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# Influence of High Pressure Homogenisation on Stability of Emulsions Containing Skipjack Roe Protein Hydrolysate

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## Abstract

**Background/Objectives:** Skipjack Roe Protein Hydrolysate (SRPH) can serve as the natural emulsifier. Nevertheless, homogenising conditions have also shown to determine the stability of emulsion. Thus, the aim of this study was to elucidate the impact of different homogenisation pressures on stability of emulsion containing SRPH. **Methods/Statistical Analysis:** Emulsions containing SRPH or sodium caseinate were prepared using varying homogenisation pressures (13.8, 20.7 and 27.6 MPa). During the 14 days of extended storage, emulsion samples were tested for particle size, flocculation factor, coalescence index, ζ-potential, creaming index, interfacial protein, confocal laser scanning microscopy and SDS-PAGE of interfacial and unabsorbed proteins respectively. **Findings:** Higher homogenisation pressure resulted in the higher decrease in particle sizes as evidenced by the decreased volume frequency distribution ($d_{43}$) ($P < 0.05$). The protein concentration at interface was increased with increasing homogenisation pressures ($P < 0.05$). Dominant interfacial proteins of SRPH stabilised emulsions had the molecular weight in the range of 7.0-16.4 kDa. During the extended storage, particle size, creaming index, flocculation factor ($F_f$) and coalescence index ($C_i$) of SRPH stabilised emulsions sharply increased, especially in emulsions prepared at 20.7 and 27.6 MPa ($P < 0.05$). Nevertheless, emulsions containing SRPH showed the lower stability, compared with those stabilised by sodium caseinate ($P < 0.05$). **Applications/Improvements:** The stability of emulsion stabilised by SRPH could be improved by homogenisation at an appropriate pressure (13.8 MPa).

**Keywords:** Emulsion, Particle Size, Roe, Skipjack Tuna, Z-Potential

## 1. Introduction

High pressure homogenisation mechanically reduces the size of particles, producing emulsion with homogeneity and high stability[1]. High-pressure homogenisation can disrupt the flocculated clusters, thereby dispersing agglomerates uniformly. Combination of intense shear, cavitation and turbulent flow conditions increases the surface activity of emulsifying molecules[2–4]. Additionally, the creaming velocity is proportional to the square of the droplet diameter and density difference[5]. Thus, the decreased average size of the oil droplet attributed by high pressure homogenisation can reduce the creaming velocity (Stokes' law) and therefore increases the stability of emulsion. Two main emulsifiers, amphiphilic macromolecules (mainly proteins) and low molecular weight emulsifiers (lecithins, monoglycerides, tweens, spans, etc.[6], have been widely used in food emulsions. Emulsifying properties of proteins or peptides is governed by their physicochemical properties such as molecular composition, conformation, viscoelasticity, etc[7]. The formation of elastic protein film at the oil-water interface plays a vital role in stabilizing emulsion against coalescence[8]. The emulsifying properties of protein have

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been successfully altered via thermal (dry heat, wet heat, microwave, etc.) chemical (succinylation and acetylation) or enzymatic methods[7].

Recently, a new food-grade natural emulsifier has gained a considerable attention. Skipjack tuna roe, a by-product of tuna canning industry, contained phosvitin and lipovitellin, which can serve as an alternative emulsifier with nutritive value[9]. Previously, Skipjack Roe Protein Hydrolysate (SRPH) with Degree of Hydrolysis (DH) of 5% using Alcalase was found to have the emulsifying property[10]. The implementation of high pressure to reduce the size of droplets and to favor the localisation of peptides at interface can be a promising approach to improve the stability of emulsion containing roe protein hydrolysate.

Thus, the aim of this study was to elucidate the impact of homogenisation at varying pressure levels on stability of emulsion containing Skipjack Roe Protein Hydrolysate.

## 2. Materials and Methods

### 2.1 Chemicals

Sodium azide ($NaN_3$) and saccharose were purchased from Fluka Chemical (Buchs, Switerland). Acridine orange, Nile blue A and Sodium Dodecyl Sulphate (SDS) were bought from Sigma Chemical Co. (St. Louis, MO, USA). Coomassie brilliant blue R-250 was obtained from Merck (Darmstadt, Germany). All reagents were of analytical grade except soybean oil, which was of commercial grade (Thanakorn Vegetable Oil Products Co. Ltd., *Samutprakan*, Thailand).

### 2.2 Preparation of Protein Hydrolysate

Frozen roes of skipjack (*Katsuwonus pelamis*) obtained from Songkhla Canning Company (Ltd.), Songkhla, Thailand, were thawed and defatted as described by Intarasirisawat et al[9]. Protein hydrolysate with 5% Degree of Hydrolysis (DH) was prepared using Alcalase as per the method of Intarasirisawat et al[10]. The obtained Skipjack Roe Protein Hydrolysate (SRPH) was lyophylised using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark), placed in polyethylene bag and kept at -20°C until use.

### 2.3 Effect of High Pressure Homogenisation on Emulsion Characteristic and Stability

Emulsion was prepared according to the method of Castellani, et al.[11] with a slight modification. SRPH (5 g) was dispersed into 100 ml of distilled water and then adjusted pH to 7.0 using 1 mol/LHCl. Ten millilitres of soybean oil were added with 100 ml of SRPH solution (oil volume fraction: 0.1). The mixture was homogenised at a speed of 10,000 rpm for 2 min using a homogeniser (Model T25 basic, IKA Labortechnik, Selangor, Malaysia). The coarse emulsions were then passed through a Microfluidics homogeniser (Model HC-5000, Microfluidizer, Newton, MA, USA) at different pressures (13.8, 20.7 and 27.6 MPa) for fifteen passes. $NaN_3$ (0.02g/100 mL) was added to the emulsions as an antimicrobial agent. The control emulsion was prepared in the same manner using 5g/100 mL sodium caseinate. All emulsion samples were then stored at room temperature (28-30°C) for 14 days. The samples were taken at day 1, 7 and 14 for analyses, except for creaming index and microstructure analyses. Creaming index was monitored at day 1, 3, 5, 7, 9, 11 and 14 and the confocal laser scanning micrograph was examined at day 1 and 14.

### 2.4 Analyses

#### *2.4.1 Particle Size Distribution*

Particle size distribution of emulsions was determined using a Liquid Particle Size Analyser (LPSA) (Model LS 230, Beckman Coulter®, Fullerton, CA, USA) as per the method of Castellani et al.[11] with a slight modification. Prior to analysis, an aliquot of emulsion (5 mL) was diluted with 10g/L Sodium Dodecyl Sulphate (SDS) solution (20 mL) in order to dissociate flocculated droplets. The surface-weighted mean particle diameter ($d_{32}$) and the volume-weighted mean particle diameter ($d_{43}$) of the emulsion droplets were measured.

#### *2.4.2 Flocculation and Coalescence*

To determine flocculation factor and coalescence index, the emulsions were diluted with distilled water in the presence and absence of 10 g/L SDS. The flocculation factor ($F_f$) and coalescence index (Ci) were calculated using the following equations:

$$C_i = \frac{(d_{43+SDS,t} - d_{43+SDS,in})\text{x}100}{d_{43+SDS,in}}$$

$$F_f = \frac{d_{43-SDS}}{d_{43+SDS}}$$

where $d_{43+SDS}$ and $d_{43-SDS}$ are the volume-weighted mean particle diameter of the emulsion droplets in the presence and absence of 10 g/L SDS, respectively; $d_{43+SDS,in}$ and

$d_{43+SDS,t}$ are the volume-weighted mean particle diameter of the emulsion droplets in the presence of 10 g/L SDS at the designated storage time.

### 2.4.3 ζ-Potential

The electrical charge (ζ-potential) of oil droplets in the emulsions was determined using a ZetaPlus zeta potential analyser (Brookhaven Instruments Corporation, Holtsville, NY, USA) at room temperature. The ζ-potential was determined by measuring the direction and velocity of droplet movement in the applied electric field. The ζ-potential of each individual sample was calculated.

### 2.4.4 Creaming

Creaming was measured according to the method of Keowmaneechai and McClements[12] with a slight modification. The emulsions (13 mL) were poured into test tubes and stored at room temperature. The volume of the separated aqueous phase at the bottom of the tube was recorded. Creaming was calculated as follows:

Creaming % = (Height of clear droplet-free phase/ Total height of the emulsion) x 100

Creaming was monitored via the kinetic formation of a clear droplet-free phase at the bottom of the sample. The percentage of creaming was plotted against storage times (0, 1, 3, 5, 7, 9, 11 and 14 days).

### 2.4.5 Confocal Laser Scanning Microscopy

Behaviors of emulsion were examined with a Confocal Laser Scanning Microscope (CLSM) (Olympus, FV300, Tokyo, Japan). The emulsion (100 µL) was suspended with 20 µL of 0.1 g/L Nile blue A and 20 µL of 0.1 g/L acridine orange in order to label lipid and protein, respectively. Five microlitres of prepared samples were smeared on the microscopy slide. The CLSM was operated in the fluorescence mode at the excitation wavelength of 533 nm and the emission wavelength of 630 nm using a Helium Neon Red laser (HeNe-R) for lipid analysis and at the excitation wavelength of 488 nm and the emission wavelength of 540 nm using a Helium Neon Green laser for protein analysis[13]. Magnification of 200x was used.

### 2.4.6 Interfacial Protein Concentration

Interfacial protein concentration of emulsions was determined according to the method of Patton and Huston[14]. Emulsion was diluted with 0.50 kg/L saccharose in the same buffer of the emulsion aqueous phase at 1:1 ratio (v/v). An aliquot of mixture (7 mL) was then carefully deposited at the bottom of a centrifuging tube containing 13.5 mL of a 0.05 kg/L saccharose solution in the corresponding buffer. These tubes were centrifuged at 3,000g for 2 h at 10°C and then immediately frozen at -20°C for 24 h. The frozen tube (-20°C) were cut to recover the two phases. Upper phase including creamed oil droplets at the top and an intermediate separating phase and lower phase or the aqueous phase located at the bottom were obtained. The protein content was determined in all fractions. Proteins in the upper phase and lower phase were adsorbed and unadsorbed proteins, respectively. The protein content of the turbid middle phase was included as the adsorbed protein. Interfacial protein concentration ($\Gamma$, mg/m$^2$) was calculated as follows:

$$\Gamma = \frac{Absorbed\ protein\ concentration (mg/ml\ of\ oil)}{Specific\ surface\ area\ S_s (m^2/ml\ of\ oil)}$$

The specific surface area of oil droplets ($S_s$ in m$^2$/mL of oil) was calculated from the surface weighted mean particle diameter ($d_{32}$ in mm) according to Walstra[15] as shown below:

$$S_s = 6/d_{32}$$

### 2.4.7 Protein Patterns

Total protein and interfacial protein were subjected to Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using 15% separating gel and 4% stacking gel according to the method of Laemmli[16]. Samples were diluted in sample buffer (0.25 mol/L Tris–HCl pH 6.8, 0.04 g/100 mL bromophenol blue and 30 g/100 mL glycerol and 6 g/100 mL SDS solution) to obtain designated protein concentration. Proteins (18 µg) determined by the Lowry's method[17] was loaded onto the gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02 g/100 ml Coomassie Brilliant Blue R-250 in 50 mL/100 mL methanol and 7.5 mL/100 mL acetic acid for 3 h and destained with 50 mL/100 mL methanol and 7.5 mL/100 mL acetic acid for 15 min, followed by 5 mL/100 mL methanol and 7.5 mL/100 mL acetic acid for 3 h. Low molecular weight markers were used for estimation of MW of protein bands.

### 2.5 Statistical Analysis

All experiments were run in triplicate. All analyses were conducted in five replications, except for interfacial protein concentration and creaming index, which were performed in triplicate. Statistical analysis was performed using one-way Analysis of Variance (ANOVA). Mean comparison was carried out using Duncan's multiple range test[18]. Analysis was performed using the SPSS package (SPSS for windows, SPSS Inc., Chicago, IL, USA)

**Table 1.** Particle size of droplets in emulsions stabilised by SRPH and sodium caseinate prepared using different pressures during storage

| Sample | Pressure used (MPa) | Storage time (days) | $d_{32}$ (µm) | $d_{43}$(µm) |
|---|---|---|---|---|
| SRPH | 13.8 | 1 | 0.36±0.07 $^{Ac}$ | 1.14±0.03 $^{Ac}$ |
| | | 7 | 1.75±0.06 $^{Ab}$ | 2.60±0.02 $^{Ab}$ |
| | | 14 | 2.14±0.03 $^{Aa}$ | 3.00±0.01 $^{Aa}$ |
| | 20.7 | 1 | 0.32±0.09 $^{Ab}$ | 0.67±0.12 $^{Bc}$ |
| | | 7 | 1.18±0.08 $^{Ca}$ | 2.01±0.01 $^{Bb}$ |
| | | 14 | 1.36±0.09 $^{Ca}$ | 2.39±0.02 $^{Ba}$ |
| | 27.6 | 1 | 0.34±0.07 $^{Ac}$ | 0.57±0.11 $^{Bc}$ |
| | | 7 | 1.37±0.05 $^{Bb}$ | 1.96±0.00 $^{Cb}$ |
| | | 14 | 1.77±0.02 $^{Ba}$ | 2.24±0.01 $^{Ca}$ |
| Sodium caseinate | 13.8 | 1 | 0.20±0.01 $^{Ab}$ | 0.36±0.02 $^{Aa}$ |
| | | 7 | 0.31±0.04 $^{Aa}$ | 0.37±0.01 $^{Aa}$ |
| | | 14 | 0.28±0.02 $^{Aa}$ | 0.38±0.01 $^{Aa}$ |
| | 20.7 | 1 | 0.20±0.02 $^{Aa}$ | 0.32±0.05 $^{ABa}$ |
| | | 7 | 0.23±0.05 $^{Ba}$ | 0.28±0.03 $^{Ba}$ |
| | | 14 | 0.23±0.03 $^{Ba}$ | 0.27±0.02 $^{Ca}$ |
| | 27.6 | 1 | 0.16±0.02 $^{Bb}$ | 0.28±0.05 $^{Ba}$ |
| | | 7 | 0.22±0.05 $^{Ba}$ | 0.33±0.04 $^{Aa}$ |
| | | 14 | 0.25±0.02 $^{Ba}$ | 0.31±0.01 $^{Ba}$ |

Data are expressed as mean ± SD (n = 5).

Different lowercase superscripts in the same column within the same pressure and sample indicate significant difference ($P < 0.05$).

Different uppercase superscripts in the same column within the same storage time and sample indicate significant difference ($P < 0.05$).

## 3. Results and Discussion

### 3.1 Particle Size Distribution

Particle size of emulsions containing SRPH and sodium caseinate prepared with different pressures (13.8-27.6 MPa) expressed as $d_{32}$ and $d_{43}$ was monitored during 14 days of storage at room temperature (Table 1). With increasing homogenisation pressure, the emulsions stabilised by SRPH or sodium caseinate had the lower $d_{32}$ ($P > 0.05$) and $d_{43}$ ($P < 0.05$). Generally, emulsions stabilised by SRPH possessed the larger size of droplets, compared with those containing sodium caseinate. At day 1 of storage, the SRPH emulsions had $d_{32}$ of 0.32-0.36 µm and $d_{43}$ of 0.57-1.14 µm. For emulsions with sodium caseinate, $d_{32}$ of 0.16-0.20 µm and $d_{43}$ of 0.28-0.36 µm were obtained. $d_{32}$ and $d_{43}$ are more sensitive to the presence of small and large particles, respectively[19]. The $d_{32}$ is inversely proportional to specific surface area. The smaller $d_{32}$ contributes to the higher specific surface area, which offers the increase in protein loads for adsorbing at interface of emulsions[20]. The $d_{43}$ can be used as the index of coalescence and flocculation. The increase in $d_{43}$ reflects the association of individual droplets into larger droplet[20]. Particle size distribution is an important parameter involving in physical properties (colour, viscosity and texture) and shelf-life of food emulsion[21].

During 14 days of storage, $d_{32}$ and $d_{43}$ of emulsion stabilised by SRPH were increased ($P < 0.05$), suggesting the coalescence of oil droplets. On the other hand, sodium caseinate could maintain the oil droplet size in emulsion during the extended storage. Differences in emulsifying properties between SRPH and sodium caseinate were plausibly due to the different constituents, conformation, hydrophobicity and chain length of peptides or proteins[20]. The employment of high pressure technique for homogenisation caused the modification of protein conformation, particularly globular protein[22]. Those proteins or peptides with more exposed hydrophobic domains likely adsorbed at interface of droplet more effectively. Adsorption of the modified macromolecule of sodium caseinate surrounding interfacial oil droplet provided steric hindrance against coalescence[23]. However, Floury et al.[24] suggested that an excessive pressure used may cause the detrimental effect on emulsifying properties of globular protein. Excessive unfolding of globular protein induced by high pressure could affect the interfacial properties of globular protein, thus enhancing coalescence. Puppo et al. [25] reported that emulsion containing soybean protein isolate (10 mg/mL) homogenised with high pressures (0.1–600 MPa) had particle size ($d_{43}$) with the range of 0.91-1.57 µm, which was most likely higher than droplet sizes observed in this study. This was possibly due to the difference in protein concentration, protein constituents, amphiphilic character, flexibility of emulsifying molecules and

interfacial film rheology[26]. Thus, particle size of droplets in emulsions was affected by emulsifiers and pressure used for homogenisation.

## 3.2 Flocculation and Coalescence

Stability of emulsions was monitored in term of flocculation factor ($F_f$) and coalescence index ($C_i$) during 14 days of storage at room temperature as shown in Table 2. For emulsions containing SRPH, the increase in pressure for homogenisation resulted in the increase in $F_f$ of emulsion after one day of storage ($P < 0.05$). For coalescence index, the increase in pressure yielded emulsion with increased coalescence index. Homogenisation pressure presumably affected the interfacial properties of the emulsifiers used. The application of higher pressure might change the conformation of the emulsifiers at interface[27].

This could lead to lower ability to decrease the interfacial tension. Therefore, the increase in $F_f$ and $C_i$ could be noticed when higher homogenisation pressure was employed. With increasing storage time, the flocculation and coalescence increased with all samples, especially in emulsions stabilised by SRPH ($P < 0.05$). The lower $F_f$ and $C_i$ of emulsions containing sodium caseinate indicated higher stability of emulsions. The ability in adsorbing at the oil-water interface, forming matrix around oil droplet and reducing interfacial tension between particles determined emulsifying property of proteins[20]. When storage time increased, emulsions with SRPH prepared using 13.8 MPa and 27.6 MPa had the increase in $F_f$ ($P < 0.05$). However, no difference in $F_f$ was found in emulsion with 20.7 MPa with increasing storage time. Similar trend was noticeable with emulsions containing sodium caseinate. The results suggested that pressures used for homogenisation affected the stability of emulsion. The coalescence index indicated that emulsion with highest stability can be achieved by using homogenisation pressure of 13.8 MPa of both SRPH and sodium caseinate.

## 3.3 Creaming Index

Creaming indexes of emulsions containing sodium caseinate and SRPH homogenised with different pressures as a function of storage time are shown in Figure 1. Within the first 7 days, emulsions containing SRPH, prepared

**Table 2.** Flocculation, coalescence and ζ-potential of emulsions stabilised by SRPH and sodium caseinate prepared using different pressures during storage

| *Sample* | *Pressure used (MPa)* | *Storage time (days)* | *Flocculation factor ($F_f$)* | *Coalescence index ($C_i$)* | *ζ-potential (mV)* |
|---|---|---|---|---|---|
| SRPH | 13.8 | 1 | 1.71±0.04 $^{Cb}$ | - | -41.63±1.36 $^{Aa}$ |
| | | 7 | 2.30±0.02 $^{Ca}$ | 128.82±1.69$^{C}$ | -38.92±1.09 $^{Ab}$ |
| | | 14 | 2.33±0.02 $^{Ca}$ | 164.06±0.64$^{C}$ | -37.22±0.91 $^{Ac}$ |
| | 20.7 | 1 | 2.76±0.34 $^{Ba}$ | - | -40.33±1.00 $^{Aa}$ |
| | | 7 | 2.74±0.01 $^{Ba}$ | 201.05±1.19$^{B}$ | -39.51±1.00 $^{Aa}$ |
| | | 14 | 2.92±0.03 $^{Ba}$ | 257.44±2.37$^{B}$ | -37.55±0.95 $^{Ab}$ |
| | 27.6 | 1 | 3.36±0.08 $^{Ab}$ | - | -40.37±0.79 $^{Aa}$ |
| | | 7 | 3.30±0.07 $^{Ab}$ | 242.42±0.62$^{A}$ | -36.05±1.39 $^{Aa}$ |
| | | 14 | 3.89±0.07 $^{Aa}$ | 291.59±1.07$^{A}$ | -35.98±2.72 $^{Ab}$ |
| Sodium caseinate | 13.8 | 1 | 1.10±0.04 $^{Bb}$ | - | -52.70±2.04 $^{Bb}$ |
| | | 7 | 1.60±0.04 $^{Aa}$ | 2.46±0.23$^{C}$ | -51.09±2.86 $^{Aab}$ |
| | | 14 | 1.63±0.08 $^{Aa}$ | 3.35±0.18$^{B}$ | -49.03±2.45$^{Ab}$ |
| | 20.7 | 1 | 1.21±0.04 $^{Aa}$ | - | -54.02±2.82 $^{Bab}$ |
| | | 7 | 1.18±0.05 $^{Ba}$ | 3.08±0.29$^{B}$ | -51.40±0.72 $^{Ab}$ |
| | | 14 | 1.19±0.04 $^{Ba}$ | 17.48±1.76$^{A}$ | -51.20±1.33 $^{Ab}$ |
| | 27.6 | 1 | 1.12±0.02 $^{Bab}$ | - | -56.58±2.62 $^{Aa}$ |
| | | 7 | 1.07±0.06 $^{Cb}$ | 9.82±0.40$^{A}$ | -51.10±1.05 $^{Ab}$ |
| | | 14 | 1.14±0.02 $^{Ba}$ | 18.09±0.76$^{A}$ | -51.13±1.36 $^{Ab}$ |

Data are expressed as mean ± SD (n = 5).

Different lowercase superscripts in the same column within the same pressure and sample indicate significant difference (P < 0.05).

Different uppercase superscripts in the same column within the same storage time and sample indicate significant difference (P < 0.05).

using pressure of 13.8 and 20.7 MPa rendered the higher creaming index than that of 27.6 MPa. Thereafter, no difference in creaming was found in all samples after day 9 of storage. The increase in creaming was related with increased flocculation and coalescence (Table 2) indicating rapid creaming in flocculated and coarser emulsion[23]. For emulsions containing sodium caseinate, creaming increased within the first 3 days of storage. Subsequently, no further change in creaming index was observed until the end of storage. This was in agreement with the smaller mean droplet diameter (Table 1) and lower $F_f$ and $C_i$ (Table 2). Creaming is an undesirable phenomenon, in which buoyant emulsion droplets form at the top of emulsion[23]. Since the separation of cream phase indicates the instability, an appropriated pressure for emulsification was required. The lower creaming rate of emulsion prepared by sodium caseinate could be explained by Stokes' law, where the decrease of average size of oil droplet contributes to the reduction of creaming velocity[28].

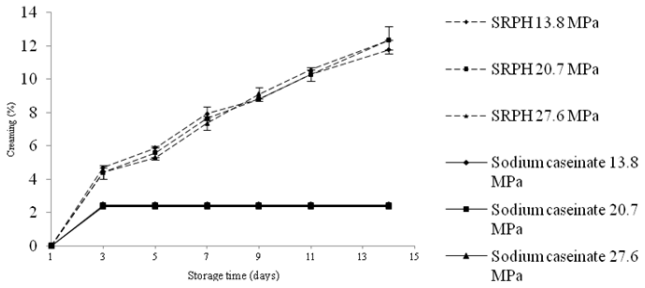



**Figure 1.** Change of creaming of emulsions stabilised by SRPH and sodium caseinate prepared using different pressures during 14 days of storage. Bars represent the standard deviation (n = 3).

## 3.4 ζ-potential

ζ-potential of emulsions containing SRPH and sodium caseinate as affected by homogenisation pressures is shown in Table 2. At the first day of storage, emulsion samples had ζ-potential values lower than -40 mV. Emulsion stabilised by sodium caseinate had ζ-potential values lower than -50 mV. Negatively charged residues on oil droplet mostly contributed to repulsion between droplets, thereby lowering coalescence. ζ-potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed droplet[29]. Emulsions exhibiting absolute ζ-potential higher than +30 mV or lower than −30 mV tend to be electrostatically stable, whilst emulsions within the range of (-30) - 30 mV tend to coagulate or flocculate[29]. It was noted that emulsion stabilised with sodium caseinate having higher ζ-potential showed higher stability. With increasing storage time, ζ-potential of all samples became decreased, especially emulsions containing SRPH. During the extended storage, the layers of protein surrounding droplets might undergo aggregation via ionic interaction as indicated by the change in ζ-potential. The insufficient electrostatic repulsion might lead to the development of flocculation and coalescence, particularly with extended storage times. Particle size of the resulting emulsions and the stability of emulsion were governed by ζ-potential surrounding droplets, which was more likely associated with the charge of proteins at interface.

## 3.5 CLSM Micrograph

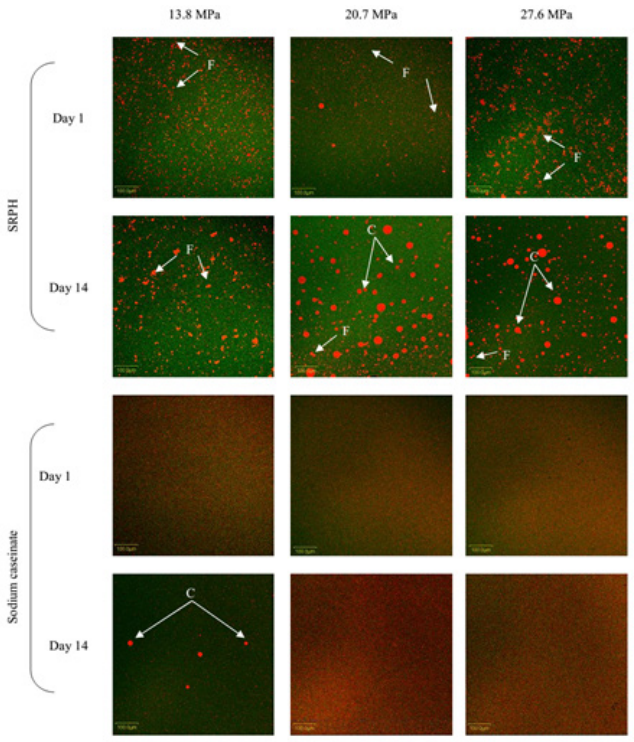



**Figure 2.** Droplet distribution of emulsions stabilised by SRPH and sodium caseinate prepared using various pressure levels. Magnification; 200x. Red and green represent lipid and protein, respectively. F; flocculated droplet, C; coalesced droplet.

Particle distribution of emulsions stabilised by SRPH or sodium caseinate, prepared with different

homogenisation pressures was visualised by CLSM (Figure 2). At the first day of storage, CLSM images depicted the particulate clusters or clumps of oil droplets (red color) in SRPH stabilised emulsions. It was noted that the smallest droplet size was found in emulsion prepared with 20.7 MPa, compared with those observed in emulsions with pressures of 13.8 and 27.6 MPa. Level of pressure applied for emulsification of sunflower oil emulsion affected distribution and size of oil droplets[4]. However, no flocs were noticeable in emulsions stabilised by sodium caseinate, regardless of pressures applied. At day 14 of storage, it was found that flocculation and coalescence took place in SRPH stabilised emulsions, particularly samples with 20.7 and 27.6 MPa. No flocculation and coalescence were observed in emulsion stabilised by sodium caseinate. Disruption of emulsion indicated by CLSM images was in accordance with the increases in $d_{32}$, $d_{43}$ (Table 1), $F_f$ and Ci (Table 2), when storage time increased. This reflected the higher stability of emulsion containing sodium caseinate as an emulsifier.

## 3.6 Interfacial Protein Concentration

Interfacial protein concentration ($\Gamma$, **mg/m²**) of emulsion stabilised by SRPH and sodium caseinate using different homogenisation pressures is presented in Table 3. Interfacial protein or adsorbed protein at interfacial area reflects the adsorption characteristic of SRPH and sodium caseinate at oil droplet interface. Generally, the employment of high pressure homogenisation can dissociate oil droplets into small particles with the increased surface area. Thus, higher amount of protein load per surface area is required for coating the newly created interface[20]. Concentration of protein at interface was influenced by surface area and size ($d_{32}$) of the oil droplets. High $\Gamma$-values of emulsions with the decreased oil surface area were coincidental with the increased $d_{32}$. The decreased $\Gamma$-values could be observed for emulsions having small $d_{32}$ (increased oil surface area). The rearrangement of protein or peptide layer might be thinner and probably approaching a monolayer[30,31]. The increase in $\Gamma$-values of the emulsions stabilised by SRPH was found with increasing homogenisation pressure and storage time. The increases in $\Gamma$-values during storage were related with increasing coalescence (Table 2). Slightly decreased $\Gamma$-values were observed for emulsions stabilised by sodium caseinate. The difference in interfacial protein level might be governed by different molecular structure, size and rearrangement of adsorbed protein molecules at the interface[32]. The higher $\Gamma$-values of emulsions containing SRPH, compared with those containing sodium caseinate, might be due to the fact that shorter peptides of SRPH could not reorient at the interface in the way that stabilised emulsions effectively. Consequently, coalescence could occur and the lower surface of oil droplet was obtained as indicated by increased $d_{32}$ (Table 1). The protein concentration at the oil-water interface was the crucial factor in the stability of the emulsions because proteins can lower the interfacial tension at the oil-water interface of the droplets. High pressure homogenisation may induce protein association or aggregation between protein in the aqueous phase and the protein that previously formed monomolecular layer at interface[28]. For SRPH stabilised emulsions, high pressure above 13.8MPa may induce the peptide aggregation, as indicated by lowered ζ-potential value. Thus, the decrease in emulsion stability was observed.

**Table 3.** Interfacial protein concentration of emulsions stabilised by SRPH and sodium caseinate prepared using different pressures during storage

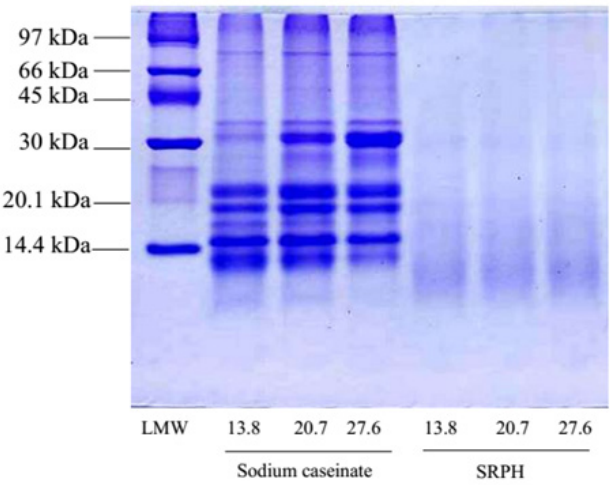
| Sample | Pressure used (MPa) | Storage time (days) | Interfacial protein concentration (mg/m²) |
|---|---|---|---|
| SRPH | 13.8 | 1 | 1.38±0.07 $^{Ca}$ |
| | | 7 | 1.83±0.03 $^{Ba}$ |
| | | 14 | 1.77±0.31 $^{Ba}$ |
| | 20.7 | 1 | 1.59±0.04 $^{Bb}$ |
| | | 7 | 2.43±0.01 $^{Aa}$ |
| | | 14 | 2.54±0.13 $^{Aa}$ |
| | 27.6 | 1 | 1.75±0.10 $^{Ab}$ |
| | | 7 | 1.83±0.01 $^{Bb}$ |
| | | 14 | 2.21±0.18 $^{ABa}$ |
| Sodium caseinate | 13.8 | 1 | 0.31±0.01 $^{Ac}$ |
| | | 7 | 0.46±0.00 $^{Aa}$ |
| | | 14 | 0.35±0. 01 $^{Ab}$ |
| | 20.7 | 1 | 0.27±0.00 $^{Ba}$ |
| | | 7 | 0.26±0.01 $^{Bb}$ |
| | | 14 | 0.20±0.00 $^{Bc}$ |
| | 27.6 | 1 | 0.16±0.01 $^{Cc}$ |
| | | 7 | 0.23±0.00 $^{Cb}$ |
| | | 14 | 0.26±0.00 $^{Ca}$ |

Data are expressed as mean ± SD (n = 3).
Different lowercase superscripts in the same column within the same pressure and sample indicate significant difference ($P < 0.05$).
Different uppercase superscripts in the same column within the same storage time and sample indicate significant difference ($P < 0.05$).

### 3.7 SDS-PAGE

Patterns of proteins and interfacial proteins under non-reducing condition are shown in Figure 3A and Figure 3B, respectively. In general, sodium caseinate contains four major proteins including $\alpha_{s1}$-casein (~23 kDa), $\alpha_{s2}$-casein (~25 kDa), $\beta$-casein (~29 kDa) and κ-casein (~19 kDa), whilst, SRPH had major peptides with MW of 5.5 and 57 kDa[10]. From Figure 3A, the absence of protein with MW of 57 kDA in SRPH with 5% DH was observed. Protein with MW of 57 kDa might be stabilised via ionic interiaction or hydrophobic effect, leading to dissociation of this protein in the presence of SDS. Protein and interfacial protein in emulsion stabilised by SRPH and sodium caseinate were slightly different. Higher band intensity of protein with MW of 30 kDa increased with increasing homogenisation pressure (Figure 3A). High pressure induced the rupture of non-covalent interactions between protein molecules, followed by the reformation of intra - and inter-molecular bonds within or between protein molecules[2] via disulphide linkage[33]. Coincidentally, proteins with MW less than 14 kDa decreased. With higher pressure, shearing became pronounced. As a result, those proteins might undergo cross-link via disulphide bond.

For interfacial protein composition, proteins with MW of 30 kDa showed the slight decrease in intensity when the higher pressure was applied for homogenisation (Figure 3B). Cross-linked protein plausibly migrated to the interface more slowly, compared with the smaller protein. Therefore, proteins especially at interface were influenced by pressure applied. This affected the localisation of protein and stiffness of protein films at interface.




(a)

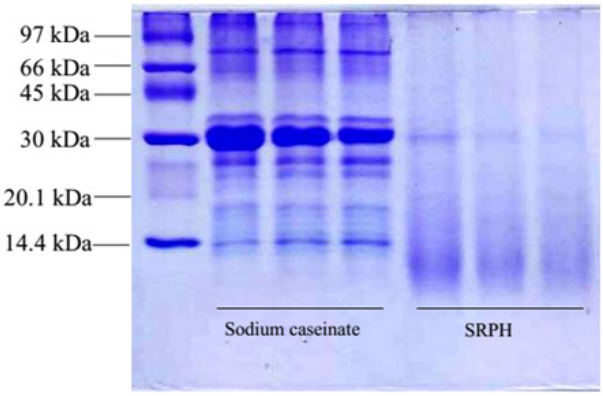



**Figure 3.** Electrophoretic patterns of total protein **(a)** and interfacial protein **(b)** of sodium caseinate and Skipjack Roe Protein Hydrolysate (SRPH) containing emulsions prepared using different pressures. Samples were loaded onto 15% running gel and 4% stacking gel in the absence of β-mercaptoethanol. LMW: Low Molecular Weight marker.

## 4. Conclusion

Stability of emulsion correlated with the homogenisation pressure. Higher homogenisation pressure reduced droplet size but decreased amount of adsorbed proteins. Emulsification at 13.8MPa could provide the highest stability of SRPH containing emulsion during 14 days of storage.

## 5. Acknowledgement

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