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Development of sandwich ELISA for testing bovine β -lactoglobulin allergenic residues by specific polyclonal antibody against human IgE binding epitopes

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Abstract

Bovine β -lactoglobulin (BLG) is the main allergen in cows' milk, and the most commonly used method for detecting BLG is enzyme-linked immunosorbent assay (ELISA). However, antibodies used in commercial ELISA kits do not recognize specifically BLG IgE epitopes. Here, an antibody specific to IgE linear epitopes for BLG was used to develop a sandwich ELISA using a rabbit anti-BLG polyclonal antibody. The linear range for BLG detection was 31.25–8,000 ng/mL and limit of detection was 1.96 ng/mL. BLG content in dairy samples was determined, and there was a good agreement between this immunoassay and reversed-phase high-performance liquid chromatography with high recovery. Additionally, BLG content in food samples had an average recovery of 104.25%. Allergenic residues were also detected in hydrolyzed infant formulas. The method developed could be a practical approach to determine BLG and its allergenic residues in food with a high degree of sensitivity, reliability and recovery.

Keywords: Bovine β -lactoglobulin, Milk allergens, Allergenic residues, ELISA

1. Introduction

Cows' milk proteins are major food allergens (Nwaru et al., 2014), and cows' milk allergy affects approximately 0.6%–2.5% of preschoolers, 0.3% of older children and teens, and fewer than 0.5% of adults (Fiocchi et al., 2010). Beta-lactoglobulin (BLG) is one of the most important allergens of the whey fraction, and accounts for 10% of total milk proteins (Hochwallner, Schulmeister, Swoboda, Spitzauer, & Valenta, 2014). Accordingly, BLG has attracted much attention in consideration of milk allergy.

A variety of methods have been used to analyze BLG. Reversed-phase high-performance liquid chromatography (RP-HPLC) was developed to separate and quantify α_{S1} -casein (α_{S1} -CN), α_{S2} -CN, β -CN, κ -CN, α -lactalbumin (ALA), and BLG, as well as determine genetic variants of β -CN, κ -CN, and BLG (Bobe, Beitz, Freeman, & Lindberg, 1998). However, RP-HPLC has low sensitivity and is only suitable for detecting native proteins. Although HPLC with mass spectroscopy can overcome some shortcomings of HPLC alone, it also requires expertise and expensive equipment (Heick, Fischer, & Pöpping, 2011). In comparison with RP-HPLC, immunoassays can easily detect both native proteins and protein fragments.

Enzyme-linked immunosorbent assay (ELISA) is the primary immunoassay method for food allergen with the advantages of being rapid, specific, and simple to operate (Zhou et al., 2013; Zhang, Lu, Ushio, & Shiomi, 2014; Costa et al., 2015). For

example, Negroni et al. (1998) developed a sandwich ELISA (sELISA) based on monoclonal antibodies to detect native and denatured BLG. Several commercial ELISA kits have been produced to satisfy demand for a rapid and reliable test for BLG. However, antibodies used in conventional commercial ELISA kits do not recognize specific human BLG IgE epitopes, resulting in inaccurate quantification of BLG allergic residues in dairy products, such as hydrolyzed infant formulas (Mariager, Sølve, Eriksen, & Brogren, 1994; Rosendal & Barkholt, 2000). In this study, a sELISA based on a polyclonal antibody specific for human IgE epitopes on BLG was developed. The effects of physical (e.g., incubation time) and chemical conditions (e.g., blocking agents and ion concentration) on the performance of the sELISA were assessed. Of this method, the limit of detection (LOD), detection range, precision, accuracy, and recovery were also evaluated. Reliability was validated against RP-HPLC.

2. Materials and methods

2.1 Materials

CNBr-activated SepharoseTM 4B resin was purchased from GE Healthcare (Uppsala, Sweden). EZ-Link® Sulfo-NHS-LC-Biotinylation Kit was obtained from Thermo Scientific Pierce (Rockford, USA). Complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), horseradish peroxidase conjugated goat anti-rabbit IgG (HRP-IgG), o-phenylenediamine (OPD), ALA (purity ≥ 85%, electrophoresis), BLG

(purity $\geq$ 90%, PAGE), α -CN (purity $\geq$ 70%, electrophoresis), β -CN (purity $\geq$ 98%, PAGE), κ -CN (purity $\geq$ 70%, PAGE), and gelatin from cold water fish were obtained from Sigma-Aldrich (St. Louis, USA). Bovine serum albumin (BSA) was purchased from Sangon Biotech (Shanghai, China). Fetal bovine serum (FBS) was acquired from Life Technologies (Gibco, USA). Polyethylene glycol (PEG) 6000 and PEG 8000 were purchased from Solarbio (Beijing, China). Horseradish peroxidase-streptavidin (HRP-streptavidin) was acquired from Neobioscience (Shenzhen, China). Ovalbumin (OVA) was provided by our laboratory (purity $\geq$ 95%, electrophoresis). Peptides (purity $\geq$ 95%, RP-HPLC) were synthesized by GL Biochem (Shanghai, China). UHT-milk and yoghurt were purchased from a local supermarket. Whey powder (Nutri Whey 800 Instant, protein: 78%) was obtained from Friesland Campina DMV B.V. (Beijing, China). All reagents were analytical grade and solutions were prepared using Milli-Q water throughout the experiments.

Crude soybean protein was prepared according to Liu et al. (2007). Similarly, peanut protein was prepared according to Hu et al. (2011). Wheat, chocolate, and candy were purchased from a local supermarket. The samples were ground into fine powder and dissolved in extraction buffer (1 g of powder in 20 mL extraction buffer, 20 mmol/L Tris-HCl, 2% Tween-20, pH 8.0). The mixture was stirred overnight at 4 °C, and then the supernatant was collected by centrifugation at $10,000 \times g$ for 10 min at 4 °C.

Whey was prepared by centrifugation and isoelectric precipitation to remove fat and CNs from UHT-milk (Stojadinovic et al., 2012).

2.2 Affinity purification of rabbit polyclonal antibodies

Epitope sequences in epitope tandem derived from BLG (tBLG), a recombinant protein containing seven human IgE linear epitopes for BLG (He et al., 2016), are shown in Table S1. All protocols involving animals were approved by the University of Nanchang Animal Care Committee, under the guideline of China Council for Animal Care (SCXK-Gan 2014-0005, China). Rabbit anti-BLG polyclonal antibody (pAb-BLG) and rabbit anti-tBLG polyclonal antibody (pAb-tBLG) were produced as described in our previous studies (Li et al., 2015; He et al. 2016). Briefly, purified BLG (0.5 mL, 2 mg/ml) and tBLG (0.5 mL, 2 mg/ml) were emulsified with CFA (0.5 mL) for the first injection. Subsequently, three booster injections were performed with 1 mg of protein (2 mg/ml) emulsified in IFA (0.5 mL) at two-week intervals. The rabbits were bled one week after the last booster, and the serum collected was stored at -20°C until use.

The pAb-BLG and pAb-tBLG were purified on a 2 mL Sepharose CNBr immunoaffinity column. The gel (0.45 g) was swelled in 30 mL of 1 mmol/L HCl at 0°C for 20 min, and washed with 1 mmol/L HCl for 20 min. The gel was washed exhaustively with coupling buffer (0.1 mol/L NaHCO_3 containing 0.5 mol/L NaCl, pH 8.3) before coupling with 15 mg of BLG in 2.5 mL coupling buffer overnight at 4°C . The washing step was performed again with 20 mL of the coupling buffer, followed by blocking with 10 mL of Tris-HCl (0.1 mol/L, pH 8.0) at room temperature for 2 h.

Subsequently, the gel was washed with 10 mL of each of the following buffers:

sodium acetate (0.1 mol/L, pH 4.0) containing 0.5 mol/L NaCl, and Tris-HCl (0.1 mol/L, pH 8.0) containing 0.5 mol/L NaCl. This step was repeated three times and was followed by washing with 10 mL of phosphate buffer saline (PBS, 140 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄ and 1.8 mmol/L KH₂PO₄, pH 7.2).

Aliquots (2 mL) of rabbit antiserum were diluted (1:1) in PBS and passed through a 0.45 µm filter. Antiserum was loaded on the Sepharose gel column by gravity. Then, the column was washed with 15 volumes of PBS. Subsequently, the elution was carried out with 10 volumes of 3 mol/L MgCl₂. The fractions were collected and concentrated using Amicon Ultra-15 centrifugal filter, and MgCl₂ was removed by washing with PBS. The purified antibodies were stored in 50% glycerol at -20 °C until use.

2.3 Biotinylation of pAb-tBLG

The purified pAb-tBLG were biotinylated using an EZ-Link[®]

Sulfo-NHS-LC-biotinylation Kit according to the manufacturer's instructions. Briefly,

13.5 µL of 10 mmol/L Sulfo-NHS-LC-Biotin was added to 0.5 mL of pAb-tBLG (2 mg/mL), followed by incubation at 0 °C for 2 h. The excess biotin reagent was removed using a desalting column, and the biotinylated pAb-tBLG in 50% glycerol were stored at -20 °C until use.

2.4 Polyclonal antibody binding to epitopes of BLG

The antibodies should bind effectively to the epitopes on BLG for successful detection of BLG allergenic residues. Allergenic residues in food are usually larger than the epitopes, and peptide should be larger than 30 amino acids for cross-linking IgE on mast cells (Knol, 2006). Seven epitopes and their blocking peptides were synthesized to analyze whether pAb-BLG and biotinylated pAb-tBLG could recognize IgE epitopes and peptides larger than an epitope. The blocking peptide was obtained by adding an amino acid at the N-terminal and C-terminal of the epitope. The sequences of epitopes and blocking peptides are shown in Table S1.

The binding capacity for IgE epitopes on BLG of pAb-BLG and biotinylated pAb-tBLG was tested by competitive inhibition ELISA. The ELISA microtiter plate was pre-coated with 100 μ L of BLG (2 μ g/mL for pAb-BLG, and 1 μ g/mL for biotinylated pAb-tBLG) in 50 mol/L sodium carbonate buffer (pH 9.6) overnight at 4 °C, and sodium carbonate buffer as the control. Peptide (70 μ L; 2, 6, 18, and 54 μ g/mL for pAb-BLG, and 1, 3, 9, and 27 μ g/mL for biotinylated pAb-tBLG) or PBS (as control) was mixed with 70 μ L of polyclonal antibody (0.5 μ g/mL for pAb-BLG, and 0.25 μ g/mL for biotinylated pAb-tBLG) and incubated for 1 h at 37 °C. The BLG pre-coated plate was washed three times with 300 μ L of PBS containing 0.05% Tween-20 (PBST), and blocked with 250 μ L of 3% gelatin in PBS for 1 h at 37 °C. After further washing, 100 μ L of polyclonal antibody pre-incubated with peptide solution or PBS was added to each well and incubated for 1 h at 37 °C. The wells

were washed again, and 100 μ L of HRP-IgG (diluted 1:5,000, for pAb-BLG) or streptavidin-HRP (diluted 1:60, for biotinylated pAb-tBLG) added to each (1 h at 37 °C). The wells were again washed three times with PBST and 100 μ L OPD substrate solution for color development was added. Samples were incubated for 15 min at 37 °C. The reaction was stopped with 50 μ L of 2 mol/L sulfuric acid, and the optical density (OD) measured at 490 nm using a microplate reader (Model 1860, Bio-Rad, USA). All peptides and antibodies were diluted in PBS. The percent inhibition (PI) values were expressed as: $PI (\%) = [1 - (A1-A0)/(A2-A0)] \times 100$ where A0, A1, and A2 represent ODs of the control, antisera with inhibitor and the antisera without inhibitor, respectively.

2.5 *sELISA procedure*

The pAb-BLG, as the capture antibody, was diluted in carbonate buffer solution immediately before use. A 96 wells microtiter plate was coated with capture antibody solution (1 μ g/mL) and incubated overnight at 4 °C. Each well was washed five times with PBS containing 0.1% Tween-20 (PBST), and blocked with 250 μ L of 4% gelatin in PBST and incubation at 37 °C for 90 min. After washing, 100 μ L of BLG (or dairy samples) was added to each well, incubated at 37 °C for 90 min, and washed again. To each well, 100 μ L of biotinylated pAb-tBLG (1 μ g/mL) was added, as the detection antibody, and incubated at 37 °C for 30 min before washed again. Subsequently, to each well streptavidin-HRP diluent was added and incubated at 37 °C for 1 h. After

washing thoroughly with PBST, to each well 100 μ L of OPD solution was added at 37 °C in the dark for 20 min for color development. The color development reaction was stopped by adding 50 μ L of 2 mol/L sulfuric acid, and OD was read at 490 nm with a Bio-Rad microplate reader. The sample, detection antibody and streptavidin-HRP were diluted with the blocking buffer.

2.6 LOD, accuracy, precision and specificity

The LOD and limit of quantitation (LOQ), accuracy, and precision for BLG detection were calculated according to the Eurachem Guide (de Bievre et al., 1998). LOD and LOQ were described as the protein concentration corresponding to the means of 10 blank values plus 3 or 10 times the standard deviations (SD), respectively. Accuracy was evaluated using bias (%), which was expressed as the difference (%) between BLG content measured using the immunoassay developed and the theoretical content. The precision of the assay was expressed as relative standard deviation of repeatability (RSDr) and reproducibility (RSDR) at a series of protein concentrations. Repeatability and reproducibility were expressed as measurement of 10 replicates at different concentrations of BLG in one day or within a month, respectively. Results were calculated as: $RSDr \text{ (or RSDR)} (\%) = SD/mean \times 100$.

The specificity of the method was evaluated with ALA, BSA, α -CN, β -CN, κ -CN, OVA, and soy, peanut and wheat proteins for cross-reactivity at 0.1, 0.5, 1.0, 2.0, 4.0, and 8.0 μ g/mL.

2.7 Detection of food samples

Reliability of the proposed method was verified by validating the BLG content in UHT-milk (defatted, and diluted 80,000 times before the test), whey and whey powder using RP-HPLC with UV detection at 220 nm (Bobe, Beitz, Freeman, & Lindberg, 1998). Moreover, BLG content in yoghurt, two chocolates (I and II), and candy were tested, and six brands of hydrolyzed infant formulas were chosen to determine the presence of epitope residues.

2.8 Tricine-SDS -PAGE

Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS -PAGE) was employed to define the molecular weight distribution of hydrolyzed infant formulas. Samples were diluted at 1:1 in sample buffer (40% sugar, 0.05 M Tris, 4% SDS, and 0.08% bromophenol blue, pH 6.8), followed by heating at 99 °C for 5 min. Electrophoresis (16.5% acrylamide/bisacrylamide for separating gel, 4% acrylamide/bisacrylamide for the stacking gel, 10% acrylamide/bisacrylamide for spacer gel between stacking and separating gels) was performed using vertical mini gel electrophoresis (Bio-Rad, USA) with 20 µL of each sample (50 µg of protein per well). Electrophoretic separation was conducted at 30 V for the stacking gel, and 100 V for the spacer and separating gels. The gels were fixed in 50% methanol containing 10% acetic acid for 20 min and stained with 0.05% Coomassie brilliant blue R-250 in

fixing solution for 30 min before being thoroughly rinsing in destaining solution (5% methanol and 7.5% acetic acid). The gels were imaged by GS-800TM Densitometer (Bio-Rad, USA).

3. Results and discussion

3.1 Characteristics of pAb-BLG and pAb-tBLG binding to IgE epitopes on BLG

The two antibodies recognized target epitopes and blocking peptides, as shown in Fig.

1. The epitopes and blocking peptides exhibited similar inhibition, and inhibition rates increased with increasing peptide concentration. As shown in Fig. 1A, for pAb-BLG, all the peptides had similar inhibition rates. For biotinylated pAb-tBLG, however, the inhibition rates of B5 and b5 were greater than that of other peptides (Fig. 1B). This difference might be due to the antigenicity of B5, which was greater than other epitopes, resulting in the production of more rabbit antibodies in response to B5. These results suggest that pAb-BLG and biotinylated pAb-tBLG bound to all epitopes and blocking peptides.

3.2 Optimization of sELISA procedure

The protocol for the sELISA was optimized step-by-step; BLG, pAb-BLG, and biotinylated pAb-tBLG were used as the antigen, capture antibody, and detection antibody, respectively.

3.2.1 Optimization of the content of capture antibody and detection antibody

Serial dilutions of capture and detection antibodies were used to determine the working concentration of each reagent. The minimum concentration used to produce an OD value of approximately 1.0 was selected. Optimal concentrations for the capture and detection antibodies were 1 µg/mL (Table S2).

3.2.2 Optimization of blocking agent and time

Non-specific binding in ELISA can result in a high background signal, and poor sensitivity and selectivity. Blocking is a key factor to reduce nonspecific binding (Huber et al., 2009). In this study, the effect of protein blockers (gelatin, BSA, and FBS) and non-protein blockers (PEG 6000 and PEG 8000) were evaluated. Tween-20 is a very common reagent used to reduce non-specific binding, particularly hydrophobic sites (Garcia, Gonzalez, Marcipar, Vega, & Gugliotta, 2014).

Accordingly, the various blocking agents were dissolved in PBST to minimize the background signal. The positive value (P, OD of sample with BLG) to negative value (N, OD of sample without BLG) (P:N) ratio (Zhou et al., 2013) was used to evaluate the effect of the different blocking agents and time (Figs. 2A and B). The results showed that gelatin was the best blocking agent and the highest P:N value was achieved with 4% gelatin. BSA is a very common blocking agent used in ELISA (Ahn, Lee, & Ko, 2014), while our results indicated that both positive and negative values were increased following blocking with BSA, the resulting P:N values were low (Fig.

2A), which might be because BSA can cause non-specific background signals (Xiao & Isaacs, 2012). ODs of positive and negative were very low when blocked with FBS (Fig. 2A). Although PEG has been reported to enhance frequency response, amplify response signal, and improve sensitivity (Wang, Lei, Li, Wu, Shen, & Yu, 2004; Holownia, Perez-Amodio, & Price, 2001), however, our results showed that PEG improved both positive value and negative signal, resulting in reduced sensitivity (Fig. 2A). Thus, 4% gelatin in PBST was used in all subsequent work, as a blocking agent. Diluting the sample, detection antibody, and streptavidin-HRP with the blocking buffer reduced background signals. Additionally, blocking time from 1 h to 2 h was evaluated. The highest P:N value was obtained at 1.5 h (Fig. 2B) and used subsequently.

3.2.3 Influence of ionic strength

Different concentrations of NaCl (100, 137, 200, 300, and 400 mmol/L) were investigated to evaluate the impact of ionic strength. Silva et al. (2013) reported that NaCl concentration had no effect on the performance of ELISA for quantifying estrogens. However, recent studies have shown that ionic strength influences significantly the antigen-antibody reaction (Garcia, Gonzalez, Marcipar, Vega, & Gugliotta, 2014; Wang, Lei, Li, Wu, Shen, & Yu, 2004). In this work, salinity also influenced the P:N value. Both positive and negative values decreased with increasing concentration of NaCl, with the maximum P:N value was obtained at 100 mmol/L

NaCl (Fig. 2C). Consequently, 100 mmol/L of NaCl was selected as the optimal ion concentration of PBS.

3.2.4 Optimization of incubation time of BLG

To optimize incubation time, set times of 45 min, 60 min, and 90 min were investigated. Results showed that the P:N value increased to a maximum at 90 min (Fig. 2D). Thus, 90 min was selected as optimal.

3.2.5 Optimization of incubation time of detection antibody

The incubation time for biotinylated pAb-tBLG also affected the P:N value. The positive value was not affected significantly by time, but the negative value increased with prolonged incubation (Fig. 2E). The P:N value declined significantly with time. Hence, 30 min was chosen as optimal for the detection antibody.

3.2.6 Optimization of incubation time of streptavidin-HRP

A series of incubation times for streptavidin-HRP were investigated (Fig. 2F). The P:N value reached a maximum when streptavidin-HRP was incubated for 1 h. Therefore, 1 h of incubation was selected as the optimal for streptavidin-HRP.

3.2.7 Optimization of incubation time of OPD solution

The positive value increased significantly when substrate reaction time was increased

(Fig. 2G), and the background signal increased slowly when developing time was more than 15 min. The highest P:N value was obtained at 20 min (Fig. 2G), which was used subsequently.

3.3 ELISA standard curve for BLG detection

The sELISA was developed to detect BLG using pAb-BLG and biotinylated pAb-tBLG as capture and detection antibodies, respectively. A calibration curve with BLG ($0.98\text{--}5.12 \times 10^5$ ng/mL) was constructed using the selected conditions (Fig. 3A). The linear range was 31.25 ng/mL to 8×10^3 ng/mL ($r^2=0.9928$, Fig. 3B). The calculated LOD and LOQ were 1.96 and 3.91 ng/mL, respectively. The sensitivity of the sELISA developed (1.96 ng/mL) was greater than those for RP-HPLC (10 μ g/mL) (Wang, Qu, Liu, & Feng, 2013) and commercial sELISA kits, based on rabbit polyclonal antibody (0.07 mg/kg) (Stumr et al., 2009), but less than that of sELISA based on monoclonal antibodies (30 pg/mL) (Negroni et al., 1998).

A mean bias value of 0.47% (Table S3) indicated excellent accuracy. The intra-assay and inter-assay precisions were (RSDr) 7.13% to 14.47% (mean=10.66%) and (RSDR) 6.39% to 16.58% (mean=11.72%) (Table S3), respectively, which suggest good precision.

3.4 Cross-reactivity of the sELISA

The specificity of the sELISA developed was evaluated. No cross-reactivity was

found when using ALA, BSA, OVA, or soy, peanut and wheat proteins (Fig. 3C).

Weak cross-reactivity was observed with κ -CN at 2 $\mu\text{g/mL}$ and α -CN and β -CN at 1 $\mu\text{g/mL}$. These results might be caused by impurities in α -CN ($\geq 70\%$) and κ -CN ($\geq 70\%$), specifically residual BLG (Mäkinen-Kiljunen & Palosuo, 1992). A previous study also showed weak cross-reactivity with CNs (Negroni et al., 1998). Moreover, cows' milk protein-sensitized mice and cows' milk allergy patients reacted to soy proteins (Ahn, Han, Nam, Park, Shin, & Lee, 2003; Smaldini et al., 2012). However, in this present study, no cross-reactivity was found between BLG and soy proteins (Fig. 3C), indicating that the sELISA developed is highly specific.

3.5 Detection of the food samples

3.5.1 Validated with RP-HPLC

Recovery experiments were performed by spiking UHT-milk, whey, and whey powder with 0.5 and 4 mg/mL of BLG. Samples were analyzed using the sELISA developed and RP-HPLC. Mean recoveries for BLG from milk, whey, and whey powder were 99.59%, 99.37%, and 101.75% (Table 1), respectively, which were also consistent with the RP-HPLC results. These findings demonstrated that the developed immunoassay could be used for BLG detection in milk and whey samples.

3.5.2 Detection of real samples

BLG content in yoghurt, chocolates, and candy were tested and fortified with BLG at

1 mg/mL (or 1 mg/g) and 4 mg/mL (or 4 mg/g). The amount of BLG in yoghurt, chocolate I, and chocolate II were 2.50 mg/ml, 5.70 mg/g, and 2.68 mg/g with average recoveries of 100.00%, 103.38%, and 106.32% (Table 2), respectively. However, BLG was not detected in candy, and the recoveries when fortified with 1 or 4 mg/g were 103.66% and 109.58%, respectively (Table 2). These results indicate that the method developed is suitable for detecting BLG in food samples with high recovery.

3.5.3 Detection of hydrolyzed infant formulas

Cows' milk allergy is the most common allergy in infancy, and hydrolyzed infant formula is a good alternative food for children with cows' milk allergy. Infant formulas are processed by hydrolysis and heating, resulting in considerable destruction of conformational epitopes (Nowak-Wegrzyn et al., 2008). Nevertheless, sequential epitopes may still exist and could be recognized by pAb-tBLG. In this work, two extensively hydrolyzed infant formulas and four partially hydrolyzed infant formulas were tested using the developed sELISA (Table 3).

BLG content varied considerably. In the four partially hydrolyzed infant formulas, BLG content was 300-fold higher than in "Tummy Care" (71,612.48 μ g/g, highest) compared with "NAN HA" (221.91 μ g/g, lowest). The amount of BLG in "NAN PRO" (518.75 μ g/g) was similar to "Precinutri (HA)" (614.08 μ g/g). However, the allergenic residual of BLG in the extensively hydrolyzed infant formulas could not be detected using the assay developed.

Tricine-SDS-PAGE analysis showed no distinct bands in the extensively hydrolyzed infant formulas (Fig. S1). This characteristic might explain why the allergenic residues were not detected by our sELISA. Moreover, several larger peptides/ proteins were found in “NAN PRO” and “Precinutri (Pepti)” compared with “NAN HA” (Fig. S1, b). This characteristic might also explain why sELISA results were higher for BLG content in “NAN PRO” and “Precinutri (Pepti)” than in “NAN HA” (Table 3). Furthermore, numerous larger peptide/ protein fragments, with molecular weights greater than 30 kDa, were detected in “Tummy Care” (Fig. S1, a and b). Bands corresponding to molecular weights greater than 18 kDa might have resulted from the aggregation of some peptides/ proteins, although a number of peptides larger than 3.4 kDa were also detected (Fig. S1, lane 6). When peptides contained two or more epitopes, and were recognized by both pAb-BLG and biotinylated pAb-tBLG, they could be detected using our sELISA, which might explain the apparent content of BLG allergenic residues in Tummy Care (Table 3).

4. Conclusion

In this study, a sELISA, based on an epitope specific polyclonal antibody, exhibited a working range between 31.25 ng/mL and 8×10^3 ng/mL for BLG testing, with a LOD of 1.96 ng/mL. Analyte recoveries for milk (95.17%–104.00%), whey (98.00%–100.61%), whey powder (98.83%–104.67%), yoghurt (98.33%–101.67%), chocolate I (102.00%–104.75%), chocolate II (102.11%–110.53%), and candy

(103.66%–109.58%) indicated good reliability. BLG allergic residues could be detected in hydrolyzed infant formulas. This study describes a reliable method for detecting, quantifying, and monitoring BLG and its allergenic residues in dairy samples.

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Caption of Figures

Fig. 1. Recognition of epitopes and blocking peptides by (A) pAb-BLG and (B)

biotinylated pAb-tBLG were tested by competitive inhibition ELISA. B1–B7

represent the seven epitopes, and b1–b7 represent the seven blocking peptides.

Results are represented as mean $\pm$ SD (n=3).

Fig. 2. Optimization of sandwich ELISA parameters: (A) blocking agents, (B)

blocking time, (C) ion concentration of PBS, (D) incubation time of sample, (E)

incubation time of detection antibody, (F) incubation time of streptavidin-HRP, and

(G) incubation time of OPD solution. Results are represented as mean $\pm$ SD (n=3).

Fig. 3. Analytical performance of the developed sandwich ELISA. Calibration curve

(A) and standard curve (B) of the optimized sandwich ELISA for BLG detection.

Cross reactivity of the sandwich ELISA method with ALA, BSA, α -CN, β -CN, κ -CN,

OVA, soy proteins, peanut protein, and wheat protein (C). Results are represented as

mean $\pm$ SD (n=3).

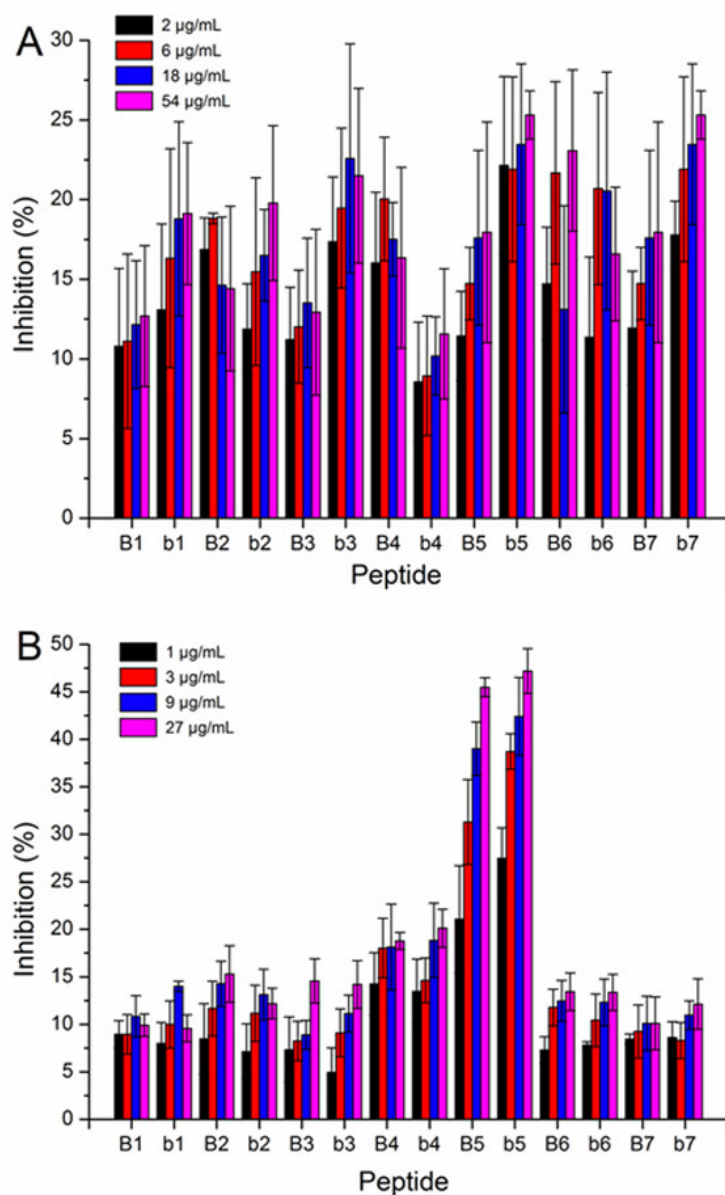


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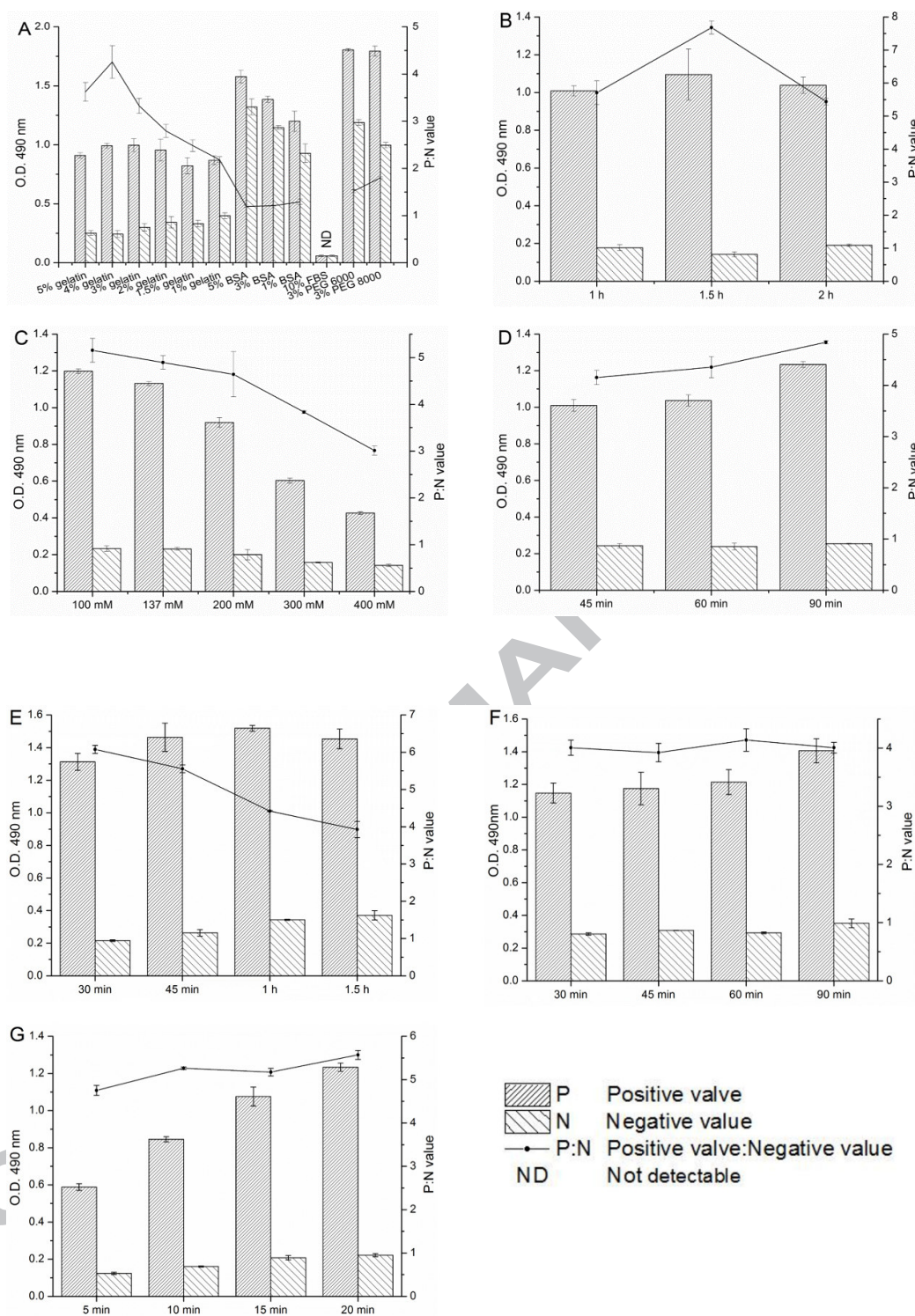


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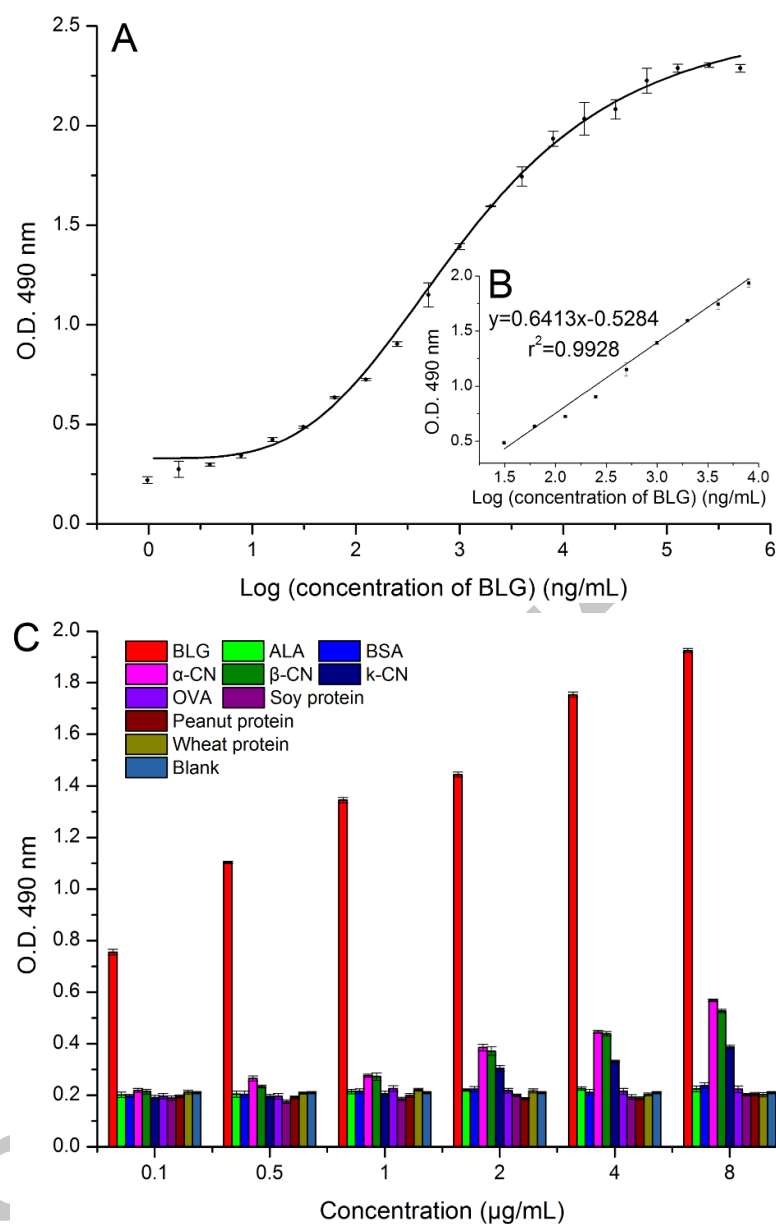


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Table 1. Recoveries of BLG in milk and whey tested by different detection methods.

Sample	Spiked concentration (mg/mL)	Detected concentration (mg/mL)		Recovery (%)	
		Sandwich	HPLC	Sandwich	HPLC
		ELISA		ELISA	
milk	0	3.48 $\pm$ 0.17	3.37 $\pm$ 0.02		
	0.5	4.00 $\pm$ 0.10	3.89 $\pm$ 0.02	104.00 $\pm$ 20.29	101.33 $\pm$ 4.16
	4	7.29 $\pm$ 0.78	7.40 $\pm$ 0.04	95.17 $\pm$ 19.53	100.42 $\pm$ 0.76
Whey	0	0.66 $\pm$ 0.04	0.63 $\pm$ 0.01		
	0.5	1.15 $\pm$ 0.06	1.22 $\pm$ 0.04	98.00 $\pm$ 12.17	114.00 $\pm$ 6.00
	4	4.69 $\pm$ 0.55	4.60 $\pm$ 0.02	100.61 $\pm$ 13.79	104.70 $\pm$ 0.11
Whey	0	1.80 $\pm$ 0.13	1.81 $\pm$ 0.01		
Powder ^a	0.5	2.32 $\pm$ 0.11	2.36 $\pm$ 0.06	104.67 $\pm$ 22.12	110.67 $\pm$ 12.22
	4	5.75 $\pm$ 0.68	5.87 $\pm$ 0.05	98.83 $\pm$ 16.97	101.43 $\pm$ 1.48

^a The whey powder with a concentration of 4 mg/mL.

Results are represented as mean $\pm$ SD (n=3).

Table 2. Recoveries of BLG in food samples tested by sandwich ELISA.

Food description	Spiked concentration (mg/mL or mg/g) ^a	Concentration (mg/mL or mg/g) ^a	Recovery (%)
Yoghurt, milk	0	2.50±0.15	
	1	3.49±0.16	98.33±15.50
	4	6.57±0.53	101.67±13.14
Chocolate I, whey powder	0	5.70±0.17	
	1	6.72±0.14	102.00±13.80
	4	9.89±0.41	104.75±10.28
Chocolate II, skim milk powder	0	2.68±0.15	
	1	3.70±0.17	102.11±16.68
	4	7.10±0.48	110.53±12.16
Candy, no milk	0	Not detectable	
	1	1.04±0.09	103.66±9.07
	4	4.38±0.44	109.58±11.06

^aThe mg/mL for yoghurt, and mg/g for chocolates and candy.

Results are represented as mean ± SD (n=3).

Table 3. BLG content in hydrolyzed formulas detected by sandwich ELISA.

Formula			BLG concentration	
Product	Manufacturer	Protein content (%)	dry weight ($\mu\text{g/g}$)	Ready-to-use formula ($\mu\text{g/L}$)
Alfaré	Nestlé	14.30	ND	ND
Precinutri (Pepti)	Dumex	11.60	ND	ND
NAN PRO	Nestlé	11.50	518.75 $\pm$ 83.01	76,083.33 $\pm$ 12174.80
NAN HA	Nestlé	9.75	221.91 $\pm$ 31.89	32,250.92 $\pm$ 4634.68
Precinutri (HA)	Dumex	11.00	614.08 $\pm$ 55.59	94,158.93 $\pm$ 8523.80
Tummy Care	Abbott	11.75	71,612.48 $\pm$ 1,747.59	9,517,298.59 $\pm$ 23,2254.70

ND: Not detectable. BLG allergenic residues did not be detected with hydrolysed

formulas protein concentration of 1.25 mg/mL.

Results are represented as mean $\pm$ SD (n=3).

Highlights

- A sandwich ELISA was developed based on an antibody specific for cows' milk developed from allergy patients IgE epitopes for beta-lactoglobulin.
- The method is linear from 31.25 ng/mL to 8 µg/mL, and the limit of detection for beta-lactoglobulin is 1.96 ng/mL.
- The sandwich ELISA developed could be applied to determine beta-lactoglobulin allergenic residues in dairy products.