Interfacial and Emulsifying Properties of Soybean Peptides with Different Degrees of Hydrolysis

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Abstract: In this study, the effects of the degree of hydrolysis on the interfacial and emulsifying properties of soybean peptides were evaluated based on surface and interfacial tension, dynamic light scattering (DLS), and freeze-fracture transmission electron microscopy (FF-TEM) analyses. Of the five evaluated soybean peptides (SP95, SP87, SP75, SP49, and SP23), those with higher degrees of hydrolysis (SP95 and SP87) did not exhibit noticeable surface-active properties in water, whereas those with relatively low degrees of hydrolysis (SP75, SP49, and SP23) exhibited remarkable surface tension-lowering activity. The latter set (SP75, SP49, and SP23) also formed giant associates with average sizes ranging from 64.5 nm to 82.6 nm above their critical association concentration (CAC). Moreover, SP23 with the lowest degree of hydrolysis exhibited excellent emulsifying activity for soybean oil, and FF-TEM analysis demonstrated that the emulsions were stabilized by a lamella-like multilayer peptide structure on the oil droplets that prevented coagulation. The peptide with the lowest degree of hydrolysis (SP23) was effective not only for soybean oil emulsification, but also for the emulsification of liquid paraffin and silicon oil that are generally difficult to emulsify.

Key words: soybean peptide, surface tension, association, emulsification, liquid paraffin, silicon oil

1 INTRODUCTION

Peptides are naturally occurring biologically active compounds¹ that have recently gained prominence as remarkable building blocks of bio-based materials²–⁴. Peptides are generally produced via enzymatic hydrolysis of various proteins, or are synthesized by the stepwise linkage of α-amino acids with coupling reagents. Other interesting features of peptides include their molecular interactions, such as hydrogen bonds, van der Waals forces, electrostatic, aromatic(π–π stacking), hydrophobic and hydrophilic interactions⁵. The synthesis of surfactant-like peptides that mimic an amphiphilic helical unit of apolipoprotein A-I (apoA-I) was successfully executed by the present authors⁶. These peptides exhibit rather high surface activity as well as solubilizing ability toward lipids, and their amphiphilicity is derived from the localization of hydrophobic and hydrophilic amino acids in their α-helical structures. It was also found that another surfactant-like peptide produced from Bacillus subtilis, so called ”surfactin", exhibits excellent surface-active properties resulting from hydrogen bonds between molecules⁷,⁸).

These observations clearly demonstrate that peptides themselves are potentially useful as natural surface-active or emulsifying agents. For practical applications of functional peptides in a wide variety of fields, peptides produced from enzymatic or chemical hydrolysis of natural-abundance proteins are considered more suitable.

Among the various peptides obtained by protein hydrolysis, soybean peptides offer the distinguishing advantages of being derived from an abundant and relatively inexpensive source of soybean proteins, and also exhibit various biological activities such as antihypertensive, anticholesterolemic, antioxidative, and anticancer activities⁹. Soy peptides obtained by hydrolysis also reportedly exhibit better emulsifying properties than that of native proteins¹⁰. Although improvement of the emulsifying properties of proteins via hydrolysis has been reported for proteins including casein¹¹, whey¹², and wheat protein¹³, little is known about the detailed interfacial and emulsifying properties of amphiphilic peptides themselves, which is a hindrance to
the widespread application of peptides.

In this study, the effect of the degree of hydrolysis of soybean peptides on their interfacial and emulsifying properties is evaluated via surface and interfacial tension, dynamic light scattering (DLS), and freeze-fracture transmission electron microscopy (FF-TEM) analyses.

2 EXPERIMENTAL

2.1 Materials

Five kinds of soybean peptides, i.e., SP95, SP87, SP75, SP49, and SP23 (high Newt AM, S, D1, DL, and DH) with different degrees of enzymatic hydrolysis of soybean proteins and native protein (New Fujipro E) were kindly supplied by Fuji Oil Co., Ltd., and used without further purification. The 15% trichloroacetic acid (TCA) solubilization rates of the peptides provided by the supplier are shown in Table 1. The 15% TCA solubilization rate indicates the percentage of peptides solubilized in 15% TCA solution, where peptides with lower solubilization rates are deemed to have a lower degree of hydrolysis\(^{14-16}\). The RP-HPLC retention times of the peptides with higher degrees of hydrolysis were confirmed to be shorter, indicating that these peptides are more hydrophilic (data not shown). Sucrose oleate (O-1570) provided by Mitsubishi-Kagaku Foods Co. was also used as a control.

Ultrapure water was used to prepare the aqueous peptide solutions, and soybean oil (Waco Co., Ltd.), liquid paraffin (Waco Co., Ltd.), and silicon oil (KF-96A-6cs Shin-Etsu Chemical Co., Ltd.) were used to produce emulsions. After adding 4 mL of oil to 1 mL aqueous peptide solution in a test tube, the emulsion was then prepared by vortexing for 5 min.

2.2 Surface and interfacial tension measurements

The surface tension of the aqueous peptide solutions was determined by the Wilhelmy plate method at 25°C using a DY-500 surface tension meter (Kyowa Kaimen Kagaku Co., Saitama, Japan), the accuracy of which was intermittently checked with ultra-pure water. The Pt plate was cleaned by flaming, and glassware was rinsed sequentially with tap water and ultra-pure water.

The interfacial tension of the oil at the water and soybean oil interface was determined at 25°C by the pendant drop method, which was performed using an automatic interfacial tensiometer (DM500, Kyowa Interface Science) in conjunction with the Drop Shape Analysis software of FAMAS ver. 2.01. A water drop was formed in soybean oil at the tip of a syringe by pressing the solution out by means of a setscrew. The drop shape analysis was performed as follows: a drop profile was extracted from the drop image; a curve-fitting program was then used to compare the experimental drop profile with a theoretical profile (the Young-Laplace method) to derive the corresponding interfacial tension value. The evolution of the drop surface tension was followed over 2 min.

2.3 Light scattering measurements

Scattering intensity measurements of the aqueous peptide solutions, which were passed through a polyvinylidene difluoride (PVDF) membrane filter (0.45 μm), were performed at 25°C with a DLS-7000 (Otsuka Electronics Co., Japan) instrument using a He-Ne laser (wavelength: 633 nm) at a scattering angle of 90°.

The size of the peptide assemblies was then evaluated via dynamic light scattering (DLS). The time-dependent correlation function of the scattered light intensity was measured at a scattering angle of 90°. The DLS intensity data were processed by using the instrumental software to obtain the hydrodynamic diameter, the polydispersity index, and the mass diffusion coefficient of the samples. The mass diffusion coefficient, \(D_m\), was derived from the decay time (\(\tau\)) of the intensity correlation function as \(D = \left(2\kappa_0\tau\right)^{-1}\), where \(k_0\) is the scattering wave vector. The hydrodynamic mass diffusion coefficient, \(D_m\), is obtained as the limit of \(D\) as \(k_0\) goes to zero. \(D_0\) is found to obey the Stokes-Einstein relation, \(D_0 = kT/6\pi\eta R_H\), where \(k\) is the Boltzmann constant, \(T\) is the absolute temperature, \(\eta\) is the viscosity of the solvent, and \(R_H\) is the hydrodynamic radius\(^{17}\).

2.4 Emulsifying properties

The emulsifying properties of soybean peptides were also examined as follows: 4 mL of soybean oil was added to 1 mL of each aqueous peptide solution (10 mg/mL) having a different degree of hydrolysis, and the mixture was emulsified by vortexing for 5 min. Soybean protein without hydrolysis treatment and sucrose ester were used as controls.

2.5 Freeze-Fracture Transmission Electron Microscopy (FF-TEM)

Freeze-fracture transmission electron microscopy (FF-TEM) was used to determine the structure of the assemblies. Select samples were frozen with liquid nitrogen at \(-189°C\). The fracture process was performed at

| Soybean peptide | SP 95 | SP 87 | SP 75 | SP 49 | SP 23 |
|-----------------|-------|-------|-------|-------|-------|
| 15% TCA solubilization rate (%) | 95.0  | 86.5  | 75.0  | 49.1  | 23.2  |

Table 1 15% TCA solubilization rate of soybean peptide.
−130°C with a JFD-9010 (JOEL, Japan) instrument, and the fractured surface was then replicated by evaporating platinum at an angle of 60°, followed by carbon at an angle of 90° to strengthen the replica. The replicate was placed on a 400 mesh copper grid subsequent to washing with water, methanol, and chloroform. The replicate was then examined and photographed using a JEM-1010 (JOEL, Japan) transmission electron microscope.

3 RESULTS AND DISCUSSION
3.1 Interfacial properties of peptides

Because the surface tension-lowering ability is the most fundamental property of amphiphilic molecules in water, the surface tension of the aqueous solutions of peptides with varying degrees of hydrolysis of the soybean proteins was first measured.

Among the five soybean peptides (SP95, SP87, SP75, SP49, and SP23) with different degrees of hydrolysis, SP95 and SP87 having higher degrees of hydrolysis, as indicated by the higher 15% TCA solubilization rate, did not exhibit noticeable surface-active properties, whereas SP75, SP49, and SP23 exhibited remarkable tension-lowering activity. LC-MS measurements revealed that SP95, with a high degree of hydrolysis, primarily comprised di- or tri-peptides. The surface tension measurements suggest that shorter peptides with 15% TCA solubilization rates more than 87% are not sufficiently long to exhibit surface activity.

Figure 1 shows the surface tension of aqueous solutions of the peptides (SP75, SP49, and SP23) with relatively low degrees of hydrolysis as a function of concentration. For all three peptides, the surface tension decreased gradually due to adsorption of the peptide at the air/water interface, and became constant at a certain concentration where the adsorption equilibrium was reached. The intersection point of the two fitted lines is defined as the critical micelle concentration (CMC) for general surfactants, above which surfactants begin to self-assemble spontaneously into micelles.

The estimated critical association concentration (CAC) and $\gamma_{\text{CAC}}$ values were respectively 0.3 mg/mL and 45.5 mN/m for SP75, 4.9 mg/mL and 41.9 mN/m for SP49, and 1.1 mg/mL and 44.9 mN/m for SP23. Although the obtained $\gamma_{\text{CAC}}$ values were not as low as that of the typical synthetic surfactant sodium dodecyl sulfate (SDS, $\gamma_{\text{CAC}} = 39.5$ mN/m), the values indicate that micelle-like associates are possibly formed above the CAC, even for the peptides obtained by protein hydrolysis.

To confirm the formation of micelle-like associates, light scattering intensity measurements were performed for the peptide solutions. Figure 1 (right axis) shows a plot of the relative scattering intensity and peptide concentration for SP75, SP49, and SP23. The scattering intensity for all peptide solutions increased sharply above the concentration of the intersection point observed from the surface tension measurements, indicating onset of self-association of the peptides, similar to the behavior of surfactant micelles at their critical micelle concentration.

DLS measurements were also performed to elucidate the size of the peptide associates. Figure 2 shows the size distribution of the peptide associates (SP75, SP49, and SP23) at a concentration of 10 mg/mL, which is above the CAC of the peptides. From the figure, the peptide associates were found to have a relatively narrow size distribution, and the average diameters of the associates were $64.5 \pm 25.8$ nm.
for SP75, 75.1 ± 31.7 nm for SP49, and 82.6 ± 33.9 nm for SP23. In general, the diameter of a micelle is several nanometers, and is twice its molecular length. Unlike general surfactants, the peptides are found to generate huge associates above the CAC by reaching their adsorption equilibrium on the surface.

3.2 Emulsifying properties of peptides

It is known that one important role of surfactants is their ability to solubilize or emulsify hydrophobic compounds in water by forming micelles above their CMC. This implies that amphiphilic peptides may also emulsify hydrophobic compounds, especially above their CAC.

The emulsifying properties of the soybean peptides were then examined; soybean protein and sucrose ester were used as controls. Figure 3 shows the visual appearance of the samples 3 days after preparation. The figure clearly indicates that SP23 with the lowest degree of hydrolysis exhibited excellent emulsifying activity, and the emulsion was more stable than that of soybean protein or even sucrose oleate as shown in Fig. 3. SP75 and SP49 were also effective for emulsification of soybean oil just after preparation, but the emulsion was not as stable as that of SP23. We then examined the stability of the emulsions prepared with SP23 below and above its CAC. Figure 4 shows the visual observation of the emulsions prepared with SP23 below (0.5 mg/mL) and above (12 mg/mL) its CAC 3 days after preparation. The figures indicate that the emulsifying ability of SP23 was markedly improved above its CAC, and the critical point of SP23 was achieved around 1.0 mg/mL, and SP23 effectively reduced the interfacial tension between soybean oil and water up to 8.1 mN/m. Chan et al. reported that the stability of emulsions prepared with hydrolyzed peptides depends on their globular structure in aqueous solution. However, the present DLS analysis indicated the similarity of the peptide globular aggregates of SP23, SP75, and SP75 in aqueous solution. Therefore, the superior emulsification ability of SP23 might be derived not from the globular structure in solution but from the peptide layer structure at the interface after adsorption.

Figure 5(a) shows the differential interference contrast (DIC) image of the emulsion composed of soybean oil and water stabilized by SP23. Large, spherical, oily droplets, with dimensions of several hundred micrometers, dispersed in water were observed. The type of emulsion (oil in water) was confirmed using Nile red as a dye. The detailed structure of the oil droplet surface was further explored by FF-TEM as shown in Fig. 5(b). Interestingly, a multilayer peptide structure that is structurally similar to the surfactant lamella structure was clearly observed on the surface of the droplets, indicating that this distinctive lamella-like structure stabilizes the emulsion.
Soybean peptide acts as an excellent emulsifier

Improvement of the emulsion stability by enzymatic hydrolysis of proteins has already been reported for abundant proteins such as soy, casein, and wheat protein, where the peptides were considered to stabilize the emulsions by promoting electrostatic repulsion or steric hindrance between emulsion droplets. In addition to these effects, the lamella-like peptide multilayer structures observed via FF-TEM are very likely to stabilize the emulsions because surfactant liquid crystals such as lamella formed at the oil/water interface are known to drastically improve the stability of emulsions by preventing droplet coalescence. Although the detailed peptide structure at the interface should be confirmed by small angle X-ray scattering (SAXS) for further study, the advantage of emulsions stabilized by liquid crystals is that they can be extended either

Fig. 3 Visual observation of emulsions at 3 days after preparation
(a) SP95 (b) SP87 (c) SP75 (d) SP49 (e) SP23. (f) Soybean protein (g) Sucrose ester.

Fig. 4 Visual observation of emulsions prepared with SP23 at 3 days after preparation.
(a) below CAC (0.5 mg/mL) (b) above CAC (12 mg/mL).

Fig. 5 (a) Optical micrograph and (b) freeze-fracture transmission electron micrograph of emulsion prepared with SP23.
hydrophobic or hydrophilic compounds.

Attempts were made to emulsify other oils such as liquid paraffin and silicon oil that are generally difficult to emulsify. **Figure 6** shows the visual appearance of the emulsions with liquid paraffin (a) and silicon oil (b) 3 days after preparation using SP23 as an emulsifier. The figure clearly indicated that soybean peptide with a low degree of hydrolysis (SP23) was effective for emulsification of not only soybean oil but also liquid paraffin and silicon oil. The obtained emulsions were stable for at least 2 weeks. This partially supports the postulate that the lamella-like peptide multilayer structures contribute to the stability of the emulsions.

The present results clearly demonstrate that an understanding of the surface and self-association behavior of peptides themselves is critical for the practical application of these species, particularly as emulsifiers.

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**4 CONCLUSION**

The effects of the degree of hydrolysis on the interfacial and emulsifying properties of soybean peptides were evaluated herein. Evaluation of five soybean peptides (SP95, SP87, SP75, SP49, and SP23) demonstrated that SP95 and SP87 having higher degrees of hydrolysis did not exhibit noticeable surface-active properties, whereas SP75, SP49, and SP23 with relatively low degrees of hydrolysis exhibited remarkable surface tension-lowering abilities for water.

Moreover, SP23 with the lowest degree of hydrolysis exhibited excellent emulsifying ability for soybean oil, and FF-TEM analysis demonstrated that a lamella-like multilayer peptide structure on the oil droplets stabilizes the emulsions by preventing coagulation. It was also found that SP23 with the lowest degree of hydrolysis could emulsify liquid paraffin and silicon oil as well as soybean oil.

These results clearly indicate that an understanding of the surface and self-association behavior of peptides themselves is crucial to the practical application of these species, particularly as emulsifiers.

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