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# Porphyran as a Functional Modifier of a Soybean Protein Isolate through Conjugation by the Maillard Reaction

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Porphyran (Por) prepared from dried nori was applied as a functional modifier of a soybean protein isolate (SPI) to conjugate with SPI from defatted soybean by the Maillard reaction (79% relative humidity and 60 °C for 7 days). Two kinds of partially denatured conjugate (Conj 45 and Conj 63) were obtained from the reaction product by sequential extraction at pH 4.5 and pH 6.3, and the respective yield and weight ratios of the SPI and Por moieties were 8.4% and 1:1 for Conj 45 and 11.7% and 1:0.16 for Conj 63. Conj 63 demonstrated improved solubility between pH 5.0 and pH 8.0, while Conj 45 exhibited substantially complete solubility over the pH range of 2.0−8.0. Conj 63 showed more tolerance against digestion with pancreatin than SPI, whereas this was lost after denaturation. Conj 63 and Conj 45 both showed a markedly higher emulsion activity index and emulsion stability than SPI, even at pH 3.0; in particular, Conj 45 exhibited outstanding emulsifying ability. Conj 63 had about a two-fold higher calcium-binding ability than SPI, and Conj 63 and Conj 45 did not aggregate with added $Ca^{2+}$ and $Mg^{2+}$. It is believed that Por could be a valuable functional modifier of SPI for providing soybean protein-based liquid foods such as beverages by conjugation through the Maillard reaction.

**KEYWORDS: Porphyran; soybean protein isolate; Maillard reaction**

## INTRODUCTION

Porphyran (Por) is known as a sulfated polysaccharide originating from the cell wall and intercellular regions of the raw laver of *Porphyra yezoensis*, a red alga, and dried nori from *Porphyra* is widely consumed as a familiar-tasting and low-energy daily food rich in dietary fiber in Japan. Por has a basic structure of altered 1,4-linked 3,6-anhydrogalactose units and 1,3-linked β-D-galactose units sometimes, respectively, occurring as the L-galactose-6-sulfate and 6-*O*-methyl derivatives (*1*, *2*). It has recently been reported that Por has such physiological functions as improving the microflora and environment of the cecum (*3*), antitumor activity (*4*), antihypertensive and anti-hyperlipidemic activities (*5*), and macrophage stimulation activity (*6*). We have previously reported that Por prepared from dried nori exhibited good emulsifying ability in terms of a high emulsifying activity index (EAI), emulsion stability (ES), and particle size distribution of the oil droplets over a wide range of pH and temperature and also in the presence of sodium chloride (*7*) and demonstrated that the outstanding emulsifying ability was caused by binding to the hydrophobic surface of an oil droplet, probably through 3,6-anhydrogalactose, and that Por could be effectively applied to stabilize liposomes (*8*). However, Por has not received more general applications. It would therefore be desirable to investigate new and useful applications based on such properties as its high solubility, low viscosity, good emulsifying ability, and charge effects of sulfate groups. We have previously established a method for conjugating proteinaceous materials such as β-lactoglobulin, poly(L-lysine), and amino acids with several such saccharides as alginic acid oligosaccharide (*9*), chitopentaose (*10*), and starches (*11*, *12*) by the Maillard reaction. This led to marked improvements in various physical and physiological properties, including a decreased immunogenicity, indicating that Por could be a valuable and a novel functional modifier of proteinaceous materials.

Soybean protein is widely used as a valuable proteinaceous material for various food products because of such functional properties as its high nutritional value, physiological functions like its antioxidative ability (*13*), and decreasing effects on the serum cholesterol level (*14*) and blood pressure (*15*) and physical functions such as its gel-forming ability explained by a mechanism involving the strand model of glycinin (*16*) and its low cost. However, soybean protein has the major limitation that its solubility drastically decreases in the acidic pH region, resulting in heavy opaqueness and easy precipitation, which in turn resulted in a loss of desirable physical properties. Several

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studies have recently been carried out to improve the physical properties of soybean protein by dry heating with glucose (*17*), dextran (*18*−*20*), and carboxymethyl cellulose (*21*), and these studies show that the Maillard reaction products of glycinin or a soybean protein isolate (SPI) with these saccharides has improved foaming ability, emulsifying ability, and stability against the heat-induced aggregation of an emulsion. However, substantially complete solubility, even in the acidic region including the isoelectric point of soybean protein, could not be achieved in these studies. Conjugation between a soybean protein hydrolysate and curdlan by the Maillard reaction also improved the emulsifying ability, gel-forming ability, and antioxidative activity (*22*). However, conjugation with curdlan is thought to not be beneficial for improving the solubility, because curdlan readily forms a gel from its dissolution state. It is generally known that soybean protein easily aggregates with such divalent cations as magnesium and calcium ions and that this unique behavior is valuable for producing a curd food (tofu). However, from the aspect of providing stable liquid types of foodlike beverages, this aggregation is a major defect; therefore, it might be desirable for soybean protein to be endowed with a proper saccharide that could inhibit such molecular assembly. In addition, because current attention demands greater bioavailability of dietary divalent cations like the calcium ion, it might also be desirable for soybean protein to be enhanced with a certain substance that could form a soluble complex with the divalent cation. However, conjugation with a neutral polysaccharide is not the answer, because it does not provide a binding site for divalent cations. It is thus believed that conjugating soybean protein with highly soluble and charged saccharides would enable these improvements to be achieved. This study, therefore, investigates the availability of Por as a functional modifier of SPI through conjugation by the Maillard reaction to improve the solubility, emulsifying ability, divalent cation-binding ability, and aggregation behavior of soybean protein.

## MATERIALS AND METHODS

**Materials.** Por was prepared according to the previous method (*9*) with some modifications. In brief, dried nori (80 g), which had been obtained by processing raw laver, *P. yezoensis*, a red alga cultivated in the Ariake Sea in Kyushu, Japan, was pulverized with a homestyle mixer, immersed in 1600 mL of distilled water, and autoclaved at 121 °C for 60 min. The centrifuged supernatant (about 8000*g* for 30 min) was passed through a G4 glass filter covered with granular activated charcoal. The filtrate was fractionated with 67% ethanol and by adding 0.1 g of $CaCl_2$ as a precipitation-promoting agent. After leaving the mixture overnight at room temperature, the precipitate was recovered by decantation and dehydrated with 99.5% ethanol and acetone to obtain crude Por by air drying. This crude Por (5 g) was dissolved in 50 mL of distilled water and then thoroughly dialyzed against distilled water. After a four-fold amount of ethanol was added and the mixture was cooled in an ice bath for 3 h, the centrifuged supernatant (about 10000*g* for 30 min) was fractionated with 80% ethanol to obtain the Por preparation by dehydration with 99.5% ethanol and acetone and subsequent air drying.

SPI was prepared by the method previously described (*23*). In brief, commercial defatted soybean flour (1 kg of Soya flour A; Nisshin Oillio Ltd., Tokyo, Japan) prepared by extraction with *n*-hexane at 60 °C was dispersed in 5 L of water at pH 8.0 (this was adjusted with 0.1 M NaOH) while stirring for 3 h at room temperature, and the dispersion was centrifuged at about 12000*g* for 30 min. The resulting supernatant was adjusted to pH 4.5 with 1 M HCl to form a precipitate. After this dissolution and precipitation process was conducted three times, SPI was obtained by spray drying the final solution (160 °C inlet temperature and 80 °C outlet temperature). The other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

**Preparation of the SPI-Por Conjugate.** SPI (40 g) was dissolved in 2 L of distilled water while the pH was adjusted to 8.0 with 1 M NaOH. Por (7 g) was dissolved in 500 mL of water and adjusted to pH 8.0 with 1 M NaOH. The SPI and Por solutions were mixed and then frozen quickly with liquid nitrogen. After lyophilization, the dried mixture was incubated at a relative humidity (RH) of 79% and 60 °C for 7 days. The reaction products were extracted in 2 L of distilled water (while the pH was adjusted to 4.5 with 1 M HCl) by stirring at room temperature for 15 h, and the suspension was centrifuged at about 10000*g* for 30 min. The resulting supernatant was dialyzed against distilled water and lyophilized to recover an SPI−Por conjugate (Conj 45). The precipitate at pH 4.5 was extracted with 2 L of distilled water (while the pH was adjusted to 6.3 with 1 M NaOH), and the resulting suspension was centrifuged at about 10000*g* for 30 min, dialyzed against distilled water, and lyophilized to recover a second SPI−Por conjugate (Conj 63). The insoluble fraction at pH 6.3 was dialyzed against distilled water and then lyophilized to recover insoluble matter (Ppt 63).

**Differential Scanning Calorimetry (DSC).** Each SPI−Por conjugate was suspended in a 0.1 M Tris-HCl buffer (pH 8.0) to give a 10% concentration, and 55 mg of the suspension was taken into a 70 $\mu$L silver DSC capsule. DSC was performed from 5 to 115 °C at a heating rate of 2 K/min by a SSC-5020 DSC-6100 apparatus (SII Nano-Technologies, Tokyo, Japan) as described by Takahashi et al. (*24*). After DSC, the lid of the capsule was removed with a clean nipper. The opened capsule and contents were dried at 110 °C for 4 h and then incinerated at 550 °C at 5 h. The sample weight in the capsule was determined from the reduced weight after incineration (*25*). This determination was performed three times.

**Determination of the Relative Solubility.** A sample was dispersed to give a 0.5% concentration in distilled water while the pH was adjusted to 8.0 with 0.1 N NaOH. The suspension was passed through a 0.45 $\mu$m membrane filter (Advantec, Tokyo, Japan), and then, the resulting filtrate was diluted to give a 0.2% solution. Aliquots (0.5 mL each) of the diluted solution were taken into centrifuge tubes and adjusted to the pH range of 2.0−8.0 in pH 1.0 increments with 0.125−1.0 M HCl. Each pH-adjusted sample was centrifuged at about 35000*g* for 10 min before the protein concentration of the supernatant was determined. A 1 N concentration of NaOH (50 $\mu$L) was added to 0.1 mL of the supernatant and then diluted 10 times with distilled water to measure the absorbance at 280 nm; SPI was used as the standard. The solubility is expressed as the ratio of the protein concentration at each pH value to that at pH 8.0. This measurement was performed three times. The relative solubility of the sample heated at 100 °C for 30 min was also evaluated in a similar manner.

**Determination of the Digestibility with Pancreatin.** The digestibility with protease of each SPI−Por conjugate was assessed with pancreatin (Sigma, St. Louis, MO). SPI and Conj 63 (35 mg, based on the protein weight) were each dissolved in 7 mL of a 0.1 M Tris-HCl buffer (pH 8.0). A 0.1% (w/v) pancreatin solution (200 $\mu$L) was added to 400 $\mu$L of the sample solution, and the reaction mixture was incubated at 40 °C for 1−24 h. After this incubation, the enzymatic reaction was stopped by heating in boiling water for 10 min prior to lyophilization to evaluate the progress of digestion by sodium dodecyl sulfate−polyacrylamide gele electrophoresis (SDS-PAGE). In addition, the sample predenatured by heating in boiling water for 10 min was also digested in a similar manner. The digestibility with pancreatin was estimated by the relative ratio of the staining strength of the SDS-PAGE pattern for each digested sample to that for the undigested sample. The experiment was performed three times.

**Measurement of the Aggregation with a Divalent Cation.** The aggregation behavior of the conjugates was evaluated according to the previous method (*26*) with some modification. In brief, the SPI and the SPI−Por conjugates were each dissolved in distilled water while the pH was adjusted to 8.0 with 1 M NaOH and was stirred to give about a 0.1% concentration. After it was passed through a 0.45 $\mu$m membrane filter (Advantec, Tokyo, Japan), the solution was diluted with distilled water adjusted to pH 8.0 with 1 M NaOH to give a concentration of 0.075% based on the protein weight. Several increments (10 or 20 $\mu$L at a time) of 0.1 M $MgCl_2$ or $CaCl_2$ adjusted to pH 8.0 with 0.1 M NaOH were added to 2 mL of the sample solution in a spectrophotometric cuvette while gently stirring, and the aggregation

was evaluated by measured the absorbance at 350 nm. This measurement was performed three times.

**Measurement of the Emulsifying Ability.** Each SPI−Por conjugate and SPI were dissolved in a 0.05 M McIlvine buffer (pH 7.0 or 3.0) to give a concentration of 0.2% as a sample weight. An oil-in-water emulsion was prepared by homogenizing 2 mL of the sample solution and 0.5 mL of corn oil in a test tube (18 mm i.d. × 85 mm) at 25 °C with a Polytron PTA-7 device (Kinematica, Switzerland) at 24000 rpm for 1 min according to the method described previously (*7*). The emulsion was diluted 100-fold with 0.1% SDS solution for 0, 10, 30, 60, and 120 min after emulsification, and the absorbance at 500 nm was measured. The EAI was calculated by the following equations (*27*):

$$\mathrm{EAI} = 2T/\phi C$$

and

$$T = 2.3A/L$$

where $A$ is the absorbance at 500 nm, $L$ (light pass) is $10^{-2}$ m, $C$ is the concentration of a sample ($10^3$ g/m$^3$), and $\phi$ (oil phase volume) is 0.2. The ES is defined as the ratio of the absorbance 120 min after emulsification to that at time 0. The evaluation was performed three times.

**Conductometric Titration.** A conductometric titration was carried out to evaluate the calcium ions bound with the SPI−Por conjugate as previously described (*28*). In brief, 10 mM $CaCl_2$ (pH 7.0) was added to 20 mL of SPI or the Conj 63 solution (pH 7.0, 1 mg/mL) in increments of 20 $\mu$L at a time, and the conductivity was measured with an ES-12 conductometer (Horiba, Kyoto, Japan).

**Analytical Methods.** The total sugar content, reducing sugar content, protein content, sulfate content, and 3,6-anhydrogalactose content of Por were, respectively, measured by the phenol−sulfuric acid method (*29*) with galactose used as the standard, the Somogyi (*30*)−Nelson method with galactose used as the standard (*31*), the Lowry method (*32*) with bovine serum albumin as the standard, the rhodizonate method (*33*) with sulfuric acid as the standard, and the resorcinol method (*34*) with fructose as the standard. The total sugar content of SPI was measured by the phenol−sulfuric acid method (*29*) with galactose used as the standard. The Por content and nitrogen content of each conjugate were, respectively, measured by the phenol−sulfuric acid method (*29*) with Por used as the standard and by a 2400II CHNS/O Ultimate analyzer (Perkin-Elmer Japan, Tokyo, Japan) with acetanilide used as the standard according to the method previously described (*35*). The isoelectric point (pI) of each conjugate was evaluated from the pH value of a sample solution that had been completely deionized in a mixed-bed ion-exchange column (Amberlite IR120 and IRA 400; Organo Co., Tokyo, Japan) according to the method previously described (*36*). SDS-PAGE for SPI was carried out under both reducing and nonreducing conditions according to the method of Laemmli (*37*), and the $\beta$-conglycinin and glycinin contents in SPI were estimated from the ratio of the corresponding bands in the densitograms as evaluated by a Densito Graph 4.0 (Atto, Tokyo, Japan) as previously described (*23*).

## RESULTS AND DISCUSSION

**Physicochemical Features of Por, SPI, and the SPI−Por Conjugates.** Por prepared by autoclaving a suspension of dried nori followed with subsequent precipitation with ethanol showed a 72.6% total sugar content, 6.9% protein content, 6.2% sulfate content, and 7.6% 3,6-anhydogalactose content. The yield was about 18% based on the weight of dried nori, higher than that (about 11%) in the previous study (*7*), and the average degree of polymerization of Por was estimated to be 44.5 by the ratio of the total sugar content to the reducing sugar content. It is thus considered that Por with relatively low molecular weight could be prepared. SPI was prepared by three-time dissolution at pH 8.0 and precipitation at pH 4.5 from the defatted soybean flour to eliminate free saccharides and then spray-drying to obtain it on a relatively large scale. The yield of SPI was about 45%. SPI showed a 2.1% sugar content due to $\beta$-conglycinin being a glycoprotein, about 92% protein content estimated from the nitrogen content by using a nitrogen coefficient of 6.53, a subunit composition of 66.5% glycinin and 33.5% $\beta$-conglycinin as evaluated by SDS-PAGE, and a pI of 5.5.

**Table 1.** Chemical Features of the Por–SPI Conjugates

| | SPI | Conj 45 | Conj 63 |
|---|---|---|---|
| total sugar content[a] (%) | 4.7 | 50.5 | 17.9 |
| Por content[b] (%) | | 45.3 | 13.2 |
| nitrogen content[c] (%) | 14.1 | 5.90 | 10.7 |
| SPI content (%) | | 45.2 | 81.3 |
| SPI:Por (weight ratio) | | 1:1 | 1:0.16 |
| *p* value[d] | 5.59 ± 0.02 | 3.77 ± 0.04 | 5.12 ± 0.02 |

[a] Measured by the phenol–sulfuric acid method (*29*) with glucose as the standard. [b] Measured by the phenol–sulfuric acid method (*29*) with Por as the standard. [c] Measured by the ultimate analysis. [d] Evaluated from the pH value of a sample solution that had been completely deionized in a mixed-bed ion-exchange column (*36*).

The dried SPI and Por mixture was then heated at 60 °C and 79% RH to prepare the SPI−Por conjugate through the Maillard reaction. This heating is thought to not denature SPI, because it is estimated to sorb about 18% water at 79% RH and has been predicted to denature at about 114 °C from the results reported previously (*23*). Consequently, the SPI−Por conjugates (Conj 45 and Conj 63) were, respectively, obtained from the dissolved fractions of the Maillard reaction product at pH 4.5 and 6.3. The yields were 8.4% for Conj 45 and 11.7% for Conj 63, whereas that for the insoluble fraction (Ppt 63) was 70.2%, because a large part of the Maillard reaction product could not be dissolved during the extraction. Conj 45 is believed to have hardly contained any free SPI because of the sparing solubility of SPI in the acidic pH range, whereas Conj 63 is estimated to have presumably contained free SPI in some degree. On the other hand, unreacted Por is thought to have remained in the conjugates to some extent. However, because free SPI and Por showed the emulsifying behavior similar to that of the control emulsion without a sample under the test conditions as described later, the conjugate preparations were used in the subsequent experiments without further purification. Conj 45 contained much more Por than Conj 63, and the weight ratios of the SPI and Por moieties were 1:1 for Conj 45 and 1:0.16 for Conj 63 (**Table 1**). This suggests that Conj 45, being rich in Por, would have better solubility than Conj 63 in the acidic pH region. The pI value for Conj 45 was lower than that of Conj 63, corresponding to the Por content. The thermal denaturation behavior was evaluated by DSC. It was reconfirmed that the DSC curve for SPI showed two clear endothermic peaks that have been assigned to the denaturation of $\beta$-conglycinin for the lower temperature peak and of glycinin for the higher temperature peak (*23*). The DSC curve for the SPI/Por mixture (Mix 45) of the same composition as that of Conj 45 showed that $T_o$ for $\beta$-conglycinin and glycinin, respectively, shifted 9 and 12 °C higher and that the transition range ($T_c - T_o$) for the denaturation of Mix 45 was narrower than for SPI, resulting in reduced enthalpy (**Table 2**). This indicates greater thermal stability for $\beta$-conglycinin and glycinin in the presence of free Por, probably due to the increasing effect of the sulfate group, because the authors have previously reported that sulfate elevated the gelatinization temperature of potato starch (*38*). Ppt 63 gave lower endothermic peaks in a temperature region similar to that for SPI. On the other hand, the DSC curves for Conj 63 and Conj 45 differed greatly as shown by the substantial disappearance of the endothermic peak of the $\beta$-conglycinin or glycinin component. It is thus considered that Conj 63 and Conj

**Table 2.** Thermal Characteristics of the SPI and SPI–Por Conjugates and SPI/Por Mixtures Evaluated by DSC[a]

| | β-conglycinin component | | | | glycinin component | | | |
|---|---|---|---|---|---|---|---|---|
| | denaturation temperature (°C) | | | | denaturation temperature (°C) | | | |
| sample | $T_o$ | $T_p$ | $T_c$ | enthalpy (mJ/mg) | $T_o$ | $T_p$ | $T_c$ | enthalpy (mJ/mg) |
| SPI | 64.8 ± 1.0 a | 71.0 ± 1.1 a | 75.4 ± 1.4 a | 2.1 ± 0.2 a | 80.4 ± 1.3 a | 86.9 ± 1.3 a | 92.9 ± 0.5 a | 5.0 ± 0.5 a |
| Conj 45 | 62.6 ± 1.3 a | 66.6 ± 0.8 b | 72.7 ± 0.5 b | 0.7 ± 0.04 b | 79.5 ± 0.5 a | | 99.7 ± 1.1 b | 1.2 ± 0.2 b |
| Mix 45 | 73.5 ± 0.6 b | 77.9 ± 0.3 c | 83.2 ± 0.9 c | 1.1 ± 0.1 c | 92.7 ± 0.2 b | 97.8 ± 0.1 b | 102.7 ± 0.5 c | 4.1 ± 0.8 a |
| Conj 63 | 73.5 ± 0.2 b | | 78.9 ± 0.9 d | 0.1 ± 0.03 d | 82.1 ± 0.4 a | 88.9 ± 0.6 a | 96.6 ± 0.7 d | 3.5 ± 0.6 c |
| Mix 63 | 64.1 ± 1.5 a | 70.3 ± 0.04 a | 74.2 ± 0.5 a | 1.6 ± 0.04 e | 82.6 ± 0.6 a | 89.4 ± 0.03 a | 96.3 ± 0.5 d | 6.6 ± 0.1 d |
| Ppt 63 | 61.2 ± 0.6 c | 70.0 ± 0.4 a | 77.8 ± 0.9 d | 2.7 ± 0.2 f | 81.1 ± 0.9 a | 85.4 ± 0.8 a | 92.6 ± 0.3 a | 1.4 ± 0.3 b |

[a] Each value is expressed as the mean ± SD ($n = 3$). Different letters in the same column show significant differences ($P < 0.05$) by Student's $t$ test.

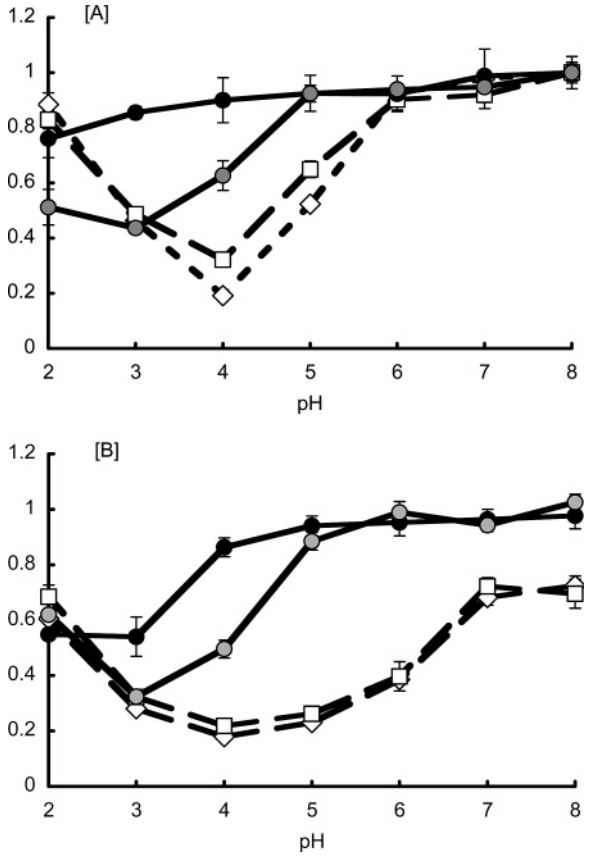


**Figure 1.** Relative solubility of the conjugates at various pH values without (**A**) or with (**B**) preheating at 100 °C for 30 min as compared with SPI and the mixture. The relative solubility is expressed as the ratio of the protein concentration of the tested solution at each pH to that at pH 8.0. Key: λ, Conj 45; ●, Conj 63: ◇, SPI; and □, Mix 45. Each value is expressed as the mean ± SD ($n = 3$).

45 were partially denatured by conjugating with Por, probably due to electrostatic repulsion of the negative charges of the sulfuric groups of the Por moiety.

**Improved Solubility.** The comparative solubility of Conj 45 and Conj 63 with SPI and Mix 45 was evaluated at different pH values. SPI and Mix 45 had similar pH-dependent solubility as shown by the remarkable decrease between pH 3.0 and pH 5.0 (**Figure 1A**). It is therefore considered that free and unbound Por, if it was contaminated, had no effect on the solubility of SPI. On the other hand, Conj 63 exhibited good solubility between pH 5.0 and pH 8.0, particularly the improved solubility (above 60%) at pH 4.0, at which SPI and Mix 45 had the minimum solubility (about 20 and 30%, respectively). Conj 45 exhibited substantially complete solubility (about 75% minimum) over the pH range of 2.0−8.0 due to the hydrophilicity endowed by the Por moiety. The effect of heating on the solubility was also investigated by using samples that had been preheated at 100 °C for 30 min. The results are expressed as the ratio of the protein concentration for the preheated sample to that for the sample without heating at pH 8.0 (**Figure 1B**). In the case of SPI and Mix 45, the solubility decreased more throughout the pH range, especially between pH 3.0 and 6.0. On the other hand, Conj 45 and Conj 63 maintained high solubility, especially between pH 3.0 and pH 6.0. It is thus concluded that conjugation with Por would be valuable for improving the solubility of SPI enabling a soybean protein-based liquid foods such as beverages to be produced.

**Reduced Digestibility.** SPI and Conj 63 were digested with pancreatin at 40 °C for 1−24 h, and the digestibility was estimated by the relative ratio of the CBB-stained strength of the SDS-PAGE pattern for each digested sample to that for the undigested sample. In the case of the samples without preheating, more than half SPI was digested during 24 h, whereas less than 30% of Conj 63 was digested (**Figure 2A**), indicating more tolerance to digestion with pancreatin than SPI, probably due steric hindrance of the conjugated Por moiety. However, the samples preheated at 100 °C for 10 min showed no significant difference between SPI and Conj 63 (**Figure 2B**), indicating the lost inhibition of the Por moiety due to protein denaturation.

**Improved Emulsifying Ability of the Conjugates.** The comparative emulsifying ability of Conj 63 and Conj 45 with SPI and Mix 63 was evaluated by the EAI and ES derived from the absorbance at 500 nm of O/W emulsions prepared at pH 3.0 and 7.0. There was no significant difference in ES between the SPI emulsion and the control emulsion (**Figure 3**), indicating no particular emulsifying ability for SPI in this evaluation system. Mix 63 showed similar EAI and ES values at pH 7.0 to those of SPI. It is thus considered that free Por could not improve the emulsion ability of SPI. On the other hand, Conj 63 and Conj 45 showed markedly higher EAI and ES values than SPI at both pH values, and in particular, Conj 45 exhibited eminently good emulsifying ability. It is thought that Conj 45 probably possessed better balance between hydrophobicity and hydrophilicity with the large amount of the conjugated Por moiety, reconfirming that the emulsifying ability of a protein could be effectively improved by conjugating with saccharide, as shown in the recent studies on conjugation of β-lactoglobulin and alginic acid oligosaccharide (*9*), ε-poly(L-lysine) and potato starch (*12*), glycinin and glucose (*17*), SPI and dextran (*18*−*20*), SPI and carboxymethyl cellulose (*21*), soy protein hydrolyzate and curdlan (*22*), ovalbumin and dextran (*39*), and β-lactoglobulin and polysaccharides (*40*) through the Maillard reaction.

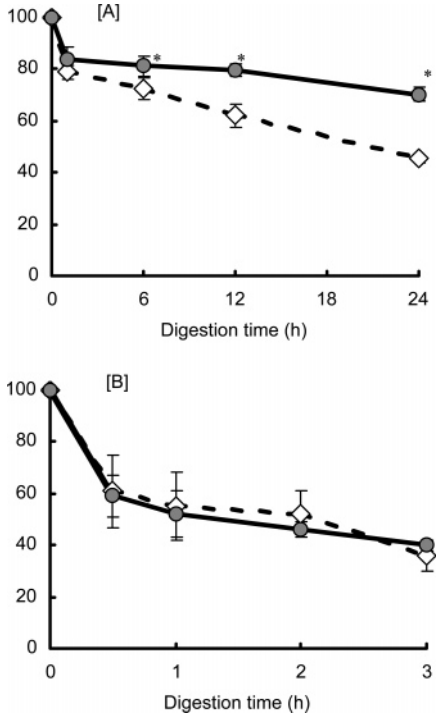


**Figure 2.** Digestibility of Conj 63 with pancreatin without (**A**) or with (**B**) preheating at 100 °C for 10 min as compared with SPI. The digestibility with pancreatin was estimated by the relative ratio of the staining strength of the SDS-PAGE pattern for each digested sample to that for the undigested sample. Each value is expressed as the mean ± SD ($n = 3$). The asterisks show significant differences at $P < 0.05$ against SPI.

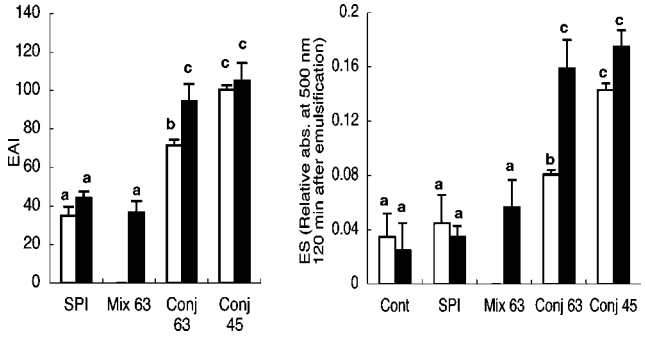


**Figure 3.** Comparative emulsifying ability of the conjugates at pH 3.0 and 7.0 with SPI and the mixture. The emulsifying ability was evaluated as the EAI and emulsifying stability (ES) derived from the absorbance at 500 nm of the O/W emulsion at pH 3.0 or pH 7.0. Key: White bars, pH 3.0; black bars, pH 7.0. Each value is expressed as the mean ± SD ($n = 3$). Different letters show significant differences at $P < 0.05$ against SPI.

**Divalent Cation-Binding Behavior of the Conjugate.** The comparative divalent cation-binding ability of Conj 63 with SPI and Mix 63 was examined at pH 7.0 by conductometric titration with $CaCl_2$ and $MgCl_2$ solutions. Conductometric titration with the $CaCl_2$ solution showed a titration curve that could be resolved into two straight lines; this enabled the end point of titration, equivalent to a complete calcium binding to a sample, to be obtained as the intersection between the two regression lines. Conj 63 exhibited significantly higher calcium-binding ability (about two-fold) than that of SPI (**Figure 4**). Because Mix 63 showed calcium ion-binding ability similar to that of Conj 63, it is considered that the improved calcium-binding ability was endowed by the Por moiety of Conj 63, similar to the improved gelatin by conjugating with low molecular weight

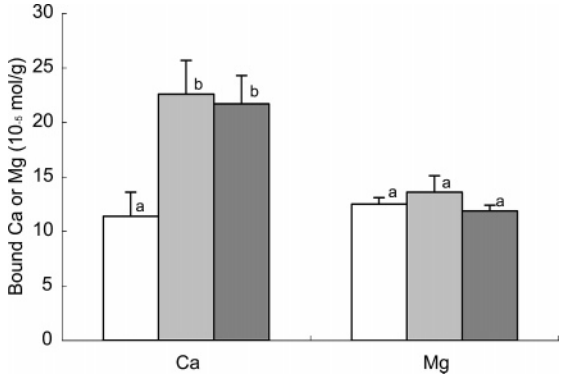


**Figure 4.** Comparative divalent metal salt-binding ability of the conjugate with PSI and the mixture. The divalent metal salt-binding ability was evaluated as the determined content of bound Ca or Mg. Key: White bars, SPI; hatched bars, Mix 63; and checked bars, Conj 63. Each value is expressed as the mean ± SD ($n = 3$). Different letters show significant differences at $P < 0.05$ against SPI.

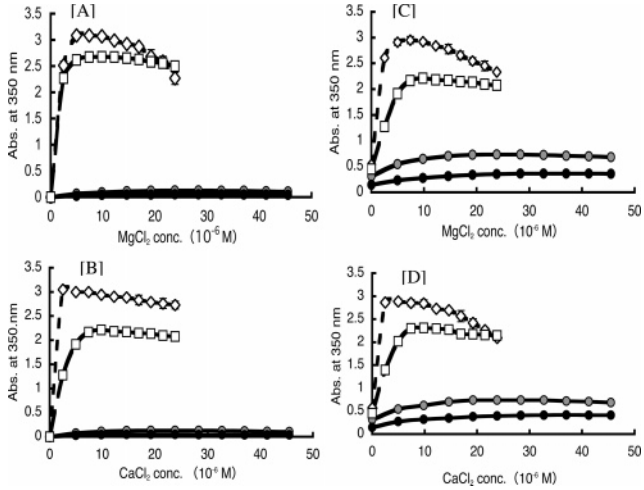


**Figure 5.** Aggregation behavior of the conjugates with divalent metal salts [$MgCl_2$ (**A** and **C**) and $CaCl_2$ (**B** and **D**)] without (**A** and **B**) or with (**C** and **D**) preheating at 100 °C for 10 min as compared with the behavior of SPI and the mixture. The divalent metal salt-induced aggregation behavior of Conj 45, Conj 63, SPI, and Mix 45 was evaluated by monitoring the absorbance at 350 nm of the respective solution at pH 8.0 with adding $MgCl_2$ or $CaCl_2$ solution. Key: ●, Conj 45; gray circle, Conj 63; ◇, SPI; and □, Mix 45. Each value is expressed as the mean ± SD ($n = 3$).

alginic acid (*35*). However, conductometric titration with $MgCl_2$ showed no significant difference in the bound magnesium content among Conj 63, Mix 63, and SPI, because the sulfate group of Por could not form a soluble complex with the magnesium ion at pH 7.0. It is thus considered that the magnesium ion-bonding ability of Conj 63 was due to the complex between the SPI moiety and the magnesium ions.

**Terminated Aggregation of the Conjugates with Divalent Cations.** The comparative divalent cation-induced aggregation behavior of Conj 45 and Conj 63 with SPI and Mix 45 was investigated by monitoring the absorbance at 350 nm of the respective solution at pH 8.0 while adding an $MgCl_2$ or $CaCl_2$ solution. SPI showed a rapid increase in the absorbance to about 2.5 with 2.5 $\mu$M $MgCl_2$ and to about 3.0 with 2.5 $\mu$M $CaCl_2$ (**Figure 5A**,**B**), and the solution then became strongly turbid, indicative of extensive aggregation. The absorbance then gradually decreased with more concentrated addition due to the further progress of aggregation. It is thus considered that SPI strongly aggregated with divalent cations, corresponding to the

formation of soybean curd (tofu) with divalent cations. The absorbance of Mix 45 also rapidly increased with added $MgCl_2$ and $CaCl_2$, except for some low levels of absorbance as compared with SPI, probably due to codissolution with free Por. It is thus considered that free Por did not substantially inhibit aggregation of SPI. On the other hand, Conj 45 and Conj 63 did not show any increase in absorbance over the divalent cation concentration range examined. Because Por could bind with $Ca^{2+}$, but not with $Mg^{2+}$ as just described, it is considered that the inability to aggregate the conjugate resulted from the high solubility of the Por moiety of the conjugate and did not result from a decrease in the actual concentration of divalent cations due to its consumption by binding with Por. It thus seems that the conjugates could provide a transparent solution that remained stable against added divalent cations. The aggregation behavior of the conjugate solution preheated at 100 °C for 10 min was also examined in the same manner, because heating induced reduced solubility as already described. Preheating resulted in large increases in the respective absorbance at 350 nm of the 0.075% SPI and Mix 45 solutions based on the protein weight to 0.565 and 0.453, whereas the increases for Conj 63 and Conj 45 were held at lower absorbance levels (0.318 and 0.147, respectively). The preheated SPI and Mix 45 solutions also showed a rapid and substantial increase in the absorbance with an added $MgCl_2$ or $CaCl_2$ solution (**Figure 5C**,**D**). However, the preheated Conj 63 and Conj 45 solutions showed only a slight increase as expected, even when $MgCl_2$ or $CaCl_2$ was added. It is therefore concluded that the aggregation behavior of SPI with divalent cations was terminated by conjugating with Por.

**Conclusion.** Por prepared from dried nori was applied as the functional modifier of a SPI from defatted soybean to conjugate with it by the Maillard reaction. Two kinds of partially denatured conjugate (Conj 45 and Conj 63) were obtained from the reaction product by sequential extraction at pH 4.5 and pH 6.3. Conj 45 and Conj 63 showed improved solubility and emulsifying ability, even in the acidic pH region; in particular, Conj 45 exhibited substantially complete solubility over the pH range of 2.0−8.0, together with outstanding emulsifying ability. Conj 63 showed more tolerance against digestion with pancreatin than SPI, whereas this was lost predenaturation. Conj 63 exhibited about two-fold higher calcium-binding ability than that of SPI, and Conj 63 and Conj 45 terminated the aggregation with added $Ca^{2+}$ and $Mg^{2+}$. It is believed that Por could be applied as a valuable functional modifier of SPI for providing soybean protein-based liquid foods such as beverages by conjugation through the Maillard reaction.

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