



## Research paper

Selection of recombinant IgE antibodies binding the  $\beta$ -lactoglobulin allergen in a conformation-dependent manner

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## ABSTRACT

Cow's milk allergy (CMA) is a common food allergy, especially among infants and young children. Approximately 85% of milk-allergic children outgrow their allergy by the age of three but the remaining 15% remain allergic. Bovine  $\beta$ -lactoglobulin (BLG) is one of the major allergens in cow's milk. There is a definite need for the specific and sensitive detection of allergenic substances. Validated methods are obligatory to demonstrate allergen contamination and even fatal hidden allergens and, thus, to prevent life-threatening conditions of allergic persons. In this study, we constructed human IgE scFv libraries from an adult milk-allergic patient and isolated the first recombinant IgE antibodies specific to a food allergen, BLG. The selection of the IgE antibody libraries with two distinct panning procedures resulted in the enrichment of four clones having different BLG-binding profiles; two of the clones recognize the native BLG whereas the other two recognize only the heat-denatured form of BLG. For further characterization, the scFv fragments were converted to Fab fragments with human IgG1 isotype. The D1 Fab fragment, binding native BLG with nanomolar affinity, also partially inhibited serum IgE binding to BLG. These BLG-specific IgE antibodies can be applied for the detection of both native and denatured BLG in cow's milk products and furthermore, for the optimization of manufacturing processes to develop safe hypoallergenic milk products.

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## 1. Introduction

Approximately 3% of infants and young children suffer from cow's milk allergy (CMA) during their first years of life (Savilahti,

1981; Host and Halken, 1990; Saarinen et al., 1999). CMA is characterized by a strong IgE response to milk proteins and clinical symptoms in skin and the gastrointestinal tract, such as atopic eczema, vomiting and diarrhea (Vaarala et al., 1995; Saarinen, 2000). Symptoms in respiratory ducts and anaphylactic shock are also possible (Host and Halken, 1990; Saarinen, 2000; Hill et al., 1999; Schrandt et al., 1993; Vanto et al., 1999). CMA is a serious problem with children because milk is an important energy source (up to 50%) for them and is not very easily replaced with non-dairy products. Nearly 85% of milk-allergic children will outgrow of their CMA by the age of three but recurrence of CMA may occur in up to one-third of older children (Sampson and Scanlon, 1989).

One of the major whey proteins and allergens in cow's milk is BLG, which belongs to the lipocalin protein family. Lipocalins are a group of a small ligand-binding proteins, mostly respiratory allergens such as Mus m 1 and Rat n 1 (mouse and rat urinary

**Abbreviations:** AFOS, alkaline phosphatase; BLG,  $\beta$ -lactoglobulin; BLG-bio, biotinylated  $\beta$ -lactoglobulin; BSA, bovine serum albumin; cDNA, complementary DNA; VH, the heavy chain variable domain of an antibody; VL, the light chain variable domain of an antibody; CDR, complementary determining region; CMA, cow's milk allergy; Fd, variable and first constant domain of a heavy chain; Fab, Fd chain and a light chain connected by a disulfide bridge; ELISA, enzyme-linked immunosorbent assay; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl  $\beta$ -D-1-thiogalactopyranoside; LB, Luria broth; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RAST, radioallergosorbent assay; SA, streptavidin; SB, super broth; scFv, single chain antibody fragment.

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proteins) (Bayard et al., 1996; Lorusso et al., 1986) and a German cockroach allergen, Bla g 4 (Arruda et al., 1995). In cow's milk BLG occurs in the form of a 36-kDa dimer with the subunits containing 162 amino acids. The 3D-structure of BLG has been determined by X-ray diffraction (Brownlow et al., 1997; Sawyer et al., 1985). It is predominantly a  $\beta$ -sheet protein with nine  $\beta$ -strands and one major  $\alpha$ -helix (Brownlow et al., 1997; Sakai et al., 2000; Kuwata et al., 1999). Like most food allergens, BLG is very stable at low pH and resistant to pepsin degradation. However, it has been reported that heat treatment can modify the native structure of BLG, leading to exposure of new antigenic sites, "hidden epitopes", and, thus, induce the synthesis of an IgE response towards non-conformational epitopes of BLG (Davis and Williams, 1998; Ehn et al., 2004; Song et al., 2005).

Reliable detection of allergens in food products is a big challenge for the food industry. Food labeling describing the potential allergenic content of the food is essential for the safety of sensitized consumers to avoid potentially life-threatening anaphylactic shocks. Traditionally, the detection of allergens is based on the use of human IgE serum pools isolated from allergic patients, polyclonal antibodies obtained from immunized animals or in some cases by monoclonal antibodies produced by hybridoma technology. Various immunological detection methods based mainly on polyclonal antibodies have been published during recent years (reviewed in van Hengel, 2007). Polyclonal antibodies from immunized rabbits have been used in a sandwich-type ELISA for the sensitive detection of BLG from cow's milk and food products (Mäkinen-Kiljunen and Palosuo, 1992). However, the polyclonal and monoclonal antibodies recognize the immunogenic epitopes of the protein. These are not necessarily the same as the allergenic epitopes recognized by the IgE antibodies that trigger the allergenic response. It would thus be preferable to use IgE antibodies for allergen detection. However, the standardization of the detection method is difficult because of variation between IgE sera collected from sensitized persons. Therefore, the development of validated commercial detection kits cannot be based on patient serum samples.

Today, it is possible to produce human recombinant IgE antibodies by the phage display technology that enables the generation of large and diverse antibody expression libraries (McCafferty et al., 1990). Previously, human IgE antibody display libraries have been constructed and recombinant allergen-specific IgE antibodies isolated against pollen, mould and latex allergens (Steinberger et al., 1996; Flicker et al., 2002; Laukkanen et al., 2003; Jakobsen et al., 2004; Andreasson et al., 2006; Persson et al., 2007). In this study, we demonstrate that also IgE antibodies specific to food allergens can be isolated from phage display libraries constructed from sensitized persons. The combinatorial human scFv phage libraries were constructed from lymphocytes of a CMA patient and recombinant IgE antibodies binding the BLG allergen in a conformation dependent-manner were isolated.

## 2. Materials and methods

### 2.1. Milk-allergic persons

Milk-allergic adult patients were diagnosed by determining the total serum IgE and the RAST classes for BLG and casein. Two of them, patients 1 and 3 (P1 and P3), suffered from immediate-

type allergic reactions such as anaphylaxis towards cow's milk. Patient 2 (P2) had suffered from milk allergy since childhood, having atopic eczema and, previously, asthma. P2 tolerated milk to some extent and also had consumed milk two days before the serum sample was collected. P1 and P3 had avoided milk and milk products because of the severity of their allergic symptoms.

### 2.2. Biotinylation and allergenicity of BLG

BLG (L3908, Sigma-Aldrich GmbH) was dissolved at 1 mg/ml in 50 mM NaHCO<sub>3</sub>, pH 8.3. Biotinylation of BLG was done by incubating 167  $\mu$ l of Sulfo-NHS-LC-biotin (Pierce) (1 mg dissolved in 1 ml of H<sub>2</sub>O) with 5 ml of the BLG solution for 30 min at room temperature (21 °C) and then removing the non-reacted biotin by dialyzing against PBS. The biotinylated BLG (BLG-bio) was filter-sterilized and the biotinylation of the protein was confirmed by immunoblotting using AFOS-conjugated streptavidin (Amersham Biosciences) for the detection (data not shown).

The binding activities of the P1-P3 sera to BLG and BLG-bio were analyzed by ELISA. The P1-P3 serum samples (1:8 dilutions) were added to Maxisorp® (Nunc) and streptavidin (SA) wells (Roche Diagnostics GmbH) coated with 1  $\mu$ g of BLG (100  $\mu$ l of 10  $\mu$ g BLG/ml 0.1 M Na-bicarbonate buffer, pH 9.6) or BLG-bio (100  $\mu$ l of 10  $\mu$ g BLG-bio/ml PBS-0.5% BSA), respectively, and incubated for 1.5 h at room temperature with gentle agitation. After the washing step, the detection was carried out with an AFOS-conjugated goat anti-human IgE antibody (Southern Biotech Associates Inc). After a 2-h incubation at room temperature with the substrate solution, *p*-nitrophenyl-phosphate (Sigma), the absorbance at 405 nm was measured (Varioscan, Thermo Electron Corporation). As a control, a serum sample from a non-allergic individual was used.

### 2.3. Construction and selection of the human IgE libraries

On the basis of skin prick and sera IgE test results, P3 was selected as the blood donor for the construction of the combinatorial IgE libraries. After the approval of the ethical committee of Helsinki University Central Hospital, a heparinized blood sample was obtained from P3, and lymphocytes were isolated according to the Ig-prime kit protocol (Novagen). Briefly, the blood sample (70 ml) was mixed with 210 ml of the lysis buffer (155 mM NH<sub>4</sub>Cl, 10 mM NH<sub>4</sub>HCO<sub>3</sub>, 0.1 mM EDTA, pH 7.4), incubated for 15 min on ice with gentle shaking and centrifuged at 450  $\times$ g for 10 min at room temperature. The supernatant was discarded and the pellet containing the white blood cells was resuspended into 10 ml of lysis buffer, incubated on ice for 5 min and centrifuged as above. The wash was repeated once more with 5 ml of lysis buffer. Finally the pellet was resuspended into 5 ml sterile PBS. RNA was isolated using Promega's RNeasy Total RNA isolation kit (Promega) according to manufacturer's protocol. First strand cDNA synthesis was carried out using the Reverse Transcription kit (Promega).

For the construction of the IgE antibody phage libraries the human IgE Fd heavy chain and kappa and lambda light chain cDNA fragments were amplified essentially as described earlier (Laukkanen et al., 2003). Each subgroup of heavy and light chains was amplified separately: 14 PCR reactions for the  $\kappa$

chain, 15 for  $\lambda$  chain and 14 for the Fd chain. The primers used for the amplification of the IgE Fd region of the heavy and whole light chain cDNAs are described in [Laukkanen et al. \(2003\)](#). A small portion of the amplified Fd fragment was cloned into the TOPO TA -vector (Invitrogen) according to the manufacturer's protocol. The IgE isotype of the amplified Fd fragments was verified by DNA sequencing analysis by ABI 3100 Genetic Analyzer (Applied Biosystems) from 10 individual transformants. Then the isolated IgE Fd and  $\kappa/\lambda$  fragments were used as templates for amplifying the IgE VH and VL  $\kappa$  and  $\lambda$  regions. In the second PCR there were 7 separate reactions for the VH region (subgroups 1–7), 6 reactions for the V $\kappa$  region (subgroups 1–6) and 6 reactions for the V $\lambda$  region (subgroups 1–6) ([Laukkanen et al., 2003](#)). The amplified VH and VL regions were pooled and cloned separately into the scFv phagemid vector as described previously ([Laukkanen et al., 2003](#)).

Two different panning strategies were used for the selection of the IgE scFv libraries. The first selection approach was carried out in solution using BLG-bio. For the BLG-bio, 0.5  $\mu$ l of a 1 mg/ml solution ( $C_{\text{final}} = 70$  nM) was incubated with 200  $\mu$ l of scFv phage libraries ( $10^{10}$  phages) in PBS–3% BSA for 1.5 hour at room temperature. Then the scFv phage–BLG solution was transferred to SA-coated wells (Roche) and incubated for 30 min at room temperature with gentle agitation. After the binding reaction, the wells were washed with 300  $\mu$ l of PBS–0.05% Tween 20 first six times without incubation, then six times with 10 seconds incubation at room temperature and finally six times with a 30 second incubation step between each wash. Finally, the bound scFv phages were eluted by adding 200  $\mu$ l of 100  $\mu$ M BLG solution and incubating for 25 min with shaking at room temperature.

The second selection approach was performed by immobilizing 1.5  $\mu$ g of BLG (in 0.1 M Na-bicarbonate, pH 9.6) onto Maxisorp® wells. The coated wells were washed three times with 300  $\mu$ l of PBS and then blocked with PBS–0.5% BSA for 1 h at room temperature. Then 100  $\mu$ l of the scFv phage libraries ( $10^{10}$  phages) were incubated for 1.5 h at room temperature and after that the wells were washed as in the first selection approach with BLG-bio. The bound scFv phages were eluted with 150  $\mu$ l of 0.1 M HCl (pH 2) for 15 min at room temperature with shaking and the eluate was neutralized with 1 M Tris, pH 8.8. For the next panning round, the eluted phage pools were infected into fresh ( $OD_{600} \sim 1$ ) XL-1 Blue cells (Stratagene) and scFv phages were amplified with the helper phage VCS-M13 (Stratagene).

#### 2.4. Screening of BLG-specific antibodies

After the selection rounds, the screening of the BLG-specific scFvs was carried out by ELISA. First, individual colonies were grown in 1 ml of Super Broth (SB) supplemented with tetracycline (10  $\mu$ g/ml), carbenicillin (50  $\mu$ g/ml) and 1% glucose using 96-deep well plates for 16 h at 30 °C with 700 rpm and 80% humidity (Infors AG CH-4103 Bottmingen). Then, the cultivations were diluted 1:50 in SB with 10  $\mu$ g/ml tetracycline and 50  $\mu$ g/ml carbenicillin and grown for 6 h at 30 °C. Antibody production was induced by adding IPTG to a final concentration of 1 mM. After a 16-h induction at 30 °C the cells were harvested by centrifugation (4000  $\times$ g, 15 min, 4 °C) and the culture supernatants containing scFv–pIII fusions (or soluble scFv cleaved from fusion

protein) were subjected to ELISA for the screening of the BLG-specific antibody clones.

Individual clones obtained from the first selection were analyzed by ELISA by immobilizing BLG-bio (0.5  $\mu$ g in PBS–0.5% BSA) onto SA-coated wells for 1 h at room temperature. The wells were washed three times with PBS and blocked with PBS–0.5% BSA for 1 h at room temperature. The scFv–pIII supernatant samples (1:2 dilutions) were incubated in the wells for 2 h at room temperature. After the washing step, the bound scFv fusions were detected using mouse anti-myc antibody (clone 9E10, ATCC # CRL-1729) and AFOS-conjugated anti-mouse Ig antibody (Bio-Rad). The screening of the individual clones from the second selection approach was performed by coating the Maxisorp plate wells with 1  $\mu$ g BLG in 0.1 M bicarbonate buffer (pH 9.6) for 16 h at 4 °C. After the coating step the ELISA was carried out as above.

The phagemid-DNA of the BLG-binding clones was isolated and the BstNI digestion pattern of the phagemid DNAs was analyzed by DNA fingerprinting. The phagemid DNAs were digested with BstNI (New England Biolabs) for 2 h at 65 °C and then analyzed on a 4% Nusieve agarose gel. The DNAs of clones with different BstNI digestion patterns were sequenced using the ABI 3100 Genetic Analyzer (Applied Biosystems). Sequence alignments were generated using DNAMAN 4.1 (Lynnon BioSoft). The CDR regions of the clones were identified by using the IMGT/V-QUEST bioinformatics tools (<http://imgt.cines.fr/>).

#### 2.5. Production and purification of the anti-BLG Fab fragments

For further characterization, all four scFv BLG-binders were cloned to human IgG1 Fab fragments and further cloned into the bacterial expression vector (pKKTac) containing a hexahistidine tag at the C-terminus of the heavy chain constant region for IMAC-purification as described earlier ([Laukkanen et al., 2003](#); [Takkinen et al., 1991](#)). The Fab fragments, A3, H2 and G1, were produced in shake flasks. Overnight cultivations (LB, 2% glucose, 100  $\mu$ g/ml ampicillin) were diluted 1:50 into 150 ml LB containing 100  $\mu$ g/ml ampicillin in 2-l shake flasks and cultivated at 37 °C and 220 rpm until the  $OD_{600}$  was 1.5. Then the production of the Fab fragments was induced with 1 mM IPTG and cultivation was continued at 30 °C with 220 rpm for 16 h. Finally the cells were harvested by centrifugation (4000  $\times$ g, 15 min, 4 °C) and the Fab fragments were purified by IMAC as described earlier ([Porath and Olin, 1983](#); [Laukkanen et al., 2003](#)).

High cell density cultivation of the D1 Fab fragment was carried out in a 4.5-litre fermenter using *E.coli* RV308 strain (ATCC 31608) as described ([Nevenen et al., 2001](#)). After the cultivation the cells were harvested by centrifugation (3963  $\times$ g, 15 min, 4 °C) and concentrated to a final volume of 1 liter using a Pellicon concentrator (Millipore) and a cellulose filter with a 10 kDa cut-off (Millipore). During the concentration, the buffer was exchanged to PBS (pH 7.4). The Fab fragment was purified using a  $\text{Cu}^{2+}$ -Chelating Sepharose Streamline column (Amersham-Pharmacia) and linear gradient elution (20–500 mM Imidazole in PBS, pH 7.4). Fractions containing the Fab fragment were pooled and the buffer was exchanged to PBS and the IMAC purification of the D1 Fab fragment was repeated in a smaller scale as described earlier ([Porath and Olin, 1983](#); [Laukkanen et al., 2003](#)).

## 2.6. Competitive ELISA

The binding activity of the purified Fab fragments was analyzed by a competitive ELISA. First the Fab fragments were incubated with different concentrations ( $C_{\text{final}} = 10, 1, 0.1, 0.01$  and  $0.001 \mu\text{M}$ ) of native or heat-treated (15 min,  $95^\circ\text{C}$ ) BLG for 1 h at room temperature. Then the samples were transferred onto SA-coated wells containing immobilized BLG-bio or onto Maxisorp® wells coated with BLG and incubated for 2 h at room temperature. After the washing steps the bound Fab fragments were detected using an AFOS-conjugated goat anti-human kappa antibody (Southern Biotech Associates Inc) by incubating for 1 h at room temperature. Finally the substrate solution was added and the absorbance values at 405 nm were measured as above.

The competitive ELISA with the patient IgE serum samples was performed as follows: the D1 Fab fragment ( $C_{\text{final}} = 0, 0.001, 0.01$ , and  $0.1 \text{ mg/ml}$ ) was incubated with serum samples for 2 h at room temperature on the SA-coated wells containing the immobilized BLG-bio. The immobilization of BLG-bio was carried out as above. Binding of the serum IgEs to immobilized BLG was detected with the AFOS-conjugated goat anti-human IgE (Southern Biotech) by incubating 1 h at room temperature and by addition of substrate solution as above. As a control, the serum of a non-allergic individual was analyzed. The functional working dilution of the serum samples was analyzed by ELISA and the serum dilutions were 1:10 for P2 and 1:8 for P1, P3 and control serum samples.

## 2.7. Immunoprecipitation assay

The immunoprecipitation assay was performed to study whether the isolated IgE antibody clones recognized BLG from cow's milk. Pasteurized, skimmed cow's milk was centrifuged ( $20\,000 \times g$ , 2 min,  $4^\circ\text{C}$ ) and the supernatant was diluted 1:4 in PBS and used for immunoprecipitation. In the immunoprecipitation assay, both heat-treated (15, 30, 60 min at  $95^\circ\text{C}$ ) and native cow's milk samples were used. The BLG-specific Fab fragments D1 and A3 were immobilized onto protein L beads (CBind™, Fluka) according to the manufacturer's instructions ( $100\text{--}150 \mu\text{g}$  protein/ $25 \mu\text{l}$  beads). Then the milk samples were incubated with protein L-Fab beads for 1 h at room temperature. After the washing step with PBS-0.05% Tween 20, elution was performed by adding first  $25 \mu\text{l}$  and then  $50 \mu\text{l}$  of  $0.1 \text{ M}$  glycine-HCl (pH 2). The eluted samples were separated on a 15% SDS-PAGE gel and stained with silver, and the desired protein bands were cut out for MS analysis (AlphaLys A/S, Denmark).

## 2.8. Analysis of the binding kinetics

The association and dissociation constants of the BLG-specific antibody D1 and A3 Fab fragments were measured by the surface plasmon resonance (SPR)-based assay (Biacore). For the affinity measurements of D1 Fab fragment, BLG-bio was immobilized onto the SA-biosensor chip (Biacore, Uppsala) using HBS-buffer ( $10 \text{ mM}$  Hepes,  $0.15 \text{ M}$  NaCl,  $3.4 \text{ mM}$  EDTA,  $0.005\%$  P20 surfactant, pH 7.4) at a concentration of  $1 \mu\text{g/ml}$  yielding a surface with approximately 400 RU (response units). The binding kinetics of the purified D1 Fab fragment at concentrations of 79,

39.5, 19.25, 9.86, 4.94, 2.46 and  $1.23 \text{ nM}$  were analyzed at a flow of  $30 \mu\text{l/min}$ . Regeneration of the BLG surface was performed with  $200 \mu\text{M}$  BLG solution. For affinity measurements of the A3 Fab fragment, heat-treated (15 min,  $95^\circ\text{C}$ ) BLG was immobilized onto the CM5-sensorchip (Biacore, Uppsala) in  $0.1 \text{ M}$  Na-acetate buffer, pH 4, at a concentration of  $10 \mu\text{g/ml}$ , yielding a surface with 800 RU. The binding kinetics of the purified A3 Fab were then analyzed at concentrations of 2000, 1000, 750, 500, 250,  $150 \text{ nM}$  and a flow of  $30 \mu\text{l/min}$ . Regeneration of the surface was performed with  $0.1 \text{ M}$  glycine-HCl, pH 3.5.

## 3. Results

### 3.1. The construction and characterization of the human IgE phage display libraries

In order to find an appropriate blood donor for the construction of the combinatorial human IgE phage display libraries, the sera from cow's milk-allergic patients (P1–P3) were tested. The total S-IgE was 237, 1206 and  $775 \text{ kU/l}$  for P1, P2 and P3, respectively. The BLG-specific IgE levels were also measured and they were 3.7, 7.2 and  $11.6 \text{ kU/l}$  for P1, P2 and P3, respectively. The IgE-binding reactivity of the human P1–P3 sera to directly immobilized or biotinylated BLG was also analyzed by ELISA (Fig. 1). P2 and P3 sera had a higher IgE response to BLG-bio than to BLG immobilized directly onto the wells whereas P1 serum showed higher binding to the directly immobilized BLG than to BLG-bio. P3, having the highest IgE response to BLG, was selected to donate blood. The scFv IgE libraries were constructed using P3 as a single donor to obtain libraries as authentic to patient-specific IgE response as possible. Total RNA was isolated from lymphocytes of P3 and cDNA syntheses with the human IgE Fd region,  $C_{\kappa}$  or  $C_{\lambda}$  specific primers were carried out. The different subgroups of the heavy and light chain genes were amplified separately to avoid a possible PCR-based bias. The IgE isotype of the amplified Fd fragments was verified by DNA sequencing of 10 individual cDNA clones (data not shown). Libraries containing  $10^6$  independent clones were cloned in an scFv format due to our earlier observation (Laukkanen et al.,

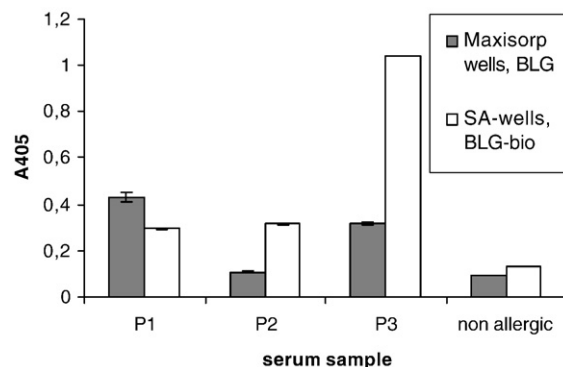


**Fig. 1.** Characterization of milk-allergic patient sera by ELISA. BLG and BLG-bio ( $1 \mu\text{g}$ ) were immobilised on Maxisorp (grey bars) and SA wells (open bars), respectively. The 1:8 dilutions of the serum samples were added and the bound serum IgE was detected with AFOS-conjugated anti-human IgE. The serum from a non-allergic person was used as a control. The results are mean values of the duplicate samples ( $\pm$  SD).

2003); the functional expression of scFv fragments in *E.coli* is better than IgE Fab fragments.

### 3.2. Isolation of BLG-specific IgE antibodies

The selection of the BLG-specific scFvs was carried out both in solution, using BLG-bio combined to elution with soluble BLG, and using BLG-coated onto Maxisorp wells combined with elution at low pH (Fig. 2). The scFv- $\kappa$  and scFv- $\lambda$  libraries were kept separately during selection. Enrichment of the BLG-specific scFv phages was more efficient during the BLG-bio selection than with immobilized BLG (data not shown).

The selection using BLG-bio resulted in the isolation of two BLG-specific scFv clones (designated D1 and H2). Almost all the BLG-specific clones screened from the second and third selection round turned out to be the same as the D1 scFv clone (83/96 and 96/96, respectively). After screening of 750 additional clones from the first selection round, the other BLG-specific clone, H2, was isolated. The selection with immobilized BLG resulted in the isolation of two BLG-specific clones (designated A3 and G1). The DNA sequences of these four clones were determined and the CDR regions are shown in Fig. 3. The amino acid sequences of the light chain CDR regions of D1, H2 and A3 were identical. The H2 clone differed only by one amino acid in framework 2 region from the D1 and A3 clones (not shown). The heavy chains of all clones were different, with especially the heavy chain CDR3 regions showing marked sequence variation (Fig. 3).

### 3.3. Characterization of BLG-specific IgE antibodies

In order to circumvent potential aggregation problems, the isolated BLG-specific scFvs were converted to Fab fragments with the constant domains of human IgG1 heavy and kappa light chains. Large-scale fermenter production of the D1 Fab fragment combined with IMAC purification resulted in a yield of 96 mg purified protein with 95% purity from 4 L of cultivation supernatant. The yield of the IMAC-purified A3 Fab fragment was about 2 mg from a 150-ml shake flask cultivation with 95% purity. A preliminary bacterial production of the G1 Fab fragment showed that the clone was poorly expressed in *E. coli*. In contrast, the H2 Fab fragment was well expressed in *E. coli*, but the ELISA test of the H2 Fab fragment supernatant samples showed only a

weak binding activity to BLG. It is possible that this particular clone, as an scFv fragment, formed multimers with consequent higher avidity and relatively good binding affinity to BLG in the first ELISA experiments.

Because of the above production and affinity problems with the G1 and H2 Fab fragments, respectively, the BLG-binding properties of the D1 and A3 Fab fragments were characterized further. Competitive ELISAs demonstrated that D1 and A3 Fab fragments recognized different forms of BLG. Native, soluble BLG inhibited binding of the D1 Fab fragment to BLG-bio more efficiently than heat-treated BLG (Fig. 4A). In contrast, binding of the A3 Fab fragment to directly immobilized BLG was more efficiently inhibited by heat-treated BLG than by native BLG (Fig. 4B). The binding kinetics of the D1 and A3 Fab fragments were analyzed by BIAcore (Table 1). The BIAcore analysis was performed in order to compare the BLG-binding properties of D1 and A3 Fab fragments. D1 Fab fragment was binding to the native BLG with higher affinity ( $K_D \sim 1.3$  nM) than A3 Fab fragment to the heat treated BLG ( $K_D \sim 142$  nM). The results are averages of three (D1) or two (A3) different measurements with seven (D1) or six (A3) different concentrations ( $\pm$  SD).

The binding properties of the D1 and A3 Fab fragments were further characterized by the immunoprecipitation assay (Fig. 5). In addition to BLG in untreated cow's milk, the D1 Fab fragment also recognized to some extent BLG in a milk sample after a 30-min heat treatment. The binding of D1 Fab fragment to BLG was abolished by longer heat treatment, while the binding to protein bands with molecular weight of about 32 kD appeared. In contrast to the D1 Fab fragment, the A3 Fab fragment recognized preferably the heat-treated BLG. The immunoprecipitated protein bands were identified by MS and it was confirmed that the 18-kDa protein precipitated by the Fab fragments was bovine BLG. The other precipitated protein band ( $\sim 32$  kD) was identified as bovine  $\alpha$ -s1-casein.

### 3.4. Inhibition by D1 Fab fragment of serum IgE binding to BLG

In order to find out whether the phage display-derived recombinant IgE D1 Fab fragment can compete with the IgE-binding of the cow's milk-allergic patient serum to BLG-bio, a competitive ELISA was performed. P1-P3 patient sera were incubated with immobilized BLG-bio together with increasing concentrations of D1 Fab fragment. In the cases of P1 and P3

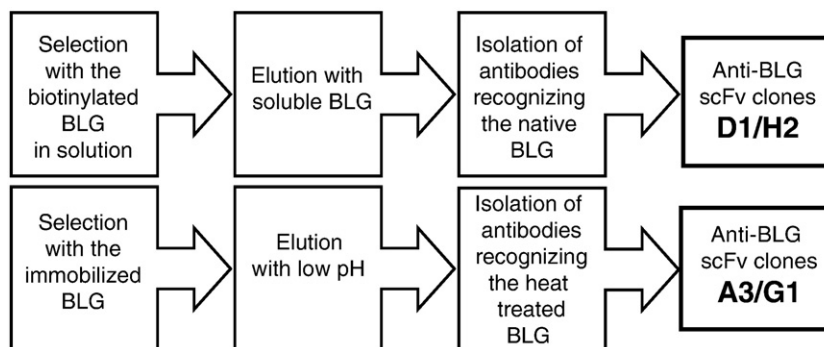


**Fig. 2.** Summary of the selection strategies used to isolate BLG-specific scFv antibodies. Two different selection strategies were used to isolate antibodies recognizing native form of BLG (D1, H2) and heat-treated BLG (A3, G1).

| scFv clone | HCDR1    | HCDR2    | HCDR3            |
|------------|----------|----------|------------------|
| D1         | GFTGHHGT | LSGSGTKT | AKAKRVGATGYFDL   |
| H2         | GFTFSYAH | ISYDGSNK | ARAGATVTTHYYGMDV |
| A3         | GYTFTGY  | INPNSGGT | ARGRWAGAFDI      |
| G1         | GGTFSSYA | IIPFGIA  | ASLATLVGDY       |

| scFv clone | LCDR1   | LCDR2 | LCDR3      |
|------------|---------|-------|------------|
| D1         | QGISSR  | AAS   | QQYHSYPWT  |
| H2         | QGISSR  | AAS   | QQYHSYPWT  |
| A3         | QGISSR  | AAS   | QQYHSYPWT  |
| G1         | QSVSSSY | AAS   | QQRSNWPPVT |

**Fig. 3.** The CDRs of the BLG-specific scFv clones. The amino acid sequences of the CDR-regions of the heavy and light chain according to IMGT/V-QUEST bioinformatics tool are shown.

sera, inhibition of the serum IgE binding to BLG was approximately 8% and 14% with 0.1 mg/ml competitor concentration, respectively. However, the D1 Fab fragment inhibited the binding of P2 serum IgE to BLG-bio by 57% with the same competitor concentration as above (Fig. 6).

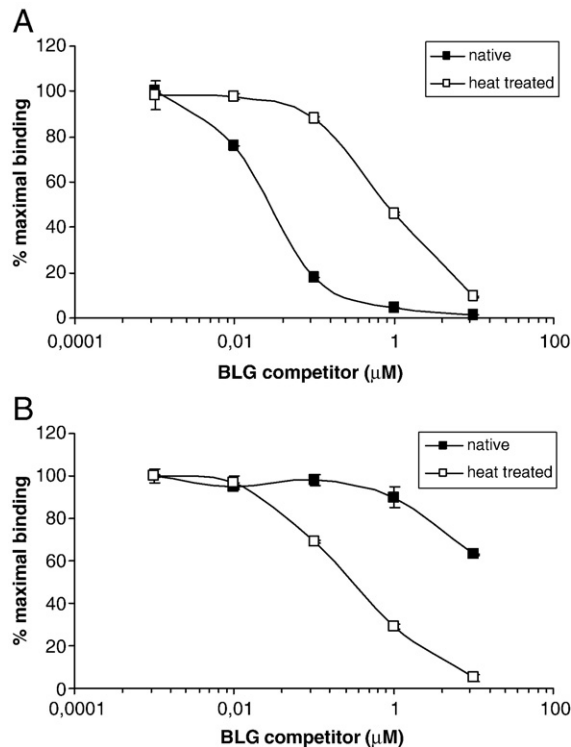


**Fig. 4.** Binding properties of the BLG-specific clones analysed by the competitive ELISA. The binding of D1 (A) and A3 (B) Fab fragments to native BLG (black squares) or heat-treated BLG (open squares). The results are mean values of the duplicate samples ( $\pm$  SD). For the heat treatment, BLG was incubated for 15 min at 94 °C before the competition ELISA was performed. See Materials and Methods for more details.

4. Discussion

This is the first report describing the isolation of recombinant human IgE antibodies against a food allergen using two different selection approaches. As a result, four antibodies with two different binding properties for the same allergen were isolated (Fig. 2). Previously, allergenic patient-specific IgE libraries have been constructed from individuals suffering from pollen or mould allergies or contact urticaria to latex gloves (Steinberger et al., 1996; Laukkanen et al., 2003; Jakobsen et al., 2004; Andreasson et al., 2006; Flicker et al., 2006). Here, the combinatorial human scFv phage libraries were constructed from the lymphocytes of an adult milk-allergic patient who had avoided milk and milk products, making the first PCR amplifications more challenging.

Binding properties of the D1 and A3 Fab fragments were characterized in more detail. Interestingly, the BLG-binding profiles of these two antibodies turned out to be distinct. The binding of D1 Fab fragment to BLG was decreased when the purified BLG or milk sample was heat treated (Figs. 4 and 5). On the contrary, the A3 Fab fragment bound well to the heat-treated BLG indicating that the A3 Fab fragment might recognize a linear rather than a conformational epitope. It has been observed previously that the direct immobilization of BLG has an effect on its conformation and, thus, on antibody binding as well (Adel-Patient et al., 2000). Here, the immobilization of BLG onto polystyrene wells at high pH (9.6) might have caused

**Table 1**  
The on/off rates and association/dissociation constants of IgE D1 and A3 Fab fragments.

|        | ka (1/Ms) $\times 10^6$ | KA (1/M) $\times 10^9$ | kd (1/s) $\times 10^{-3}$ | KD (M) $\times 10^{-9}$ |
|--------|-------------------------|------------------------|---------------------------|-------------------------|
| D1 Fab | (1.62 $\pm$ 0.83)       | (1.04 $\pm$ 0.62)      | (1.61 $\pm$ 0.2)          | (1.30 $\pm$ 0.66)       |
| A3 Fab | (0.072 $\pm$ 0.036)     | (0.009 $\pm$ 0.007)    | (8.05 $\pm$ 0.2)          | (142 $\pm$ 88)          |

The values are averages  $\pm$  SD of three (D1) or two (A3) independent runs with seven (D1) or six (A3) different Fab concentrations.

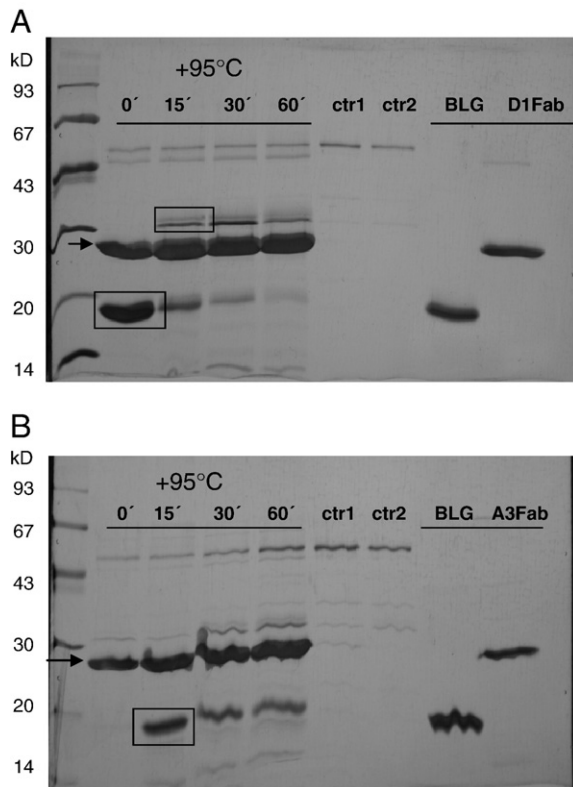


**Fig. 5.** Immunoprecipitation using the D1 (A) and A3 (B) Fab fragments and cow's milk samples. The Fab fragments were immobilised on protein L beads and then incubated with the milk samples without or with heat treatment (0, 15, 30 or 60 min at 95 °C). After washing steps, the samples were analysed on a 15% SDS-PAGE gel and stained with silver. The boxes indicate the protein bands that were cut out for MS-analysis. Arrows indicate the Fab fragments. Ctr = protein L beads without the immobilized Fab incubated with 0' (1) and 60' (2) heated BLG sample. As a control, 1 µg of native BLG (Sigma) and D1/A3 Fab were used.

a partial denaturation of the allergen and lead to the exposure of new hidden allergenic epitopes, mimicking the heating process (Chen et al., 2006).

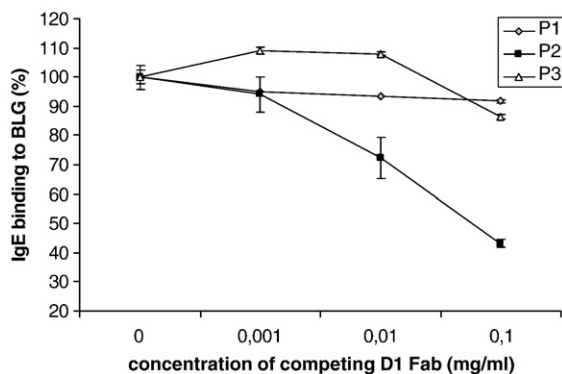


**Fig. 6.** Inhibition of the serum IgE binding to BLG by D1 Fab fragment. The serum samples (P1, P2 and P3) together with increasing concentrations of the D1 Fab fragment were added onto the immobilized BLG-bio. The bound IgEs were detected with the AFOS-conjugated anti-human IgE. The results are mean values of the duplicate samples ( $\pm$  SD).

The effect of heat-treated BLG on the serum IgE binding ability has been studied previously (Davis and Williams, 1998; Ehn et al., 2004; Haddad et al., 1979). The authors reported that an extensive heat treatment (60 min, 95 °C) substantially decreased the IgE binding to BLG and cow's milk. By using mouse monoclonal IgG antibodies, Song and co-workers determined the thermal denaturation region of BLG (Song et al., 2005). They suggested that BLG undergoes conformational rearrangements during a heating process and, thus, new antigenic epitopes were exposed. It has also been reported that heat-treated BLG may expose new allergenic epitopes (Davis and Williams, 1998; Haddad et al., 1979) and that hypoallergenic milk formulas based on hydrolyzed protein content have caused reactions in some allergic children (Mäkinen-Kiljunen and Sorva, 1993; Rosendal and Barkholt, 2000). Our results may explain why the IgE reactivity of milk and milk products is retained even after extensive heating. They indicate that IgE antibodies able to bind denatured BLG may be present in the IgE repertoire of a milk allergic-person.

The immunoprecipitation revealed when the milk was heated,  $\alpha$ -s1-casein was also precipitated by the D1 Fab fragment and to some extent by the A3 Fab fragment (Fig. 5). Caseins ( $\alpha$ -s1 and 2,  $\beta$  and  $\kappa$ ) are major allergens of cow's milk (Bernard et al., 1998) and P3, who donated blood for the library construction, has been diagnosed to have high casein-specific IgE by RAST (class 3). BLG can bind to casein micelles, especially to  $\kappa$ -casein when the milk is heated (Zittle et al., 1962).

The BLG-specific D1 Fab fragment was able to inhibit the binding of patient serum IgE to BLG (Fig. 6). Surprisingly, the strongest inhibition of the binding of serum IgE to BLG was seen with the P2 serum sample, even though the IgE scFv libraries were constructed using the P3 blood sample. The P1 and P3 serum samples showed only weak inhibition of IgE binding to BLG with the highest D1 Fab concentration. Based on these results, it is likely that BLG has at least one additional IgE epitope and inhibition of the binding of the bivalent, polyclonal IgE antibody pool by a monoclonal monovalent Fab fragment is not efficient as described earlier (Steinberger et al., 1996). Although the RAST result of patient P2 is undoubtedly positive and the patient's IgE level in serum is high, there is a possibility that the IgE antibodies of P2 have lower affinity to BLG.

Currently, the immunological detection of food allergens is mainly based on the use of non-homogeneous and non-standardized polyclonal antibody pools from immunized animals or IgE serum pools from allergic patients (Poms et al., 2004). Validated methods are obligatory to ensure that food labeling fulfils legal demands. Sensitive detection methods are also needed when developing and manufacturing hypoallergenic food products to reveal even trace amounts of contaminating allergens.

In this study, the recombinant antibody recognizing native BLG with the nanomolar affinity was developed. Preliminary ELISA-based experiments demonstrated that the BLG detection limit was ten times less sensitive than achieved using sandwich ELISA and the BLG-specific rabbit polyclonal serum (Mäkinen-Kiljunen and Palosuo, 1992). On the other hand, better assay sensitivity can always be reached by optimizing assay conditions e.g. by direct labeling of the antibodies with fluorescent labels.

The antibodies recognizing the allergenic epitopes of BLG have great potential not only for allergen detection from various

food products and consumer goods but also for studying the development of an allergic immune response and the IgE antibody-allergen immunocomplexes (Niemi et al., 2007).

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