Reduced immunogenicity and endowed antimicrobial activity in β-lactoglobulin by preparing edible bioconjugate

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Received: 27 May 2022 / Accepted: 10 November 2022 / Published online: 17 November 2022
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Abstract β-lactoglobulin (BLG) and ε-polylysine (PL) were bound by using microbial transglutaminase. Dextran (Dex) was further conjugated to the BLG-PL conjugate by the Maillard reaction. Confirmation of conjugation was carried out by SDS-PAGE. From the results of isoelectric focusing, it was revealed that the isoelectric point of the BLG-PL conjugate was shifted to the basic side as compared with native BLG. Immunogenicity of BLG in BALB/c mice was lowered by conjugation with PL and further lowered by conjugation with Dex. By conjugation with PL and PL-Dex, antibacterial activity against Staphylococcus aureus was endowed to BLG. Because the conjugation method in this study is a safe method, it is valuable in that it can be applicable to food processing.

Keywords β-Lactoglobulin · Neoglycoconjugate · Functional improvement · Protein conjugation

Introduction

β-lactoglobulin (BLG) is a major whey protein of Mw 18 kDa consisting of 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 the protein is categorized as a member of the lipocalin superfamily (Sawyer and Kontopidis 2000).

In terms of food science, BLG is considered to be a valuable protein with various functional properties such as emulsifying (Shimizu et al. 1985), foaming (Phillips et al. 1995) and gelling properties (Foegeing et al. 1992) as well as high content of essential amino acids (McKenzie 1971).

BLG belongs to lipocalin super family. BLG has a β-barrel structure (Sawyer and Kontopidis 2000) 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 (Virtanen et al. 1999). In fact, 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). Therefore, it is strongly hoped 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 is superior to other hypoallergenic methods in that it can simultaneously achieve reduced allergenicity and improved functional properties (thermal stability, solubility, emulsifying ability, etc.) while maintaining the physiological functions of proteins (Hattori 2002).

In our previous report, we conjugated BLG with PL by using microbial transglutaminase (MTGase) (EC2.3.1.13) and enhancement of emulsifying property and reduction of immunogenicity were simultaneously achieved. In the present study, we further conjugated dextran to achieve further reduction of immunogenicity. In addition, we investigated antimicrobial activity of the conjugates. We will describe reduction of immunogenicity of BLG and endowment of antimicrobial activity to BLG.

Materials and methods

Materials

e-Polylysine was a gift from Chisso Corporation (Tokyo Japan) (average polymerization degree: 30). Activa TG-K (EC2.3.2.13) was a gift from Ajinomoto Co. (Tokyo, Japan).

Isolation of BLG

BLG (genotype AA) isolated from fresh milk of a Holstein cow according to the method of Armstrong et al. (1967) was further purified by ion-exchange chromatography in a DEAE-Sepharose Fast Flow column (2.5 ID×50 cm; GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Crude BLG was applied to the column and eluted by a 0–500 mM NaCl linear gradient in a 0.05 M imidazole buffer at pH 6.7 and a flow rate of 1.0 mL/min. The eluted protein was detected by the absorbance at 280 nm. The major fraction was dialyzed against distilled water and lyophilized. The purity of BLG was confirmed by SDS-PAGE.

Preparation and purification of the BLG-PL conjugate

Preparation and purification of the BLG-PL conjugate were carried out as described previously (Yoshida et al. 2022). 0.2% solution of MTGase was used as MTGase for the enzymatic reaction. Activa TG-K (Ajinomoto Co., Tokyo, Japan) was used as a source of MTGase. Activa TG-K (5 g) was dissolved in 0.1 M imidazole buffer (pH 7.0, 25 mL) and centrifuged at 4 °C at 18,000 rpm for 30 min. Enzyme titer of Activa TG-K was 100 units/g. The supernatant was collected, filtrated with paper, and dialyzed overnight against the same buffer to remove calcium lactate and dextrin. MTGase-catalized reaction was carried out under the condition of BLG:PL = 1:3. BLG and PL were dissolved in 0.2 M imidazole buffer (pH 7.0, 20 mL). 200 mg of BLG and 126 mg of PL were used for conjugation. Enzymatic reaction was started by adding 20 mL of MTGase in this solution. The reaction mixture was incubated at 37 °C for 48 h.

After enzymatic reaction, the solution was dialyzed against 0.1 M imidazole buffer (pH 7.7) at 4 °C overnight and adjusted to pH to 7.7 and loaded on CM Sepharose Fast Flow column (2.4 ID×45 cm; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) to remove free BLG, PL, and MTGase. The column was suspended in equilibrium with 0.1 M imidazole buffer (pH 7.7), eluted with 200 mL of the same buffer, and eluted with a 0–1.0 M NaCl gradient. Absorbance was observed at 230 nm and 280 nm to detect proteins and peptides. The purity of the conjugate was confirmed by SDS-PAGE. Purified BLG-PL fraction was lyophilized and the fraction containing excess PL was purified again in the same way.

Preparation and purification of the BLG-PL-Dex conjugate

Purified BLG-PL and Dex were dissolved in distilled water at the molar ratio of 1:5. 80 mg of the BLG-PL conjugate and 180 mg of Dex were dissolved in 25 mL of distilled water and lyophilized. The dried mixture was allowed to stand for 24 h at 50 °C at a relative humidity of 79% to perform the Maillard reaction.
After the Maillard reaction, crude BLG-PL-Dex conjugate was dissolved in 0.1 M imidazole buffer at pH 7.7. The solution was loaded on a CM-Sepharose Fast Flow column (2.4 ID × 45 cm; GE Healthcare, Uppsala, Sweden) to remove free BLG-PL and Dex. This column was suspended in equilibrium with 0.1 M imidazole buffer (pH 7.7), eluted with 500 mL of the same buffer, and eluted with 0.1 M imidazole buffer containing 1.0 M NaCl. Absorbance at 280 nm was detected. A small fraction was sampled from the main fraction and SDS-PAGE led to confirmation of the purity of the BLG-PL-Dex conjugate. The fraction of purified BLG-PL-Dex conjugate was dialyzed against distilled water and lyophilized.

Electrophoresis

SDS-PAGE was carried out in 4% stacking gel and 15% separating gel following the method of Laemmli (1970). Prior to electrophoresis, sample proteins were heated at 100 °C for 5 min in SDS-PAGE sample buffer. The gel electrophoresis was carried out at 20 mA constant current. The gels were stained with Coomassie brilliant blue (CBB) and periodic acid Schiff (PAS).

Isoelectric focusing (IEF) was performed by PhastSystem™ (Amersham Biosciences, Buckinghamshire, UK) (Kramlová et al. 1986). Gels were loaded with 4 μL of 1 mg/mL and 0.5 mg/mL sample solution using IEF 3–9 (GE Healthcare Bio-sciences AB, Uppsala, Sweden).
Sweden). After electrophoresis, the protein was fixed in a fixing solution for IEF (20% trichloroacetic acid solution) for 5 min, and the gel was washed with distilled water for 2 min 3 times. After dyeing for 30 min with a gel staining solution for IEF (a mixture of Phast Gel Blue R solution and decolorizing solution for IEF), it was decolorized with a decoloring solution for IEF (30% methanol/10% acetic acid).

Immunization

Female BALB/c (Clea Japan, Tokyo, Japan) at 6 weeks of age (seven animals per group) were immunized intraperitoneally with 100 μg (as a protein) of BLG, BLG-PL or BLG-PL-Dex emulsified in Freund’s complete adjuvant (Difco Laboratories, MI, USA). Fourteen 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 7 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 Tokyo University of Agriculture and Technology (R03-186, July 29th, 2021).

Enzyme-linked immunosorbent assay (ELISA)

Non-competitive ELISA was carried out as follows. BLG, BLG-PL, and BLG-PL-Dex were dissolved in PBS at a protein concentration of 0.1 mg/mL (100 μL) and added to the wells of a polystyrene microtitration plate (Maxisorp, Nunc, Roskilde, Denmark), and the plate was incubated overnight at 4 °C to coat the wells with each antigen. After removal of the solution, each well was washed three times with 200 μL of PBS-Tween (PBS containing 0.05% Tween 20). A 125 μL amount of a 1% ovalbumin/PBS solution was added to each well, the plate was incubated at 25 °C for 2 h, and then the plate was washed three times with 200 μL of PBS-Tween. A 100 μL amount of an antiserum diluted with PBS was added to each well, and the plate was incubated at 25 °C for 2 h. After three washing, 100 μL of alkaline phosphatase-labeled rabbit anti-mouse immunoglobulin 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. 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 a 5 M sodium hydroxide solution (20 μL) to each well to stop the reaction, the absorbance at 405 nm was measured with an MPR-A4i microplate reader (MPR-A4i, Tosoh, Tokyo, Japan).

Statistical analysis

In the evaluation of immunogenicity of BLG and the conjugates, statistical analysis was performed on the obtained results by Student's test.

Evaluation of antimicrobial activity of the BLG-PL and BLG-PL-Dex conjugate

Antimicrobial activity of the BLG-PL and BLG-PL-Dex conjugate was evaluated by using L-shaped test tube. Growth of Staphylococcus aureus in LB medium containing PL and the conjugate was investigated. 2.5 mL of LB medium was pipetted into an L-shaped test tube and autoclaved, and then antimicrobial material for measurement was added according to the final concentration. The final concentration of PL was 0, 10, 40 μg/mL. Staphylococcus aureus preculture solution was inoculated in the liquid medium in the L-shaped test tube to which the antibacterial material for measurement was added, and cultured at 37 °C with shaking, and the densitometer (DEN-1 B, WAKEN B TECH, Kyoto, Japan) was used to measure the turbidity at 550 nm.

Results and discussion

Preparation and purification of the BLG-PL conjugate

The BLG-PL conjugate was purified by CM chromatography (Fig. 1a). Since BLG and MTGase exist as anions, they do not adsorb to the column at pH 7.7, and the BLG-PL conjugate exist as a cation which
adsorbs to the column. Therefore, F3-9 were considered to be the peak region of the BLG-PL conjugate. The eluate of F1 to 9 was subjected to SDS-PAGE (Fig. 1b). From the bands of F1 and F2, it was confirmed that BLG and MTGase were eluted. In F3 to 9, two bands appeared near the molecular weight of 30,000, and these bands are considered to be the BLG-PL conjugate (BLG: PL = 1:1 and 1:2). In our previous study (Yoshida et al. 2022), amino acid analysis was performed to calculate that the average
molecular weight of the BLG-PL conjugate was 22,900. The molecular weight obtained by SDS-PAGE is considered to be different from the molecular weight obtained by amino acid analysis because proteins with high basicity have low electrophoretic mobility. Therefore, in this study as well, future experiments were conducted with the average molecular weight of the BLG-PL conjugate to be 22,900. Bands were also detected in the higher molecular weight region, which were considered to be dimer of the BLG-PL conjugate and the BLG-PL conjugate crosslinked by the transglutaminase reaction. As shown in above results, the BLG-PL conjugate could be purified by cation exchange chromatography.

Preparation and purification of the BLG-PL-Dex conjugate

In this study, Dex was further conjugated with BLG-PL conjugate. We chose Dex for conjugation because Dex is homogenous in molecular weight and sequence and suitable for a model study. The Maillard reaction product of the BLG-PL conjugate and Dex was subjected to CM chromatography (Fig. 2a). The eluate of the obtained peak region was subjected to SDS-PAGE (Fig. 2b, c). The Maillard reaction product before subjecting to CM chromatography was also subjected to SDS-PAGE. Preparation and purification of the BLG-PL-Dex conjugate were confirmed by CBB staining and also by PAS staining. After dialysis and lyophilization, the sample was used as the BLG-PL-Dex conjugate in subsequent experiments. Although the obtained Maillard reaction product was heterogeneous in molecular weight, it is considered that it is due to the difference in the number of saccharides bound and the polymerization of the compounds produced in the Maillard reaction.

Isoelectric focusing

Isoelectric focusing was carried out to investigate the changes in isoelectric point of BLG by conjugating with PL and Dex (Fig. 3). The isoelectric point of the BLG A mutant was 5.2, but in the BLG-PL conjugate, bands appeared in the range of 6.55 to 7.00 and 8.10 to 8.45, and it was confirmed that the band was greatly shifted to the basic side. This result is considered to be due to the binding of PL, which is a basic amino acid polymer. Also, the bands from 6.55 to 7.00 are conjugates with BLG: PL = 1:1 and the bands from 8.10 to 8.45 are considered to be conjugates with BLG: PL = 1:2. The band of the BLG-PL-Dex conjugate appeared around 7.30 to 8.45. This is considered to be due to the fact that basicity was imparted as compared with BLG due to the binding of PL.

Generally, glycosylation displaces basic amino acid side chains, so the basicity decreases causing a shift of the isoelectric point towards the acidic side. Therefore, isoelectric focusing is suitable as an index for evaluating glycated products. Bands of the BLG-PL-Dex conjugate were slightly shifted to the acidic side than the band confirmed by the BLG-PL conjugate. From these results, it was also confirmed that the BLG-PL-Dex conjugate was prepared.

Reduced immunogenicity of BLG by conjugation with PL and Dex

Changes in immunogenicity of BLG by conjugation with PL and Dex in BALB/c mice were evaluated by non-competitive ELISA (Fig. 4a). As a result, anti-BLG response was significantly lowered by conjugation with PL ($p < 0.05$). And anti-BLG response was further lowered by further conjugation with Dex ($p < 0.01$). BLG-PL conjugate was coated on
the solid phase and the ability to produce antibodies against the BLG-PL conjugate was evaluated. Novel immunogenicity of BLG was not observed after conjugation with PL (Fig. 4b). Novel immunogenicity after further conjugation with Dex was also evaluated (Fig. 4c) and the results showed that novel immunogenicity after conjugation with Dex was not observed. We also investigated with C57BL/6 mice and obtained similar results (data not shown). Conjugation with PL and Dex was considered to be an effective method to reduce immunogenicity of BLG without inducing novel immunogenicity.

Evaluation of antimicrobial activity of the BLG-PL and the BLG-PL-Dex conjugate

Antibacterial activity of the BLG-PL and BLG-PL-Dex conjugate was evaluated by using L-shaped test tube (Fig. 5). In the medium containing polylysine alone, proliferation of *S. aureus* was completely inhibited at 10 μg/mL. In the medium containing the BLG-PL conjugate, growth suppression was observed at a PL equivalent of 10 μg/mL, and was suppressed completely at 40 μg/mL. In the medium containing the BLG-PL-Dex conjugate, growth suppression was not observed at a PL equivalent of 10 μg/mL, but growth suppression was confirmed at 40 μg/mL. These results indicated that antimicrobial activity of PL was endowed to BLG by conjugation with PL. However, its antimicrobial activity was weakened in the order of PL, BLG-PL, and BLG-PL-Dex. ε-PL is considered to adsorb on the cell surface of bacteria to exfoliate the outer membrane of bacteria resulting in abnormal distribution of cytoplasm and injury to bacterial cells (Shima et al. 1984). Therefore, it was considered that Lys residue as the source of PL basicity was used for binding due to the binding of BLG and Dex, the charge decreased, and the amount of PL
Acknowledgements

We thank CHISSO CORPORATION (Tokyo, Japan) for presenting ε-polylysine. We thank Ajinomoto Co. (Tokyo, Japan) for presenting Activa TG-K (E.C.2.3.2.13). This work was supported in part by JSPS KAKENHI Grant Number JP22580126.

Author contributions

TY: Conceptualization, Data curation, Writing manuscript, Funding acquisition, SK: Conceptualization, Data curation, Writing manuscript, RI, KH, YM: Data curation, Writing manuscript, MH: Conceptualization, Writing manuscript, Funding acquisition.

Data availability

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

Declarations

Conflict of interest

The authors have no conflicts of interest to declare.

References

Armstrong JM, McKenzie HA, Sawyer WH (1967) On the fractionation of beta-lactoglobulin and alpha-lactalbumin. Biochim Biophys Acta 147:60–72. https://doi.org/10.1016/0005-2795(67)90090-6

Chen FM, Lee JH, Yang YH, Lin YT, Wang LC, Yu HH, Chiang BL (2014) Analysis of α-lactalbumin-, β-lactoglobulin-, and casein-specific IgE among children with atopic diseases in a tertiary medical center in northern Taiwan. J Microbiol Immunol Infect 47:130–136. https://doi.org/10.1016/j.jmii.2012.08.009

Foegeding EA, Kuhn PR, Hardin CC (1992) Specific divalent cation-induced changes during gelation of β-lactoglobulin. J Agric Food Chem 40:613–619. https://doi.org/10.1021/jf00023a011

Hattori M (2002) Functional Improvements in Food Proteins in multiple aspects by conjugation with saccharides: case studies of β-lactoglobulin-acidic polysaccharides conjugates. Food Sci Technol Res 8:291–299. https://doi.org/10.3136/fstr.8.291

Kramlová M, Přistoupil Tí, Fričová V, Kraml J (1986) First experience with the use of the Pharmacia PhastSystem for the characterization of hemoglobulins by isoelectric focusing. J Chromatogr A 367:443–445. https://doi.org/10.1016/S0021-9673(00)94868-4

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685. https://doi.org/10.1038/227680a0

McKenzie HA (1971) Milk proteins. Academic Press, New York, pp 257–330

Phillips LG, Hawks SE, German JB (1995) Structural characteristics and foaming properties of β-lactoglobulin: effects of shear rate and temperature. J Agric Food Chem 43:613–619. https://doi.org/10.1021/jf00051a011

Concluding remarks

In this study, we prepared the BLG-PL and BLG-PL-Dex conjugates with modified functionality by using MTGase and the Maillard reaction. Immunogenicity of BLG was lowered by conjugation with PL, and further lowered by further conjugation with Dex. Antimicrobial activity could be endowed to BLG by conjugation with PL. Since the conjugation method carried out in this study is a safe method, this method is considered to be very valuable in that it would be applicable for food processing.

Fig. 5 Growth pattern of Staphylococcus aureus subsp. in the presence of PL, BLG-PL and BLG-PL-Dex. Antimicrobial activity of the BLG-PL and BLG-PL-Dex conjugate was evaluated by using L-shaped test tube. Growth of Staphylococcus aureus in LB medium containing PL and the conjugate was investigated. 2.5 mL of LB medium was pipetted into an L-shaped test tube and autoclaved, and then antimicrobial material for measurement was added according to the final concentration. The final concentration of PL was 0, 10, 40 μg/mL; ●, broth; ○, BLG (40 μg/mL); ▲, PL (10 μg/mL); △, PL (40 μg/mL); ▼, BLG-PL (PL equivalent 10 μg/mL); ▼, BLG-PL (PL equivalent 40 μg/mL); ■, BLG-PL-Dex (PL equivalent 10 μg/mL); ▽, BLG-PL-Dex (PL equivalent 40 μg/mL)

adsorbed on the cells of the fungus decreased. However, antimicrobial activity could be added to BLG by conjugation with PL in this study. The conjugates obtained in this study is promising as an antibacterial agent that can be used for foods.

Concluding remarks

In this study, we prepared the BLG-PL and BLG-PL-Dex conjugates with modified functionality by using MTGase and the Maillard reaction. Immunogenicity of BLG was lowered by conjugation with PL, and further lowered by further conjugation with Dex. Antimicrobial activity could be endowed to BLG by conjugation with PL. Since the conjugation method carried out in this study is a safe method, this method is considered to be very valuable in that it would be applicable for food processing.
Sawyer L, Kontopidis G (2000) The core lipocalin, bovine β-lactoglobulin. Biochim Biophys Acta 1482:136–138. https://doi.org/10.1016/S0167-4838(00)00160-6

Shima S, Matsuoka H, Iwamoto T, Sakai H (1984) Antimicrobial action of epsilon-poly-L-lysine. J Antibiot 37:1449–1455. https://doi.org/10.7164/antibiotics.37.1449

Shimizu M, Saito M, Yamauchi K (1985) Emulsifying and structural properties of β-lactoglobulin at different pHs. Agric Biol Chem 49:189–194. https://doi.org/10.1080/00021369.1985.10866680

Virtanen T, Zeiler T, Rsutinainen J, Mäntyjärvi R (1999) Allergy to lipocalins: a consequence of misguided T-cell recognition of self and nonself? Immunol Today 20:398–400. https://doi.org/10.1016/S0167-5699(99)01515-7

Yoshida T, Tanemura M, Shimizu A, TanakaKurokawa HS, Takahashi K, Hattori M (2022) Functional improvements in β-lactoglobulin by preparing edible conjugate with microbial transglutaminase. Biosci Biotechnol Biochem 86:390–396. https://doi.org/10.1093/bbb/zbab220

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