Preparation and Characterization of Ovalbumin–Dextran Conjugate Having Excellent Emulsifying Properties

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Soluble protein–carbohydrate conjugates have been prepared by coupling ovalbumin to cyanogen bromide activated dextran. The ovalbumin–dextran conjugate shows a broad distribution of molecular weight, from 250 to several thousand kilodaltons. The emulsifying properties of the ovalbumin–dextran conjugate were much higher than of native ovalbumin and were superior to those of commercial emulsifier, especially in high salt concentration and in acidic pH. Conjugates of dextran with other proteins, such as lysozyme and soy protein, also showed excellent emulsifying properties. Thus, it was suggested that the protein–dextran conjugate can be used as a soluble macromolecule emulsifier for food industry.

Recently, much attention has been directed toward the preparation of new functional proteins. A number of studies to improve protein functionality have involved chemical modification such as acylation or alkylation (Kinsella, 1976; Sen et al., 1981; Nieto and Palacian, 1983), esterification or amidation (Mattarella and Richardson, 1983; Mattarella et al., 1983) and deamidation (Wu et al., 1976; Matsudomi et al., 1985) and the covalent attachment of carbohydrates (Waniska and Kinsella, 1984; Kitabatake et al., 1985) and fatty acids (Haque and Kito, 1983). In addition to these chemical modifications, the enzymatic modifications of food protein (Watanabe et al., 1981a,b; Nio et al., 1985; Motoki et al., 1986) have also been investigated to make a functional protein. Among the numerous studies on the functional properties of chemically modified proteins, there is little information on the functionality of the conjugates of proteins with polymeric biomaterials. We have reported that the emulsifying and foaming properties of denatured proteins are greatly enhanced (Kato et al., 1981). However, the denatured proteins are usually insoluble in aqueous solution. If the denatured proteins are obtained in soluble form, they can be used as functional macromolecules. Thus, we tried to prepare the conjugates of proteins with soluble polysaccharides to make a functional biomaterial. This paper describes the excellent emulsifying properties of ovalbumin–dextran prepared by coupling ovalbumin to cyanogen bromide activated dextran.

MATERIALS AND METHODS

Materials. Dextran (average molecular weight 75 000), glycine, and cyanogen bromide were purchased from Wako Pure Chemical Industries. Serum albumin (bovine) was purchased from Sigma Chemical Co. Sephadex G-100 was purchased from Pharmacia Fine Chemicals. Ovalbumin was prepared from fresh egg white by the crystallization method in sodium sulfate and recrystallized five times (Kekwick and Cannan, 1936). Lysozyme was prepared from fresh egg white by a direct crystallization method and recrystallized five times (Alderton and Fevold, 1946). Soy protein was prepared from defatted soybean meal by the acid crystallization method at pH 4.8 (Thanh et al., 1975). SunSoft SE-11 and Q-18S were supplied from Taiyo Kagaku Co., Ltd.

Preparation of Ovalbumin–Dextran Conjugate. Ovalbumin–dextran conjugate was prepared by the method of Marshall and Rabinowitz (1976). To a stirred solution of dextran (2.5 g) in water (250 mL), adjusted to pH 10.7 with 500 mM sodium hydroxide solution, was added cyanogen bromide (0.625 g), followed by a second addition of cyanogen bromide (0.625 g) 30 min later. The pH was maintained at 10.7 during this process by addition of sodium hydroxide solution (500 mM). Thirty minutes after the second addition of cyanogen bromide, the pH was adjusted to 9.0 by addition of 100 mM hydrochloric acid solution. After dialysis at 4 °C for 2 h, against 4 L of sodium carbonate solution, pH 9.0 (prepared by addition of 1.0 M sodium carbonate solution to distillized water until pH 9.0), ovalbumin (0.50 g) was added. The pH was maintained at 9.0 during addition of ovalbumin, by addition of sodium carbonate solution (200 mM). Coupling of ovalbumin to cyanogen bromide activated dextran was then allowed to proceed during 12 h at 4 °C. After this period, the solution was dialyzed for 2 h at room temperature against 4 L of sodium carbonate solution (prepared as above), and then 20 mL of glycine solution (100 mg/mL) was added. After the mixture was allowed to stand for a further 12 h at 4 °C, the product was lyophilized.

Gel Filtration of Ovalbumin–Dextran Conjugate. To further purify ovalbumin–dextran conjugate, gel filtration of the conjugate was performed on a column (100 × 2 cm) of Sephadex G-100. Elution was carried out with 5 mM acetate buffer, pH 5.0, containing 10 mM sodium chloride, and 3.0-mL of fractions were collected automatically. Fractions of conjugate (32–48) were collected, dialyzed against deionized water, and lyophilized.

Determination of the Molecular Weight of Ovalbumin–Dextran Conjugate. Ovalbumin-dextran conjugate solution (0.1%) in 67 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl was applied to a high-performance gel chromatography system, connected in tandem a TSK gel G4000SW column (Toyo Soda Co.; 0.75 × 60 cm) and a TSK gel G6000PW column (Toyo Soda; 0.75 × 60 cm), at a flow rate of 0.3 mL/min. Elution from columns was monitored with a low-angle laser light scattering photometer (LS-8; Toyo Soda) and then with a precision differential refractometer (RI-8; Toyo Soda). The molecular weight of ovalbumin–dextran conjugate was estimated from the ratio of the output of a low-angle laser light scattering photometer, $(\text{output})_{LS}$, to that of a refractometer, $(\text{output})_{RI}$, by eq 1 (Takagi and Hizukuri,

$$MW = k(\text{output})_{LS}/(\text{output})_{RI} \quad (1)$$

1984), where k is a constant depending on the instrumental and experimental conditions and is determined by using standard sample. Native dextran was used in this experiment as a molecular weight standard. The weight-average molecular weight of the ovalbumin–dextran con-

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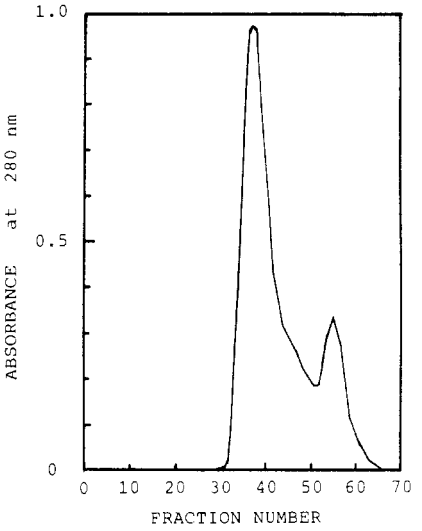

Figure 1. Elution pattern of ovalbumin–dextran conjugate on a Sephadex G-100 column.

Table I. Binding Ratio of Ovalbumin–Dextran Conjugate

binding ratio	ovalbumin	dextran
weight	1	5
molar	1	3

jugate was determined by eq 2 (Takagi and Hizukuri, 1974), where $(\text{area})_{LS}$ and $(\text{area})_{RI}$ are the total areas in the elution peak of the LS photometer and the refractometer, respectively.

$$MW = k(\text{area})_{LS}/(\text{area})_{RI} \quad (2)$$

Measurement of Emulsifying Properties. The emulsifying properties were determined by the method of Pearce and Kinsella (1978). To prepare emulsion, 1.0 mL of corn oil and 3 mL of protein solution in 0.1 M phosphate buffer, pH 7.4, were shaken together and homogenized in an Ultra Turrax (Hansen & Co.) at 12 000 rpm for 1 min at 20 °C. A 50-μL emulsion sample was taken from the bottom of the container after different times and diluted with 5 mL of 0.1% sodium dodecyl sulfate solution. The absorbance of diluted emulsion was then determined at 500 nm. The emulsifying activity was determined from the absorbance measured immediately after emulsion formation. The emulsion stability was estimated by measuring the half-time of the turbidity of emulsion.

RESULTS

Figure 1 shows the gel filtration pattern of ovalbumin–dextran conjugate on a column of Sephadex G-100. The majority of the protein peaks were eluted at the void volume of the column. As a control experiment ovalbumin was treated with a same condition used in the preparation of ovalbumin–dextran conjugate. The peak of the control ovalbumin was eluted at fractions 50–63. Thus, the peak eluting at void volume of fractions 34–48 was collected as the ovalbumin–dextran conjugate. The fraction thus obtained was lyophilized after dialysis against deionized water. It was used for the analysis of the binding ratio, the estimation of the molecular weight, and the measurement of the functional properties.

Table I shows the binding ratios of ovalbumin–dextran conjugate. From the absorbance at 280 nm of 0.1% (w/v) native ovalbumin and 0.1% (w/v) ovalbumin–dextran conjugate, the binding ratio was estimated. The ovalbumin to dextran binding molar ratio was estimated to be 1/3.

The molecular weight of the conjugate was determined by the low-angle laser light scattering technique combined with high-performance gel chromatography. Figures 2 and 3 show a typical elution pattern of native dextran and ovalbumin–dextran conjugate, respectively, obtained with the low-angle laser light scattering photometer and the precision differential refractometer. Noisy trace indicates the elution patterns obtained with the low-angle laser light scattering photometer, and smooth trace indicates the elution patterns obtained with the precision differential refractometer. There was a time lag, indicated by a double-heated arrow, between the two recordings for a particular fraction. The lag was determined as the distance between the two peaks recorded when standard ovalbumin was injected. The elution patterns of dextran and ovalbumin–dextran conjugate are so broad that the molecular weight cannot be determined exactly. However, the low-angle laser light scattering technique can estimate the

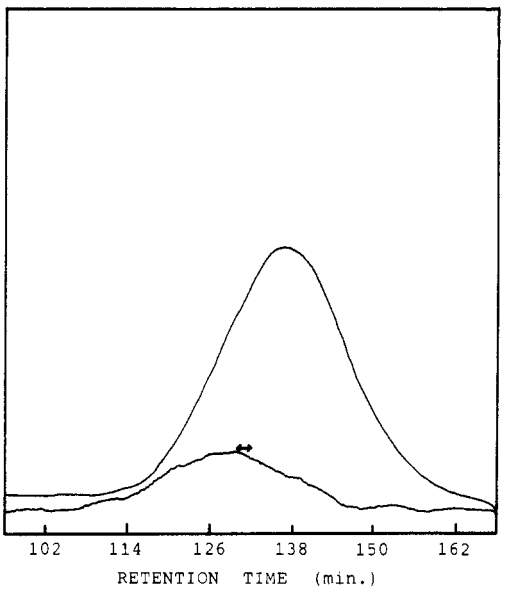

Figure 2. Elution patterns of dextran on a TSK gel G4000SW + a TSK gel G6000PW column obtained with the low-angle laser light scattering photometer (noisy trace) and the precision differential refractometer (smooth trace). A double-headed arrow indicates the time lag between two tracings.

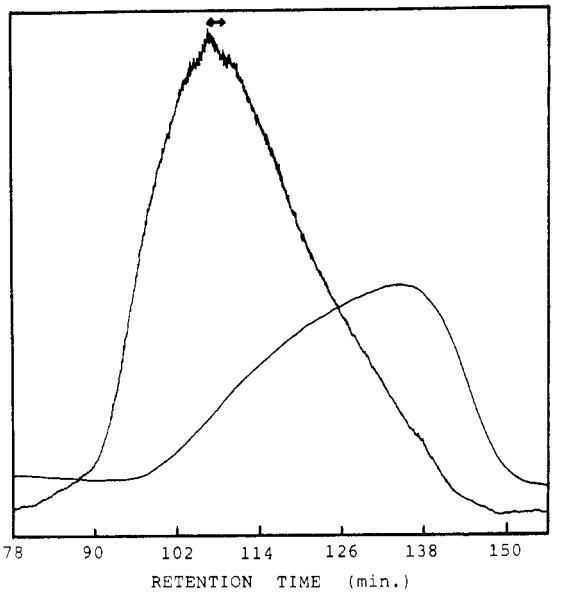

Figure 3. Elution patterns of ovalbumin–dextran conjugate on a TSK gel G4000SW + a TSK gel G6000PW column obtained with the low-angle laser light scattering photometer (noisy trace) and the precision differential refractometer (smooth trace). A double-headed arrow indicates the time lag between two tracings.

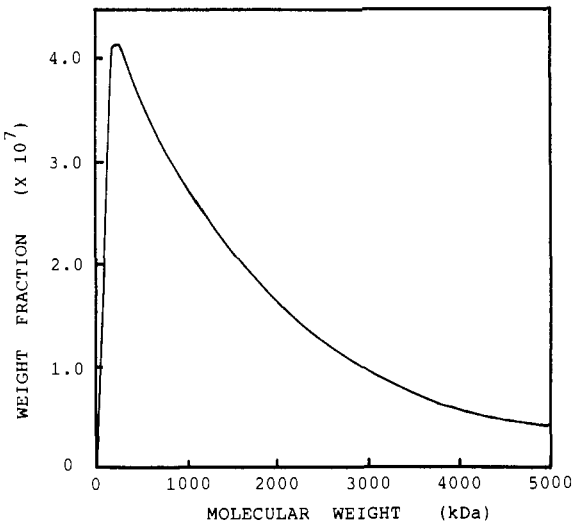

Figure 4. Molecular weight distribution curve of ovalbumin–dextran conjugate estimated from HPLC patterns in Figure 3.

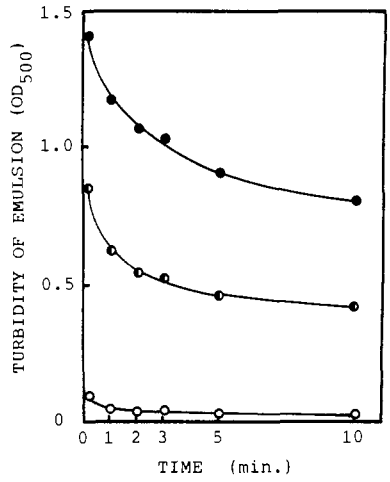

Figure 5. Emulsifying property of ovalbumin–dextran conjugate in 0.1 M phosphate buffer, pH 7.4, in oil in water emulsion (O/W = 1/3): ●, 0.5% conjugate; ◐, 0.1% conjugate; ○, 0.1% ovalbumin.

molecular weight at each elution position. From the weight-average molecular weight of native dextran and $(\text{area})_{LS}/(\text{area})_{RI}$ in Figure 2, the constant, k, was determined to be 3.4×10^5. By using the constant, $k = 3.4 \times 10^5$, and eq 1, the molecular weight of ovalbumin–dextran conjugate was estimated at each elution time in Figure 3. Figure 4 shows the molecular weight distribution of the conjugate. According to the molecular weight distribution curve of the conjugate, the molecular weight of the major population ranges from 250 to 1000 kDa. On the other hand, estimating from $k(\text{area})_{LS}/(\text{area})_{RI}$ in Figure 3, the weight-average molecular weight of ovalbumin–dextran conjugate was 700 kDa.

Figure 5 shows the emulsifying property of native ovalbumin and ovalbumin–dextran conjugate. The emulsifying property of the conjugate was enhanced much more than that of native ovalbumin even if the conjugate concentration is 0.1%. The values of emulsifying activity of 0.5% ovalbumin–dextran conjugate, 0.1% ovalbumin–dextran conjugate, and 0.1% native ovalbumin were 1.420, 0.860, and 0.102, respectively. The emulsion stability of the conjugate was also enhanced much more than native ovalbumin. The half-time of the turbidity of the emulsion, showing the emulsion stability, of the conjugate was more than 10 min, while that of ovalbumin is less than 1 min. Table II shows the emulsifying activity of the mixture of various ratios of dextran and ovalbumin. The total concentration of the mixture is 0.1%. The value of emulsifying activity of only dextran was 0.31. From these results, it is suggested that not the noncovalent ovalbumin–dextran mixture but the covalent ovalbumin–dextran conjugate generates the excellent emulsifying properties. Figure 6 shows the comparison of the emulsifying property of ovalbumin–dextran conjugate with that of ovalbumin–pectin conjugate. Ovalbumin–pectin conjugate was prepared by the same method as ovalbumin–dextran conjugate. But, in the case of ovalbumin–pectin conjugate, the emulsifying property was not improved. The value of emulsifying activity of ovalbumin–pectin conjugate was 0.110.

Figure 7 shows the comparison of emulsifying property of ovalbumin–dextran conjugate with that of commercial emulsifiers. The commercial emulsifiers used in the ex-

Table II. Emulsifying Activity of the Mixture of Ovalbumin and Dextran

mixing ratio: ovalbumin/dextran	emulsifying act. (OD_{500})	mixing ratio: ovalbumin/dextran	emulsifying act. (OD_{500})
1/0	0.100	1/4	0.060
1/1	0.060	0/1	0.310
1/2	0.050		

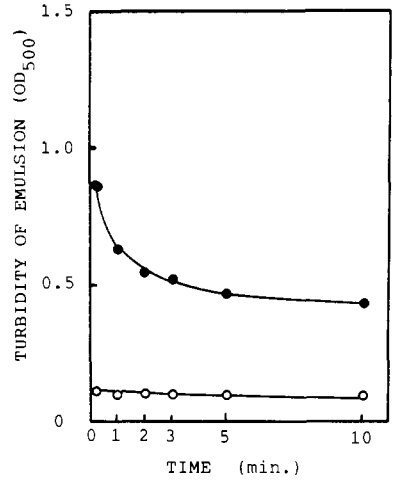

Figure 6. Comparison of the emulsifying property of ovalbumin–dextran conjugate with that of ovalbumin–pectin conjugate in 0.1 M phosphate buffer, pH 7.4, in oil in water emulsion (O/W = 1/3): ●, 0.1% ovalbumin–dextran conjugate; ○, 0.1% ovalbumin–pectin conjugate.

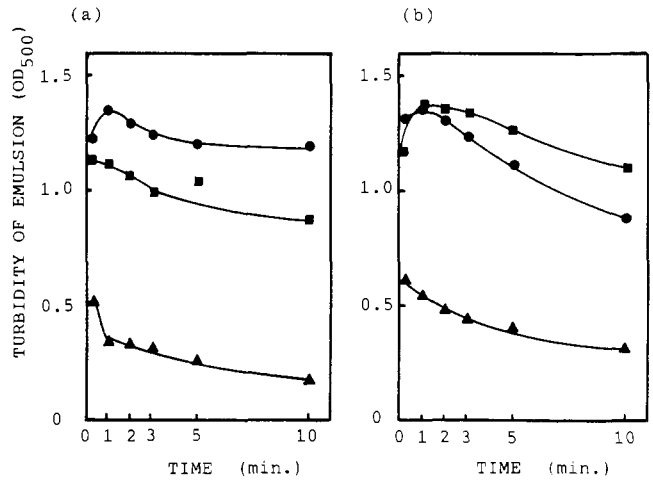

Figure 7. Emulsifying property of ovalbumin–dextran conjugate and commercial emulsifier in the emulsion system where emulsifiers were added to water phase (a) and oil phase (b): ●, 0.1% ovalbumin–dextran conjugate; ■, 0.1% Sunsoft SE-11; ▲, 0.1% Sunsoft Q-18S.

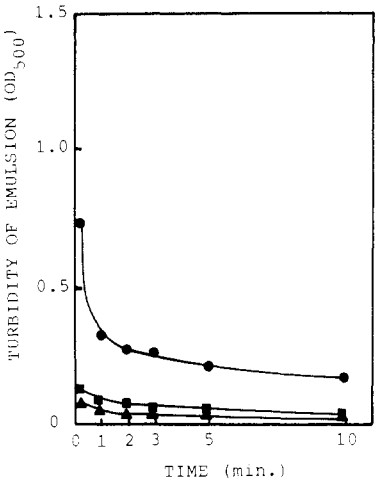

Figure 8. Effect of salt on the emulsifying property of ovalbumin–dextran conjugate and commercial emulsifier in oil in water emulsion (O/W = 1/3), with 10% NaCl contained in the water phase: ●, 0.1% ovalbumin–dextran conjugate; ■, 0.1% Sunsoft SE-11; ▲, 0.1% Sunsoft Q-18S.

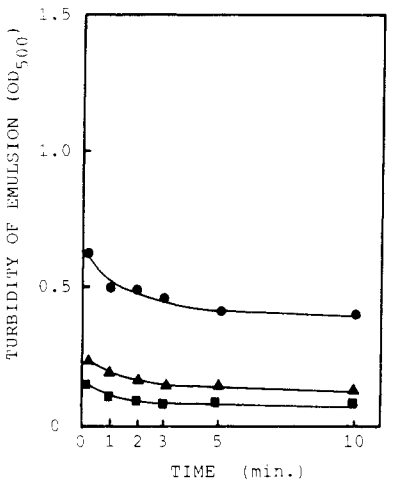

Figure 9. Effect of acid on the emulsifying property of ovalbumin–dextran conjugate and commercial emulsifier in oil in water emulsion (O/W = 1/3), with 1% citric acid contained in the water phase, pH 2.3: ●, 0.1% ovalbumin–dextran conjugate; ■, 0.1% Sunsoft SE-11; ▲, 0.1% Sunsoft Q-18S.

periment are SunSoft SE-11 and Q-18S. The former is sucrose–fatty acid ester and the latter is polyglycerin ester. The comparison of the emulsifying properties was done in two emulsion systems consisting of equivolumes of water and oil. In one system the emulsifier was added to the water phase, and in another system it was added to the oil phase. The ovalbumin–dextran conjugate is suitable for both emulsion system.

Figure 8 shows the effect of salt on the emulsifying property of oil in water emulsion. NaCl (10%) was contained in the water phase. The values of emulsifying activity of ovalbumin–dextran conjugate, SE-11, and Q-18S were 0.734, 0.130, and 0.077, respectively. Compared to the commercial emulsifiers, the ovalbumin–dextran conjugate showed better emulsifying properties in the presence of 10% NaCl.

Figure 9 shows the effect of acid on the emulsifying property of oil in water emulsion. Citric acid (1.0%) was added to the water phase, pH 2.3. As well as in the presence of salt, ovalbumin–dextran conjugate was stable under acidic pH. The values of emulsifying activity of ovalbumin–dextran conjugate, SE-11, and Q-18S were 0.630, 0.150, and 0.235, respectively.

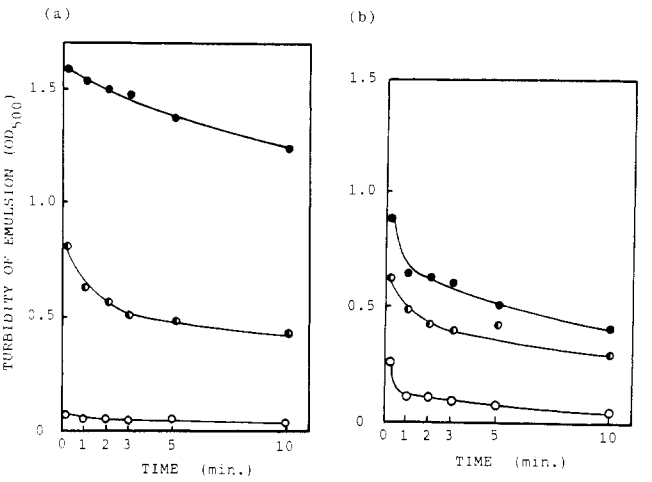

Figure 10. Emulsifying property of lysozyme–dextran conjugate (a) and soy protein–dextran conjugate (b) in 0.1 M phosphate buffer, pH 7.4, in oil in water (O/W = 1/3): ●, protein–dextran conjugate (0.1% protein concentration); ◐, protein–dextran conjugate (0.1% conjugate concentration); ○, 0.1% protein.

Table III. Effect of Heat Denaturation on the Emulsifying Activity of Protein–Dextran Conjugates

conjugate[a]	emulsifying act. (OD_{500})
ovalbumin–dextran	
untreated	1.420
heated	1.610
lysozyme–dextran	
untreated	1.585
heated	1.845
soy protein–dextran	
untreated	0.887
heated	1.200

[a] The concentration of conjugates is 0.1% at protein concentration. Heat denaturation was carried out at 90 °C.

The dextran conjugates with other food proteins were prepared, and their emulsifying properties were measured to extend the application to the food industry. Lysozyme and soy protein were used as food proteins. Figure 10 shows the emulsifying property of lysozyme–dextran conjugate and soy protein–dextran conjugate. From these results, even if proteins other than ovalbumin were conjugated with dextran, the emulsifying properties of the conjugates seem to be improved.

Table III shows the value of emulsifying activity of untreated and heated conjugates. Protein concentration was 0.1%. Turbidity was not observed by heat denaturation at 90 °C. The value of emulsifying activity of heated conjugates was higher than that of nonheated conjugates.

DISCUSSION

The functional macromolecule was made by covalently coupling ovalbumin to dextran. The weight-average molecular weight of ovalbumin–dextran conjugate was estimated to be about 700 kDa, while the major population of the conjugate was 250–1000 kDa according to the molecular weight distribution curve. Estimating from the binding ratio of the conjugate, 1 mol of ovalbumin seems to bind with 3 mol dextran. From the above results, the possible scheme of the conjugate is proposed as follows: The subunit of this conjugate is composed of 1 mol of ovalbumin and 3 mol of dextran (250 kDa), which is extended as prolonged polymers.

The emulsifying properties of ovalbumin–dextran conjugate were superior to those of dextran, native ovalbumin, and ovalbumin and dextran mixture. But the emulsifying property of ovalbumin–pectin conjugate was not improved.

Dextran has a highly branched network structure. On the other hand, pectin has a single-stranded chain structure. This structural difference probably affects the formation of the emulsion of the conjugates. The macromolecule branched structure may be efficient to wrap the oil drops to result in the excellent emulsifying properties.

The emulsifying properties of the ovalbumin–dextran conjugate were comparable or superior to those of the commercial emulsifier. The emulsifying properties of the conjugate were almost the same as SunSoft SE-11 in water or in 0.1 M phosphate buffer, pH 7.0. Interestingly, the emulsifying properties of the conjugate were superior to those of the commercial emulsifier in the presence of salt and in acidic conditions. Another advantage is that the ovalbumin–dextran conjugate is soluble in both water and oil. In addition, the heat-stable property of this conjugate is suitable for heat treatment and sterilization in food systems.

As mentioned above, the ovalbumin–dextran conjugate can be used in the food industry as a new functional emulsifier that is soluble, macromolecular, and stable in acid or in the presence of high concentrations of salt.

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Composition, Solubility, and Electrophoretic Patterns of Proteins Isolated from Kerman Pistachio Nuts (*Pistacia vera* L.)

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Extraction of protein from two pistachio sources (Kerman and Turkish) was performed with different solvents. The results showed the maximum amount of protein extracted with 0.5 M NaCl solution. The other solvents in sequential extraction, e.g. water, 60% (v/v) *tert*-butyl alcohol, and 0.1 M sodium borate (pH 10)/1% (w/v) $NaDodSO_4$/1% (v/v) 2-mercaptoethanol (2-ME), extracted little or no protein. The Kjeldahl data indicated that 66% of the total protein was globulin (NaCl soluble) while albumins (H_2O soluble), glutelins (sodium borate soluble), and prolamins (*tert*-butyl alcohol soluble), respectively, contributed 25, 7, and 2% of the total protein. Fractionation of the protein by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) showed three of the four solubility fractions had complex polypeptide compositions with molecular weights of 12–68K. However the protein profiles of all fractions were very similar except for the alcohol extract which did not produce any distinct bands. Two-dimensional electrophoretic analyses revealed about 100 protein spots when the meal was extracted directly with 6 M urea and 1% 2-ME, and the two pistachio sources showed similar polypeptide patterns.

The pistachio is one of the favorite tree nuts of the world. It is native to the Middle East region and was introduced to most of the Mediterranean countries and recently (1960) to the United States. Several species of the genus *Pistacia* are referred to as pistachio, but only the fruits of *Pistacia vera* attain sufficiently large size to be acceptable to consumers as edible nuts. Pistachio imports to the United States annually were in the vicinity of 15000 metric tons and came mainly from Iran (10–12000 metric tons) while the rest came from other countries (e.g., Turkey) (Woodroof, 1979). However, in recent years, an increase in local production has made the United States the world's second largest producer of pistachio nuts. The USDA reported in 1985 that the total U.S. production had reached 15500 metric tons, only 8600 metric tons being imported and almost exclusively (98.1%) from Iran. The

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