**Note**

Preparation and Immunological Characterization of β-Lactoglobulin-Amylose Conjugate

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β-Lactoglobulin (BLG), a major allergen of cow’s milk, was conjugated with the N-hydroxysuccinimide ester of the amylose-glycylglycine adduct (AG-ONSu) to reduce its immunogenicity, and the biochemical and immunological properties of the resulting conjugate (AG-BLG) were studied. The conjugate was prepared by modifying BLG with AG-ONSu, and was purified in a Sephadex G-100 column. The analytical data for AG-BLG indicated that 10.5 moles of AG-ONSu, with a mean molecular weight of 2,800, was covalently attached to the amino groups of the BLG molecule. Conjugation with AG-ONSu greatly decreased the reactivity of BLG with anti-BLG polyclonal antibodies owing to its shielding action for epitopes on the protein’s surface. These findings suggest that AG-ONSu can be used advantageously to suppress the hypersensitivity mediated by IgG antibodies in milk allergy.

**Key words:** β-lactoglobulin; immunogenicity; amylose; protein-carbohydrate conjugate

We have conjugated bovine β-lactoglobulin (BLG), a major allergen in cow’s milk, a relatively small protein of 162 residues with a molecular weight of 18.4 kDa,¹ with an active dextran derivative under mild conditions to reduce its immunogenicity and to produce a low-allergenic milk.² In the resulting conjugate (DG-BLG), the active dextran derivative greatly decreased the reactivity of BLG with anti-BLG polyclonal antibodies and suppressed their production in vivo owing to its shielding action for epitopes on the protein’s surface.

However, given the fact that Streptococcus mutans is a major pathogen in human dental caries,³⁻⁵ DG-BLG might facilitate the development of dental caries. S. mutans has enzymes that polymerize glucose, identified from sucrose, and add branch structures to linear dextran. The latter enzymes convert soluble dextran to an insoluble form and produce biofilms on tooth surfaces. The production of biofilms by S. mutans is thought to be a critical trigger in the formation of dental caries. Dextran biofilms allow the active dextran derivative greatly decreased the formation of acid-forming bacteria to the tooth enamel and thus acid dissolution of tooth enamel. Therefore, although inhibiting the production of dextran and the formation of biofilms by S. mutans is thought to be important to prevent dental caries, the dextran molecules in DG-BLG can be used in the human mouth as a substrate of the enzymes converting soluble dextran to insoluble form when DG-BLG is administered. It is thought to be more appropriate to use other functional polysaccharides, such as amylose, in the per-oral feeding of BLG-carbohydrate conjugate. Considering the molecular size of BLG, a polymer length of marketed amylose generally makes it possible to control the physical properties of the resulting conjugate easily.

We have developed a method of synthesizing an active amylose derivative (AG-ONSu), an N-hydroxysuccinimide ester of the amylose-glycylglycine adduct (AG) that is effective at reducing the immunogenicity of proteins as a conjugating material to specific proteins.⁶ Hence, in this study we prepared BLG conjugate (AG-BLG) with AG-ONSu and examined to determine whether conjugation with AG-ONSu would reduce the immunogenicity of BLG as compared with DG-ONSu. Skimmed milk was obtained from Snow Brand Milk Products (Sapporo, Japan). Bovine BLG was purified from skimmed milk by the method described by Ebeler et al. and Erhardt, with slight modifications.⁷ Anti-BLG polyclonal antibodies prepared previously in mice using native BLG as soluble antigen were used in enzyme-linked immunosorbent assay (ELISA).² All the other reagents used in this study were of high-quality analytical grade.

BLG was conjugated with AG-ONSu under mild conditions. Briefly, AG-ONSu (76 mg) was added to a solution of BLG (10 mg in 7 mL of a 0.1 M Na-borate buffer, pH 8.0) over a period of 30 min with stirring, and the reaction proceeded at 4°C for 12 h. The mixture was

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Abbreviations: BLG, β-lactoglobulin; DG-BLG, BLG-dextran conjugate; AG, amylose-glycylglycine adduct; AG-ONSu, N-hydroxysuccinimide ester of AG; AG-BLG, BLG conjugated with AG-ONSu; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TNBS, trinitrobenzenesulfonate
then dialyzed against a 10 mM Na-borate buffer (pH 8.8) containing 0.1 M NaCl at 4°C, and then samples were chromatographed in a Sephadex G-100 column (69 cm) equilibrated with the same buffer. The column was developed at 4°C. The eluate was monitored at 280 nm, and samples (0.1 mL) of each fraction were tested and measured at 490 nm by the phenol-sulfuric acid method. As shown in Fig. 1, three major peaks (peaks 1–3) were obtained by monitoring the absorbance at 280 nm in the gel chromatography of AG-BLG on Sephadex G-100. The absorption at 490 nm made it possible for a peak (peak 4) with a sugar-positive nature to be detected. The fractions that included peak 2 that demonstrated absorbance at both 280 nm and 490 nm were found to contain AG-BLG. On the other hand, the fractions that included peaks 1 and 3 were considered to contain BLG cross-linked with residual dicyclohexyl-carbodiimide in the synthesis process of AG-ONSu and monomeric BLG, respectively, since the fractions with both peaks showed absorbance only at 280 nm. Hence, the fractions shown by a horizontal bar, which appeared to contain only AG-BLG without unmodified BLG, were pooled as AG-BLG. The inset shows the SDS–PAGE patterns for AG-BLG pooled in peak 2 (lane A) and native BLG (lane B). Electrophoresis was performed in 15% polyacrylamide gel at a constant current in 0.1% SDS and 50 mM Tris in a 38 mM glycine solution, and proteins were stained with Coomassie Brilliant Blue R250.

The number of free amino groups in AG-BLG was determined by the trinitrobenzenesulfonate (TNBS) method to characterize the conjugate. The TNBS method indicated that 5.5 moles of amino group/mole of AG-BLG was available for trinitrophenylation (data not shown). This implies that an average of 10.5 of the 16 amino groups (one α-amino group of the N-terminal leucine residue and 15 ε-amino groups of lysine residues1) in BLG were modified with AG-ONSu. To gain a better understanding of the conjugate and to establish more efficient control of the immunogenicity of the antigen proteins, we must identify the amino groups to which AG-ONSu predominantly forms covalent bonds.

To examine the effects of modification with the amylose derivative on antigen recognition by the anti-BLG polyclonal antibodies, the reactivities of AG-BLG and BLG were measured by ELISA using polyclonal antibodies produced by BALB/c mice. As shown in Fig. 2, the reactivity of native BLG with the anti-BLG polyclonal antibodies was measured by ELISA, as described in the text. The concentration of BLG was measured spectro photometrically using an absorption coefficient of ε = 1.743 × 10⁶ M⁻¹·cm⁻¹ at 280 nm. The same value was used for AG- and DG-BLG, because they showed UV absorption spectra identical to that of native BLG.
IgG polyclonal antibodies was markedly decreased by modification with AG-ONSu. The differences in the estimated IC₅₀ values from the immunogenicity curves between AG-BLG (1.85 × 10⁻² mg/mL) and native BLG (5.07 × 10⁻⁴ mg/mL) were large. Since AG-ONSu in AG-BLG is attached to BLG in such a manner as to shield its epitopes and thereby prevent anti-BLG antibodies from accessing the antigens, it is suggested that the epitopes (I, Tyr42-Ile56; II, Glu62-Thr76; and III, Ala139-Pro153) of BLG were not easily recognized by the anti-BLG antibodies. Although we must also determine in further study the reactivities of AG-BLG and native BLG with anti-AG-BLG IgG antibodies prepared using AG-BLG as soluble antigen, these findings clearly indicate that the covalent attachment of AG-BLG to BLG effectively decreased the immunogenicity of BLG. Since expansion of the shielded area of proteins with modifying materials in general reduces the immunogenicity of proteins, the molecular weight of the modifying materials is an important factor as well as the modification rate. When enzymes and food proteins are conjugated, there is a possibility of disadvantages, such as a decrease in catalytic activity and changes in taste. We must have to examine in further study the most suitable condition to conjugate BLG with AG-ONSu. Since BLG has been reported to improve resistance against proteolysis by conjugating with DG-ONSu, AG-BLG is also expected to be resistant against them. Hence, we must also clarify the metabolism of AG-BLG in detail as to the effects of digestive enzymes.

Moreover, the immunogenicity curve of AG-BLG was almost identical to that of DG-BLG, and the IC₅₀ value of AG-BLG was close to that of DG-BLG (2.75 × 10⁻² mg/mL), as shown in Fig. 2. Since the development of dental caries can be aggravated by the use of conjugates with DG-ONSu, as mentioned above, we suggested that AG-ONSu can be used to prepare a protein-carbohydrate conjugate for the prevention of dental caries.

Amylose is industrially supplied by the following methods: (i) hydrolyzing amyllopectin included in natural starch enzymatically, and (ii) extracting selectively from natural starch by organic solvents. However, the branch structure remains to a slight extent and the amylose distribution of molecular weight is broad in the amylose obtained by these methods. The cost of manufacturing amylose is also high. Mass production of a pure linear amylose has been not achieved. Recently, an enzyme-synthesizing method for amylose using sucrose phosphorylase (EC 2.4.1.17) and glucan phosphorylase (EC 2.4.1.1) was developed. Complete linear amylose with a desirable molecular weight can be prepared by controlling the enzyme reaction strictly. Moreover, one of the advantages of synthesizing amylose enzymatically is that cheap sucrose and cellobiose are used as ingredients. Because high-quality amylose is expected to be stably supplied to the market at a comparatively low price, it is also desirable for the preparation of AG-BLG.

The immunological results obtained from our protein-carbohydrate conjugate might promote the development of low-allergenic milk, in which BLG is conjugated with AG-ONSu. The resulting low-allergenic milk and skimmed milk might be useful for improving allergies in babies and infants by the above-mentioned immunological tolerance effect. Further characterization of AG-BLG for the development of less immunogenic foods and the application of this derivative to other proteins are currently being investigated.

References