Purification and Characterization of β-Lactoglobulin Genetic Variant A and B by Using Preparative Elution Electrophoresis and Isoelectric Focusing

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Introduction

β-lactoglobulin (βLG) is a major whey protein found in the milk of cows and other ruminants, deer, bison, and buffalo. βLG is also found in some nonruminants, such as pigs,[1] horses,[2] dogs, dolphins,[3] cats,[4] and whales. However, βLG is not found in human milk.[5, 6, 7, 8] βLG is a glycoprotein which exists at the normal pH of bovine milk as a dimer with a molecular weight of 36,000, and consists of two monomeric subunits with molecular weight of 18,000 (162 residues). Several genetic variants of βLG have been detected,[9] of which the bovine phenotype A and phenotype B are most predominant. The bovine βLG A variant differs from the βLG B variant by only two amino acids: aspartate-64 (Asp) and valine-118 (Val). These amino acids are substituted by glycine (Gly) and alanine (Ala) in the B variant. All variants contain five cysteine residues, four of which are involved in forming intrachain disulfide bridges.

The function of βLG is not yet clear, although binding and transport of retinol, small hydrophobic ligands and fatty acid in postnatal animals, have been suggested as possible functions, since its three-dimensional structure is essentially similar to that of human retinol-binding protein in serum.[10, 11, 12, 13, 14, 15] βLG is considered as one of the main allergenic components in bovine milk,[16, 17, 18] and therefore the modification of βLG is considered as a promising treatment for milk allergy.[19] βLG also has good emulsifying and foaming properties, and therefore offers a good model for elucidation of the adsorption characteristics of proteins at a surface. βLG is also a model protein for studying the denaturation mechanism.[20, 21] These properties are important in the milk industry and bovine milk allergy.

Purification and analytical separation of the genetic variants of bovine βLG have been carried out by using different chromatographic methods, *e.g.* ion exchange chromatography with DEAE cellulose and recently HPLC,[22, 23, 24, 25, 26] and also by different electrophoretic methods.[27] Many of the above separation methods are either uneconomical or too time-consuming to be used routinely for large scale purification and phenotyping of dairy cow populations.

We report here a novel method which can be used for both preparative and analytical scale purification of βLG A and βLG B variants with native PAGE by using continuous elution electrophoresis.

Methods

SAMPLE PREPARATION; NATIVE BLG

A mixture of the phenotypes A and B from bovine milk (approximately 80%, lyophilized powder, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in deionized water, pH 7.0 (20 mg/300 µl), and the protein concentration of the solution was measured both at 280 nm (Shimadzu UV-1201 spectrophotometer) $\varepsilon^{1\%}1$ cm=9.5[28] and by using Bio-Rad's Protein Assay (Bio-Rad, Richmond, CA, USA) modifying the method of Bradford[29] at 595 nm. For preparative electrophoresis (PE) 0.0625 M Tris-HCl (100 µl), pH 6.8, containing 25% glycerol and bromophenol blue 0.012% (final concentration) were added to the βLG solution.

STANDARDS

βLG variants A, B and A/B from bovine milk (Sigma Chemical Co., St. Louis, MO, USA), bovine a-lactalbumin (Sigma Chemical Co., St. Louis, MO, USA), a mixture of caseins (Sigma Chemical Co., St. Louis, MO, USA), and bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA), 10 mg each were dissolved in 10 ml deionized water. Protein concentrations of the standards were estimated, and the EF samples were prepared as described above. Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) low molecular weight standards, kaleidoscope polypeptide standards, and isoelectric focusing (IEF) standards were from Bio-Rad (Bio-Rad, Richmond, CA, USA).

PREPARATIVE CONTINUOUS ELUTION PAGE ELECTROPHORESIS

Buffers used in preparative native PAGE were those described by Ornstein-Davis.[30] The electrophoresis equipment used was Bio-Rad's Model 491 Prep Cell. The separating gel (110 x 37 mm) was 15% acrylamide/0.4% bisacrylamide in 0.375 M Tris-HCl (final concentration), pH 8.8 (catalyst concentrations in resolving gel were 0.025% APS/0.025% TEMED). The stacking gel (10 x 37 mm) was 3% acrylamide/0.08% bisacrylamide in 0.125 M Tris-HCl (final concentration), pH 6.8. The running buffer was 0.025 M Tris-base, 0.19 M glycine, pH 8.3. Before sampling loading, an

electrophoresis field was applied at constant power at 5 W for 30 minutes Thereafter 400 µl sample was injected and PE was run at constant power of 12 W for 4,000 Vh for 19 h at 4 °C. Elution flow rate was 0.75–1.0 ml/min and fractions of 5 ml were collected. Elution of βLG variants was monitored at 280 nm with a UV detector (Bio-Rad BioLogic LP workstation, Model 2128).

Protein concentrations of the eluted fractions were estimated by measuring the optical absorption of the protein fractions at 280 nm (Shimadzu UV-1201). The eluted fractions were analyzed by analytical SDS-PAGE and isoelectric focusing (IEF). According to these results, two combined fractions were each pooled and dialyzed against deionized water overnight at 4 °C. After dialysis, the two fractions were concentrated by lyophilization (Christ LMC-1, model beta 2–16), and then analyzed further by using analytical SDS-PAGE, isoelectric focusing (IEF), and immunoblotting (IB).

ANALYTICAL ELECTROPHORESIS

The molecular weights of the proteins in the two fractions, separated with the Model 491 Prep Cell, were estimated by SDS-PAGE under reducing conditions modifying the method of Laemmli.[31] Briefly, gels containing a 12–20% acrylamide separating gel and a 3% stacking gel were loaded with the samples (0.5–5 µg protein/well), mixed with sample buffer (1:1), and 10 µl sample was injected per well. The gels were run (PROTEAN® II electrophoresis cell) at 20 mA constant current, 3 W, 185 Vh at RT with 0.024 M Tris/0.192 M glycine/0.1% SDS, pH 8.3 as a running buffer. Bovine βLG containing the A and B variants as also the A and the B variant (Sigma) and molecular weight standards (Bio-Rad SDS-PAGE low range and kaleidoscope polypeptide standards) were run simultaneously. The gels were stained with 0.025% Coomassie® Brilliant Blue R-250 (B-0149, Sigma Chemical Co., St. Louis, MO, USA) in 10% isopropanol/7% acetic acid, and were destained with 50% isopropanol/10% acetic acid.

ANALYTICAL IEF

The isoelectric points of the two βLG fractions separated on the Model 491 Prep Cell, were analyzed by modifying the method obtained from Bio-Rad (IEF Ready Gel Application Guide). IEF was performed on Bio-Rad IEF Ready Gels (pH 5–8) with 20 mM NaOH as a cathode buffer and 7 mM phosphoric acid as an anode buffer on Bio-Rad's Mini Prep Cell apparatus. The two βLG fractions and the standards were dissolved in distilled water containing 10% glycerol (1 mg/ml). The run was performed at 5 W constant power and 500 V_{max}. for 2.5 h. The gels were stained and destained as described above.

IMMUNOBLOTTING

The two βLG fractions, from preparative electrophoresis, were also characterized by polyclonal antiserum to βLG (Nordic Immunologic, Tilburg, Netherlands). Standards were as described above. Sample proteins, separated by PAGE or IEF, were electrophoretically blotted into nitrocellulose (0,45 µm nitrocellulose membrane, Hybond™-C, Amersham®) by the method of Towbin.[32] The electrophoretic blotting was performed with 25 mM Tris, 192 mM glycine, pH 8.3, at 30 V overnight. Residual binding sites were blocked with 1.0% bacto-gelatin for 2 h at RT, and the nitrocellulose sheet was then washed three times with PBS Tween 20 (0.05%), 10 minutes each. To detect the βLG specific proteins, the nitrocellulose sheet was incubated with the polyclonal antiserum to βLG (5 µl/50 ml PBS/0.05% Tween 20) for 2 h at RT, washed three times as above, and then incubated with horseradish peroxidase-coated second antiserum (10 µl/50 ml PBS Tween 20 (0.05%) for 2 h at RT and washed as above. The sheet was stained with 4CN (4-Chloro-1-Naphthol) according to Bio-Rad's instructions.

Results

SEPARATION OF BOVINE BLG PHENOTYPES BY CONTINUOUS ELUTION ELECTROPHORESIS

Figure 1 shows an elution profile of bovine βLG A/B separated by preparative continuous electrophoresis as described in Methods. The fractions were analyzed by analytical SDS-PAGE, and then pooled: peak I (fractions 35–43) and peak II (fractions 47–55) and concentrated. Total protein recovery of the combined fractions was 78% (15.6 mg/20 mg) while protein concentration of peak I (fractions 35–43) was 0.190 mg/ml (total 7.05 mg) and that of peak II (fractions 47–55) was 0.160 mg/ml (total 8.5 mg).

CHARACTERIZATION OF THE TWO COMBINED FRACTIONS SEPARATED BY THE MODEL 491 PREP CELL

Molecular weights of the pooled fractions analyzed by SDS-PAGE under reducing conditions are shown in Figure 2A. The two eluted fractions each have the same molecular weight as bovine βLG A/B, according to their mobility in SDS-PAGE. The two fractions show only one single band by electrophoresis.

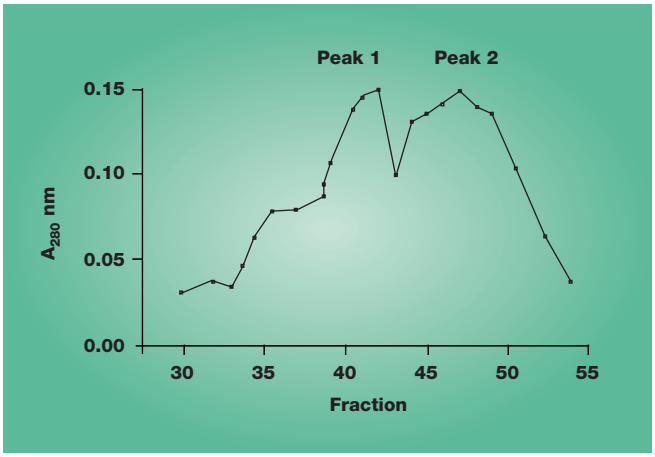

Fig. 1. *Elution profile of bovine βLG A/B by preparative continuous EF. Peaks: peak I (fractions 35–43) and peak II (fractions 47–55).*

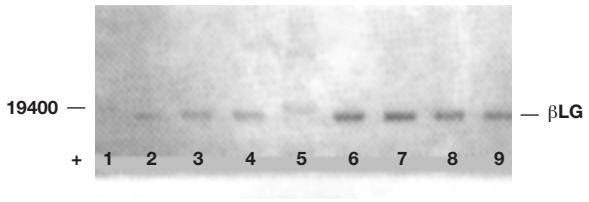

Fig. 2A. *Analysis of Model 491 Prep Cell purification samples by SDS/PAGE. Lanes 1 and 5 are molecular weight standards (1.5 µl). All other lanes are either commercial βLG or Model 491 Prep Cell purified βLG loaded at 1 µg per lane. Lanes 6 and 7 are loaded with the pooled fractions 35–43 (peak 1) from the Prep Cell, and lanes 8 and 9 are loaded with the pooled fraction 47–55 (peak 2). For comparison, lane 2 is βLG A, lane 3 is βLG B, and lane 4 is βLG A/B. The bands are visualized with Coomasie Blue R-250.*

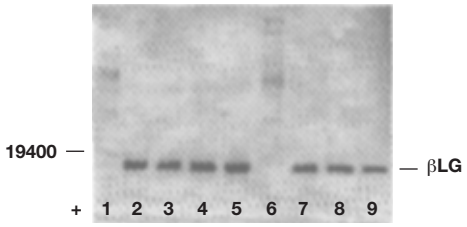

Fig. 2B. *Analysis of Model 491 Prep Cell purification samples by Immunoblot. Lanes 1 and 6 are molecular weight standards (1.5 µl). All other lanes are either commercial βLG or model 491 Prep Cell purified βLG loaded at 1 µg per lane. Lanes 4 and 5 are loaded with the pooled fractions 35–43 (peak 1) from the Prep Cell, and lanes 2 and 3 are loaded with the pooled fraction 47–55 (peak 2). For comparison, lane 9 is βLG A, lane 8 is βLG B, and lane 7 is βLG A/B. The bands are visualized as described in Methods, Immunoblotting with 4CN.*

identification of the combined fractions was confirmed by western blot with polyclonal antibodies to bovine βLG. Immunoblotting shows that βLG A/B and both eluted fractions are recognized by antiserum to βLG (Figure 2B).

Since IEF is the most sensitive method for charge distinction within different phenotypes, the isoelectric points of the two eluted fractions, separated by continuous native PAGE, were estimated also by isoelectric focusing at pH range 5–8. Commercial bovine βLG phenotype standards A, B, and A/B were analyzed for comparison. Previous data (Tulp *et al.,*[33] Conti *et al.*[34]) shows that the difference between the isoelectric points (pI) between the phenotypes βLG A and βLG B is only 0.3 pH unit. Figure 3 shows that bovine βLG A/B focused into two bands with pIs of 5.1 and 5.3. Peak I (fractions 35–43) has the same pI as phenotype βLG A (5.3), and peak II (fractions 47–55) has the same pI as phenotype βLG B (5.1).

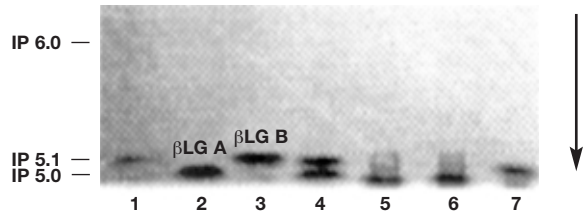

Fig. 3. *Analysis of Model 491 Prep Cell purification samples by isoelectric focussing. Lanes 1 is IEF ready gel standards (2 µl). All other lanes are either commercial βG or Model 491 Prep Cell purified (LG loaded as indicated. Lanes 5 and 6 are loaded with 5 µg of pooled fractions 35–43 (peak 1) from the Prep Cell, and lanes 7 is loaded with 0.5 µg pooled fraction 47–55 (peak 2). For comparison, 5 µg of commercial βLG isotypes is loaded as follows: lane 2 is βG A, lane 3 is βLG B, and lane 4 is βG A/B. The bands are visualized with Coomassie Blue R-250.*

Conclusions

We have developed a novel method for purifying and separating bovine βLG A and B variants by preparative electrophoresis. The molecular weight and the isoelectric points of the eluted peak 1 (fractions 35–43) and the eluted peak 2 (fractions 47–55) separated by continuous electrophoresis, were analyzed by analytical SDS-PAGE and IEF. Since the molecular weight and the isoelectric points of peak 1 were the same as those of βLG A, and the molecular weight and the isoelectric point of fraction II the same as those of bovine βLG B, we suggest that the eluted fraction I and the eluted fraction II are bovine βLG A and B variants, respectively. Both fractions were recognized with antiserum to bovine βLG A/B. The molecular weight and isoelectric points of other milk proteins, used as controls, were different from those of the eluted fractions and were not recognized by antiserum to bovine βLG (data not shown). The results confirm that the two eluted fractions are specific βLG phenotypes and do not contain any impurities. The method could be used both in analytical and preparative scale in milk industry and when studying bovine milk allergy.

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