Bioscience, Biotechnology, and Biochemistry, 2023, Vol. 87, No. 4, 426-433

https:/doi.org/10.1093/bbb/zbac210
Advance access publication date: 28 December 2022
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Site-specific glycosylation and single amino acid substitution dramatically reduced the immunogenicity of β-lactoglobulin

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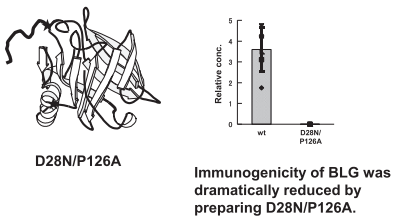
ABSTRACT

To reduce the immunogenicity of β-lactoglobulin (BLG), we prepared recombinant BLG which has both site-specific glycosylation and single amino acid substitution (D28N/P126A), and expressed it in the methylotrophic yeast *Pichia pastoris* 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 D28N/P126A was conjugated with a ∼4 kDa high-mannose chain. D28N/P126A retained ∼61% of the retinol-binding activity of BLG. Structural analyses by circular dichroism (CD) spectra, intrinsic fluorescence, and Enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies indicated that the surface structure of BLG was slightly changed by using protein engineering techniques, but D28N/P126A was covered by high-mannose chains and substituted amino acid without substantial disruption of native conformation. Antibody responses to the D28N/P126A considerably reduced in C57BL/6 mice. We conclude that inducing both site-specific glycosylation and single amino acid substitution simultaneously is an effective method to reduce the immunogenicity of BLG.

Graphical Abstract



Immunogenicity of β-lactoglobulin was dramatically reduced by site-specific glycosylation and single amino acid substitution.

Received: 28 October 2022; **Accepted:** 21 December 2022

Keywords: β-lactoglobulin, neoglycoconjugate, single amino acid substitution, reduced immunogenicity, protein engineering

Abbreviations: BLG: β-lactoglobulin; CBB: Coomassie Brilliant Blue; PAS: periodic acid–Schiff; SDS–PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; wt: wild-type

β-Lactoglobulin (BLG), a major whey protein of M_r 18400, comprises 162 amino acids and possesses two disulfide bridges and one free cysteine residue (McKenzie 1971). 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 this protein is categorized as a member of the lipocalin superfamily (Flower 1996).

In the context of food science, BLG is a valuable protein with various functional properties such as gelling (Mulvihill and Kinsella 1987), foaming (Waniska and Kinsella 1988), and emulsifying properties (Shimizu *et al*. 1985) as well as high content of essential amino acids (McKenzie 1971).

BLG has a β-barrel structure (Papiz *et al*. 1986; Brownlow *et al*. 1997), which is a common feature among lipocalins. This kind of molecule has high allergenic potential, and several allergens of animal origin belong to the lipocalin superfamily (Virtanen *et al*. 1999). Indeed, BLG is one of the major allergens in cow's milk allergy and about 47% of milk allergy patients are sensitive to this protein (Chen *et al*. 2014). 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 (Magi *et al*. 2004; Lucas *et al*. 2008), these methods destroy the physiological functions of the proteins and bring about problems with their taste (Kilara and Panyam 2003). 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.

The present study is based on two of our previous studies: (1) preparation of recombinant BLGs glycosylated by protein engineering led to reduced antibody production (Tatsumi *et al*. 2012), and (2) preparation of recombinant BLGs substituted single amino acid by protein engineering brought about reduced T cell response (Yoshida *et al*. 2022). ^{28}Asp is adjacent to one of the major epitopes of BLG (Hattori *et al*. 2000) and reduction in B-cell response is brought about by the introduction of saccharides (Tatsumi *et al*. 2012). And the substitution of ^{126}Pro to Ala leads to low T-cell response (Yoshida *et al*. 2022). In this study, we prepared recombinant BLG (D28N/P126A) which was prepared by site-specific glycosylation and single amino acid substitution, and analyzed its conformational and immunological properties.

Materials and methods

Strains and plasmids

Escherichia coli DH5α was used as the host strain to construct vectors for the expression of wild-type (wt) and D28N/P126A (Totsuka *et al*. 1990). The expression vector used was pPICZαA (Invitrogen, Carlsbad, CA). Plasmid pBB29/BLG was used as the source of the wt BLG gene for site-directed mutagenesis (Totsuka *et al*. 1990). *Pichia pastoris* KM71H was used as the host strain for protein expression.

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 *Bst*XI) were obtained from Toyobo (Osaka, Japan). PCR products were purified by PCR purification kit (QIAGEN).

Media

Escherichia coli DH5α cells were grown in Luria-Bertani (LB) medium containing ampicillin (100 mg/L). Minimal glycerol medium containing histidine (MGYH; 1.34% yeast nitrogen base [YNB], 1% glycerol, 4×10^{-5}% biotin, 0.004% histidine) and minimal methanol + histidine (MMH; 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol, 0.004% histidine) were used for growing *P. pastoris* and producing D28N/P126A, respectively. Yeast extract peptone dextrose sorbitol (YPDS; 1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar, 100 μg/mL Zeocin) was used for screening of *pPICZαA* transformants.

Construction of expression vector

The expression vector of D28N/P126A was prepared with QuickChange Site-Directed Mutagenesis Kit (Stratagene, CA, USA). The sample reactions were mixed according to the manufacturer's manual as indicated below; 5 μL of 10x reaction buffer, 0.5 μL of DNA template (pPICZαA-D28N; 50 ng/μL), 1.56 μL of oligonucleotide primers (100 ng/μL), respectively, 1 μL of dNTP mix, 1 μL *PfuTurbo* DNA polymerase (2.5 U/μL), and ddH_2O to a final volume 50 μL. This mixture was incubated with PCR at 95 °C for 30 min, and then subjected to 16 cycles of 95 °C for 30 s (denaturation), 55 °C for 1 min (annealing), and 68 °C for 4 min (extension). After incubation, *Dpn* I was added 1 μL to the products. *Dpn* I treated DNA was transformed to XL1-Blue Supercompetent Cells. pPICZαA-D28N/P126A produced by the transformation was magnified with PCR.

Transformation of *P. pastoris*

Pichia pastoris KM71H was transformed with the mutant BLG/pPICZαA digested with *Bst*X 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 BLG in *P. pastoris*, a single colony of mutant BLG strains of *P. pastoris* were incubated in 100 mL of MGYH at 28 °C with shaking until the OD600 was 2-6. The cells were collected by centrifugation at 1000 x *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% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and bands were detected by staining with Coomassie Brilliant Blue (CBB) and Schiff reagent, and by Western blotting.

SDS–PAGE

SDS–PAGE was carried out under denaturing conditions using a 4% stacking gel and 15% separating gel, following the method of Laemmli (1970). Electrophoresis was carried out at 20 mA constant current, and the gels were stained with CBB and the Schiff reagent. Periodic acid–Schiff (PAS) staining was carried out under the following conditions: 12.5% trichloroacetic acid was used for fixing proteins in the gel, and 1% orthoperiodic acid was used for oxidation of 1,2-glycolic groups of carbohydrates. The gel was stained in Schiff reagent in the dark and then washed with 0.5% potassium pyrosulfite.

Western blotting

After SDS–PAGE, the proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane. Nonspecific binding sites were blocked by incubating the membrane in PBS–Tween (PBS containing 0.05% Tween 20) containing 1% ovalbumin (OVA). A BLG-specific mouse monoclonal antibody (mAb 31A4) (Hattori *et al.* 1993) was used as the primary antibody. Horseradish peroxidase-labeled anti-mouse IgG (GE Healthcare, Buckinghamshire, UK) was used as the secondary antibody.

Fermentation of *P. pastoris*

Transformed *P. pastoris* cells (wt, D28N/P126A) were cultured in 6 L of MGYH for 24 h at 28 °C with shaking until the OD600 became 2-6, and the cells were collected by centrifugation at 1000 x *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 D28N/P126A

After the culture supernatant was adjusted to pH 6.7 with 25% ammonia, the supernatant was recovered by centrifugation at 7500 x *g* and filtered through a 0.65 μM PVDF membrane. The supernatant was dialyzed against 0.05 N imidazole buffer (pH 6.7) and applied to a DEAE Sepharose Fast Flow column (2.5 ID x 30 cm, GE Healthcare) equilibrated with the same buffer. D28N/P126A was eluted with a linear gradient of 0-1 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. Protein was detected and analyzed as described above. Fractions containing protein were dialyzed against distilled water and then lyophilized and stored at −30 °C.

Endoglycosidase H treatment

To confirm glycosylation, D28N/P126A was 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 U/mL Endo-H (Sigma–Aldrich, Steinheim, Germany) 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 BLGs was carried out using the Pharmacia Phast System (GE Healthcare) (Kramlová *et al.* 1986). The protein bands were detected by CBB staining.

Circular dichroism

Circular dichroism (CD) spectra of BLGs were measured with a J-720 spectropolarimeter (Jasco, Tokyo, Japan), using a cell with 1.0 mm path length (13). Each sample was dissolved in phosphate buffered saline (PBS; a 0.1 M phosphate buffer at pH 7.0 containing 0.04 M NaCl and 0.02% NaN_3) at a protein concentration of 0.1 mg/mL.

Fluorescence measurement

The intrinsic fluorescence of 0.1 mg/mL BLGs dissolved in PBS was measured under excitation at 283 nm using an RF 5300 PC instrument (Shimadzu, Kyoto, Japan) (Hattori *et al.* 1993).

Retinol-binding activity of the recombinant BLGs

The retinol-binding activities of BLGs were measured by fluorescence titration (Futterman and Heller 1972; Cogan *et al.* 1976). Aliquots (2 mL, 0.1 mg/mL) of each protein in PBS were put into a cuvette, and 5 μL increments of 1.73×10^{-4} M retinol in ethanol were added. Fluorescence was measured using an RF 5300 PC instrument with excitation at 330 nm and emission at 470 nm.

Immunization

In the experiments for antibody production, female C57BL/6 mice (Clea Japan, Tokyo, Japan) at 6 weeks of age (six animals per group) were immunized intraperitoneally with 100 μg (as a protein) of BLG (wt, D28N/P126A) emulsified in Freund's complete adjuvant (Difco Laboratories, MI, USA). A total of 14 days after the primary immunization, the mice were boosted with 100 μg of protein emulsified in Freund's incomplete adjuvant (Difco Laboratories). Blood samples were collected from mice seven days after the secondary immunization and stored at 4 °C for 24 h to form a clot. Antisera were collected from each blood sample after clot formation.

This study was performed in conformance with the guidelines for the care and use of experimental animals established by the ethics committee of the Tokyo University of Agriculture and Technology (R03-186, July 29, 2021).

Enzyme-linked immunosorbent assay (ELISA)

Wt and D28N/P126A were dissolved in PBS at a protein concentration of 0.1 mg/mL, and 100 μL was added to the wells of a Maxisorp polystyrene microtitration plate (Nunc, Roskilde, Denmark), which was then incubated overnight at 4 °C to coat the wells with antigen. After removal of the solution, each well was washed three times with 200 μL of PBS–Tween and 125 μL of 1% ovalbumin/PBS solution was added to each well, and the plate was then incubated at 25 °C for 2 h and washed three times

Table 1. Primers used in site-directed mutagenesis of BLG and in sequencing of pBS recombinant BLG cDNA.

Primer	Sequence
BLG Forward	5′-AGGCTCGAGAAAAGACTCATCGTCACCCAG-3′ *Xho* I site
BLG Reverse	5′-TCCTCTAGAGGGGCCAAGGCT-3′ *Xba* I site
D28N Forward	5′-ATGGCGGCCAGCAACATCTCCCTGCTG-3′ D28N
D28N Reverse	5′-CAGCAGGGAGATGTTGCTGGCCGCCAT-3′ D28N
P126A Forward	5′-TGGTCAGGACCGCTGAGGACGACGAC-3′ P126A
P126A Reverse	5′-GTCGTCGTCCTCAGCGGTCCTGACCA-3′ P126A
M13 Forward	5′-CCCAGTCACGACGTTGTAAAACG-3′ M13 binding site
M13 Reverse	5′-AGCGGATAACAATTTCACACAGGAAAC-3′ M13 binding site

with 200 µL of PBS–Tween. Antibody (100 µL of antiserum or mAb) diluted with PBS was added to each well, and the plate was incubated at 25 °C for 2 h. After three washes, 100 µL of alkaline phosphatase-labeled rabbit anti-mouse immunoglobulin (Dako, A/S, Glostrup, Denmark) diluted with PBS–Tween was added to each well. The plate was incubated at 25 °C for 2 h, and then the wells were washed three times. A total of 100 µL of 0.1% *p*-nitrophenyl phosphate disodium salt dissolved in a 1 M diethanolamine hydrochloride buffer (pH 9.8) was added to each well, and the plate was incubated at 25 °C. After the addition of 20 µL of 5 M sodium hydroxide to each well to stop the reaction, the absorbance at 405 nm was measured with an MPR-A4i microplate reader (Tosoh, Tokyo, Japan).

To determine the immunogenicity of the recombinant BLGs, a standard curve was made with serially diluted anti-BLG antiserum pooled from C57BL/6 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 recombinant BLGs using anti-BLG mAbs (mAbs 21B3, 31A4, 61B4, and 62A6) as probes (Hattori *et al.* 1994). The equilibrium constant (K_{AS}) for the interaction of a mAb with wt, D28N/P126A, and RCM-BLG was calculated according to the method of Hogg *et al.* (1987), from the results of competitive and noncompetitive ELISA.

Results and discussion

Production of recombinant BLGs

Pichia pastoris wt expression system was constructed in previous study (Tatsumi *et al.* 2012), and *P. pastoris* D28N/P126A expression system was constructed in this study. Primers used in this study are shown in Table 1. Cell-free supernatants from transformed *P. pastoris* cultures were analyzed by SDS–PAGE (CBB and PAS staining) and Western blotting. As shown in Figure 1, the supernatant of cells expressing wt showed a major and a minor band in CBB staining and Western blotting. The molecular weights of these bands differed by ~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 supernatant of D28N/P126A was detected as broad bands by CBB staining, PAS staining, and Western blotting. As the length of high-mannose chain conjugated to D28N/P126A is variable, the presence of α-factor signal sequence may not be observed. The high-molecular polysaccharides that commonly appeared in the yeast expression system as a result of PAS staining were not detected in the medium, and were therefore, considered to be yeast-derived substances and foreign substances that should be removed by purification.

Purification of recombinant BLGs

The results of SDS–PAGE analysis indicated that the supernatants from transformed *P. pastoris* (wt, D28N/P126A) contained the desired recombinant BLG proteins. However, they also contained high-molecular-weight material and nonglycosylated BLGs (in the case of D28N/P126A) whose removal was considered to be necessary. Therefore, wt and D28N/P126A were purified by anion-exchange chromatography (Figure 2). The peak fractions of wt were dialyzed against distilled water and lyophilized. In the case of D28N/P126A, there were two different peaks. SDS–PAGE analysis indicated the bigger one (F1; fraction numbers 39-46) contained only the glycosylated protein (Figure 3). Thus, they were dialyzed against distilled water and lyophilized.

The lyophilized proteins were quantitated by UV spectrophotometry. The final yields, per liter of MMH, of the wt and D28N/P126A were ~50 mg and 44 mg respectively.

Chemical properties of recombinant BLGs

The molecular weights of purified wt and D28N/P126A were estimated by SDS–PAGE to be 18.8 kDa and ~23 kDa (Figure 3). To

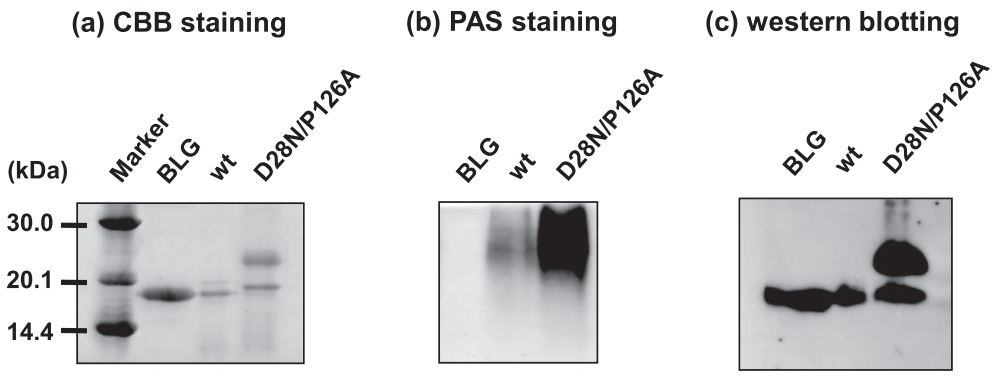


Figure 1. SDS–PAGE and Western blotting analysis of the recombinant BLGs produced by *P. pastoris* after fermentation. The recombinant BLGs (wt and D28N/P126A) were stained with CBB (a) or Schiff reagent (b) after SDS–PAGE, and were detected by Western blotting (c).

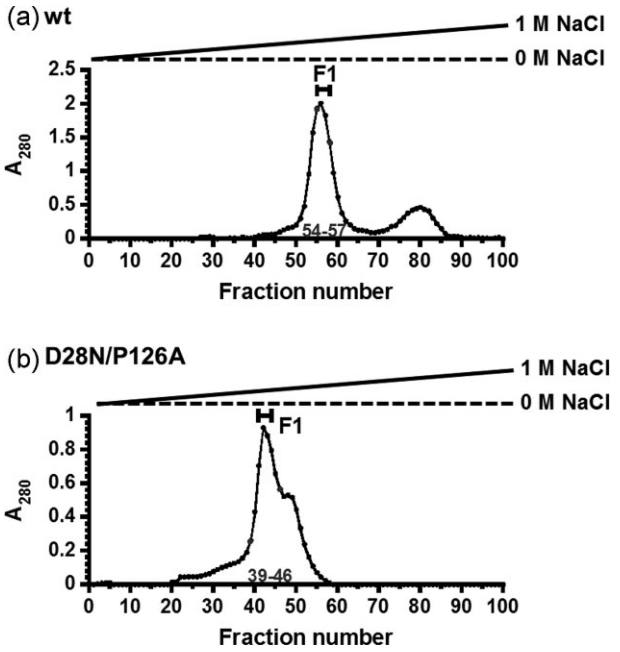


Figure 2. Anion-exchange chromatograms of wt and D28N/P126A. (a) wt; (b) D28N/P126A. Conditions: column, DEAE Sepharose Fast Flow (GE Healthcare, Buckinghamshire, UK); column size, 2.5 ID x 30 cm; flow rate, 5 mL/min; elution, 0-1.0 M NaCl gradient elution in 0.05 M imidazole buffer (pH 6.7). To detect the protein, the absorbance was monitored at 280 nm.

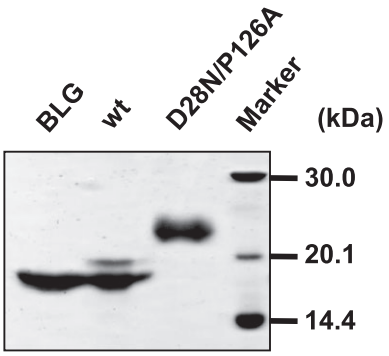


Figure 3. SDS–PAGE analysis of the purified recombinant BLGs. The recombinant BLGs (wt and D28N/P126A) were stained with CBB.

confirm purified D28N/P126A were glycosylated, it was digested with Endo-H and subjected to SDS–PAGE. On endoglycosidase H treatment of D28N/P126A, the band at about 23 kDa was replaced by a single band at about 19 kDa (Figure 4). These results indicate that the 23 kDa D28N/P126A contained a 4 kDa high-mannose chain.

Furthermore, isoelectric focusing was carried out to evaluate the *pI* value of D28N/P126A (Figure 5). Wt showed two bands just as SDS–PAGE analysis. The wt band located on lower position showed a similar *pI* value to that of native BLG ($pI = 5.2$), whereas the thin band located on upper position showed a reduced *pI* value. These results may reflect the conjugation with α-factor signal sequence (Glu–Ala–Glu–Ala). The *pI* values of D28N/P126A were higher ($pI = 4.8, 5.0$) than that of wt. It is suggested that this shift of *pI* resulted from shielding of NH_2 group by high-mannose chain conjugated.

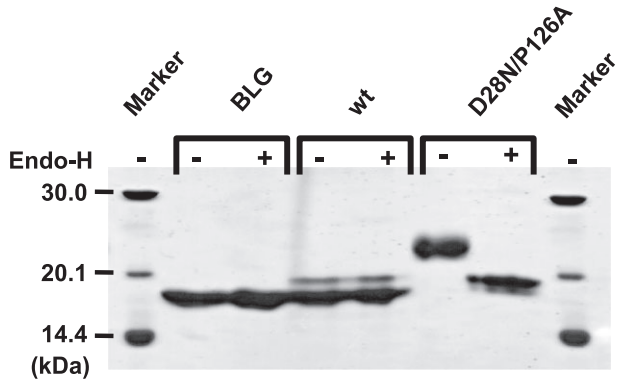


Figure 4. Endoglycosidase H digestion of the recombinant BLGs. SDS–PAGE analysis. (Left to right) lane 1, marker; 2, BLG; 3, BLG + Endo-H; 4, wt; 5, wt + Endo-H; 6, D28N/P126A, 7, D28N/P126A + Endo-H; 8, marker. BLG and wt were not affected by Endo-H digestion. D28N/P126A was shifted to 19 kDa affected by Endo-H digestion.

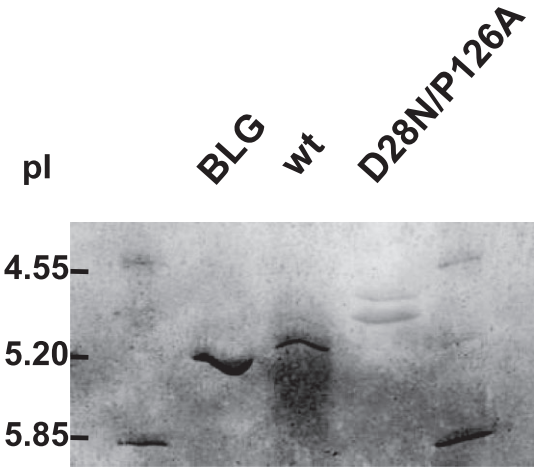


Figure 5. IEF pattern of the recombinant BLGs. IEF was carried out with Phast Gel System (GE Healthcare, Buckinghamshire, UK) and Phast Gel IEF 3-9 (GE Healthcare, Buckinghamshire, UK).

Structural features of recombinant BLGs

CD spectra of wt and D28N/P126A are shown in Figure 6a. Reduced carboxymethylated (RCM)-BLG was used as a denatured protein reference. The CD spectrum of wt showed a negative maximum at 215 nm, indicating that BLG is rich in β-sheet structure. In the case of D28N/P126A, the minimum was blue-shifted to 209 nm. This indicates high-mannose chain conjugation and/or single amino acid substitution leaded to slight change of the secondary structure of D28N/P126A.

The intrinsic fluorescence emission spectra of the wt and D28N/P126A are shown in Figure 6b. 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 wt and D28N/P126A indicate that the maximum emission wavelength of D28N/P126A was lower than that of wt. This decrease is considered to have been due to the shielding effect by high-mannose chain conjugation and/or single amino acid substitution.

The retinol-binding activity of wt was ~61% of that of native BLG (Figure 6c). The retinol-binding activity of D28N/P126A

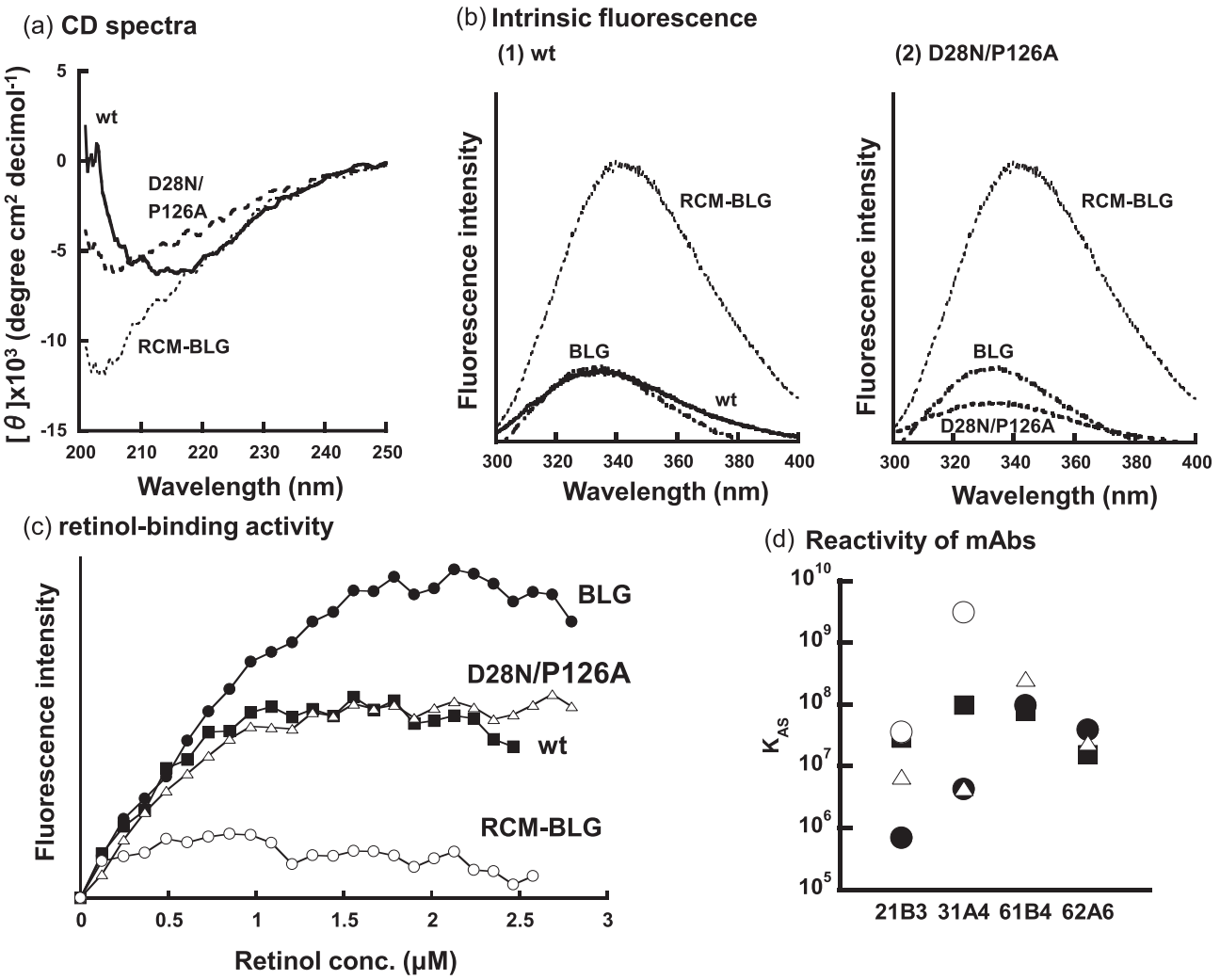


Figure 6. Structural analyses of recombinant BLGs. (a) CD spectra of the recombinant BLGs. CD spectra of BLG, wt, and D28N/P126A were measured with a spectropolarimeter, using a cell with a 1.0 mm path length. Protein concentration was 0.01% in PBS. (b) Intrinsic fluorescence spectra of the recombinant BLGs. The intrinsic fluorescence spectra of BLG, wt, D28N/P126A were measured under an excitation wavelength of 283 nm. Protein concentration was 0.01% in PBS. (c) Retinol-binding activity of the recombinant BLGs. The retinol-binding activities of BLG, wt, D28N/P126A were measured by fluorescence titration. Small (5 μL) increments of 4.95×10^{-5} M retinol in ethanol were added to a cuvette containing 2.0 mL of 0.01% protein in PBS. The fluorescence was measured with excitation at 330 nm and emission at 470 nm. •, BLG; ■, wt; △, D28N/P126A; , RCM-BLG. (d) Equilibrium constants (K_{AS}) of anti BLG mAb binding to the recombinant BLGs. K_{AS} values were calculated from the results of competitive and noncompetitive ELISA according to the procedure of Hogg *et al.* (1987). •, BLG; ■, wt; △, D28N/P126A; , RCM-BLG.

was similar to that of wt. These results indicate that the region involved in retinol-binding in the BLG (^{19}Trp, ^{70}Lys) has been changed as a result of protein engineering techniques, but the structure of this region was not changed by glycosylation and amino acid substitution.

Local conformational changes in D28N/P126A were evaluated by competitive ELISA with four anti-BLG mAbs (21B3, 31A4, 61B4, and 62A6) as probes (Hattori *et al.* 1993). 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 ^{15}Val-^{29}Ile (β-sheet region), ^{8}Lys-^{19}Trp (random coil, and short helix), ^{125}Thr-^{135}Lys (α-helix region), and close to the epitope for 61B4, respectively. MAbs 21B3 and 31A4 bound more strongly to RCM–BLG (the denatured form 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 wt and D28N/P126A are shown in Figure 6d. The reactivity of mAbs 21B3 and 31A4 to wt and that of mAbs 21B3 to D28N/P126A were slightly stronger than that for the binding to native BLG. Therefore, the conformation around ^{15}Val-^{29}Ile and ^{8}Lys-^{19}Trp is considered to have been slightly changed. On the other hand, the reactivity of mAbs 61B4 and 62A6 to wt and D28N/P126A was similar to that for the binding to native BLG. Therefore, the conformation around ^{125}Thr-^{135}Lys is considered to have been maintained.

D28N/P126A thus maintained the native-like protein structure, although a little collapse in the structure was observed.

Immunogenicity of recombinant BLGs

The immunogenicity of wt and D28N/P126A in C57BL/6 mice was evaluated by measuring the reactivity of diluted antisera (3×10^3-fold dilution) with antigen (wt or D28N/P126A) adsorbed to the solid phase of a microtitration plate by noncompetitive ELISA (Figure 7). The anti-wt antibody response in C57BL/6 mice immunized with D28N/P126A was reduced remarkably than that

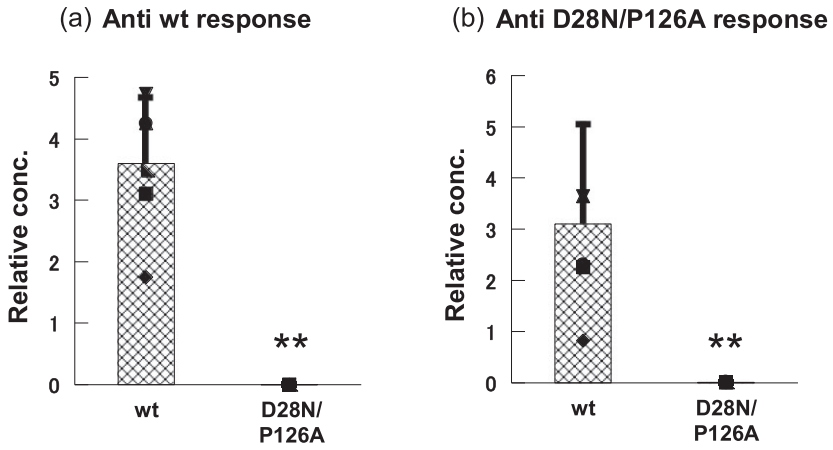


Figure 7. Immunogenicity of the recombinant BLGs in C57BL/6 mice. The anti-wt (a) and anti-D28N/P126A (b) response after the secondary immunization were evaluated by noncompetitive ELISA. A significant difference compared with anti wt serum is determined by Student's *t*-test. It is indicated by double asterisks ($P < .01$).

in mice immunized with wt (Figure 7a). The level of production of antibody specific for D28N/P126A was evaluated by applying D28N/P126A as the coating antigen on the solid phase (Figure 7b). As antisera from mice immunized with D28N/P126A showed the antibody titer was reduced remarkably than that of the anti-wt antisera, novel immunogenicity by glycosylation and single amino acid substitution is considered not to have emerged.

Conclusion

We prepared in this study, recombinant D28N/P126A BLG mutant by site-specific glycosylation and single amino acid substitution. Conformation of D28N/P126A almost maintained native BLG conformation, and it had the retinol-binding activity of BLG. D28N/P126A had very low immunogenicity without novel immunogenicity. The method developed in this study is considered to be epoch-making to reduce the allergenicity of BLG without inducing novel immunogenicity. D28N/P126A is considered to be applicable as a food additive with low allergenicity.

Acknowledgments

We are very grateful to Emeritus Professor Shuichi Kaminogawa of The University of Tokyo for his help in the ELISA experiments.

Data availability

The data underlying this article are available in the article and also from the corresponding author upon request.

Author contribution

M.E., K.I., T.I.: data curation, writing manuscript; T.Y., M.T., M.H.: conceptualization, funding acquisition, writing manuscript.

Funding

This work was supported in part by JSPS KAKENHI Grant Number JP17580107.

Disclosure statement

No potential conflict of interest was reported by the authors.

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