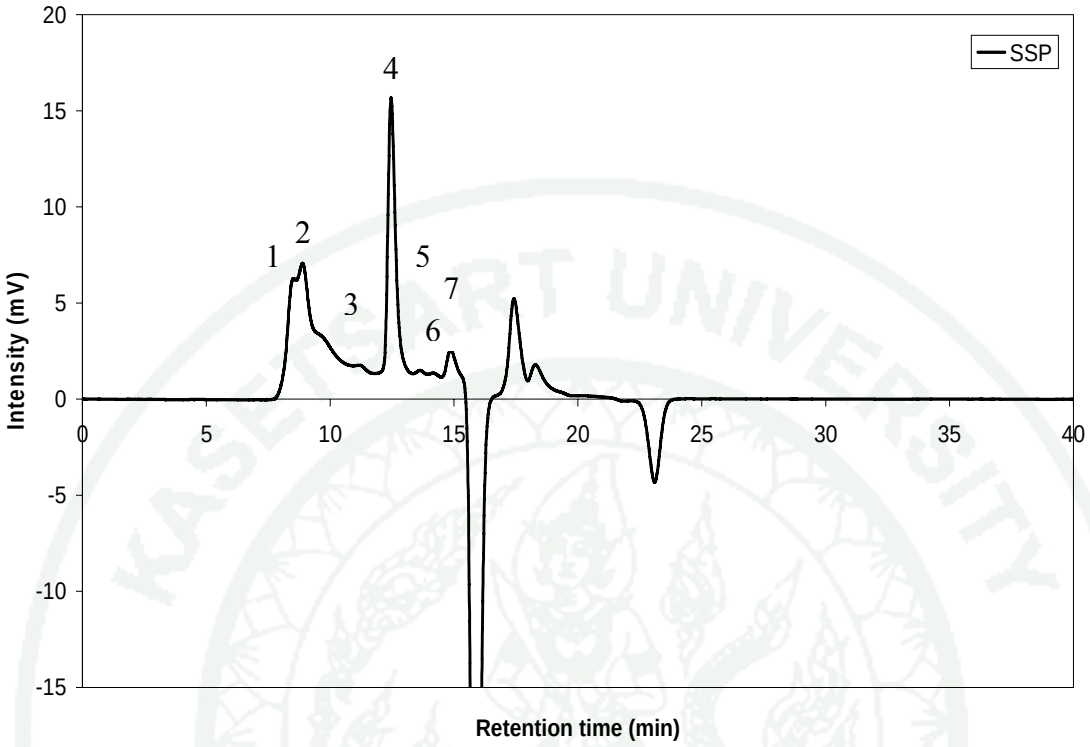
Molecular weight standard was performed with the same condition for analysis of SSP and PH-SSP. The larger molecular weight of standard is, the faster standard comes out (less retention time). Then, plot standard curve of retention time against log MW of standard. MWs of sample were calculated by the equation from the standard curve.

**Appendix Table 1** Retention time of molecular weight standard of polysaccharide.

| Standard | MW (Da) | Retention time (min) |
|---|---|---|
| α-L-Rhamnose | 164.2 | 16.37 |
| Glucose | 180 | 15.61 |
| Maltose | 360 | 15.59 |
| (D)-+-Raffinose | 594.52 | 14.75 |
| Shodex STD P-5 | 5900 | 12.51 |
| Shodex STD P-20 | 22800 | 11.04 |
| Shodex STD P-100 | 112000 | 9.34 |



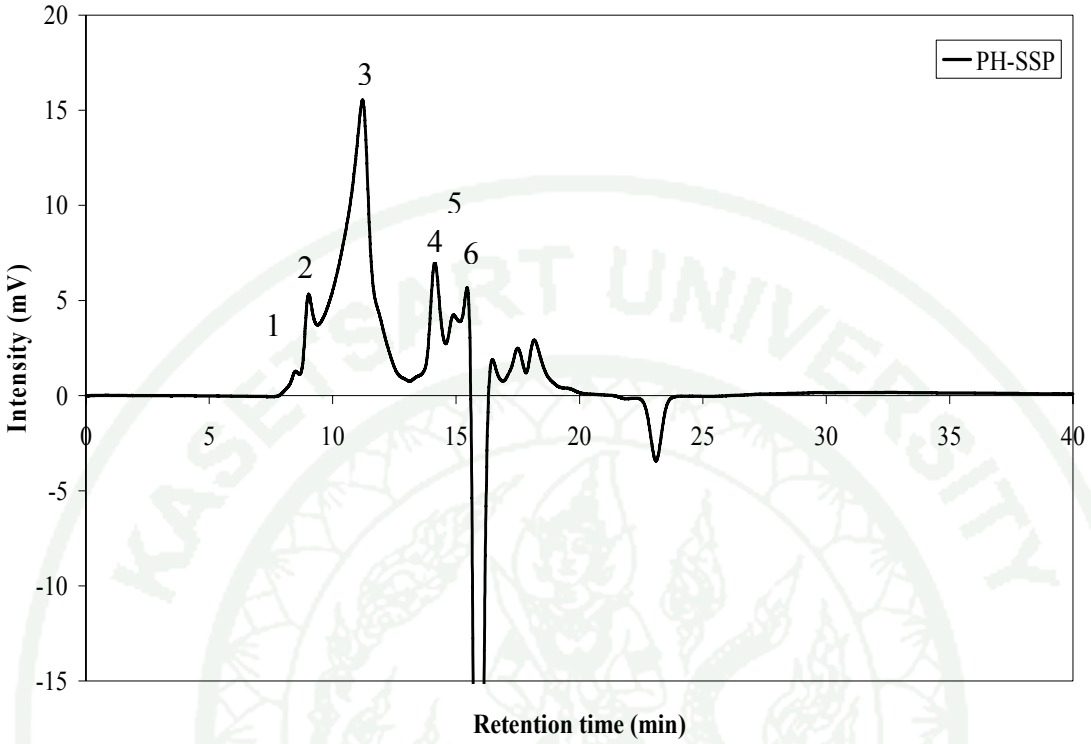

**Appendix Figure 3** Standard curve of MW standard for polysaccharides.




**Appendix Figure 4** Size exclusion chromatogram of SSP on TSKgel G-3000 $PW_{XL}$ column. Elution was monitored by refractive index detector.

**Appendix Table 2** Retention time of SSP and MW (kDa) which was calculated by using standard pullulan and sugar.

| Peak No. | Retention time (min) | MW (kDa) |
|---|---|---|
| 1 | 8.51 | 253 |
| 2 | 8.88 | 178 |
| 3 | 11.16 | 19.9 |
| 4 | 12.45 | 5.79 |
| 5 | 13.61 | 1.90 |
| 6 | 14.13 | 1.16 |
| 7 | 14.85 | 0.58 |



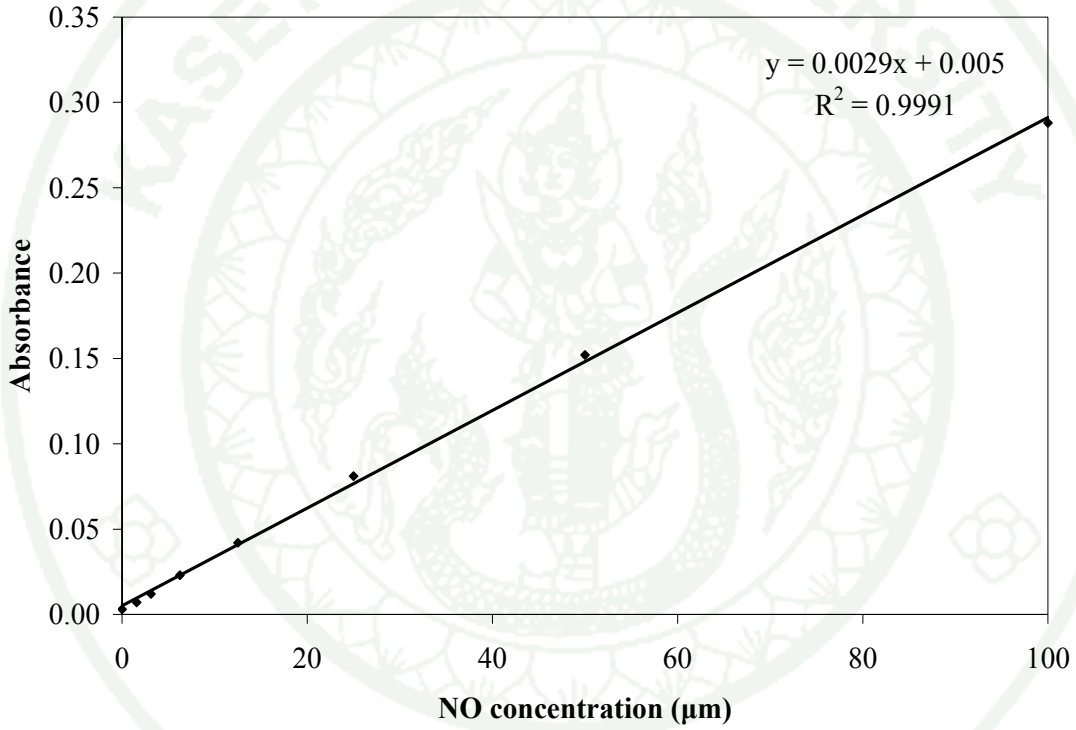

**Appendix Figure 5** Size exclusion chromatogram of PH-SSP on TSKgel G-3000 $PW_{XL}$ column. Elution was monitored by refractive index detector.

**Appendix Table 3** Retention time of PH-SSP and MW (kDa) which was calculated by using standard pullulan and sugar.

| Peak No. | Retention time (min) | MW (kDa) |
|---|---|---|
| 1 | 8.49 | 258 |
| 2 | 9.05 | 151 |
| 3 | 11.19 | 19.4 |
| 4 | 14.13 | 1.16 |
| 5 | 14.91 | 0.55 |
| 6 | 15.45 | 0.32 |

**7. Determination of nitric oxide production by macrophage**

To determine the amount of nitric oxide. The standard curve was plotted the absorbance at 540 nm against sodium nitrite content of standard solution. Then, calculate the amount of nitric oxide produced by macrophage by using the equation from the standard curve of sodium nitrite.




**Appendix Figure 6** Standard curve of nitric oxide (NO) determination.

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Fafaungwithayakul, N., P. Hongsprabhas and P. Hongprabhas. 2009. Emulsion stability of soy-based liquid emulsion containing dietary fiber. *In* **Proceedings of 11th Agro-Industry Conference: Food Innovation Asia Conference 2009**. 18-19 June 2009. Bangkok, Thailand.