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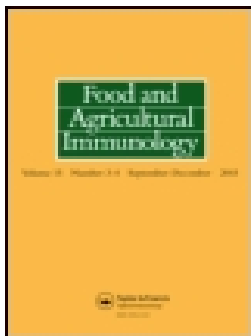
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Preparation, immunological characterization and polyclonal antibody development for recombinant epitope tandem derived from bovine β -lactoglobulin

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ABSTRACT

Bovine β -lactoglobulin (BLG) is one of the major allergens in cow's milk. Epitopes play a key role in food allergy, while most of the antibodies developed do not specifically recognize the IgE-binding epitopes. Here, a tandem derived from human IgE linear epitopes of BLG (tBLG) was designed according to the principles of epitope-based vaccine methodology, expressed in *Escherichia coli*, and a polyclonal antibody (pAb-tBLG) was developed. The recombinant tBLG protein preserved the antigenicity and potential allergenicity of epitopes. The pAb-tBLG recognized all the IgE epitopes of tBLG, and showed a high specificity, but it had weak cross-reactivity with caseins. We concluded that pAb-tBLG can detect of BLG and may be useful for identifying allergenic residues in dairy products.

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Milk allergy; β -lactoglobulin; epitope tandem; polyclonal antibody; ELISA

Introduction

Cow's milk, regarded as one of the eight major allergic food (Poms, Klein, & Anklam, 2004), is one of the most common and wide-spread allergy sources, especially in infants (Høst & Halcken, 1990). Approximately 0.6–2.5% of preschoolers, 0.3% of older children and teens, and less than 0.5% of adults suffer from cow's milk allergy (CMA) (Fiocchi et al., 2010), and approximately 85% of children with milk allergy become tolerant by school age (Wood, 2003). Cow's milk contains more than 30 types of proteins, and all of which have can potentially induce allergic reactions. β -lactoglobulin (BLG) accounts for approximately 10–15% of total milk proteins and represents a serious health risk as it is known as one of the major milk allergens (Restani, Ballabio, Di Lorenzo, Tripodi, & Fiocchi, 2009). BLG belongs to lipocalin family and consists of 162 amino acids with a molecular mass of 18.3 kDa. The secondary structure of native BLG is composed of nine anti-parallel β -sheets, forming a central calyx (Tavel, Moreau, Bouhallab, Li-Chan, & Guichard, 2010), making it stable and highly resistant to gastrointestinal

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digestion (El-Zahar et al., 2005), especially in children whose digestive system is still not fully developed.

Epitopes, including B and T cell epitopes, are the basic cause of allergic reactions. In general, B cell epitopes comprise two forms, conformational and sequential epitopes, both of which can cause allergic patients to generate specific IgE antibodies. Given that milk and dairy products often undergo processing, such as boiling, baking, and hydrolysis, the tertiary structure of proteins could be destroyed (Nowak-Węgrzyn et al., 2008), leading to the destruction of most conformational epitopes. By contrast, sequential epitopes may still exist until the amino acid sequence of the protein is destroyed. Most studies focused on linear IgE epitopes of BLG, for example, one major IgE-binding linear epitope (AA97-108) of BLG was identified using radioimmunoassay with 16 sera from CMA patients by Ball et al. (1994). Eight IgE epitopes, namely, peptides AA1-8, AA9-14, AA25-40, AA41-60, AA84-91, AA92-100, AA102-124, and AA149-162, from enzymatically hydrolyzed BLG were identified with sera from 46 CMA patients by Sélo et al. (1999). And seven IgE epitopes, namely, peptides AA1-16, AA31-48, AA47-60, AA67-78, AA75-86, AA127-144, and AA141-152, were identified using spot technology with sera from 19 milk-allergic patients by Järvinen, Chatchatee, Bardina, Beyer, and Sampson (2001). In addition, peptides AA 58-77, AA 76-95, and AA 121-140 were recognized by more than 75% of CMA children ($n = 16$) by Cerecedo et al. (2008). T cell epitopes are also important when the allergens are presented to the immune system. In previous studies, peptides AA 67-75, AA 71-79, and AA 80-88 of BLG were identified as dominant T cell epitopes in a mouse model (Totsuka, Ametani, & Kaminogawa, 1997). Moreover, seven major human T cell epitopes were identified, including AA1-21, AA14-29, AA30-47, AA47-67, AA77-97, AA97-117, and AA 142-162 of BLG (Inoue et al., 2001).

Epitope-based peptide vaccines have been developed in recent years with stability, low-cost, high specificity, and efficient antigen presentation (Giri, Ugen, & Weiner, 2004). The vaccines consist of B cell epitope, T cell epitope, and linker. The role of B cell epitopes is to trigger humoral immune response, whereas the role of T cell epitope is to induce cellular immune response. In general, linker sequences were inserted between different epitopes to make each epitope possible to induce immune response (Tian et al., 2014). For example, based on this principle, recombinant linear neutralizing epitope tandem of human enterovirus 71 and polypeptides derived from capsid protein VP1 were expressed in *Escherichia coli*, followed by producing specific mouse neutralization antibodies (IgG) against the recombinant protein (Jang et al., 2014; Li et al., 2014). Moreover, specific antibodies were produced against all the epitopes of a recombinant epitope tandem protein derived from *Helicobacter pylori* urease (Guo et al., 2014). Accordingly, this kind of antibody can be produced for detecting epitopes *in vitro*.

Currently, the mostly antibodies used to detect BLG and BLG allergenic residues are not specifically against human IgE epitopes on BLG. Nevertheless, we aimed to prepare a recombinant epitope tandem derived from reported epitopes on BLG (tBLG), partially characterize its immunoreactivity, and develop specific polyclonal antibody (pAb) for further detection usage. In this work, tBLG was designed according to the principles of epitope-based peptide vaccine development, followed by expression in *E. coli* BL21 (DE3) pLysS as a fusion protein. The IgG- and IgE-binding ability of the recombinant tBLG was determined by indirect enzyme-linked immunosorbent assay (ELISA) with specific rabbit pAb and sera from CMA patients, respectively. Competitive inhibition

ELISA was carried out to estimate the binding ability of pAb-tBLG to epitopes of tBLG, and the sensitivity and specificity of pAb-tBLG.

Materials and methods

Materials

The tBLG DNA fragment was synthesized by Takara (Dalian, China). The restriction enzymes, *Bam*HI and *Eco*RI were purchased from MBI Fermentas (Lithuania), and T4 DNA ligase was purchased from Takara (Dalian, China). Ampicillin, isopropyl β -D-1-thiogalactoside (IPTG), reduced glutathione, cell culture reagents, and 4-chloro-1-naphthol were purchased from Sangon Biotech (Shanghai, China). Complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), protease inhibitor cocktail, horseradish peroxidase conjugated goat anti-rabbit IgG (HRP-IgG), horseradish peroxidase conjugated goat anti-human IgE (HRP-IgE), and o-phenylenediamine (OPD) were purchased from Sigma (St. Louis, MO, USA). Glutathione S-transferase (GST) affinity chromatography column (GSTrap HP) was purchased from GE Healthcare (Uppsala, Sweden). Peptides (purity $\geq 95\%$, high-performance liquid chromatography) were synthesized by GL Biochem (Shanghai, China). All other reagents were of analytical grade and were obtained from commercial sources.

CMA patients' sera were kindly provided by Dr Luo (Department of Pediatrics, First Affiliated Hospital of Guangxi Medical University, China), and stored at -80°C until use.

Design of tBLG expression vector

Construction of a tandem derived from human IgE epitopes of BLG (tBLG) was performed according to Han (Han, Gao, Li, & Chen, 2008). Briefly, seven major B cell IgE-binding linear epitopes on BLG, namely B1, B2, B3, B4, B5, B6, and B7, were selected as a part of the tandem according to Järvinen's work (Järvinen et al., 2001). And a T cell epitope (AA79–99) was also selected, because it was the main T cell epitope of both BALB/c mice (Totsuka et al., 1997) and human (Inoue et al., 2001). All the epitope sequences are shown in Table 1. T cell epitope locates in the N-terminal, and lysine–lysine (KK) as a

Table 1. The amino acid sequences of seven B cell epitopes, one T cell epitope of tBLG, and the blocking peptides.

Peptide	Abbreviation	Amino acid sequences
B cell epitopes	B1	AA1~8 (LIVTQTMK)
	B2	AA31~48 (LLDAQSAPLRVYVEELKP)
	B3	AA47~60 (KPTPEGDLLEILLQK)
	B4	AA67~78 (AQKKIIAEKTKI)
	B5	AA75~86 (KTKIPAVFKIDA)
	B6	AA127~144 (EVDDEALEKFDKALKALP)
	B7	AA141~152 (KALPMHIRLSFN)
Blocking peptides	b1	AA1~9 (LIVTQTMKG)
	b2	AA30~49 (SLLDQASAPLRVYVEELKPT)
	b3	AA46~61 (LKPTPEGDLLEILLQKW)
	b4	AA66~79 (SAQKKIIAEKTKIP)
	b5	AA74~87 (EKTIPAVFKIDAL)
	b6	AA126~145 (PEVDDEALEKFDKALKALPM)
	b7	AA140~153 (LKALPMHIRLSFNP)
T cell epitope	T	AA77~97 (KIPAVFKIDALNENKVLVLDT)

linker was inserted between T cell epitope and its adjacent B cell epitope. Glycine (G) was inserted between the B cell epitopes. Linkers (KK and G) were used to retain the immunologic ability of each epitope and avoid generation of new epitopes (Yano et al., 2005). When both the scores of hydrophilicity and the antigenicity of tBLG were more than 0 and the values of surface accessibility was more than 1 (according to the prediction of DNASTar software), the peptide is more probably to be an epitope (Emini, Hughes, Perlow, & Boger, 1985; Jameson & Wolf, 1988; Kyte & Doolittle, 1982). The secondary structure of tBLG was predicted by a network server-SOMPA, of which β -turn and random coil regions were more likely to be epitopes (Lu, Wan, & Wu, 2003). The optimal portfolio epitope combination of tBLG was as follows: T-K-K-B1-G-B6-G-B5-G-B3-G-B7-G-B2-G-B4.

Restriction sites were added to the N-terminal and C-terminal of the designed tBLG. The tBLG gene was inserted into the vector pMD-19T to form a new recombinant vector named pMD19-T-tan. The plasmid was confirmed by gene sequencing and was transferred into *E. coli* JM109. The *Bam*HI-*Eco*RI fragment (tBLG) of pMD19-T-tan was ligated with *Bam*HI and *Eco*RI sites of pGEX-4T-1 (GE Healthcare, USA) to construct expression plasmid pGEX-4T-1-tan, followed by transforming into *E. coli*, BL21 (DE3) pLysS strain. The positive BL21 (DE3) pLysS cell with vector pGEX-4T-1-tan was selected by double digestion using *Bam*HI-*Eco*RI restriction enzyme mapping, ampicillin resistance test (100 μ g/mL), and the plasmid was confirmed by gene sequencing.

Expression of recombinant tBLG

The positive *E. coli* BL21 (DE3) pLysS cells were inoculated in 5 mL of Luria–Bertani (LB) medium containing 100 μ g/mL ampicillin, followed by incubation at 37°C overnight under shaking at 220 rpm. The pre-cultured cells were refreshed in 100 mL of LB medium containing 100 μ g/mL ampicillin and grew at 37°C under shaking at 220 rpm until the optical density reached $OD_{600} = 0.6$. To optimize the expression parameters, different incubation time (2, 4, 6, and 8 h), incubation temperature (37°C, 30°C, 25°C, 23°C, and 18°C), and final concentrations of inducer IPTG (1, 0.7, 0.5, 0.3, and 0.1 mM) were investigated. The incubated cells were harvested by centrifugation at 14,087 $\times$ g for 5 min at 4°C, and the pellet was re-suspended in phosphate buffer saline (PBS), containing protease inhibitor cocktail, followed by lysing by sonication at 200 W (60 times for 3 s per run). The lysed cells underwent centrifugation at 14,087 $\times$ g for 30 min at 4°C, and the crude protein was in the collected supernatant.

Purification of recombinant protein

Recombinant protein was purified by GSTrap HP according to the manufacturer's protocol. Briefly, the column was equilibrated with five column volumes of binding buffer. The sample was loaded onto the column at a flow rate of 1 mL/min, and the unbound constituent was washed by five column volumes of binding buffer, followed by elution of the bound protein by five column volumes of elution buffer (50 mM of Tris-HCl, 20 mM of reduced glutathione, pH 8.0, flow rate of 1 mL/min). The eluted proteins were collected and the concentration of the GST fusion protein was measured by the Lowry method. After GST affinity purification, the GST fusion protein was further separated on

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gel, followed by concentration using Amicon Ultra-15 centrifugal filter devices according to the manufacturer's instructions.

Preparation of polyclonal antibody

All animal-use protocols were approved by the University of Nanchang Animal Care Committee under the guideline of China Council for Animal Care. Two male Japanese big-eared rabbits (eight-week-old) were purchased from Jiangxi Administration Committee of Laboratory Animal and fed with a milk-free diet. After acclimating for one week, the animals were subcutaneously immunized with 1 mg of the recombinant tBLG protein emulsified in CFA. Two weeks later, the rabbits were re-immunized with 1 mg of the recombinant tBLG emulsified in IFA at two-week intervals. One week after the last injection, rabbits were bled through the femoral artery. The whole blood samples were clotted at room temperature for 1 h, followed by centrifugation at 4000×g for 5 min to collect serum. The serum was named pAb-tBLG and then stored at –20°C until use.

SDS–PAGE and western blotting

Samples were dissolved (1:1 v/v) in loading buffer containing 100 mM of Tris-HCl with pH 8.0, 20% (v/v) glycerol, 1% (w/v) SDS, 1% (v/v) β -mercaptoethanol, and 0.02% (w/v) bromophenol blue, followed by heating at 100°C for 5 min. Subsequently, they were loaded on a 4% acrylamide stacking gel and 12% acrylamide separating gel with a current of 8 and 16 mA, respectively. This method was performed in a vertical mini gel electrophoresis cell (Bio-Rad, USA). The gels were stained with Coomassie brilliant blue R-250, followed by destaining by thoroughly rinsing with destaining solution (7.5% acetic acid and 5% methanol). Finally, the gels were imaged by GS-800™ Densitometer (Bio-Rad, USA).

After SDS–PAGE, the purified recombinant tBLG proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane at 50 mA for 1 h. The membrane was blocked with 3% gelatin in Tris-buffered saline (TBS) buffer for 1 h at 37°C, followed by washing three times with TBS containing 0.05% Tween 20 (TBST). After that, the membrane was incubated with rabbit anti-BLG polyclonal antibody (pAb-BLG, 1:3000 dilutions in TBS) overnight at 4°C. After further washing, HRP-IgG (1:5000 dilutions in TBS) was added and incubated for 1 h at 37°C. Subsequently, the membrane was transferred into an appropriate volume of 4-chloro-1-naphthol solution and protected from light for 15 min at 37°C.

Igg- and IgE-binding ability of the recombinant tBLG protein

The antigenicity and potential allergenicity of epitopes preserved in the recombinant tBLG were tested by the binding ability of tBLG to IgG and IgE, respectively. A microtiter plate was coated with recombinant tBLG (1.56 μ g/mL) or BLG (2.5 μ g/mL) in 50 mM sodium carbonate buffer (pH 9.6) incubated overnight at 4°C. The wells were washed three times with PBS containing 0.05% Tween 20 (PBST). This washing system was used after each incubation step. After blocking with 3% gelatin in PBS for 1 h at 37°C, 100 μ L antisera

(pAb-tBLG or pAb-BLG was diluted from 1:1000 to 1:256,000 in PBS to test the IgG-binding ability of tBLG, and human sera were diluted five-fold in 1% PBST for testing the IgE-binding ability of tBLG, PBS as control) were added and incubated for 1 h at 37°C, followed by incubation with 100 µL of HRP-IgG or HRP-IgE (diluted 1:5000 in PBS) for 1 h at 37°C. Peroxidase activity on the wells was detected using OPD and H₂O₂ as enzyme substrates. Color development was stopped with 50 µL of 2 M sulfuric acid, and the optical density was measured at 490 nm using a microplate reader (Bio-Rad, USA).

The titer of antisera was defined as: the maximum dilution factor that yielded $P/N > 2.1$, and $P > 0.2$ ($n = 3$). P and N represented the optical density of positive (antisera) and the optical density of negative (PBS), respectively.

The pAb-tBLG binding to epitopes of BLG

Recognition of the human IgE epitopes on tBLG by pAb-tBLG is a prerequisite for successful detection of BLG and BLG allergic residues in dairy products. In addition, allergenic residues are always larger than epitopes, and for IgE cross-linking, peptide should be larger than 30 amino acids (Aalberse & Crameri, 2011; Knol, 2006). In order to analyze whether pAb-tBLG could recognize epitopes and the peptide larger than an epitope, seven epitopes and their blocking peptides were synthesized. The blocking peptide was the peptide that is obtained by adding an amino acid at the N-terminal and C-terminal of the epitope, respectively. The blocking peptide sequences are shown in Table 1.

The binding ability of pAb-tBLG to the epitopes and blocking peptides was evaluated using competitive inhibition ELISA. The microtiter plate was pre-coated with 4 µg/mL of BLG and incubated overnight at 4°C, and sodium carbonate buffer was added as control. Seventy microliter of pAb-tBLG was mixed with 70 µL of peptide or PBS and incubated for 1 h at 37°C. The BLG pre-coated plate was washed three times with PBST, then blocked for 1 h at 37°C. After a further washing step, 100 µL of pAb-tBLG, pre-incubated with peptide solution or PBS, was added and incubated for 1 h at 37°C. After the wells were washed again, 100 µL of HRP-IgG (diluted 1:5000) was added to each well, and incubated for 1 h at 37°C. The following procedures were identical to indirect ELISA. The percent inhibition (PI) values were expressed as the following equation:

$$PI(\%) = [1 - (A1 - A0)/(A2 - A0)] \times 100\%,$$

where A_0 , A_1 , and A_2 represented the optical density of control, the optical density of antisera with inhibitor, and the optical density of antisera without inhibitor, respectively.

Sensitivity and specificity of pAb-tBLG

The cross-reactivity (CR) of pAb-tBLG with other milk allergens, ovalbumin (OVA), and soybean proteins was evaluated with competitive inhibition ELISA, the ELISA procedures as described above. The 50% inhibition levels (IC_{50}) was used to determine the CR% as follows: $CR\% = IC_{50} (BLG)/IC_{50} (inhibitor) \times 100$ (Chen, Huang, Li, Kong, & Huai, 2013).

Results and discussion

Construction of recombinant plasmid pGEX-4T-tan

The recombinant plasmid pGEX-4T-tan was digested with the restriction enzymes, and the tBLG DNA fragment was located between 400 and 500 bp (data not shown). In addition, the sequencing results also showed that the recombinant plasmid pGEX-4T-tan contains the full gene of tBLG (data not shown). Therefore, these results indicate that the recombinant plasmid pGEX-4T-tan was successfully constructed.

Expression of recombinant tBLG protein

After double digestion and ligation, pGEX-4T-tan was transformed into BL21 (DE3) plysS. First, expression of tBLG protein was investigated at 37°C by induction with 1 mM of IPTG. An apparent band with a molecular weight of approximately 40 kDa was observed, and the recombinant protein content reached the maximum after 4 h of IPTG induction (Figure 1(a), line 4). Subsequently, different temperatures were chosen to optimize the yield of the soluble recombinant tBLG protein, and the optimum condition was induction with 0.3 mM IPTG at 23°C for 4 h. Western blotting showed that pAb-BLG could react with the recombinant protein (Figure 1(b)), further indicating that the tBLG fusion protein was successfully expressed.

Purification of recombinant tBLG protein

The SDS-PAGE pattern of purified GST-tagged protein is shown in Figure 3(a). Most nonspecific proteins were eluted by binding buffer (Figure 2(a), lane 3). The tagged

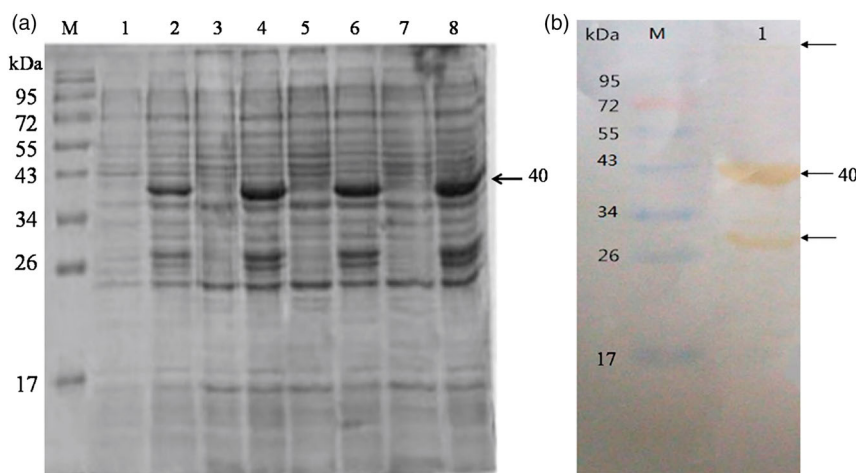


Figure 1. (a) SDS-PAGE analysis of the recombinant tBLG protein under different induction conditions. M: markers; lanes 1, 3, 5, and 7: incubation without IPTG for 0, 2, 4, and 6 h, respectively; lanes 2, 4, 6, and 8: induction with IPTG for 2, 4, 6, and 8 h, respectively. (b) Western blotting analysis of the expressed tBLG protein using pAb-BLG as the primary antibody. M: markers; lane 1: sample after induction with 0.3 mM of IPTG at 23°C for 4 h. All the samples were the supernatant of lysed cells. The recombinant protein band and the visible blotting images are indicated by arrows.

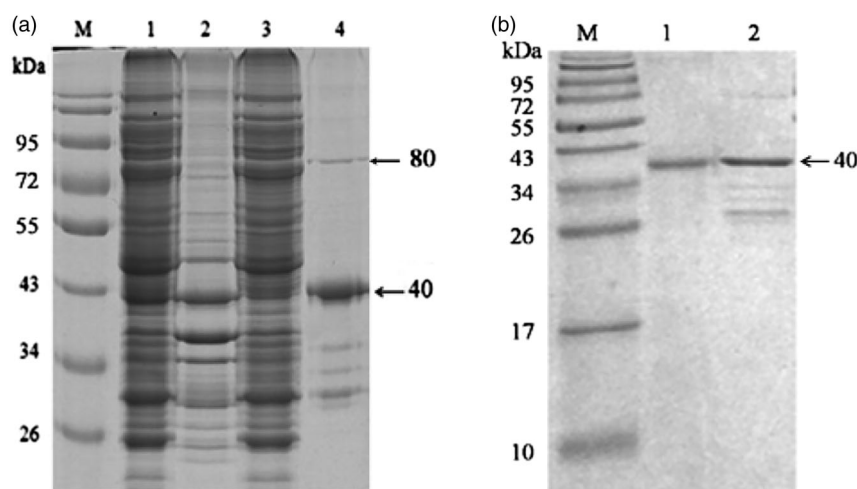


Figure 2. SDS-PAGE analysis of recombinant tBLG by two-step purification. (a) Recombinant tBLG purified with GSTrap HP. M: markers; lanes 1 and 2: supernatant and precipitate of cell lysates of *E. coli* after centrifugation; lane 3: flow-through protein of the column; lane 4: prepared proteins by affinity purification. (b) Isolated recombinant protein from the SDS-PAGE gel. M: markers; lane 1: purified protein from the gel; lane 2: purified protein with GSTrap HP.

protein bound to the GST affinity column was eluted with five column volumes of elution buffer (Figure 2(a), lane 4). However, some contaminants remained. Since GST contains exposed cysteine residues that could promote the oxidative aggregation of GST fusion proteins, resulting in retaining the proteins' ability to bind to the affinity resin (Walls & Loughran, 2011), two tBLG recombinant proteins formed a homodimer of about 80 kDa (Figure 2(a), lane 4). The recombinant tBLG protein was further prepared by isolating from the gel, and its purity is higher than that purified by GSTrap HP (Figure 2(b)).

IgG-binding ability of the recombinant tBLG protein

The titers of sera from two rabbits were determined by indirect ELISA using recombinant tBLG as a coating antigen. Immunization was performed five times with recombinant tBLG, and the titers of sera from rabbits A and B reached 1:128,000 and 1:64,000, respectively. Thus, the sera of rabbit A were chosen for the following research.

Although western blotting showed that pAb-BLG could react with recombinant tBLG (Figure 1(b)), indicating the similar antigenicity between recombinant tBLG and BLG, we need further evaluation to compare the IgG-binding ability of recombinant tBLG protein and BLG. For this, an indirect ELISA test was carried out to assess the CR between recombinant tBLG and BLG with pAb-BLG and pAb-tBLG. As shown in Figure 3(a), the binding ability of pAb-BLG to BLG was almost the same as that of pAb-tBLG to recombinant tBLG, while pAb-BLG reacted more strongly with BLG than recombinant tBLG. This finding may be due to the fact that pAb-BLG can recognize not only linear epitopes but also conformational epitopes of BLG. In addition, the reaction between pAb-tBLG and tBLG was stronger than that between pAb-tBLG and BLG. One possible reason is that a GST tag was inserted in recombinant tBLG, and GST could be involved in the reaction.

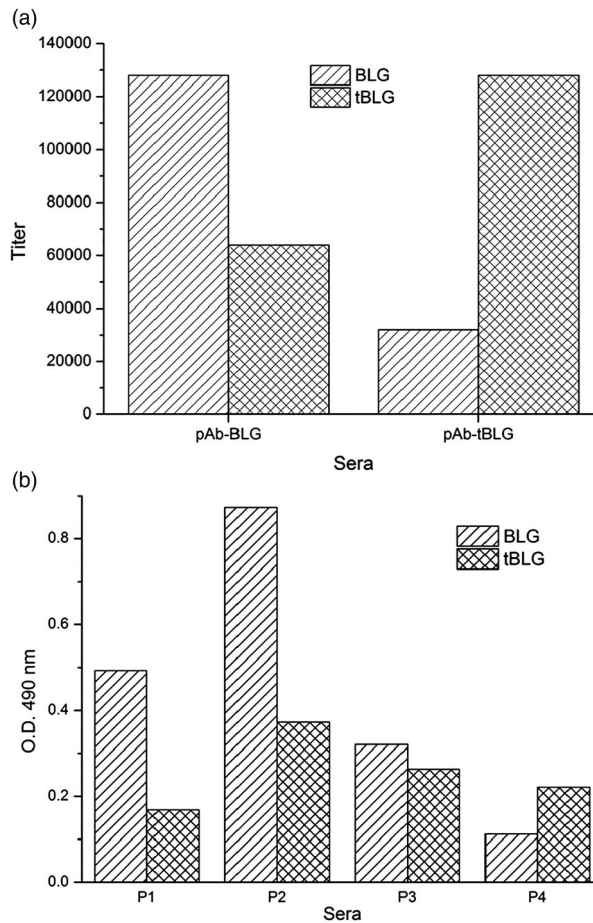


Figure 3. (a) Cross-reactivity between recombinant tBLG and BLG defined by indirect ELISA by using pAb-tBLG or pAb-BLG as a primary antibody. (b) IgE-binding ability of tBLG and BLG was defined by indirect ELISA with sera from CMA patients. P1–P4 represent sera from four CMA patients.

These results strongly indicated that the antigenicity of the human IgE-binding linear epitopes of BLG was preserved in the recombinant tBLG, and pAb-tBLG efficiently recognized epitopes on BLG and recombinant tBLG protein.

IgE-binding ability of recombinant tBLG

To determine whether the allergenicity of epitopes was preserved in the recombinant tBLG, the IgE-binding to tBLG was estimated by indirect ELISA with sera from CMA patients. As shown in Figure 3(b), both the recombinant tBLG and native BLG were recognized by all the four CMA patients' sera. However, the reactivity of three out of four sera (P1, P2, and P3) to BLG was higher than their reactivity to recombinant tBLG. This finding may be due to the fact that the major IgE-binding epitopes of BLG were conformational (Jarvinen et al., 2002). Moreover, individual differences directly cause the different immune reaction among the four patients and BLG/tBLG. Nevertheless, native BLG

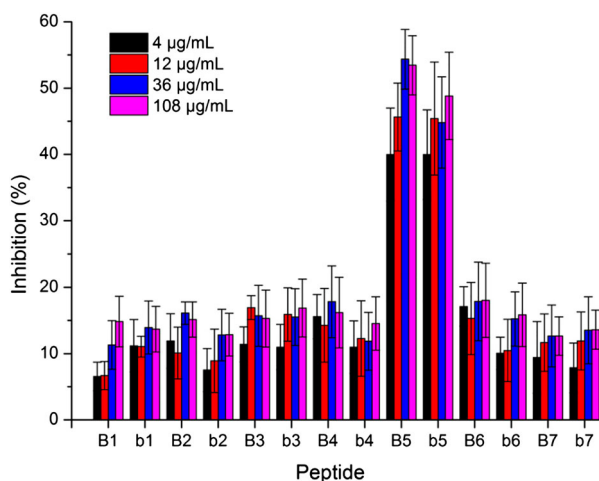


Figure 4. Binding characteristics of pAb-tBLG to epitopes and blocking peptides at different concentrations (4, 12, 36, and 108 µg/mL) were defined by the indirect competition ELISA test. B1–B7 represent seven epitopes, and b1–b7 represent seven blocking peptides. Data are represented as mean ± standard deviation ($n = 3$).

and recombinant tBLG possess similar IgE-binding ability, indicating that the allergenicity of human B cell epitopes of BLG was preserved well in the recombinant tBLG protein.

Characteristics of pAb-tBLG binding to epitopes

Even though the indirect ELISA indicated that the antigenicity and allergenicity of human B cell epitopes of BLG were preserved well in tBLG, and pAb-tBLG could recognize epitopes presented in BLG and recombinant tBLG, these results did not demonstrate that the pAb-tBLG could recognize all the seven human IgE epitopes of tBLG. The binding ability of pAb-tBLG to the epitopes and blocking peptides was analyzed by competitive inhibition ELISA (Figure 4). The result shows that pAb-tBLG recognized all the epitopes and the blocking peptides, pAb-tBLG has similar binding ability for blocking peptides compared with epitopes, and the inhibition was increased with the increase in the concentration of peptide. Except B5, the other six epitopes have the similar inhibition. The inhibition of B5 was significantly higher than the others, which we hypothesize to be because the antigenicity of B5 was stronger than the others, resulting in rabbit to produce higher levels of antibodies in response to B5. Anyway, the result has been suggested that pAb-tBLG was able to bind to all the epitopes and blocking peptides.

Antibody sensitivity and specificity

The sensitivity and specificity of pAb-tBLG were defined by competitive inhibition ELISA. The sensitivity of pAb-tBLG was expressed as IC_{50} value of BLG, and the specificity of pAb-tBLG was defined as the CR with other allergens. IC_{50} and CR (%) data for each allergen are shown in Table 2, the IC_{50} of BLG was 0.38 µg/mL, and there was no CR with α -lactalbumin (α -LA), bovine serum albumin (BSA), OVA and soybean proteins. However, there was weak CR with α -casein (α -CN), β -casein (β -CN), and κ -casein (κ -CN). This may

Table 2. CR of pAb-tBLG with food allergens.

Inhibitor	IC ₅₀ (μg/mL)	CR (%)
BLG	0.38	100
α-LA	ND	0
α-CN	18.98	2.02
β-CN	23.99	1.6
κ-CN	19.89	1.93
BSA	ND	0
OVA	ND	0
Soybean proteins	ND	0

ND: Not detected, no inhibition with a protein concentration of 100 μg/mL.

be because α-CN and κ-CN (both ≥ 70% pure) contained residual BLG (Mäkinen-Kiljunen & Palosuo, 1992). Another study showed that two anti-BLG monoclonal antibodies also had a weak CR with CNs (Negroni et al., 1998). Nevertheless, the results indicated that the pAb-tBLG had high specificity.

Potential application of pAb-tBLG for detecting BLG allergenic residues

Hypoallergenic infant formulas are processed by hydrolysis and heat treatment. However, residual potentially allergenicity cannot be determined solely based on peptide size distribution (Knipping et al., 2014). ELISA was the traditional method for determination of BLG allergenic residue content in hypoallergenic infant formulas, but the actual quantification of BLG content was dependent on the specific test antibody used. In previous studies, anti-BLG polyclonal antibodies were used to measure the content of BLG in hypoallergenic infant formulas (Mäkinen-Kiljunen & Sorva, 1993; Oldæus, Björkstén, Einarsson, & Kjellman, 1991). And indirect competitive ELISA based on either BLG monoclonal antibody or polyclonal antibody was then established. However, the results showed that the BLG content measured by monoclonal antibody assay is about 10 times lower compared with the polyclonal antibody assay (Mariager, Sølvé, Eriksen, & Brogren, 1994). Moreover, a 1:1 mixture of antibodies to native and heat-denatured BLG was used to detect BLG content in infant formulas. The content of residual BLG antigenicity may be overestimated by ELISA assays using polyclonal antibodies because peptides rather than native BLG are detected (Rosendal & Barkholt, 2000). Furthermore, BLG content in hypoallergenic formulas will be lower when measured by monoclonal antibody because only one epitope on BLG is reactive (Mariager et al., 1994). In addition, most of these antibodies did not specifically recognize human IgE epitopes on BLG. Therefore, a new anti-BLG antibody was needed for the detection of BLG allergenic residues in hydrolyzed milk formulas. In this study, a polyclonal antibody directed against human IgE linear epitopes of BLG was prepared. Given that this antibody could recognize human IgE linear epitopes, it may overcome the drawbacks of polyclonal antibody and monoclonal antibody described above. Therefore, this antibody may serve as a potential suitable antibody for the detection of BLG allergenic residues in hydrolyzed milk formulas.

Conclusion

In conclusion, to our knowledge, this study is the first to report the construction, expression, and purification of human IgE linear epitopes of BLG as a tandem. Specific

antibody which can recognize all the human IgE epitopes of tBLG was developed. Indirect ELISA indicated that recombinant tBLG preserved the antigenicity and allergenicity of epitopes. The pAb-tBLG could not only recognize all the seven B cell epitopes of tBLG, but also had the similar reactivity to their blocking epitopes. It was highly specific, but had a weak CR with CNs. For the first time, the polyclonal antibody against human IgE epitopes on BLG was developed. This antibody could be used to detect BLG and BLG allergenic residues in dairy products and may be useful in the development of hypoallergenic infant formulas.

Disclosure statement

No potential conflict of interest was reported by the authors.

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