Introducing Site-Specific Glycosylation Using Protein Engineering Techniques Reduces the Immunogenicity of β-Lactoglobulin

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To reduce the immunogenicity of β-lactoglobulin (BLG), we prepared wild-type bovine BLG variant A (wt) and three site-specifically glycosylated BLGs (D28N, D137N/A139S, and P153A), and expressed them in the methylotrophic yeast Pichia pastoris (BLG), we prepared wild-type bovine BLG variant A (wt) and three site-specifically glycosylated BLGs (D28N, D137N/A139S, and P153A), and expressed them in the methylotrophic yeast Pichia pastoris [Saccharomyces cerevisiae] by fusion of the cDNA to the sequence coding for the α-factor signal peptide from Saccharomyces cerevisiae. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis indicated that the glycosylated BLGs were conjugated with a ~4 kDa high-mannose chain. Each glycosylated BLG retained ~80% of the retinol-binding activity of BLG. Structural analyses by intrinsic fluorescence, CD spectra, and ELISA with monoclonal antibodies indicated that the surface structure was slightly changed by using protein engineering techniques, but that the site-specific glycosylated BLGs were covered by high-mannose chains without substantial disruption of wt conformation. Antibody responses to the glycosylated BLGs tended to be weaker in BALB/c, C57BL/6, and C3H/He mice. We conclude that site-specific glycosylation is an effective method to reduce the immunogenicity of BLG, and that masking of epitopes by high-mannose chains is effective to reduce immunogenicity.

Key words: β-lactoglobulin; neoglycoconjugate; functional improvement; protein conjugation; protein engineering

β-Lactoglobulin (BLG), a major whey protein of Mr 18,400, comprises 162 amino acids and possesses two disulfide bridges and one free cysteine residue. Although the physiological function of BLG still remains unclear, it is tentatively considered to be the binding and transportation of small hydrophobic ligands such as retinol and fatty acids, and the protein is categorized as a member of the lipocalin superfamily. In the context of food science, BLG is a valuable protein with various functional properties such as gelling, foaming and emulsifying properties as well as a high content of essential amino acids. BLG has a β-barrel structure, which is a common feature among the lipocalins. This kind of molecule has high allergenic potential, and several allergens of animal origin belong to the lipocalin superfamily. Indeed, BLG is a potent milk allergen, and ~82% of milk allergy patients are sensitive to this protein. It is therefore highly desirable to develop new methods that would reduce the allergenicity of BLG. Although attempts to reduce the allergenicity of proteins have been made by enzymatic digestion and denaturation, these methods destroy the physiological functions of the proteins and bring about problems with their taste. In contrast, protein conjugation can simultaneously achieve reduced allergenicity and improved functional properties (such as thermal stability, solubility, and emulsifying ability) while maintaining the physiological functions of proteins.

In our previous studies, we have focused on neoglycoconjugates of BLG. BLG was conjugated with carboxymethyl dextran or chitosan by means of a water-soluble carbodiimide and with acidic oligosaccharides (alganic acid oligosaccharides or phosphoryl oligosaccharides) by the Maillard reaction. These conjugates showed lower immunogenicity than native BLG. However, the binding sites in the protein were limited, and could not be chosen in these methods because saccharides were able to bind only to amino groups. Therefore, we adopted protein engineering techniques (site-directed mutagenesis) to glycosylate BLG at specific amino acid residues.

In this study, we constructed the yeast expression system for wild-type (wt) and glycosylated BLGs by protein engineering, and analyzed their conformational and immunological properties.

Materials and Methods

Strains and plasmids. Plasmid pBB29/BLG was used as the source of the wt BLG gene for site-directed mutagenesis. Plasmid cloning was done using Escherichia coli DH5α. Recombinant proteins were expressed in Pichia pastoris KM71H using an expression plasmid pPICZαA (Invitrogen, Carlsbad, CA).

DNA manipulation. Plasmid DNA was purified by PEG precipitation, LiCl precipitation and phenol-chloroform extraction, or using a QIAGEN plasmid preparation kit (QIAGEN, Santa Clarita, CA). Restriction enzymes (Xho I, Xba I, and BstXI) were obtained from Toyobo (Osaka, Japan). PCR products were purified by PCR purification kit (QIAGEN).

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Abbreviations: BLG, β-lactoglobulin; wt, wild-type; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue; PAS, periodic acid-Schiff
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Media. E. coli DH5α cells were grown in Luria-Bertani (LB) medium containing ampicillin (100 μg/mL). MGYH (minimal glycerol medium containing histidine; 1.34% yeast nitrogen base (YNB), 1.34% YNB, 4 × 10^{-3}% biotin, 0.004% histidine) and MMH (minimal methanol + histidine; 1.34% YNB, 0.004% histidine) were used for growing P. pastoris and producing recombinant BLG, respectively. YPDS (yeast extract peptone dextrose (Table 1). In this method, two-step PCR reactions were performed as performed using the products of the first PCR (upstream and primer using pBB29/BLG as a template. The second PCR was performed using the forward (or reverse) mutagenic primer and the 3’ flanking (BLG-Reverse) 5’ flanking primer included 5’ flanking primer of the first PCR (upstream and downstream fragments) as megaprimer together with 5’ and 3’ flanking primers. The products from the second PCR were purified using the QIAGEN PCR purification kit (QIAGEN), digested using XbaI and XhoI, and then cloned into the XhoI and XbaI sites of pBluescript SK(−). The BLG portions of the expression cassettes were sequenced using M13 forward and reverse primers. Plasmids containing the correct mutant BLG cDNAs were digested with XhoI and XbaI, and the BLG frames were inserted into the XhoI and XbaI sites of pPICZaA.

Construction of expression vectors. We used 4 oligonucleotide primers for each of the site-directed mutagenesis: forward and reverse mutagenic primers, a 5’ flanking primer and a 3’ flanking primer (Table 1). In this method, two-step PCR reactions were performed as described previously. Briefly, the first PCR was done with the forward (or reverse) mutagenic primer and the 3’ (or 5’) flanking primer using pBB29/BLG as a template. The second PCR was performed using the products of the first PCR (upstream and downstream fragments) as megaprimer together with 5’ and 3’ flanking primers.

The products from the second PCR were purified using the QIAGEN PCR purification kit (QIAGEN), digested using XhoI and XbaI, and then cloned into the XhoI and XbaI sites of pBluescript SK(−). The BLG portions of the expression cassettes were sequenced using M13 forward and reverse primers. Plasmids containing the correct mutant BLG cDNAs were digested with XhoI and XbaI, and the BLG frames were inserted into the XhoI and XbaI sites of pPICZaA.

Transformation of P. pastoris. P. pastoris KM71H was transformed with the mutant BLG/pPICZaA digested with BsuX I. Transformation of P. pastoris and screening for integrated vector, methanol utilization phenotype and protein expression were done with the Easy Select Pichia Expression Kit (Invitrogen, Carlsbad, CA).

Screening for mutant BLG expression. To express recombinant and mutant BLG in P. pastoris, single colonies of recombinant P. pastoris were incubated in 100 mL of MGYH at 30 °C with shaking until the OD_{600} was 2–6. The cells were collected by centrifugation at 6000 g and resuspended in 10 mL of MMH. Methanol was added every 24 h to a final concentration of 0.5% in this medium with shaking. Secretion of mutant BLG into the culture medium was monitored using 15% SDS–PAGE (see below) and bands were detected by staining with Coomassie Brilliant Blue (CBB) and Schiff reagent, and by western blotting.

SDS–PAGE and western blotting. SDS–PAGE was carried out under denaturing conditions using a 4% stacking gel and 15% separating gel, following the method of Laemmli. After electrophoresis, CBB staining and periodic acid–Schiff (PAS) staining was carried out.

In western blotting, the proteins were electroblotted onto a PVDF membrane after SDS–PAGE and detected using a BLG-specific mouse monoclonal antibody (mAb31A4) and horseradish peroxidase-labelled anti-mouse IgG (GE Healthcare, Buckinghamshire, UK) as described previously.

Fermentation of P. pastoris. Transformed P. pastoris cells (wt, D28N, D137N/A139S and P153A) were cultured in 6 L of MGYH for 24 h at 30 °C with shaking until the OD_{600} became 2–6, and the cells were collected by centrifugation at 1000 g and resuspended in 600 mL of MMH. The transformed P. pastoris cells were further cultured for 144 h, during which methanol was added every 24 h to a final concentration of 0.5%.

Purification of mutant BLGs. After the culture supernatant was adjusted to pH 6.7 with 25% ammonia, the supernatant was recovered by centrifugation at 7,500 × g and filtered through a 0.65 μm PVDF membrane. The supernatant was dialyzed against 0.05 M formic acid buffer (pH 4.0), after which the precipitate was separated by centrifugation at 7,500 × g and dissolved in 0.05 M imidazole buffer (pH 6.7). The solution was purified by anion and cation exchange chromatography. The supernatant was then dialyzed against 0.05 M imidazole buffer (pH 6.7) and applied to a DEAE Sepharose Fast Flow column (2.5 ID × 30 cm, GE Healthcare) equilibrated with the same buffer. Mutant BLGs were eluted with a linear gradient of 0 to 0.5 or 1 M NaCl in 0.05 M imidazole buffer (pH 6.7). Protein was detected by measuring the absorbance at 280 nm, and protein-containing fractions were analyzed by SDS–PAGE and stored at 4 °C. The peak fractions of the glycosylated BLGs were dialyzed against 0.05 M formic acid buffer (pH 4.0) and applied to a CM Sepharose Fast Flow column (2.5 ID × 30 cm, GE Healthcare) equilibrated with the same buffer. The glycosylated BLGs were eluted with a linear gradient of 0 to 1 M NaCl in 0.05 M formic acid buffer (pH 4.0). Protein was detected and analyzed as described above. Fractions containing protein were dialyzed against distilled water and then lyophilized and stored at −30 °C.

Table 1. Primers Used in Site-Directed Mutagenesis of BLG and in Sequencing of pBS Recombinant BLG cDNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>5’ flanking (BLG-Forward)</td>
<td>5'-AGGCCTCGAGAAGAAGGAGGAGCCCCAGCTATCCTGGTACGGCAGCCAGATG-3’</td>
</tr>
<tr>
<td></td>
<td>XhoI site</td>
</tr>
<tr>
<td>3’ flanking (BLG-Reverse)</td>
<td>5'-TCTCTAGAGGGGCAAGGCT-3’</td>
</tr>
<tr>
<td></td>
<td>XbaI site</td>
</tr>
<tr>
<td>D28N-Forward</td>
<td>5’-TCTTGGCATGCGGCCGAGCACACATCTCCCTGCTGAGCACGGGATAGTGGTGTCGTCGCCGCAATGGCAAGG-3’</td>
</tr>
<tr>
<td></td>
<td>D28N</td>
</tr>
<tr>
<td>D28N-Reverse</td>
<td>5’-ACTCTGGCGTCCAGCCAGGAGATGTGGTGACGTCGCCGCAAATGGCAAGG-3’</td>
</tr>
<tr>
<td></td>
<td>D28N</td>
</tr>
<tr>
<td>D137N/A139S-Forward</td>
<td>5’-AGGCCTGGAGAATTTCACAAACTCTCCAGAGCCAGCCCAGTCG-3’</td>
</tr>
<tr>
<td></td>
<td>D137N/A139S</td>
</tr>
<tr>
<td>D137N/A139S-Reverse</td>
<td>5’-ATGGCAGAGGCTCTGAGGATTGTTGAGAAATTTCCAGCCGCTCGG-3’</td>
</tr>
<tr>
<td></td>
<td>D137N/A139S</td>
</tr>
<tr>
<td>P153A-Forward</td>
<td>5’-CACATCCCAGGCCTGCTTCCAGACGCTCACCGAGGAGGGCAGGTG-3’</td>
</tr>
<tr>
<td></td>
<td>P153A</td>
</tr>
<tr>
<td>P153A-Reverse</td>
<td>5’-GCACGCTGCTCCAGCGCTGGGAGAGTTGAGTGTAAGGACGGCAAGG-3’</td>
</tr>
<tr>
<td></td>
<td>P153A</td>
</tr>
<tr>
<td>M13 Forward</td>
<td>5’-CCCAGTACAGGCAAGTTGAAAAACG-3’</td>
</tr>
<tr>
<td></td>
<td>M13 binding site</td>
</tr>
<tr>
<td>M13 Reverse</td>
<td>5’-AGCGGATAAACAAATTCACACAGGAAAC-3’</td>
</tr>
<tr>
<td></td>
<td>M13 binding site</td>
</tr>
</tbody>
</table>

5’ flanking primer included 5’ terminal sequence and XhoI site. 3’ flanking primer included 3’ terminal sequence and XhoI. Mutant primers (D28N, D137N/A139S, P153A) had base sequence for N-linked glycosylation site. M13 primer was used in sequencing reaction. M13 primer binding sites were included in pBS.
munosorbent assay (ELISA) was performed as described previously. Phosphate buffered saline (PBS; 0.11 M phosphate buffer at pH 7.0) was measured under excitation at 283 nm using an RF 5300PC instrument (Shimadzu, Kyoto, Japan). PBS was measured with a J-720 spectropolarimeter (Jasco, Tokyo, Japan), using 0.1 mg/mL.

Antisera were collected from each blood sample after clot formation. Female BALB/c, C57BL/6 and C3H/He mice (Clea University of Agriculture and Technology). This study conformed to the guidelines for the care and use of experimental animals established by the ethics committee of Tokyo University of Agriculture and Technology.

**Results and Discussion**

**Production of BLG mutants**

A *P. pastoris* BLG wt and mutant (D28N, D137N/A139S, P153A) expression system was constructed in this study. Our purpose was to introduce saccharides in the vicinity of the B cell epitopes in BLG clarified in our previous study. Cell-free supernatants from transformed *P. pastoris* cultures were analyzed by SDS–PAGE (CBB and PAS staining) and western blotting. As shown in Fig. 1A, the supernatant of cells expressing wt BLG displayed a major and a minor band in CBB staining and western blotting. The molecular weights of these bands differed by about 1 kDa. These results suggest that α-factor (Glu-Ala-Glu-Ala) signal sequence conjugated to wt was cleaved nonuniformly and that two different secreted protein molecules existed. The supernatants of D28N and P153A also showed two bands by CBB staining and western blotting. Although the supernatant of D137N/A139S

**Fig. 1.** SDS–PAGE and Western Blotting Analysis of Recombinant BLGs.

A, after fermentation. The recombinant BLGs (wt, D28N, D137N/A139S, P153A) were stained with CBB (a) or Schiff reagent (b) after SDS–PAGE, and were detected by western blotting (c). B, the wt supernatant after adjustment to pH 4.0. Recombinant *P. pastoris* cells expressing wt BLG were incubated at 30 °C until OD600 became 2–6. Methanol was added to the culture every 24 h to a final concentration of 0.5% for 144 h. Lane 1, wt supernatant; lane 2, the supernatant separated from the wt supernatant after dialysis against 0.05 M formic acid buffer (pH 4.0); lanes 3, 4, the precipitate was separated from wt supernatant dialyzed against 0.05 M formic acid buffer (pH 4.0), which solved by 0.05 M formic acid buffer (pH 6.7) (lane 4). Samples were stained with CBB (a) or Schiff reagent (b). C, the purified recombinant BLGs. The recombinant BLGs (wt, D28N, D137N/A139S and P153A) were stained with CBB.

**Endoglycosidase H treatment.** To confirm glycosylation in mutant proteins, the glycosylated BLGs were digested with endoglycosidase H (Endo-H), which specifically cleaves the high-mannose core of N-linked glycoproteins. The glycosylated BLGs (1 mg/mL) in 100 mM acetate buffer (pH 5.5) were incubated with 20 mU/mL Endo-H at 37 °C for 24 h. The proteins were then resolved by 15% SDS–PAGE and detected by CBB staining.

**Isoelectric focusing.** Isoelectric focusing (IEF) of the mutant BLGs was carried out using the Pharmacia Phast System (GE Healthcare). The protein bands were detected by CBB staining.

**Spectroscopic analysis.** CD spectra of the mutant BLGs were measured with a J-720 spectroptol fluorimeter (Jasco, Tokyo, Japan), using a cell with a 1.0 mm path length. Each sample was dissolved in phosphate buffered saline (PBS; a 0.11 M phosphate buffer at pH 7.0 containing 0.04 M NaCl and 0.02% NaN3) at a protein concentration of 0.1 mg/mL.

**Retinol-binding activity of mutant BLGs.** The equilibrium constant (Kd) for the interaction of a mAb with BLG, wt, D28N, D137N/A139S and P153A was calculated according to the method of Hogg et al. from the results of competitive and noncompetitive ELISA.

**Table 1.** Molecular masses (kDa) of the mutant BLGs.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Molecular Mass (kDa)</th>
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<tbody>
<tr>
<td>wt</td>
<td>20.1</td>
</tr>
<tr>
<td>D28N</td>
<td>14.4</td>
</tr>
<tr>
<td>D137N/A139S</td>
<td>30.0</td>
</tr>
<tr>
<td>P153A</td>
<td>27.2</td>
</tr>
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</table>

Briefly, BLG and mutant BLGs was adsorbed to the wells of polystyrene microtitration plate, which were then blocked by 1% ovalbumin/PBS solution. An antibody (100 μL of antiserum or mAb) diluted with PBS was added to each well, and then detected by alkaline phosphatase-labeled rabbit anti-mouse immunoglobulin. An alkaline phosphatase substrate 5-nitrophenyl phosphate disodium salt dissolved in a 1 M diethanolamine hydrochloride buffer (pH 9.8) was added to each well, and the reaction was stopped by adding 5 M sodium hydroxide solution. Absorbance at 405 nm was measured with a microplate reader.

To determine the immunogenicity of the glycosylated BLGs, a standard curve was made with serially diluted anti-BLG antiserum pooled from each strain of mice. The antibody titer was calculated from the standard curve and expressed as relative concentration.

A competitive ELISA was carried out to investigate local conformational changes in the BLG mutants, using anti-BLG mAbs (mAbs 21B3, 31A4, 61B4, and 62A6) as probes, and to evaluate the antigenicity of the glycosylated BLGs. The equilibrium constant (Kd) of the interaction of a mAb with BLG, wt, D28N, D137N/A139S and P153A was calculated according to the method of Hogg et al., from the results of competitive and noncompetitive ELISA.

**Enzyme-linked immunosorbent assay (ELISA).** Enzyme-linked immunosorbent assay (ELISA) was performed as described previously. As shown in Fig. 1B, the supernatant of cells expressing wt BLG displayed a major and a minor band in CBB staining and western blotting. The molecular weights of these bands differed by about 1 kDa. These results suggest that α-factor (Glu-Ala-Glu-Ala) signal sequence conjugated to wt was cleaved nonuniformly and that two different secreted protein molecules existed. The supernatants of D28N and P153A also showed two bands by CBB staining and western blotting. Although the supernatant of D137N/A139S

**Appendix**

A cell with 1.0 mm path length.
was detected as single band by CBB staining, two bands were detected by western blotting. These results indicate that each supernatant contained mixed types of BLG mutant molecules.

The supernatants of D28N, D137N/A139S, and P153A each showed a single band by PAS staining (Fig. 1A–b), indicating that each contained a single glycoprotein. However, since all supernatants were stained broadly in the high-molecular-weight area by PAS, these supernatants apparently also contained impurities from *P. pastoris*.

**Purification of the BLG mutants**

The results of SDS–PAGE analysis indicated that the supernatants from transformed *P. pastoris* (wt, D28N, D137N/A139S, and P153A) contained the desired glycosylated mutant BLG proteins. However, they also contained high-molecular-weight material and nonglycosylated BLGs (in the case of the glycosylated BLG) whose removal was considered to be necessary. Therefore, the wt and glycosylated BLGs were purified by ion-exchange chromatography. First, the cell-free culture supernatant pH was adjusted to 4.0 and the precipitate was recovered by centrifugation. The high-molecular-weight substances impurities from *P. pastoris* in the precipitate fraction were only detected as slight PAS staining, but were abundant in the supernatant fraction (Fig. 1B). These results suggested that the precipitate fraction was largely free of impurities from *P. pastoris*. Then, the precipitate fractions were purified by anion-exchange chromatography using DEAE Sepharose Fast Flow columns. The peak fractions of wt BLG were dialyzed against distilled water and lyophilized. The lyophilized proteins were shown), they were further purified by cation-exchange chromatography using CM Sepharose Fast Flow columns. The peak fractions were purified by anion-exchange chromatography using DEAE Sepharose Fast Flow columns. The peak fractions of the BLG mutants were found by SDS–PAGE analysis to contain only the nonglycosylated BLGs (data not shown), they were further purified by cation-exchange chromatography using CM Sepharose Fast Flow columns. The peak fractions were dialyzed against distilled water and lyophilized. The lyophilized proteins were quantitated by UV spectrophotometry. The final yields, per liter of MMH, of the BLG wt and mutants were about 50 mg (wt), 42 mg (D28N), 10 mg (D137N/A139S), and 45 mg (P153A).

**Chemical properties of the glycosylated BLGs**
The molecular weights of purified BLGs were estimated by SDS–PAGE to be 18.6 kDa (wt) and about 23 kDa (D28N, D137N/A139S, P153A) (Fig. 1C). To confirm that the purified mutant BLGs were glycosylated, they were digested with Endo-H and subjected to SDS–PAGE. On Endo-H treatment of the glycosylated mutant BLGs, the band at about 23 kDa was replaced by a single band at about 19 kDa (Fig. 2). These results indicate that the 23-kDa glycosylated mutant BLGs contained a ~4-kDa high-mannose chain.

Furthermore, isoelectric focusing was carried out to evaluate the pl value of BLG mutants (Fig. 3A). BLG wt showed two bands just as SDS–PAGE analysis. The lower wt band showed a similar pl value to that of native BLG (pl = 5.2), whereas the upper band showed a reduced pl value. These results may reflect the conjugation with α-factor signal sequence (Glu-Ala-Glu-Ala). The pl values of each glycosylated BLG were lower (D28N and D137N/A139S, pl = 4.8, 5.0; P153A, pl = 4.6, 4.9) than that of BLG. In addition, the pl value of D137N/A139S was lower than that of nonglycosylated D137N/A139S (pl = about 5.2) (Fig. 3B). The difference in pl value is likely to result from conjugation with oligosaccharides.

**Structural features of the glycosylated BLGs**
CD spectra of wt, D28N, D137N/A139S, and P153A are shown in Fig. 4A. The CD spectrum of wt BLG showed a negative maximum at 215 nm, indicating that BLG is rich in β-sheet structure. As the spectra of BLG mutants were similar to the spectrum of BLG, the structure of the β-sheet region in BLG were considered to have been maintained after conjugation with saccharides.

![Fig. 2. Endo-H Digestion of Recombinant BLGs.](image)

**Fig. 2.** Endo-H Digestion of Recombinant BLGs.
SDS–PAGE analysis (left to right): lane 1, marker; 2, BLG; 3, BLG+Endo-H; 4, wt; 5, wt+Endo-H; 6, D28N; 7, D28N+Endo-H; 8, D137N/A139S; 9, D137N/A139S+Endo-H; 10, P153A; 11, P153A+Endo-H. BLG and wt were not affected by Endo-H digestion. The glycosylated BLGs (D28N, D137N/A139S and P153A) were shifted to 18kDa affected by Endo-H digestion.

![Fig. 3. IEF Pattern of the Recombinant BLGs.](image)

**Fig. 3.** IEF Pattern of the Recombinant BLGs.
IEF was carried out with Phast Gel System (GE Healthcare, Buckinghamshire, UK) and Phast Gel IEF 4-6.5 (GE Healthcare, Buckinghamshire, UK). A, lane 1, BLG; 2, wt; 3, D28N; 4, D137N/A139S; 5, P153A; B, lane 1, BLG; 2, wt; 3, D137N/A139S; 4, nonglycosylated D137N/A139S.
The intrinsic fluorescence emission spectra of the BLG and its mutants are shown in Fig. 4B. Reduced carboxymethylated (RCM)-BLG was used as a denatured protein reference. As the conformation of BLG changes, the fluorescence intensity increases with the red shift of the wavelength for maximum emission. When excited at 283 nm, native BLG exhibited a fluorescence emission maximum wavelength of 333 nm. The fluorescence emission spectra of the BLG recombinants indicate that the maximum emission wavelength of the BLG mutants was indistinguishable from that of BLG. Therefore, the conformation around the Trp residues (19Trp, 61Trp) of the BLG mutants had maintained the native form. In addition, the peak intensity for D137N/A139S and P153A was lower than that for BLG, wt and the other BLG mutants. This decrease is considered to have been due to the shielding effect by specific amino acid substitutions or glycosylation.

The retinol-binding activity of wt and each BLG mutant was 75% of that of native BLG (Fig. 5). The retinol-binding activity of each glycosylated BLG was similar to that of wt. These results indicate that the region involved in retinol binding in the BLG (19Trp, 70Lys) has been changed as a result of protein engineering techniques, but the structure of this region was not changed by glycosylation.

Local conformational changes in the BLG mutants were evaluated by competitive ELISA with four anti-BLG mAbs (21B3, 31A4, 61B4, and 62A6) as probes. These mAbs can detect subtle conformational changes in local areas of the BLG molecule by determining the affinity change. The epitope regions for mAb 21B3, 31A4, 61B4, and 62A6 are 15Val-29Ile (β-sheet region), 8Lys-19Trp (random coil, and short helix), 125Thr-135Lys (α-helix region), and the second region close to the epitope for 61B4, respectively. MAbs 21B3 and 31A4 bound more strongly to RCM-BLG (the denatured form of BLG) than to native BLG or the other mutants.
of BLG), while mAb 61B4 and 62A6 bound preferentially to native BLG. The equilibrium constants ($K_{AS}$) for the binding of anti-BLG mAbs to the BLG mutants are shown in Fig. 6. The reactivity of mAbs 21B3 and 31A4 to the BLG mutants was slightly stronger than that for the binding to BLG. Therefore, the conformation around $^{135}$Val-$^{138}$Ile and $^{135}$Lys-$^{137}$Trp is considered to have been slightly changed. On the other hand, the $K_{AS}$ for binding of mAb 61B4 and 62A6 were different among the BLG mutants; the binding to wt and D28N were stronger than that to native BLG, that to D137N/A139S was a little stronger than that to native BLG, and that to P153A was lower than that to native BLG. Therefore, the conformation around $^{135}$Thr-$^{135}$Lys in D137N/A139S is the ratio of the absorbance at the step of ELISA in the presence of competitive antigen to the absorbance in the absence of competitive antigen. $B/B_0$ values were calculated from the results of competitive and noncompetitive ELISA according to the procedure of Hogg et al. (26).

**Fig. 6.** Equilibrium Constants ($K_{AS}$) of Anti-BLG mAb Binding to the Glycosylated BLGs.

$K_{AS}$ values were calculated from the results of competitive and noncompetitive ELISA according to the procedure of Hogg et al. (26). ○, BLG; ▲, wt; △, D28N; □, D137N/A139S; △, P153A.

**Immunological properties of the glycosylated BLGs**

The antigenicity of each BLG mutant in BALB/c, C57BL/6 and C3H/He mice was evaluated by competitive ELISA, using anti-BLG antisera (Fig. 7). BLG was adsorbed to the solid phase, and BLG, wt, D28N, D137N/A139S, and P153A were used as competitive antigens. Although anti-BLG antisera were elicited by immunization with native BLG, they showed higher affinity to the denatured form of BLG (RCM-BLG) than to the native form in BALB/c, C57BL/6, C3H/He mice.22) Therefore, conformationally altered forms of BLG are considered to have high antigenicity. The reactivity of the anti-BLG antiserum with each BLG mutant was slightly greater than with BLG. This may be due to conformational changes in BLG as a result of protein engineering techniques, but this conformational change hardly occurred by glycosylation.

The immunogenicity of each BLG mutant in BALB/c, C57BL/6 and C3H/He mice was evaluated by measuring the reactivity of diluted antisera (BALB/c and C57BL/6: 3 × 10$^{-5}$-fold dilution; C3H/He: 3 × 10$^{-2}$-fold dilution) with antigen (BLG, wt, D28N, D137N/A139S, and P153A) adsorbed to the solid phase of a microtitration plate by noncompetitive ELISA (Fig. 8). The anti-wt antibody response in these three strains of mice immunized with each glycosylated mutant BLG tended to be lower than that in mice immunized with wt (Fig. 8A-a, B-a and C-a). The level of production of antibody specific for each glycosylated mutant BLG was evaluated by applying each glycosylated BLG as the coating antigen on the solid phase (Fig. 8A-b-d, B-b-d and C-b-d). As antisera from mice immunized with each glycosylated mutant BLG showed an antibody titer similar to or lower than that of the anti-wt antisera, novel immunogenicity by glycosylation is considered not to have emerged. Although the precise mechanism responsible for the reduction of immunogenicity of proteins by conjugation with saccharides remains unclear, we have found that BLG-carboxymethyl dextran conjugates with higher saccharide content showed lower immunogenicity in our previous study.12,13) One of the mechanisms for the low immunogenicity of glycosylated BLG in this study is considered to be shielding of the epitopes by the conjugated saccharides which allows the escape from recognition by the immune system. Other plausible mechanisms are the induction of regulatory T cells27) and the reduced susceptibility of the conjugates to endosomal/lysosomal enzymes in antigen-presenting cells.28,29) Further studies focusing on these aspects should be carried out.

As shown in this study, glycosylation by using protein engineering techniques should be valuable for reducing the immunogenicity of BLG without inducing novel immunogenicity.

**Fig. 7.** Reactivity of Anti-BLG Antisera to the Glycosylated BLGs in Three Mouse Strains.

Reactivity of each anti-BLG antiserum obtained from BALB/c (a), C57BL/6 (b), C3H/He (c) mice to BLG and recombinant BLGs (wt, D28N, D137N/A139S, P153A) was measured by competitive ELISA. $B/B_0$ is the ratio of the absorbance at the step of ELISA in the presence of various concentrations of a competitive antigen to the absorbance in the absence of competitive antigen. ○, BLG; ▲, wt; △, D28N; □, D137N/A139S; △, P153A.
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References


Fig. 8. Immunogenicity of the Glycosylated BLGs in Three Mouse Strains.
A, BALB/c mice. B, C57BL/6 mice. C, C3H/He mice. The anti-wt (a), anti-D28N (b), anti-D137N/A139S (c), and anti-P153A (d) response after the secondary immunization were evaluated by noncompetitive ELISA. A significant difference compared with anti-wt serum as determined by Student’s t-test is indicated by single asterisk (p < 0.05) or two asterisks (p < 0.01).
Reduced Immunogenicity of a Protein by Protein Engineering