Review

Functional Improvements in Food Proteins in Multiple Aspects by Conjugation with Saccharides: Case Studies of β-Lactoglobulin-Acidic Polysaccharides Conjugates

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Many studies on the conjugates of proteins and saccharides (neoglycoproteins) have been carried out over the past 20 years to improve the function of proteins. However, most have been carried out by attachment or elimination of low-molecular weight molecules for single purposes. The structure-function relationship of the conjugates has not been well understood, and little is known about the improvement of proteins by conjugation with polysaccharides. We sought to achieve functional improvements in food proteins in multiple aspects by conjugation with charged polysaccharides. We chose bovine β-lactoglobulin (β-LG) as the target protein for improvement of the function and attached carboxymethyl dextrans (CMDs) of different molecular weight (10, 40, 70 and 162 kDa) covalently. After conjugation and purification, we explored the structure of the β-LG-CMD conjugates to prove that we could prepare conjugates while maintaining the native-like structure of protein molecule. By conjugation with CMDs, enhancement of thermal stability, improvement in the emulsifying properties and reduction of immunogenicity of β-LG could be achieved. Increases in the CMD content and net charge were viewed as the major factors in the improved emulsifying properties. Conjugation with high saccharide content using polysaccharides of higher molecular weight is believed to be effective in reduced immunogenicity of β-LG.

Keywords: β-lactoglobulin, carboxymethyl dextran, lipocalin, protein conjugation, functional improvement, emulsification, reduced immunogenicity.

1. Introduction: What is neoglycoprotein?

Among natural resources, there are many hybrid compounds including saccharides, so called glycoconjugates, such as glycoproteins, glycopeptides and glycolipids. Protein-saccharide conjugates mentioned in this review originally do not exist in nature and are artificially prepared by chemical modification techniques. Such conjugates are designated neoglycoconjugates (Kawaguchi et al., 1981).

Studies on the chemical modification of proteins started in the 1950s. Pioneering works in this field were carried out for identification of amino acids responsible for the function of proteins and structural analyses of proteins in aqueous solutions, and there were many findings on the structure-function relationships by the early works. Proteins also have been important for human beings as materials for various purposes in food, clothing and shelter. Applications for their use have been developed on the basis of these structure-function relationships. Because the structure of each protein is very complicated and there are many varieties, the understanding of proteins is far from perfect and their current applications as materials depend on the traditional blend techniques. Although proteins are widely utilized as representative biopolymers, materials with new functionality have become strongly desired in recent years to cope with the increasing variety of demands. Effective utilization of unutilized or low-utilized proteins has also captured wide interest, and much attention has been paid to the preparation of proteins with new functions, and in particular, protein modification has captured wide interest. In the late 1970s, new research projects to prepare novel protein conjugates with saccharides and synthetic polymers started. The purpose of these studies was to enhance the merits and improve the defects of proteins and to apply such conjugates for use in such fields as medical, pharmaceutical, engineering and agricultural sciences. Representative protein conjugates with synthetic polymers include the conjugates of proteins and polyethylene glycol (Inada et al., 1995; Veronese, 2001) and immobilized enzymes (Taylor, 1991; Liang et al., 2000). On the other hand, many studies to improve the functions of proteins by conjugation with various saccharides were also conducted: alkylation (Nieto et al., 1983; Sen et al., 1981) esterification (Mattarella & Richardson, 1983), amidation (Mattarella et al., 1983), deamidation (Matudomi et al., 1985; Wu et al., 1976), lipophilization (Aki & Nakai, 1990a; 1990b), covalent attachment of gluconic acid (Kitabatake et al., 1985), cross-linking with transfutaminate (Nio et al., 1985; Motoki et al., 1987) and so on. An overview of the studies on protein-saccharide conjugates shows that most of the studies have been carried out by attachment or elimination of low-molecular weight molecules. Little is known about the improvement of proteins by conjugation with polysaccharides. Although these studies spotlighted functional improvements in proteins, structural studies of the conjugates are still inadequate. Because protein structure is essential for the expression of functions, accumulation of structural studies of the conjugates is essential for designing novel conjugates with novel or...
improved functions. In addition, most of these studies to date have been carried out for a single purpose.

We thought that conjugation of protein with a polymer is more effective to improve protein functions than conjugation with low-molecular weight components. In particular, multiple improvements to protein functions can be expected by conjugating with a charged polymer due to the difference in molecular weight or charge of the chemical species conjugated. By exploring the structure of the conjugates after preparation and purification, fundamental insights on the functional improvements of proteins by conjugation methods can be obtained. Broad structural information of novel conjugates is important to establish universal methods for functional improvement of useful proteins.

In this review, I introduce our work and discuss the possibility of functional improvements in food proteins in multiple aspects.

2. Designing of functional improvements in food proteins in multiple aspects

As a target protein, we chose the milk protein β-lactoglobulin (β-LG). β-LG is a predominant whey protein of 18 kDa with two disulfide bridges, as well as free cysteine (McKenzie, 1971). Many physicochemical and physiologlcal studies on this protein have been carried out: its higher ordered structure (Townend et al., 1967; Papiz et al., 1986; Gu & Brady, 1992; Papiz et al., 1982; Brownlow et al., 1997; Kuwata et al., 1999), unfolding (Alexander & Pace, 1971; Sawyer et al., 1971, Kaminogawa et al., 1989; Laligant et al., 1994; Dufour et al., 1994; Katou et al., 2001), refolding (Hattori et al., 1993; Creamer, 1995; Hamada et al., 1996; Ragona et al., 1999; Forge et al., 2000; Kuwata et al., 2001), polymerization and gelation behavior (Mulvihill & Kinsella, 1987; Laligant et al., 1991; Poegeding et al., 1992), foaming and emulsifying properties (Shimizu et al., 1985; Waniska & Kinsella, 1988), immunological response (Kurisaki et al., 1982; Kurisaki et al., 1985; Takahashi et al., 1988; Takahashi et al., 1990; Tsuji et al., 1993; Totsuka et al., 1997; Mantyjarvi et al., 2000) and so on. This protein consists of nine antiparallel β-sheets and one α-helix to form a calyx shaped β-barrel structure, and it is categorized as a member of the lipocalin superfamily (Flower, 1996; Sawyer & Kontopidis, 2000). Although the function of β-LG still remains unclear, it is tentatively considered to be the binding and transportation of small hydrophobic ligands such as retinol, fatty acids, and so on (Pérez & Calvo, 1995).

β-LG is believed to be a valuable protein in terms of food science because of its various useful functional properties such as emulsifying, foaming and gelling properties (Shimizu et al., 1985; Waniska & Kinsella, 1988; Poegeding et al., 1992) as well as plenty of essential amino acids (McKenzie, 1971). However, it does have some defects, the most serious being that it is a potent allergen of milk allergy: about 82% of milk allergy patients are sensitive to β-LG (Spies, 1973). And, so far as its functional properties are concerned, it loses emulsifying properties in the acidic pH region or in the presence of salt. So 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 planned the preparation of a neoglycoconjugate of β-LG.

To improve various aspects of its functions, we planned to achieve simultaneously the enhancement of heat stability, improvement of emulsifying properties under unfavorable conditions and reduction of immunogenicity while maintaining the whole structure and retinol binding ability of β-LG. Our strategy for achieving this is as follows. 1) Improvement of emulsifying properties under unfavorable conditions: By conjugation with polysaccharides, the protein would be endowed with hydrophilicity and its amphiphilic property would be enhanced. If charged polysaccharides were employed for conjugation, ion-exchange activity would be given to the protein, which would lead to better emulsifying properties in the presence of salt. Endowment of charge would bring about a change in isoelectric point which would contribute to the improvement of emulsifying properties in the acidic region. 2) Reduction of allergenicity: Low allergenicity would be gained by conjugation of a protein with materials with low antigenicity and immunogenicity. Saccharides were thought to be suitable for this purpose, particularly polysaccharides which would be more effective than conjugation of a protein with low-molecular-weight saccharides because polysaccharides would cover the epitope regions of the allergens more effectively (Sehon, 1982). 3) Enhancement of heat stability: When proteins denature in aqueous solution, water molecules around their surface are responsible for transferring heat flow. To hydrate and structurize such water molecules by conjugation with hydrophilic molecules like polysaccharides was expected to be effective in enhancing the heat stability of proteins.

Because many kinds of saccharides could be candidates for conjugation, we considered the following points: 1) molecular weight, 2) charge, 3) homogeneity, and 4) antigenicity and immunogenicity. Charged polysaccharides which have been scarcely used for conjugation so far were thought to be suitable to achieve multiple functional improvements. Homogeneity of polysaccharides was also important because proteins which are originally homogeneous would become heterogeneous after conjugation with polysaccharides. In addition, polysaccharides with low antigenicity and immunogenicity were preferable for improving the immunological property of proteins. Hence, we chose carboxymethyl dextran (CMD) for conjugation with β-LG. We used CMDs of different molecular weight (10, 40, 70 and 162 kDa) to evaluate the effect of molecular weight on the functional changes in β-LG. These CMDs were named CMD 10, CMD 40, CMD 70 and CMD 162, respectively.

As methods to improve protein functions, chemical modification and DNA recombinant technology are available. We chose chemical modification to prepare neoglycoproteins because there are many established methods and a high yield of products could be expected. Since our purpose was to create novel protein-polysaccharide conjugates with new or improved functions while retaining the native-like protein structure, the technique used to conjugate protein with polysaccharides should be a moderate whole structure and retinol binding ability of β-LG. Our strategy for achieving this is as follows. 1) Improvement of emulsifying properties under unfavorable conditions: By conjugation with polysaccharides, the protein would be endowed with hydrophilicity and its amphiphilic property would be enhanced. If charged polysaccharides were employed for conjugation, ion-exchange activity would be given to the protein, which would lead to better emulsifying properties in the presence of salt. Endowment of charge would bring about a change in isoelectric point which would contribute to the improvement of emulsifying properties in the acidic region. 2) Reduction of allergenicity: Low allergenicity would be gained by conjugation of a protein with materials with low antigenicity and immunogenicity. Saccharides were thought to be suitable for this purpose, particularly polysaccharides which would be more effective than conjugation of a protein with low-molecular-weight saccharides because polysaccharides would cover the epitope regions of the allergens more effectively (Sehon, 1982). 3) Enhancement of heat stability: When proteins denature in aqueous solution, water molecules around their surface are responsible for transferring heat flow. To hydrate and structurize such water molecules by conjugation with hydrophilic molecules like polysaccharides was expected to be effective in enhancing the heat stability of proteins.

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![Fig. 1. Expected reaction mechanism for β-LG-CMD conjugate formation with carbodiimide. EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.](image-url)
one. In our research, we chose water-soluble carbodiiimide for conjugation between β-LG and CMDs. As shown in Fig. 1, this substance activates carboxyl groups of CMDs and binds amino groups of proteins under a mild condition.

3. Functional changes in β-LG by conjugation with CMD of 10 kDa

Possible functional improvements were evaluated using the conjugates of β-LG and CMD of low-molecular weight (10 kDa) (Hattori et al., 1994; Nagasawa et al., 1996a). In the preparation of these conjugates, various reaction conditions of β-LG, CMD and EDC (ratio of mixing amount, order of addition, reaction temperature and reaction pH) were examined, and the two methods shown in Fig. 2 were established. In method 1, EDC exceeded the total amount of carboxyl residues of β-LG and CMD, while in method 2, the molar ratio among amino groups of β-LG, EDC and carboxyl groups of CMD was 1 : 10 : 10. Because the ratio of CMD to β-LG in method 2 was greater than that in method 1, we expected that the conjugate obtained in the method 2 would contain more saccharide than that obtained in method 1. Obtained conjugates were named Conj. 10A and Conj. 10B after the molecular weight of CMD used for conjugation. Each conjugate was purified by salting-out with (NH₄)₂SO₄ and DEAE-chromatography. Formation of the β-LG-CMD conjugates was confirmed by SDS-PAGE and isoelectric focusing. Properties of the β-LG-CMD conjugates are summarized in Table 1.

The composition of the two kinds of conjugate determined by their absorbance at 280 nm and by the phenol-sulfuric acid method indicated that the molar ratio of CMD to β-LG was about 5 : 1 for Conj. 10A and 2 : 1 for Conj. 10B. The molecular weight of each β-LG-CMD conjugate was evaluated to be 280 kDa and 250 kDa for Conj. 10A and Conj. 10B, respectively, by size exclusion chromatography. Because the molecular weights of β-LG and CMD are 18,400 and 10,000, Conj. 10A and Conj. 10B were thought to be composed of 14 molecules of β-LG and 3 molecules of CMD, and of 10 molecules of β-LG and 5 molecules of CMD, respectively.

We also explored the conformation of the conjugates by four methods: retinol-binding activity measurement, CD spectra, intrinsic fluorescence and ELISA with monoclonal antibodies (mAbs) (Kaminogawa et al., 1987; 1989; Hattori et al., 1993). Retinol-binding activity of each conjugate was similar to that of β-LG. And the structure concerning retinol binding, which includes 19Trp and 70Lys, was determined to maintain native structure. This result suggested that the whole structure of β-LG had not collapsed. By CD spectra measurement, slight loss in secondary structure was observed with each conjugate. Trp fluorescence of β-LG was lowered after conjugation with CMD, indicating that the surface of β-LG was shielded by CMD in the conjugates. This shielding effect was notable with Conj.10B which contained more saccharides than Conj. 10A. By ELISA with anti-β-LG mAbs, conformation around 125Thr, 135Lys, which is α-helix region, maintained the native structure, while subtle conformational changes around 15Val-29Ile, which is β-sheet region, were observed. This region was considered to be exposed after conjugation with CMD. In a word, although a slight collapse in secondary structure was observed, each conjugate maintained its native protein structure as we had expected.

We investigated the functional improvements in β-LG by conjugation with CMD of 10 kDa in the three aspects of thermal stability, emulsifying ability and immunogenicity. The thermal stability was evaluated by differential scanning calorimetry (DSC). Denaturation temperature for the conjugates was about 87°C and 92°C, which are much higher than that of native β-LG. Kita-batake et al. (1985) reported that covalent binding of gluconate

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**Table 1.** Properties and function of the β-LG-CMD conjugates.

<table>
<thead>
<tr>
<th>Structural features</th>
<th>β-LG</th>
<th>Conj. 10A</th>
<th>Conj. 10B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mw</td>
<td>18 kDa</td>
<td>280 kDa</td>
<td>250 kDa</td>
</tr>
<tr>
<td>β-LG : CMD (mole)</td>
<td>5.2</td>
<td>1 : 5</td>
<td>2 : 1</td>
</tr>
<tr>
<td>pI</td>
<td>4.7</td>
<td>4.8</td>
<td>maintained</td>
</tr>
<tr>
<td>Retinol-binding activity</td>
<td>maintained</td>
<td>maintained</td>
<td></td>
</tr>
<tr>
<td>Intrinsic fluorescence</td>
<td>native state</td>
<td>native state</td>
<td></td>
</tr>
<tr>
<td>Conformation around Trp</td>
<td>native state</td>
<td>native state</td>
<td></td>
</tr>
<tr>
<td>Fluorescence intensity</td>
<td>lowered</td>
<td>lowered</td>
<td></td>
</tr>
<tr>
<td>CD spectrum</td>
<td>changes in β-sheet structure</td>
<td>changes in β-sheet structure</td>
<td></td>
</tr>
<tr>
<td>ELISA with mAbs</td>
<td>15Val-29Ile</td>
<td>subtle conformational change (exposed)</td>
<td>subtle conformational change (exposed)</td>
</tr>
<tr>
<td>125Thr, 135Lys</td>
<td>native state</td>
<td>native state</td>
<td></td>
</tr>
<tr>
<td>Function</td>
<td>Denaturation temperature</td>
<td>72.6°C</td>
<td>86.5°C</td>
</tr>
<tr>
<td>Emulsifying ability</td>
<td>in the presence of salt</td>
<td>lost</td>
<td>improved</td>
</tr>
<tr>
<td>in the acidic region</td>
<td>lost</td>
<td>improved</td>
<td>improved</td>
</tr>
<tr>
<td>Immunogenicity</td>
<td>reduced</td>
<td>reduced</td>
<td></td>
</tr>
<tr>
<td>Novel immunogenicity</td>
<td>emerged</td>
<td>emerged</td>
<td></td>
</tr>
</tbody>
</table>
The emulsifying ability of the \( \beta \)-LG-CMD conjugates in the neutral pH range was evaluated as the emulsion stability of an emulsion of oleic acid and each \( \beta \)-LG-CMD conjugate (Pearce & Kinsella, 1978). Figure 3 shows the stability of these emulsions as a function of time. The emulsions with each CMD conjugate were more stable than that with \( \beta \)-LG alone (Kinsella, 1978). Figure 3 shows the stability of these emulsions.

![Fig. 3. Emulsion stability of O/W emulsions prepared with the \( \beta \)-LG-CMD conjugates. 0.5 ml of oleic acid was emulsified with 2 ml of each \( \beta \)-LG-CMD conjugate solution (0.01% as a protein) at pH 7.0 at 25°C. Emulsion stability was measured by the turbidity at 500 nm of the emulsion 200 min after emulsification.](image)

The effect of pH on the emulsifying ability of the \( \beta \)-LG-CMD conjugates was evaluated in BALB/c, C57BL/6 and C3H/He mice by measuring the reactivity of antisera with antigen (\( \beta \)-LG, RCM-\( \beta \)-LG, Conj. 10A & Conj. 10B) by noncompetitive ELISA (Hattori et al., 2000a). The anti-\( \beta \)-LG antibody response was markedly low in the three strains of mice immunized with each \( \beta \)-LG-CMD conjugate (Fig. 4a). Effective reduction in immunogenicity of \( \beta \)-LG was observed with Conj. 10B. When each \( \beta \)-LG-CMD conjugate was used as the coating antigen on the solid phase to evaluate the productivity of the specific antibody for each conjugate (Figs. 4b, c), both anti-Conj. 10A antisera and anti-Conj. 10B antisera showed a higher antibody titer than anti-\( \beta \)-LG antisera. These results indicate the emergence of novel immunogenicity in \( \beta \)-LG as a result of conjugation with CMD. The conformational changes in \( \beta \)-LG due to conjugation with CMD may have brought about new epitopes. Berzofsky (1985) reported that the hydrophobic region tended to become an epitope. New epitopes may have emerged as a result of exposure of the hydrophobic region, buried in the native state, upon conjugation with CMD. However, the decrease in immunogenicity of \( \beta \)-LG observed after conjugation with CMD surpassed the emergence of novel immunogenicity.

4. Role of the polysaccharide content and net charge on high emulsifying ability of of \( \beta \)-LG-CMD conjugates

CMDs of different molecular weight (40, 70, 162 kDa) were conjugated with \( \beta \)-LG to investigate the mechanism of improvement in emulsifying ability of \( \beta \)-LG by such conjugation (Nagawa et al., 1996b). The \( \beta \)-LG-CMD conjugates with different saccharide content and net charge were prepared using method 2 as in preparation of Conj. 10B. The weight ratios (\( \beta \)-LG : CMD) in the conjugates were about 1 : 1, 1 : 1.5 and 1 : 2 for Conj. 40, Conj. 70 and Conj. 162. After reacting with CMD of higher molecular weight, CMD content was higher in the conjugates. The pl for Conj. 40, Conj. 70 and Conj. 162 was 4.54, 4.45 and 4.20, respectively, the degree of shift of pl increasing with increasing CMD content of the conjugates.

The effect of NaCl on the emulsifying ability of the \( \beta \)-LG-CMD conjugates was evaluated by measuring the absorbance 30 min after emulsification at pH 7.0 (Fig. 5). \( \beta \)-LG almost lost its emulsifying ability in the presence of 0.2 M NaCl; in contrast, the \( \beta \)-LG-CMD conjugates maintained their ability at a high level in the presence of 0.2 M NaCl, and Conj. 162 retained a high emulsifying ability even in the presence of 0.5 M NaCl.

The effect of pH on the emulsifying ability of the conjugates...
was also evaluated by measuring the absorbance 30 min after emulsification (Fig. 6). β-LG showed good emulsifying ability in the neutral pH range, but completely lost this ability at pH 4.0 and 5.0; the β-LG-CMD conjugates, on the other hand, showed very high ability even at these pH values. This was especially seen at pH 5.0, where they maintained an emulsifying ability similar to that of β-LG at pH 7.0. Conj. 40, Conj. 70 and Conj. 162 maintained higher emulsifying ability than the conjugates with CMD of 10 kDa (Conj. 10A and Conj. 10B), described in the previous section (Nagashawa et al., 1996a).

The emulsifying ability of β-LG under unfavorable conditions (in the presence of salt and in the acidic pH region) could be markedly improved by conjugating with CMD of different molecular weights. To analyze emulsifying properties of the β-LG-CMD conjugates in detail, the results of those conjugates including Conj. 10A and Conj. 10B were compared with others. The addition of hydrophilicity, the negative charge and the conformational change of β-LG by conjugation with CMD could be the reasons the β-LG-CMD conjugates showed much higher emulsifying ability than β-LG did under unfavorable conditions.

The relationship between the emulsifying ability at pH 7.0 and CMD contents of the β-LG-CMD conjugates is shown in Fig. 7. The emulsifying properties of the conjugates were evaluated by the EAI (emulsifying activity index) value (Fig. 7a) and the emulsion stability 30 min after emulsification (Fig. 7b). From these plots, it was found that the CMD content had a high positive correlation with both indices ($r = 0.991$, Fig. 7a; $r = 0.994$, Fig. 7b). This strongly suggested that addition of the physical properties of CMD (hydrophilicity, negative charge, viscosity and so on) greatly contributed to the improved emulsifying ability of β-LG; in particular, the addition of hydrophilicity was believed to be one of the most important factors enhancing this ability. When an emulsion was prepared with a β-LG-CMD conjugate, the hydrophobic regions of the β-LG molecule would be adsorbed to the surface of oil droplets, while the CMD chains would be oriented to the aqueous phase and stabilize the oil droplets by hydration with the surrounding water. This assumption is suggested by electron microscopic views of the exterior surface of human milk fat globules (Buchheim, 1986), where polysaccharide chains of glycoprotein adsorbed to the surface of the milk fat globules appear to orient to the aqueous phase.

The addition of a negative charge from CMD can be expressed by pl values of the β-LG-CMD conjugates. Figure 8 shows the relationship between the pl value for each conjugate and the emulsifying ability in the presence of NaCl at pH 7.0 as monitored by EAI (Fig. 8a), and the emulsion stability 30 min after emulsification (Fig. 8b). In relation to EAI, it was found that pl had high correlation with the emulsifying activity among all β-LG-CMD conjugates ($r = 0.944$ (0 M NaCl), $r = 0.981$ (0.2 M NaCl), and $r = 0.745$ (0.5 M NaCl)). However, some plots for β-LG were off the straight lines in the presence of 0.2 M and 0.5 M NaCl, since the emulsion prepared with β-LG broke down quickly (Fig. 8a). It was found that pl was also highly correlated with the emulsion stability (Fig. 8b); $r = 0.990$ (0 M NaCl), $r = 0.998$ (0.2 M NaCl), and $r = 0.999$ (0.5 M NaCl). Since the emulsion was prepared at pH 7.0, the net charge of the emulsifier would increase with decreasing pl value. Accordingly, it is suggested that the net charge of the emulsifier played an important role in displaying higher emulsifying ability. The increase in net charge of protein by conjugation with CMD is thought to have inhibited the flocculation of oil droplets by electrostatic repulsion.

The relationship between the difference in molar ellipticity at 216 nm for each conjugate and β-LG and the emulsifying activity (EAI) was plotted to examine the effect of the conformational change. The difference in molar ellipticity at 216 nm is considered to reflect the degree of conformational change in the β-sheet region. However, high correlation between the conformational change in the β-sheet region and the emulsifying properties was not found.

Addition of the physical properties of CMD polysaccharide is believed to have contributed to the improved emulsifying properties of β-LG, rather than the effect of the conformational change; increases in CMD content and net charge are therefore the major
factors in these improved properties. Conjugation with CMD of high molecular weight which led to the increase in polysaccharide content and net charge was very effective in improving the emulsifying ability of β-LG under unfavorable conditions.

5. Reduced immunogenicity of β-LG by conjugation with CMD of different molecular weight

As described, the immunogenicity of β-LG could be reduced by conjugation with CMD of 10 kDa, however, novel immunogenicity occurred in both Conj. 10A and Conj. 10B. Because Conj. 10B with a greater amount of saccharide whose surface was effectively covered by CMD showed an effective reduction in immunogenicity, the conjugation method to bring about effective covering of the surface of β-LG by CMD was considered promising to achieve reduced immunogenicity without emergence of novel immunogenicity. To cover the surface of a protein molecule effectively, conjugation of polysaccharides with higher molecular weight was predicted to be effective. Hence, we prepared β-LG-CMD conjugates with CMDs of different molecular weight (40 and 162 kDa) on a large experimental scale (Kobayashi et al., 2001). Obtained conjugates were named Conj. 40 and Conj. 162, and the molar ratio of β-LG to CMD was 8 : 1 and 7 : 1, respectively. Because these conjugates had similar saccharide content, they were appropriate to use in evaluating the influence of the molecular weight of polysaccharides used in preparation of conjugates on the immunological properties of proteins after conjugation. The results of structural analyses clarified that the surface of β-LG in each conjugate was effectively covered by CMD without great disruption of native conformation, this was particularly true of the surface of Conj. 162.

Fig. 7. Relationship between the emulsifying properties in the presence of NaCl at pH 7.0 and pI of the β-LG-CMD conjugates. ●, 0 m NaCl; ■, 0.2 m NaCl; ▲, 0.5 m NaCl. The emulsifying properties of the β-LG-CMD conjugates were evaluated by EAI as the emulsifying activity (a) and by the absorbance 30 min after emulsification as the emulsifying stability (b).

Fig. 8. Relationships between the CMD content and emulsifying properties at pH 7.0, and pI values of the β-LG-CMD conjugates. The emulsifying properties of the β-LG-CMD conjugates were evaluated by EAI as the emulsifying activity (a) and by the absorbance 30 min after emulsification as the emulsifying stability (b).
To evaluate immunogenicity of the β-LG-CMD conjugates, three strains of mice (BALB/c, C57BL/6 and C3H/He) were immunized with β-LG or the conjugates (Conj. 40 and Conj. 162) and the reactivity of obtained antisera was evaluated by non-competitive ELISA. As shown in Fig. 9a, the anti-β-LG antibody response was low in the mice immunized with each β-LG-CMD conjugate. The reduction of immunogenicity was more effective in the case of Conj. 162 than in the case of Conj. 40. Emergence of novel immunogenicity after conjugation with CMD was also investigated in an assay where each β-LG-CMD conjugate was applied as a coating antigen on the solid phase (Fig. 9b and c). Both anti-Conj. 40 antisera and anti-Conj. 162 antisera showed similar antibody titer to the anti-β-LG antisera and no statistically significant difference was observed. Novel immunogenicity in β-LG did not emerge after conjugation with CMDs of 40 and 162 kDa.

In our immunization system, anti-β-LG antisera showed higher affinity to the denatured form of β-LG (RCM-β-LG) than to the native material in all mouse strains tested, despite their being elicited by immunization with native β-LG. Anti-β-LG antisera were believed to recognize linear epitopes rather than conformational epitopes. Thus, it is important to clarify the linear epitope profiles of β-LG and the β-LG-CMD conjugates and to learn how immunogenicity of β-LG was reduced by conjugation with CMD. The B cell epitopes of β-LG and the β-LG-CMD conjugates was analyzed by ELISA using overlapping 15-mer multipin peptides (Fig. 10). The linear epitope profiles of the conjugates were proven to be similar to those of β-LG in the three strains of mice, whereas the antibody response to the epitopes was reduced. The results clearly demonstrated the reduction of the immunogenicity of β-LG by conjugation with CMD. In Fig. 10, plausible carbohydrate-binding sites (47Lys, 60Lys, 77Lys, 100Lys and/or 101Lys and 138Lys) are indicated with triangles (Morgan et al., 1997; Leonil et al., 1997; Fogliano et al., 1998; Hattori et al., 2000a; Siciliano et al., 2000). The response to the epitope regions in the vicinity of these Lys residues in each conjugate prepared in our study was lowered in the three strains of mice. These results showed that one mechanism responsible for reduced immunogenicity by conjugation with CMD was the shielding of the epitopes in β-LG by CMD which allows the epitopes to escape recognition by the immune system.

In conclusion, the conjugation with CMD of higher molecular weight was effective to reduce the immunogenicity of β-LG without inducing novel immunogenicity, and the effective shielding of epitopes by CMD was thought responsible for the reduced immunogenicity. These, together with the results obtained earlier (Hattori et al., 2000a), indicate that conjugation with high saccharide content using polysaccharides of higher molecular weight is most effective in reducing immunogenicity of proteins.

6. Concluding remarks

As shown in this review, multiple functional improvements in β-LG could be achieved by conjugation with CMDs of different molecular weight. Application of these research results to food is
our goal. However, the conjugates prepared by EDC cannot be used for food because of safety and legal regulations. Whether such conjugates are practically applicable to food or not is a very important point. However, we believe that establishment of a scientific and technological basis to achieve functional improvements in food proteins is currently very important before their practical use. To apply novel conjugates as food materials, the safety of conjugates should be guaranteed and the cost for their preparation should be solved. Safe methods to prepare conjugates should also be developed; in this context, the Maillard reaction and enzymatic reactions are promising. Kato et al. achieved many functional improvements in lysosome by conjugation with polysaccharides (dextran, galactomannan and xylloglucan) by the Maillard reaction: enhanced thermal stability, improved emulsifying properties and antimicrobial properties (Kato & Kobayashi, 1991; Nakamura et al., 1991; 1992a). They also improved the solubility of wheat gluten and antioxidative activity of ovalubumin by conjugation with dextran or galactomannan (Kato et al., 1991; Nakamura et al., 1992b). We also prepared conjugates of β-LG with alginic acid and alginic lyase-lysatase by the Maillard reaction and improved thermal stability, emulsifying properties and aggregating property of β-LG (Hattori et al., 1996; 1997). As a conjugation method with enzymatic reactions, Longo and Combes (1995) used levansucrase to lengthen glycosidic chains after the attachment of sucrose to lysozyme with α-Lac1996; 1997). As a conjugation method with enzymatic reactions, Longo and Combes (1995) used levansucrase to lengthen glycosidic chains after the attachment of sucrose to lysozyme with cyanoagen bromide. Development of new conjugation methods using enzymes is also desirable. Choice of saccharides for conjugation is very important and there are many possibilities. We have also achieved various functional improvements in β-LG by conjugation with cationic saccharides and carboxymethyl cyclodextrin (Hattori et al., 2000b; 2000c). The conjugation method described in this review is superior in that it enables multiple functional improvements such as improvements in functional properties and reduced immunogenicity of proteins simultaneously. Further studies on the mechanisms responsible for the improved functions by this method will lead to practical use of protein conjugates. I hope that our studies will contribute to the development of novel foods with new functions.

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References


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