Functional Improvement of β-Lactoglobulin by Conjugating with Alginate Lyase-Lysate

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Bovine β-lactoglobulin−alginic acid oligosaccharide (β-LG−ALGO) conjugate was prepared by the Maillard reaction to improve the function of β-LG. The molar ratio of β-LG to ALGO in the conjugates was 1:7. The isoelectric point of the conjugate was 4.55, which is lower than that of β-LG. Fluorescence studies suggested that the conformation around Trp had changed in the conjugate and that the surface of the conjugate was covered with saccharide chain. Structural analyses with monoclonal antibodies indicated that the conformation around Val15−Ile29 (β-sheet) in the conjugate had changed, while native structure was maintained around Thr125−Lys135 (α-helix). By conjugation with ALGO, β-LG was endowed with high heat stability. The emulsifying activity and the aggregating property of β-LG was improved in the conjugate.

Keywords: *β-Lactoglobulin; alginic acid; alginate lyase; neoglycoconjugate; functional improvement; acidic polysaccharide; protein conjugation; emulsification; solubility; retinol-binding; lipocalin*

INTRODUCTION

β-Lactoglobulin (β-LG) is a major whey protein of M_r 18 400 with two disulfide bridges, as well as free cysteine containing plenty of essential amino acids (McKenzie, 1971). The results of X-ray crystallography (Papiz et al., 1986) and protein sequence determinations (Pervaiz and Brew, 1985) have shown remarkable similarity between β-LG and plasma retinol binding protein. The function of β-LG is tentatively thought to be the binding and transportation of retinol, and it is categorized as a member of the lipocalin super family (Åkerstrom and Lögdberg, 1990). It is also well-known that β-LG has good emulsifying properties above pH 5 (Shimizu et al., 1985). However, the emulsifying activity of β-LG decreases in the acidic pH region. Although β-LG is thought to be a valuable protein according to nutritional science, β-LG is known as a potent allergen of milk allergy, about 82% of milk allergy patients being sensitive to β-LG (Spies, 1973).

Hence, it is strongly desirable to develop a new method that would decrease the allergenicity and enhance the functional properties of β-LG. To achieve this, we have been studying on the neoglycoconjugates of β-LG. Studies on the neoglycoconjugates of proteins have been widely investigated in this decade, and various improvements to the functional properties of proteins have been reported. As far as β-LG is concerned, several studies on the conjugates of β-LG have been carried out, focusing on the improvement of solubility, heat stability, foaming properties, and emulsifying properties (Mattarella et al., 1983; Kitabatake et al., 1985; Waniska and Kinsella, 1988; Mattarella and Richardson, 1983; Akita and Nakai, 1990a,b; Bertrand-Harb et al., 1990; Creuzenet et al., 1992). We have reported that the conjugate of β-LG and carboxymethyldextran showed improved emulsifying properties and immunogenicity (Hattori et al., 1994; Nagasawa et al., 1996). We could prepare β-LG−alginic acid (ALG) conjugates by using a water-soluble carbodiimide and the Maillard reaction (Hattori et al., 1996). However, in the case of the β-LG−ALG conjugates, the conformation of β-LG decayed and the retinol binding activity was reduced. In the β-LG−ALG conjugates, the conformational decay of β-LG is thought to be due to the electrical repulsion of high-density polyanion. We thought that the conjugation of β-LG with oligosaccharides from alginic acid (ALGO) with low degree of polymerization would be effective to prevent this decay in the structure of β-LG because these oligosaccharides have low amount of anion.

In the present study, we prepared the β-LG−ALGO conjugate by the Maillard reaction. We describe the unique properties of the β-LG−ALGO and the conformational changes in β-LG brought about by conjugating with ALGO.

MATERIALS AND METHODS

Materials. Alginate lyase-lysate (ALGO, $\overline{DP} = 4$) was supplied by Meiji Seika Co. Ltd. (Tokyo, Japan). The other reagents were of special grade commercially available.

Preparation of β-LG and RCM-β-LG. β-LG (genotype AA) was isolated from fresh milk of Holstein cow supplied by the dairy farm of Tokyo University of Agriculture and Technology (Tokyo, Japan) according to the method of Armstrong et al. (1967) and was purified by ion-exchange chromatography in a DEAE-Sephacel column (2.5 i.d. × 50 cm; Pharmacia, Uppsala, Sweden). Crude β-LG was applied to the column and eluted by 0−500 mM NaCl linear gradient elution in a 0.05 M imidazole buffer at pH 6.7 at a flow rate of 1.0 mL/ min. The eluted protein was detected by the absorbance at 280 nm. The major fraction was dialyzed against distilled water and lyophilized. The purity of β-LG was confirmed by polyacrylamide gel electrophoresis (PAGE) according to the method of Davis (1964). RCM-β-LG was prepared by reducing the disulfide bonds in β-LG with 2-ME and then by carboxymethylating the free sulfhydryl groups with sodium iodoacetate as described previously (Kaminogawa et al., 1989).

Preparation of the β-LG−ALGO Conjugate. The β-LG−ALGO conjugate was prepared by the Maillard reaction. ALGO (200 mg) and β-LG (200 mg) were dissolved in 100 mL of distilled water and then lyophilized. The mixture was incubated at 50 °C at a relative humidity of 79% in a desiccator containing a saturated KBr solution for 24 h. After dialyzing against distilled water and lyophilizing, a crude β-LG−ALGO conjugate was obtained.

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Purification of the β-LG−ALGO Conjugate. Free ALGO was removed by salting-out. The crude sample was dissolved in distilled water, ammonium sulfate was added to a final concentration of 5 M, and the solution was adjusted to pH 5.8. The precipitate was recovered by centrifuging (20 000 rpm, 30 min) at 20 °C. The purified β-LG−ALGO conjugate was obtained after dialyzing against distilled water and lyophilizing.

Size Exclusion Chromatography (SEC). The molecular weight of the β-LG−ALGO conjugate was measured by SEC. A TSKgel G5000PW$_{XL}$ column (7.8 i.d. × 300 mm, Tosoh) was equilibrated with a 0.067 M phosphate buffer containing 4 M guanidine hydrochloride at pH 7.0. The β-LG−ALGO conjugate (500 μg) in 100 μL was applied to the column and eluted at a flow rate of 0.5 mL/min. The absorbance was monitored at 280 nm.

Isoelectric Focusing. Isoelectric focusing of the β-LG−ALGO conjugate was performed by using the Pharmacia-Phast System (Kramlova et al., 1986). The protein bands were detected by staining with Coomassie Brilliant Blue.

Measurement of the Retinol Binding Activity of the β-LG−ALGO Conjugate. The retinol binding activity of the β-LG−ALGO conjugate was evaluated by fluorescence titration (Futterman and Heller, 1972; Cogan et al., 1976; Hattori et al., 1993). Retinol in ethanol was added to a 2.0 mL solution in a cuvette containing 2.0 mg of protein in PBS. Small increments (5 μL at a time) of the retinol in ethanol at 3.06×10^{-4} M were added to the cuvette with a micropipet. The fluorescence was measured by a Shimadzu RF-510 instrument (Kyoto, Japan) with excitation at 330 nm and emission at 470 nm.

Fluorescence Measurement. The fluorescence of the β-LG−ALGO conjugate dissolved in PBS at 0.001% (as a protein concentration) was measured under an excitation wavelength of 283 nm by a Shimadzu RF-510 instrument (Kyoto, Japan).

Competitive Enzyme-Linked Immunosorbent Assay (ELISA). Competitive ELISA was carried out as described previously (Kaminogawa et al., 1987, 1989; Hattori et al., 1993). A β-LG solution (100 μL at 0.01%) dissolved in PBS was added to a polystyrene microtitration plate (Nunc), before the plate was incubated at 25 °C for 2 h. After the β-LG solution had been removed and the well washed three times with 125 μL of PBS−Tween, 125 μL of a 1% ovalbumin solution was added. The plate was again incubated at 25 °C for 2 h and washed. β-LG, RCM-β-LG, or the β-LG−ALGO conjugate at various concentrations (50 μL) was added to the well just before adding an mAb (50 μL), and the plate was further incubated at 25 °C for 2 h. After removing the solution and washing, 100 μL of a 0.1% solution of *p*-nitrophenyl phosphate disodium salt in a 1 M diethanolamine hydrochloride buffer (pH 9.8) was added as a substrate, and the plate was incubated for the last time at 25 °C for 30 min. The reaction was stopped by adding 20 μL of 6 M sodium hydroxide, and an assay was performed by reading the absorbance at 405 nm. The binding constants of mAb with β-LG, RCM-β-LG, and the β-LG−ALGO conjugate were calculated by the method of Hogg et al. (1987), based on the competitive and noncompetitive ELISA results.

Differential Scanning Calorimetry (DSC). The β-LG−ALGO conjugate was dissolved in PBS (pH 7.0) at a protein concentration of 5.0%. A 50 μL portion of this solution was sealed in a silver DSC cell, and DSC was conducted to determine the denaturation temperature and enthalpy as described previously (Takahashi et al., 1988; Hattori et al., 1994). DSC curves were recorded on a Seiko SSC-5020 DSC-100 instrument (Tokyo, Japan) programmed at a heating rate of 2 K/min in the temperature range from 20 to 100 °C. The reference sample was distilled water.

Evaluation of the Emulsifying Ability of the β-LG−ALGO Conjugate. The β-LG−ALGO conjugate was dissolved in McIlvaine buffer (pH 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0) or McIlvaine buffer (pH 7.0) containing 0.1 or 0.2 M NaCl to give a concentration of 0.1% (as protein). Oleic acid (0.5 mL) was emulsified with 2 mL of β-LG−ALGO conjugate solution by a Polytron PTA-7 (Kinematica, Switzerland) at 24 000 rpm for 1 min at 25 °C. The emulsion stability was measured by the absorbance at 500 nm of the emulsion 100-fold diluted with a 0.1% SDS solution (Pearce and Kinsella, 1978).

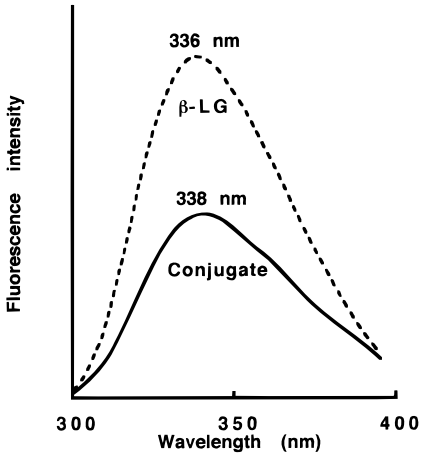

Figure 1. Intrinsic fluorescence of the β-LG−ALGO conjugate. The emission spectrum of the β-LG−ALGO conjugate was measured with an excitation wavelength of 283 nm: (- - -) β-LG; (—) β-LG−ALGO conjugate.

The emulsifying activity of the β-LG−ALGO conjugate was evaluated by spectroturbidity according to the method of Pearce and Kinsella (1978). The emulsifying activity is expressed as emulsifying activity index (EAI). EAI being calculated by the formula $EAI = 2T/\phi c$, where T (turbidity) = $2.3A/l$ (A being the absorbance at 500 nm and l (light path) = 10^{-2} m) and c is the concentration of protein (10^3 g/m^3), with ϕ (oil phase volume) = 0.2.

Evaluation of the Aggregating Ability of the β-LG−ALGO Conjugate. The aggregating ability of the β-LG−ALGO conjugate by heating was evaluated as follows. β-LG−ALGO conjugate was dissolved in a 0.067 M phosphate buffer (pH 7.0) containing 0.1 M NaCl at 5.0 mg/mL (as protein) and put into a test tube (∅ 10 mm, and 1 mm glass thickness). The conjugate solution was heated at a constant temperature (80, 90, or 100 °C) for 10, 20, 30, or 60 min. After cooling to room temperature and centrifuging (3000 rpm for 15 min), the supernatant was filtered and subjected to SEC. A TSKgel G3000PW$_{XL}$ column (7.8 i.d. × 300 mm, Tosoh) was equilibrated with a 0.067 M phosphate buffer containing 0.3 M NaCl at pH 7.0. An aliquot (70 μL) of the supernatant was applied to the column and eluted at a flow rate of 0.5 mL/min. The absorbance was monitored at 280 nm. The aggregating ability of the β-LG−ALGO conjugate was evaluated by measuring the residual native protein by SEC.

RESULTS AND DISCUSSION

Chemical Features of the β-LG−ALGO Conjugate. Isoelectric focusing was carried out to confirm covalent binding between β-LG and ALGO. The β-LG−ALGO conjugate had a relatively distributed isoelectric point (pI, 4.2−4.8), the pI value for the main component of the β-LG−ALGO conjugate being 4.55, which is lower than that of native β-LG (pI 5.2). This result strongly suggests that β-LG and ALGO were covalently bound to each other. The composition of the conjugate determined by Bradford method (Bradford, 1976) and by the phenol−sulfuric acid method (Dubois et al., 1956) indicated that the molar ratio of β-LG to ALGO in the conjugate was 1:7. The molecular weight of the β-LG−ALGO conjugate was evaluated to be 23 500 by SEC.

Structural Features of the β-LG−ALGO Conjugate. The fluorescence emission spectrum of the β-LG−ALGO conjugate is shown in Figure 1. When excited at 283 nm, native β-LG exhibited a fluorescence emission maximum at 336 nm. It has previously been clarified that the fluorescence intensity increased with

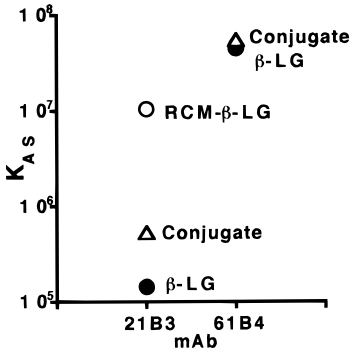

Figure 2. Binding constants of anti-β-LG mAbs to the β-LG−ALGO conjugate: (●) β-LG; (△) β-LG−ALGO conjugate; (○) RCM-β-LG.

Table 1. Thermal Characteristics of the β-LG−ALGO Conjugate[a]

	denaturation temp (°C)			denaturation enthalpy (mJ/mg)
	T_o	T_p	T_f	
β-LG−ALGO	74.2	86.5	102.5	18.2
β-LG	68.1	72.8	77.8	11.7
mixture	68.6	73.3	78.4	13.7

[a] T_o, onset temperature; T_p, peak temperature; T_f, final temperature. Mixture: mixture of β-LG and ALGO in the same ratio as that in the β-LG−ALGO conjugate.

red-shift of the wavelength for maximum emission as the conformation of β-LG changed (Kaminogawa et al., 1989; Hattori et al., 1993). In this experiment, the β-LG−ALGO conjugate exhibited the emission maximum wavelength of 338 nm. Hence, the conformation around the Trp residues (Trp19 and Trp61) of the conjugate seems to have been changed from that of native β-LG. The fluorescence intensity of the β-LG−ALGO conjugate was about 55% of native β-LG. This decrease is thought to have been due to the shielding effect by the oligosaccharide chain bound to β-LG in the conjugate (Hattori et al., 1994).

The conformational difference between the β-LG−ALGO conjugate and native β-LG was evaluated by using anti-β-LG mAbs as probes. We have previously shown that mAbs can detect the subtle conformational differences in local areas within the β-LG molecule during unfolding and refolding by determining the affinity change (Kaminogawa et al., 1989; Hattori et al., 1993). The properties of mAbs used in this experiment are summarized as follows. The epitope regions for mAbs 21B3 and 61B4 are Val15−Ile29 (β-sheet region) and Thr125−Lys135 (α-helix region), respectively. MAb 61B4 reacts preferentially to native β-LG, while mAb 21B3 reacts more strongly to RCM-β-LG (the denatured form of β-LG).

Binding constants of these mAbs to the β-LG−ALGO conjugate are shown in Figure 2. The reactivity of mAb 21B3 to the conjugate was stronger than that to the native material, while the reactivity of mAb 61B4 to the conjugate was similar to that to the native material. In the β-LG−ALGO conjugate, the conformation of Val15−Ile29 (β-sheet region) are thought to have been changed from the native form, while Thr125−Lys135 (α-helix region) is thought to have maintained native conformation. In the β-LG−ALGO conjugate, Val15−Ile29 (β-sheet region), which exists internally in the native material (Papiz et al., 1986), is thought to have been exposed. Val15−Ile29 is considered to be a hydrophobic region by adopting the hydrophilicity value proposed by Hopp and Woods (1983) to β-LG. It can be expected that

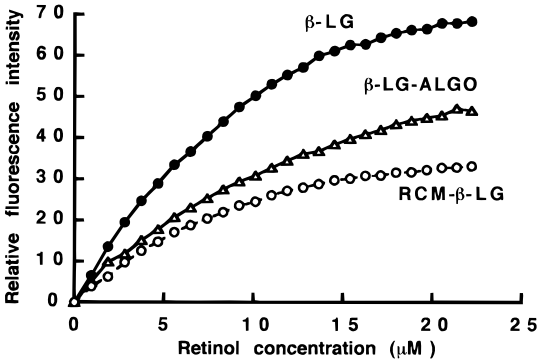

Figure 3. Retinol binding activity of the β-LG−ALGO conjugate: (●) β-LG; (△) β-LG−ALGO conjugate; (○) RCM-β-LG.

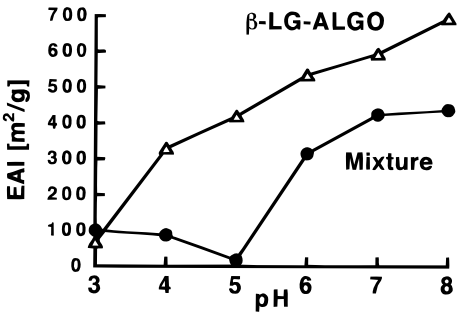

Figure 4. Emulsifying activity index of the β-LG−ALGO conjugate at different pH: (●) mixture (mixture of β-LG and ALGO in the same ratio as that in the β-LG−ALGO conjugate); (△) β-LG−ALGO conjugate.

the β-LG−ALGO conjugate would be more hydrophobic than native β-LG and may have better emulsifying ability.

Thermal stability of the β-LG−ALGO conjugate was evaluated by DSC. The thermal characteristics of the β-LG−ALGO conjugate are summarized in Table 1. ALGO was thermally inactive throughout the temperature range for the present DSC run (data not shown). The denaturation temperature (T_p) and denaturation enthalpy (ΔH) for a mixture of β-LG and ALGO in the same ratio as that of in the β-LG−ALGO conjugate are similar to the values for native β-LG. T_p for the β-LG−ALGO conjugate was about 87 °C, which is much higher than that of native β-LG. Kitabatake et al. (1985) have reported that covalent binding of gluconate to the amino groups of β-LG enhanced the solubility of β-LG at high temperatures. Conjugation of saccharides to a protein is thought to bring about an improvement in the heat stability of a protein. ΔH for the β-LG−ALGO conjugate increased to about 156% of the value for native β-LG. This increase in ΔH which indicates the wide range of thermal transition in β-LG is thought to have mainly been due to the increased structural changes in β-LG−ALGO conjugate by heating.

Retinol Binding Activity of the β-LG−ALGO Conjugate. β-LG is one of the proteins in the lipocalin superfamily, and its biological function has been proposed to be the transport and uptake of retinol into suckling animals (Åkerstrom and Lögdberg, 1990). β-LG dimer can actually bind two molecules of retinol (Fugate and Song, 1980). The possibility whether this retinol binding activity of β-LG would be maintained in the conjugate was investigated by fluorescence titration (Futterman and Heller, 1972; Cogan et al., 1976; Hattori et al., 1993). Although the retinol binding activity of β-LG was lowered a little by conjugation with ALGO, the conjugate actually had retinol binding activ-

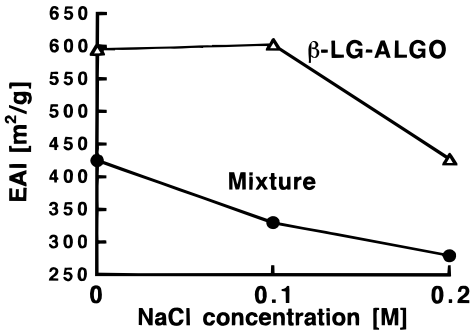

Figure 5. Emulsifying activity index of the β-LG−ALGO conjugate in the presence of NaCl: (●) mixture (mixture of β-LG and ALGO in the same ratio as that in the β-LG−ALGO conjugate); (△) β-LG−ALGO conjugate.

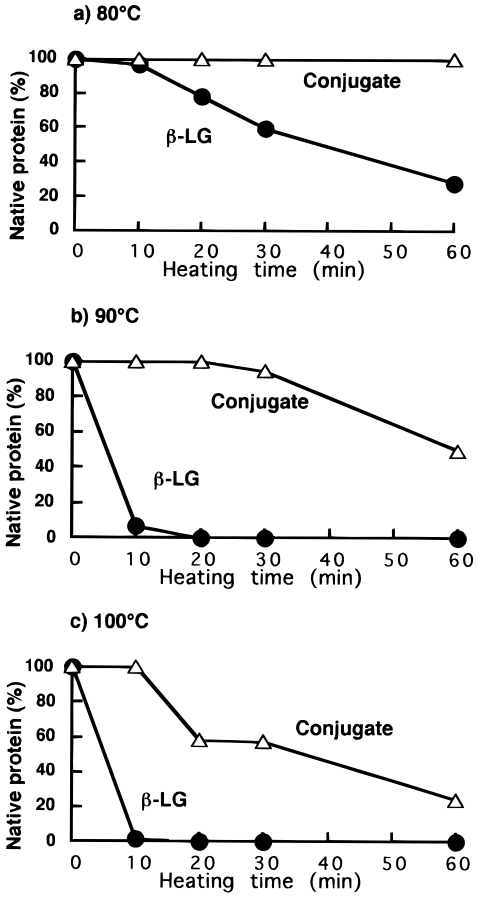

Figure 6. Aggregation of the β-LG−ALGO conjugate by heating at pH 7.0: (●) β-LG; (△) β-LG−ALGO conjugate.

ity (Figure 3). The difference in retinol binding activity is thought to have been brought about by the conformational changes in β-LG induced by conjugation with ALGO.

Emulsifying Ability of the β-LG−ALGO Conjugate. The emulsifying ability of the β-LG−ALGO conjugate at pH 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 was evaluated as the emulsifying activity index (EAI) of an emulsion of oleic acid and the β-LG−ALGO conjugate (Figure 4). The β-LG−ALGO conjugate showed better emulsifying activity than that of the native material above pH 4.0. Conjugation with ALGO is thought to have induced the exposure of hydrophobic region(s) and enhanced the flexibility of β-LG molecule that enabled the interaction with oleic acid when emulsification was carried out. The former possibility is supported by the results of our structural analysis that the highly hydrophobic region in β-LG (Val15−Ile29) was exposed at the surface by conjugating with ALGO. For the latter possibility, Shimizu et al. (1985) have suggested that the denaturability (flexibility) plays an important role in the emulsifying ability of β-LG. By conjugating with ALGO, the β-LG portion is thought to have been more susceptible to surface denaturation than native β-LG due to the high mobility of ALGO (Hattori et al., 1994). In addition to these factors, since ALGO is rich in net charge, the β-LG−ALGO conjugate covering oil droplets may inhibit their flocculation by electrostatic repulsion.

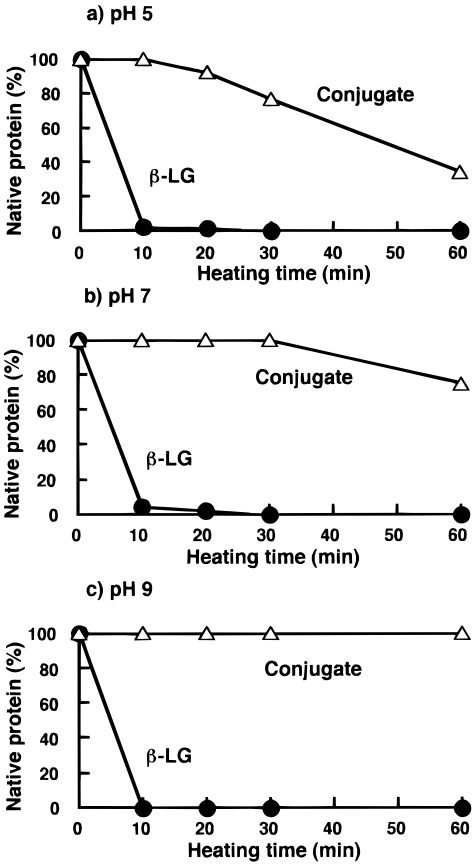

Figure 7. Aggregation of the β-LG−ALGO conjugate at different pH values: (●) β-LG; (△) β-LG−ALGO conjugate. Aggregation of the β-LG−ALGO conjugate and β-LG was evaluated at 90 °C.

The emulsifying ability of the β-LG−ALGO conjugate in the presence of NaCl at pH 7.0 was evaluated as the EAI value of an emulsion of oleic acid and the β-LG−ALGO conjugate (Figure 5). The β-LG−ALGO conjugate showed better emulsifying activity than β-LG because the conjugate was rich in net charge that displayed a strong protective function against shielding of the electrical charge in the emulsifier.

Aggregating Property of the β-LG−ALGO Conjugate. Changes in the aggregating property of β-LG by conjugation with ALGO were investigated. It is well-known that β-LG aggregates after heating and that this aggregation occurs more easily in the alkaline pH region than in the acidic pH region (McSwiney et al., 1994). β-LG−ALGO and β-LG were each dissolved in a 0.067 M phosphate buffer (pH 7.0) containing 0.1 M NaCl at 5.0 mg/mL (as protein), and each solution was heated at 80, 90, or 100 °C. After cooling to room temperature, centrifuging, and filtering, each supernatant was subjected to SEC to evaluate the residual native protein (Figure 6). Aggregation of β-LG occurred at 80 °C, while the β-LG−ALGO conjugate did not aggregate. When

heating was done at 100 °C, aggregation of the conjugate was depressed. The effect of pH value on the aggregating property of β-LG−ALGO and β-LG was also evaluated after heating at 90 °C in pH range of 5−9 (Figure 7). In the case of β-LG, almost all portions aggregated after heating for 10 min. However, in the case of the β-LG−ALGO conjugate, aggregation was depressed at all pH values. It was clarified that the aggregating property of β-LG was depressed by its conjugation with ALGO. These results are thought to have been due to the enhanced thermal stability, steric hindrance, and electrostatic repulsion brought about by conjugation with ALGO.

Concluding Remarks. In this study, we could prepare the β-LG−ALGO conjugate by using the Maillard reaction. Although conformational changes in β-LG occurred by its conjugation with ALGO, the emulsifying activity of β-LG was improved and aggregation was depressed. It is strongly hoped that the β-LG−ALGO conjugate prepared in this study will contribute to developing the utilization of these abundant resources as food additive.

ABBREVIATIONS USED

β-LG, β-lactoglobulin; RCM-β-LG, reduced and carboxymethylated β-lactoglobulin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PBS−Tween, PBS containing 0.05% Tween 20; 2-ME; 2-mercaptoethanol; ALGO, oligosaccharide from alginic acid (alginate lyase-lysate); $\overline{DP}$, degree of polymerization.

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