

Identification of epitopes within beta lactoglobulin recognised by polyclonal antibodies using phage display and PEPSCAN

S.C. Williams ^{a,*}, R.A. Badley ^a, P.J. Davis ^a, W.C. Puijk ^b, R.H. Meloen ^b

^a Unilever Research, Colworth Laboratory, Colworth House, Sharnbrook, Bedford MK44 1LQ, UK

^b Institute for Animal Science and Health (ID-DLO), Research Head Office, Edelhertweg 15, Postbus 65, 8200 AB, Lelystad, Netherlands

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Abstract

Two different epitope mapping techniques were used to identify linear epitopes recognised by polyclonal IgG antibodies from rabbits immunised with bovine beta lactoglobulin (BLG), which is generally regarded as a major allergen in milk. The first, PEPSCAN, was used to investigate the binding of several rabbit polyclonal antisera to sequential overlapping peptides (12-mers) across the sequence of BLG. Each peptide was synthesized on a different polypropylene PIN, and a standard ELISA procedure was used to locate which of these peptides bound the antibodies under investigation. Comparisons of PEPSCANs for antisera from six different rabbits showed that each rabbit recognized a similar set of epitopes within BLG. PEPSCAN analysis also showed that polyclonal antibodies from the mouse recognize a set of epitopes similar to those recognized by the rabbit. The second epitope mapping technique is known as phage display and utilizes libraries of randomized short peptides fused to the coat proteins of filamentous phage as a source of epitopes for analysis. A gene VIII phage display library was used in this study with constrained nonapeptides, which were screened for epitopes recognized by affinity purified rabbit anti-BLG IgG. Immobilised rabbit anti-BLG IgG was screened in two separate experiments, each consisting of three rounds of panning. For each separate experiment, a sensitive phage ELISA was used to screen several hundred single phage clones for binding to anti-BLG IgG immobilised on microtiter plates. As a result, a number of positive phage were identified from the two separate screens of the library (19 different peptides were isolated, which resembled four different regions of BLG). The identified sequences were found to constitute a subset of the linear epitopes recognized by the PEPSCAN technique. The coordinates of the crystal structure of BLG were used to display mapped epitopes on its structure. This study has permitted detailed mapping of the major linear antigenic regions within BLG recognised by IgG antibodies from immunised rabbits and mice. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Bovine beta lactoglobulin; Epitope mapping; Phage display; PEPSCAN; Species/animals

1. Introduction

Beta lactoglobulin (BLG) is the major whey protein in the milk of various species including cows, sheep, goats, horses and pigs, but is not present in

Abbreviations: BLG, Bovine beta-lactoglobulin

* Corresponding author.

humans or rodents (for reviews, see Godovac-Zimmermann and Braunitzer, 1987; Perez and Calvo, 1995). Some studies have been carried out on the antigenicity of BLG and have involved investigation of the binding of polyclonal rabbit antisera to chemically modified and denatured intact BLG, or large fragments of proteolytically cleaved BLG (Otani et al., 1985; Otani and Hosono, 1987; Takahashi et al., 1990). BLG is one of the major allergens in cow's milk allergy, which affects between 2 and 7.5% of infants (for review, see Schwartz, 1991) and limited work has been carried out on the molecular regions, which may be important in allergy (Huang et al., 1985; Adams et al., 1991; Ball et al., 1994). Our work involves study of defined antigenic determinants (epitopes) within BLG, which are recognised by polyclonal antibodies from different immunised species using two epitope mapping techniques.

BLG has been crystallized and has a structure which is very similar to retinol-binding protein, i.e., eight strands of anti-parallel beta-sheet twisted into a cone-shaped barrel and flanked on one side by an alpha helix (Papiz et al., 1986; Monaco et al., 1987). In fact, both proteins are described as members of a superfamily of proteins known as the lipocalins, which are reported to be involved in the transport of small hydrophobic molecules (Perez and Calvo, 1995; Flower et al., 1993; Cho et al., 1994). Although the function of BLG is largely unknown, accumulating evidence exists to suggest that it is involved in the binding of retinol and fatty acids (Cho et al., 1994; Frapin et al., 1993).

In recent years, new technology has been developed, which permits the construction of large libraries of peptides for the study of interactions between biologically active molecules (for reviews, see Houghten, 1993; Scott and Craig, 1994; Gallop et al., 1994). One of the major uses of these libraries has been the discovery of antigenic determinants or epitopes.

The epitope mapping technique of phage display was pioneered by George Smith (Smith, 1985; Parmley and Smith, 1988) and exploits the molecular biology of filamentous phage for the expression of small peptides. Phage display peptide libraries are made by shotgun cloning of random oligonucleotides into the 5' ends of either the pIII or pVIII genes of filamentous phage. In this way, large repertoires of

random peptides have been expressed on the surface of phage as fusions with the phage coat proteins pIII or pVIII (Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith, 1990; Felici et al., 1991; Felici et al., 1993; Luzzago et al., 1993). The wide variety of uses for random peptide phage libraries has recently been reviewed (Barbas, 1993; Scott and Craig, 1994; Schatz, 1994; Cortese et al., 1995). Although most of the work carried out with phage display libraries has been in screening monoclonal antibodies, Folgori et al. (1994) showed the uses of screening polyclonal antibodies from patients immunised with human hepatitis B virus surface antigen (HBsAg).

Chemical synthesis of peptides can be used to produce random sets of peptides for epitope mapping (Geysen et al., 1987; Houghten et al., 1991; Lam et al., 1991). The non-random approach of synthesizing overlapping peptides of known amino acid sequence (i.e., PEPSCAN) has also had many applications (Geysen et al., 1984, 1985; Meloen et al., 1991). Using the PEPSCAN technique, synthetic overlapping peptides of a given length (e.g., 9–12 amino acids in length), which cover the entire sequence of a given protein are synthesized on plastic pins and used for epitope mapping by conventional ELISA (for review, see Meloen et al., 1995). Among its many applications, the overlapping peptide strategy has been used for the characterisation of epitopes recognized by polyclonal and monoclonal antibodies to viral, bacterial and parasitic proteins. Human IgE antibodies recognising the allergenic wheat protein α -amylase (Walsh and Howden, 1989) and the house dust mite allergen Der pII (Van t'Hof et al., 1991), have also been studied.

In this study, we report details of dominant antigenic regions within bovine beta lactoglobulin, which are recognised by polyclonal antibodies raised in rabbits and mice. Comparisons of antigenic regions recognised by different animals of the same species have been made, as well as between different species (rabbits and mice). Visualisation of the position of the antigenic regions within the crystal structure of the original antigen has also been carried out, and shows that the majority of epitopes recognised are present on the surface of the native antigen. Although the PEPSCAN and phage display epitope mapping techniques are often used to study regions recognised by monoclonal antibodies, we have also

shown that existing methodology works equally well for the study of polyclonal antibodies.

2. Materials and methods

2.1. Materials

Bovine beta-lactoglobulin B (BLG) was obtained from Sigma Chemical Company, Poole, UK (Code: L8005) as was an antiserum raised in rabbits to bovine whey proteins (Code: W3501). An additional rabbit antiserum to beta-lactoglobulin B was obtained from Nordic Immunochemicals (Tilburg, Netherlands). For PEPSCAN experiments detection of rabbit antibodies to BLG was carried out with peroxidase-conjugated swine anti-rabbit immunoglobulin conjugate and the detection of mouse antibodies with peroxidase-conjugated rabbit anti-mouse immunoglobulin (Code numbers P217 and P260; Dako). Peptide 1 corresponding to amino acids 120–135 (QCLVRTPEVDDEALEK) of BLG was synthesised by Ms L. Law at the Institute of Food Research, Norwich and Peptide 2 corresponding to amino acids 81–91 (CFKIDALNENKV) of BLG was synthesised by Dr. M. Munns (Peptide and Protein Research Consultants) at the University of Exeter, UK. The affinity-purified sheep anti-M13 bacteriophage antibodies used in phage ELISAs came from C.P. Laboratories, Bishop's Stortford, UK and M13K07 helper phage were purchased from Gibco-BRL Life Technologies, Paisley, Scotland. CNBr-activated Sepharose 4B used for coupling of BLG for affinity purification of anti-BLG antibodies was obtained from Pharmacia, Milton Keynes, UK.

2.2. Immunisation of rabbits and mice with bovine beta lactoglobulin B (BLG)

Two eight-week old White New Zealand rabbits were immunised by injection at multiple subcutaneous sites with beta lactoglobulin B (1 ml of 1 mg/ml in phosphate-buffered saline, PBS mixed with 1 ml of Freund's complete adjuvant). The rabbits were then boosted 1 month later by subcutaneous injections with 1 ml of BLG (1 mg/ml in PBS) mixed with 1 ml of incomplete Freund's adjuvant. A test bleed of 5 ml was taken after a further

10–14 days and the serum analysed for the presence of anti-BLG specific antibodies by ELISA as outlined below. One month later, a further boost was carried out and anti-BLG antibodies were affinity purified (see below) from the bleed taken 10 days later.

BALB/c mice were immunised at subcutaneous and intraperitoneal sites with 50 μ l of BLG (1 mg/ml in PBS) mixed with 50 μ l of Freund's complete adjuvant. A booster injection was carried out two weeks later with the same quantity of BLG mixed with incomplete Freund's adjuvant, and a test bleed was taken 1 week later. A further boost was given 2 weeks later, and the bleeds taken after a further week were tested for the presence of anti-BLG specific antibodies by ELISA.

2.3. Detection of specific anti-BLG antibodies by ELISA

Whole rabbit or mouse bleeds were tested for anti-BLG antibody activity using ELISA. Greiner 96-well microtitre plates were coated with BLG (100 μ l/well; 10 μ g/ml in PBS) overnight at 4°C. The plates were then blocked with PBS containing 0.1% (v/v) Tween 20 and 1% (w/v) gelatin (PBSTG) for 1 h at room temperature. Serial doubling dilutions (from 1/1000) of rabbit serum in PBSTG (100 μ l/well) were made and incubated with the BLG-coated plates for 2 h at room temperature. After three washes with PBST (PBS containing 0.1% Tween 20), alkaline phosphatase conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse immunoglobulin; Zymed, Cambridge Bioscience, Cambridge, UK) were added (100 μ l/well, diluted 1/1000 in PBSTG) and incubated for 2 h at room temperature. After further washing, assays were developed with 200 μ l/well of *p*-nitrophenyl phosphate (1 mg/ml; Sigma: N2765) in 1 M diethanolamine, 1 mM MgCl_2 , pH 9.6. Absorbance values were read at 410 nm after 10–20 min incubation at room temperature.

2.4. Affinity purification of rabbit anti-BLG antibodies

Beta lactoglobulin (BLG) was coupled to CNBr-activated Sepharose 4B following the manufacturer's

instructions and used for affinity purification of anti-BLG antibodies from whole rabbit antiserum. Briefly, a Pharmacia glass column (8×1 cm containing 2 ml of CNBr-activated Sepharose coupled to 6 mg of BLG) was pre-equilibrated with 10 ml of PBS at room temperature. The anti-BLG rabbit serum (2 ml) was then loaded onto the column and incubated for 20 min at room temperature. The BLG–Sepharose was washed with 10 column volumes of PBS followed by 2 column volumes of 10 mM sodium phosphate, 0.5 M NaCl, pH 7.4. A further wash (5 ml) with Milli Q water was carried out to remove traces of sodium phosphate buffer before elution of bound anti-BLG antibodies with 3 M MgCl_2 , (pH 7.4, adjusted with 1 M Tris). Fractions of 1 ml were collected and samples were analysed for anti-BLG activity by ELISA as above. The pooled, purified fractions were used to coat Immuntubes (Nunc) for panning of the pVIII9aa-cys phage library, for PEPSCAN analysis and for phage ELISAs as detailed below.

2.5. PEPSCAN assays

A set of 151 peptides corresponding to 12-mers spanning the amino acid sequence of BLG (each peptide overlapping with the previous peptide by 11 amino acids) were synthesised on polyethylene pins using established PEPSCAN methodology (Geysen et al., 1984, 1987; Geysen, 1985, 1990; Maeji and Geysen, 1991; Bray et al., 1990). The polyethylene pins were held on racks, which had a 96-well format compatible with conventional ELISA plates. The peptides were tested for their ability to bind rabbit and mouse anti-BLG polyclonal antibodies using ELISA. The ELISA procedure was carried out as follows (Geysen et al., 1987). Non-specific binding sites on the pins were blocked by incubation in blocking buffer (PBS containing 5% ovalbumin, 5% horse serum and 1% Tween 80) in the wells of a Sterilin microtitre plate ($150 \mu\text{l}/\text{well}$) for 30 min at room temperature. Anti-BLG antiserum (rabbit or mouse) was then diluted in blocking buffer ($100 \mu\text{l}/\text{well}$) and incubated overnight at 4°C . After careful washing ($\times 3$) with PBS containing 1% (v/v) Tween 80 at room temperature the pins were transferred to wells containing $100 \mu\text{l}$ of the relevant anti-immunoglobulin horse radish peroxidase conju-

gate (see Section 2 for details; diluted 1/1000 in blocking buffer) and incubated for 1 h at 37°C . After further washing, the presence of antibody was detected by incubation in fresh substrate solution (50 mg ABTS and $20 \mu\text{l}$ of 30% (w/w) H_2O_2 solution in 100 ml of 0.1 M sodium phosphate/0.08 M citrate buffer, pH 4.0). Plates were read at 405 nm. In order to remove bound antibody from pins for reuse sonication was carried out in a polyethylene container (wall approximately 2 mm thick) containing 10 mM sodium phosphate buffer, 1% (w/v) sodium dodecyl sulphate and 0.1% (w/v) betamercaptoethanol (added prior to sonication) in a 60-l, 2000-W sonicating water bath for 60 min at 70°C . This was followed by careful rinsing of the pins with double-distilled water and then re-sonication in double-distilled water for a further 1 h at 70°C . Pins were stored in a plastic container at -20°C .

2.6. pVIII9aa-cys nonapeptide phage library

The phage library described by Felici et al., 1991 and Luzzago et al., 1993 was used in this study. The library consists of random nonapeptides fused to the major coat protein pVIII, so that several hundred peptides are displayed on each phage particle. In addition, each peptide is flanked by two cysteines, so that at least some of these cysteines form disulphide bonds (Luzzago et al., 1993).

2.7. Screening of phage library

Affinity selection of phage was performed by a combination of the methods of Folgori et al., 1994 and Parmley and Smith, 1988. Polystyrene tubes used for panning (Immuntubes from Nunc) were coated either with affinity-purified anti-BLG antibodies ($20 \mu\text{g}$) in 2 ml of coating buffer (0.1 M NaHCO_3 , pH 9.0) or with coating buffer only overnight at 4°C . After three washes with Tris-buffered saline (TBS; 50 mM Tris–HCl, 140 mM NaCl, pH 7.4) both tubes were incubated with 4 ml of blocking buffer (TBS containing 10 mg/ml ovalbumin) for 4 h at room temperature. The pVIII9aa-cys library sample obtained from Dr. Alfredo Nicosia (Institute of Molecular Biology, Pomezia, Rome) was shown to have a titre of 1×10^{13} transducing units/ml (TU/ml) by infection of logarithmic XL1

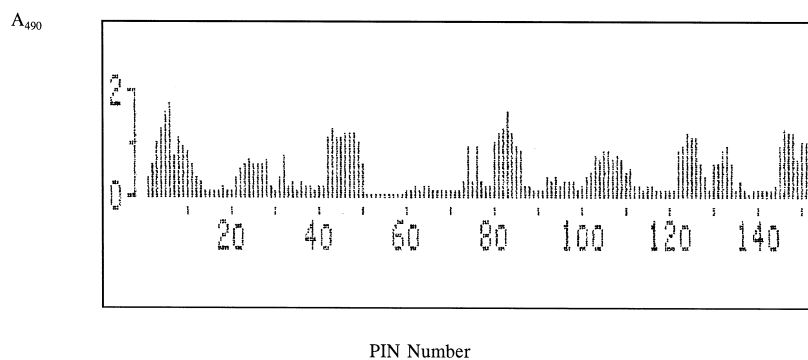
Blue bacteria. Aliquots ($10\ \mu\text{l}$; 1×10^{11} TU) from the donated phage suspension were added to the antibody-coated and un-coated polystyrene tubes each containing 1 ml of TBS and 1 mg/ml ovalbumin and incubated overnight at 4°C . Unbound phage were removed by 15 washes (each of 4 ml) with TBS containing 0.5% (v/v) Tween 20 (TTBS) followed by 5 washes with TBS at room temperature. Bound phage were eluted by incubation of washed panning tubes with 1 ml of elution buffer (0.1 M HCl, pH 2.2, adjusted with glycine, containing 1 mg/ml ovalbumin) for 12 min at room temperature. The eluted phage were transferred to 1 ml polypropylene tubes and neutralised with 60 μl of 2 M Tris (pH not adjusted). Aliquots (200 μl) of 1 M Tris-HCl, pH 7.4 were also added to the panning tubes for neutralisation. The eluted neutralised phage particles (1 ml) were used for infection of 9 ml of logarithmic XL1-Blue bacteria in 2TY media (Bactotryptone, 20 g, Bacto-yeast, 5 g, NaCl, 0.5 g made up to 1 l in distilled water) containing 1% (w/v) glucose. Logarithmic XL1-Blue bacteria (4 ml) were also added directly to the neutralised panning tubes. Infection was carried out for 30 min at 37°C with no shaking. The infected bacteria were then pooled (total volume 13 ml), ampicillin was added (to 100 $\mu\text{g}/\text{ml}$), and the cultures incubated overnight with shaking at 37°C . A small aliquot (10 μl) of infected bacterial cells was removed prior to overnight incubation for titration (diluted 10^{-2} to 10^{-6} in 2TY/Amp/Glucose) on SOBAG agar (Bactotryptone, 20 g, Bacto-yeast, 5 g, NaCl, 0.5 g, Bacto-agar, 15 g, 1 M MgCl_2 , 10 ml, 1% (w/v) glucose and ampicillin (100 $\mu\text{g}/\text{ml}$) made up to 1 l in distilled water). An aliquot (150 μl) of the overnight XL1-Blue culture infected with phage eluted from the panning tube coated with anti-BLG antibodies was then added to 15 ml of 2TY containing 1% (w/v) glucose and 100 $\mu\text{g}/\text{ml}$ ampicillin and grown to logarithmic phase. The cells were then superinfected with M13K07 helper phage (1×10^{11} phage/ml) and incubated for 30 min without shaking at 37°C . This was followed by centrifugation for 20 min at $3000 \times g$ and resuspension of the cell pellet in 200 ml of 2TY containing 100 $\mu\text{g}/\text{ml}$ ampicillin and 25 $\mu\text{g}/\text{ml}$ kanamycin. The bacterial culture was then incubated overnight at 37°C with shaking. Bacterial cells were then pelleted by cen-

trifugation ($3000 \times g$; 15 min) and the phage particles in the supernatant precipitated by addition of 40 ml of PEG/NaCl (2.5 M NaCl containing 20% (w/v) polyethylene glycol 8000) and incubation on ice for 1 h. The phage suspension was then spun at $15\,000 \times g$ for 20 min at 4°C , and the resulting pellet resuspended in 20 ml of TBS. A further PEG precipitation was carried out by addition of 4 ml of PEG/NaCl and incubation on ice for a further 20 min. The final phage pellet was dissolved in 2 ml of TBS which resulted in phage titres of the order of 1×10^{13} TU/ml. These phage particles were added directly to fresh panning tubes and the entire panning procedure repeated a further two times. The entire screening protocol (three rounds of panning) was repeated after the first screen to test whether further peptide epitopes could be isolated by screening the anti-BLG antibodies a second time.

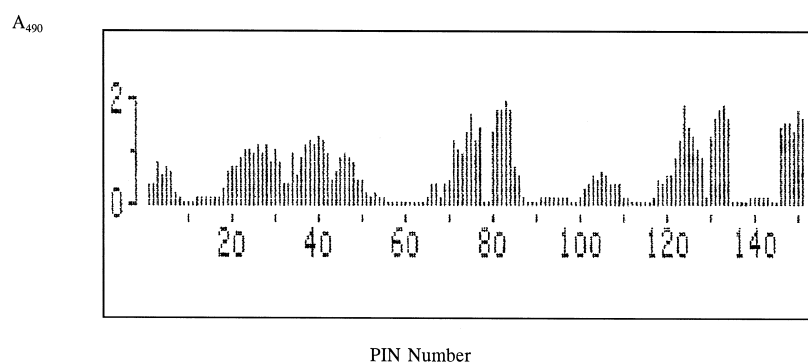
2.8. Phage ELISAs

The output from the third round of panning was plated out on SOBAG agar and incubated overnight at 37°C . Random individual bacterial colonies (~ 200) were picked and added to the wells of 96-well microtitre plates (Sterilin) each containing 200 μl of 2TY, 1% (w/v) glucose and ampicillin (100 $\mu\text{g}/\text{ml}$). The microtitre plates were incubated overnight with shaking at 37°C . The following day, aliquots from each well (20 μl) were added to the wells of fresh microtitre plates each containing 200 μl of 2TY, 1% (w/v) glucose, 100 $\mu\text{g}/\text{ml}$ ampicillin and incubated with shaking for 1 h at 37°C . At the next stage, 25 μl of 2TY containing ampicillin (100 $\mu\text{g}/\text{ml}$), 1% (w/v) glucose and 10^9 M13K07 helper phage were added to each well. The plates were incubated for 30 min at 37°C without shaking, followed by further incubation for 1 h at 37°C with shaking. The plates were then spun at $800 \times g$ for 20 min at room temperature, the supernatant aspirated off and the cell pellet resuspended in 200 μl of 2TY containing ampicillin (100 $\mu\text{g}/\text{ml}$) and kanamycin (20 $\mu\text{g}/\text{ml}$). Incubation with shaking at 37°C was then carried out overnight. Centrifugation of overnight cultures in the wells of microtitre plates was carried out ($800 \times g$; 20 min) and phage-containing supernatants (100 μl) were added to sheep anti-M13 bacteriophage coated microtitre plates

Rabbit 1



Rabbit 2



Rabbit 3

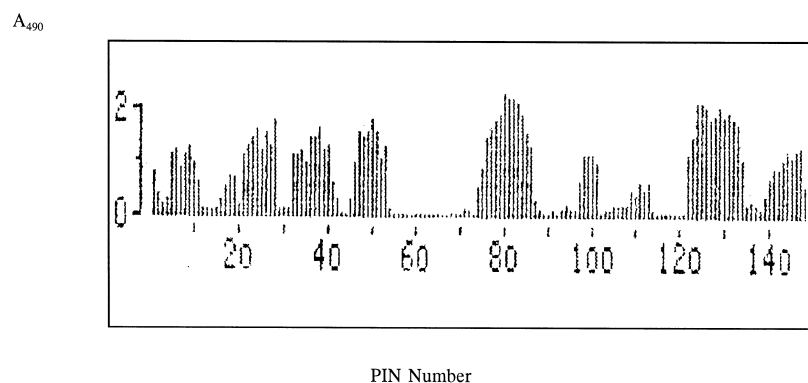
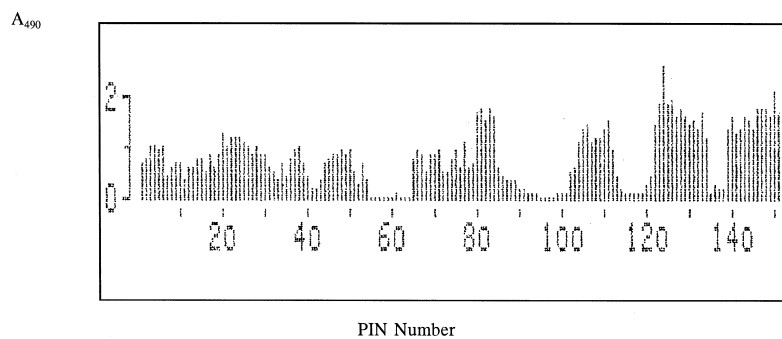
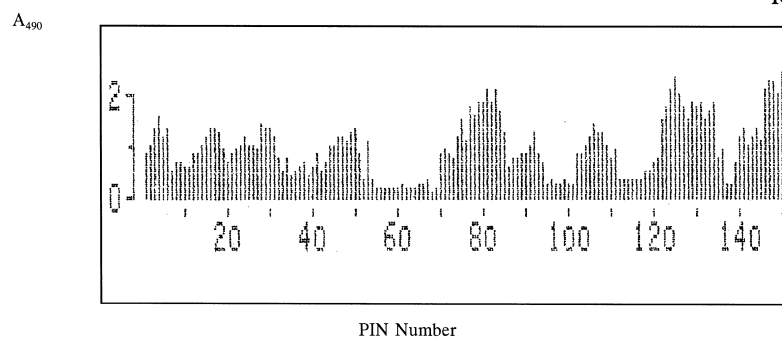


Fig. 1. PEPSCAN analysis of six anti-BLG rabbit antisera. Rabbits 1 and 2 were immunised with Sigma BLG B as described. The other rabbit anti-BLG antisera were obtained as follows: Rabbit 3 was raised at the Institute of Food Research, Norwich, and Rabbit 4 was immunised at Laboratoire Associe INRA-CEA. Rabbits 5 (Sigma) and 6 (Nordic) were obtained as described in Section 2. Rabbit serum 7 was a bleed from rabbit 2 taken prior to immunisation. All antisera were diluted 1/200 in PEPSCAN blocking buffer for analysis except for rabbit 5 which was diluted 1/300.

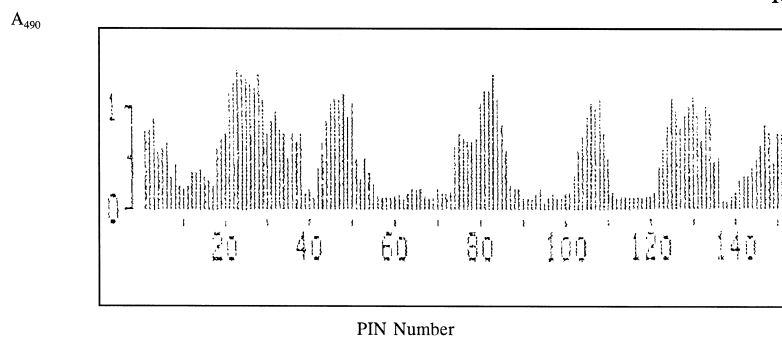
Rabbit 4



Rabbit 5



Rabbit 6



Rabbit 1 Pre-bleed

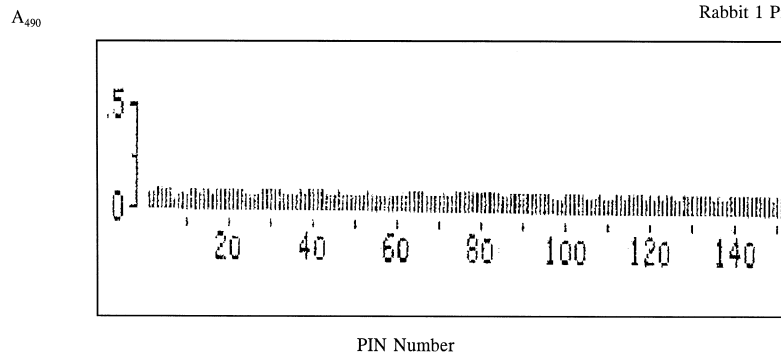


Fig. 1 (continued).

(Greiner, high bind). Purified sheep anti-M13 antibody-coated plates were prepared by overnight incubation (100 μ l/well; 10 μ g/ml) at 4°C in binding buffer (0.1 M NaHCO₃, pH 9.0). Blocking was carried out with PBST containing 10 mg/ml ovalbumin (200 μ l/well) for 1 h at room temperature. After removal of unbound phage from sheep anti-M13-coated plates by five washes with PBST, affinity purified rabbit anti-BLG antibodies were added (2 μ g/ml in PBST containing 10 mg/ml ovalbumin; 100 μ l per well). Incubation was carried out for 2 h at room temperature. Alkaline phosphatase conjugated goat anti-rabbit immunoglobulin (100 μ l/well) was then added at a dilution of 1/1000 (in PBST, 10 mg/ml ovalbumin) and incubated for a further 2 h at room temperature. The assay was developed with 100 μ l/well of *p*-nitrophenyl phosphate (1 mg/ml) in 1 M diethanolamine, 1 mM MgCl₂, pH 9.6 and the absorbance values read at 410 nm after 30 min.

2.9. DNA sequencing

Double-stranded phagemid DNA was purified from bacterial cultures (50 ml) infected with positive phage clones using the Qiagen plasmid purification kit according to the manufacturer's instructions. Sequencing was carried out on an Applied Biosystems automated sequencer (Model 373A, version 1.2.0) using the oligonucleotide primer: TTT CCC AGT CAC GAC GTT G so that analysis of the inserted DNA sequence encoding the random nonamer could be made. Amino acid sequences from phage clones, which gave positive signals in phage ELISA were compared to the primary sequence of beta lactoglobulin (Godovac-Zimmermann and Braunitzer, 1987)

2.10. Representation of discovered epitopes on the crystal structure of BLG

The coordinates of an updated version of the bovine BLG crystal structure (Brownlow et al., 1997) were displayed using BIOSYM/MSI Insight II Molecular Modeling software (Version 95.0) installed on a Silicon Graphics workstation. The software was used to represent the dimeric molecule as one of its monomers, and those epitopes discovered

by the pVIII9aa-cys phage peptide library and by the PEPSCAN technique were highlighted.

3. Results

3.1. Antisera against BLG

Antisera to BLG raised in rabbits and mice were found to contain anti-BLG antibodies detectable by ELISA. Rabbit antibodies were purified by affinity chromatography on immobilised BLG as described in Section 2, and the fractions eluted were tested for anti-BLG activity. Unbound material, which passed through the column, was found to be devoid of anti-BLG antibodies, but fractions eluted with 3 M MgCl₂ showed binding to BLG-coated microtitre plates (data not shown). Peak fractions 3–6 were pooled and dialysed extensively against TBS (final protein concentration 130 μ g/ml).

3.2. PEPSCAN assays of rabbit anti-BLG antisera

Results from PEPSCANs of antibodies derived from rabbits 1 and 2 are shown in Fig. 1. In addition, results from four anti-BLG rabbit antisera obtained from various other sources are shown in Fig. 1. As a negative control non-immune rabbit serum was analysed (pre-bleed from rabbit 2) and the scan is also shown in Fig. 1. No visible peaks were present in this trace.

BLG appeared to be a good immunogen in rabbits, since there was a large number of different peaks in the PEPSCANs, i.e., a large proportion of the BLG peptides are recognised by the rabbit anti-BLG antisera. In addition, it is also apparent in Fig. 1 that similar linear epitopes were recognised by each rabbit.

When monoclonal antibodies, which recognise linear epitopes, are analysed by PEPSCAN, scans are usually very clear with sharp peaks often spanning only a few peptides. The core epitope can then be easily identified by the amino acids common to these peptides (Worthington and Morgan, 1994). However, in the case of polyclonal antisera, the scans obtained are more complex with more frequent and wider peaks. As can be seen in Fig. 1, we obtained some quite wide peaks for the anti-BLG antisera, e.g., see

rabbit 5. The broad peaks may be explained by the fact that different antibodies bind to epitopes which overlap, so that a number of adjacent peptides have high signals. There are also regions that do not

exhibit any peaks, and these regions are also common to all of the rabbit sera tested.

In order to analyse whether the scans obtained represented epitopes recognised by an anti-BLG IgG

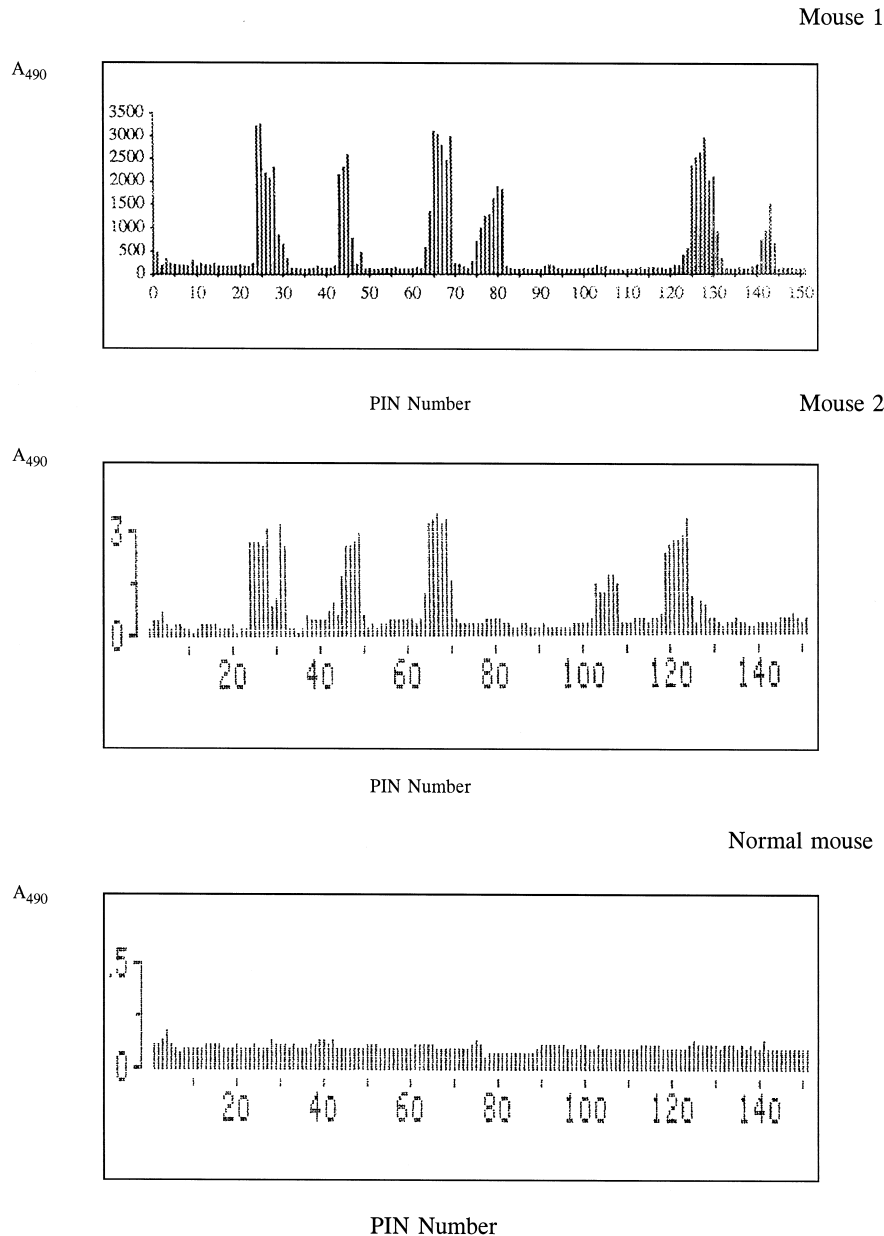


Fig. 2. PEPSCAN analysis of anti-BLG mouse antisera and pre-immune mouse serum. Mouse 1 was immunised using the procedure outlined in Section 2. Mouse 2 was immunised at the Institute of Food Research, Norwich and normal mouse serum (shown at the bottom of figure) was obtained from Sigma, Poole, Dorset.

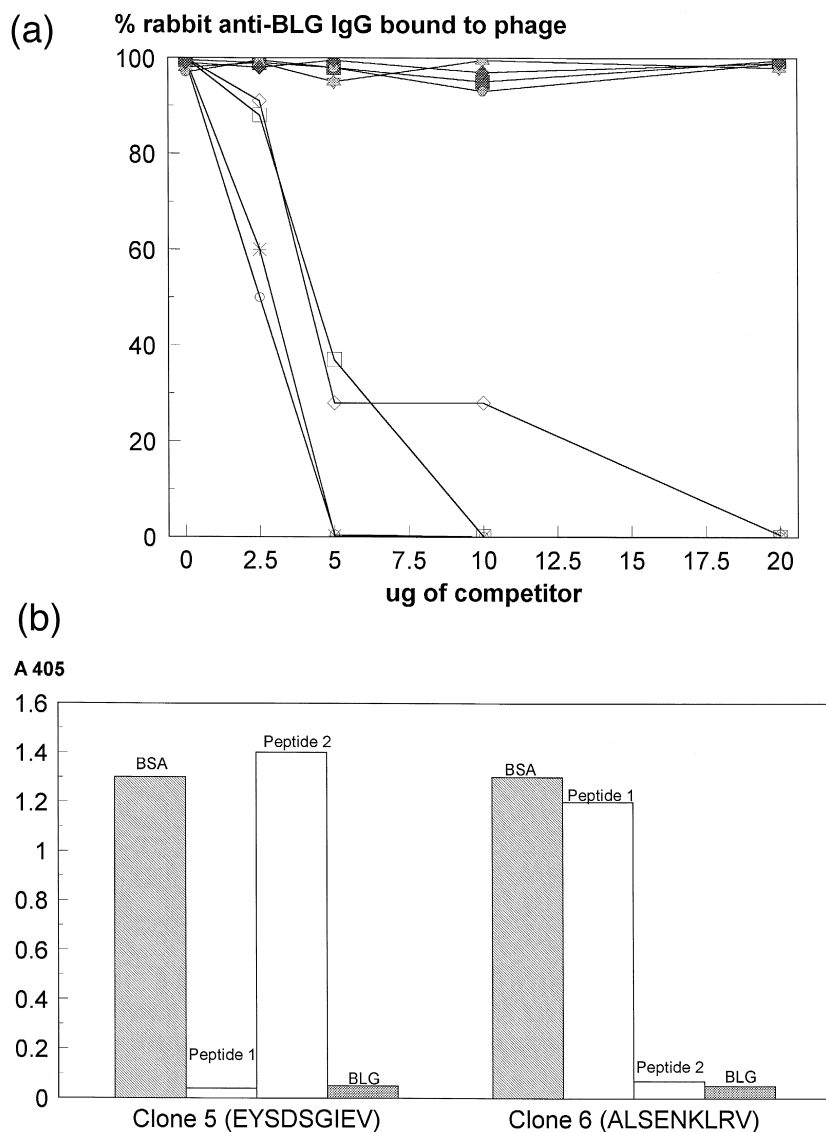


Fig. 3. Effect of intact BLG (a) and synthesised peptides corresponding to the linear regions of BLG (b) on the binding of rabbit anti-BLG IgG to individual phage clones. (a) The phage ELISA was carried out as described in Section 2, except that serial two-fold dilutions of BLG (100 μ l; maximum conc. 200 μ g/ml), BSA or PBST only were added to wells containing positive phage clones captured by immobilised sheep anti-M13. Affinity purified rabbit anti-BLG antibodies were then added immediately to each well (2.5 μ l from purified stock, 130 μ g/ml in Tris-buffered saline). Detection of rabbit antibody bound to individual phage clones was then made using the standard alkaline phosphatase anti-rabbit IgG conjugate used for all other phage ELISAs. Phage clones were chosen so that one peptide would be tested from each of the peptide groups shown in Table 1. The effect of whole BLG on the binding purified anti-BLG to phage clones is shown as follows: clone 5 (EYDSGIEV) from Group 1 ($\diamond$), clone 6 (ALSENKLRV) from Group 2 ($\square$), clone 15 (FTPEGDHRP) from Group 3 ($\circ$) and clone 17 (SDIDLLSQV) from Group 4 (*). The same experiments carried out in the presence of an irrelevant competitor (BSA) are shown by filled-in symbols. (b) Inhibition experiments were carried out as for (a) except that antibody binding to individual phage clones (clone 5 [EYDSGIEV] and clone 6 [ALSENKLRV]) was tested in the presence of 250 μ g/ml (100 μ l, in PBS) of Peptide 1 (QCLVRTPEVDDEALEK), Peptide 2 (CFKIDALNENKV), BLG or BSA.

response, a re-scan for rabbit 2 (Fig. 1) was carried out with an anti-rabbit immunoglobulin alkaline phosphatase conjugate (Zymed: 04-1622) specific for rabbit IgG, which has been shown not to recognise rabbit IgM and IgA classes. The scan (not shown) exhibited the same pattern of peaks as that obtained with the anti-rabbit immunoglobulin horseradish peroxidase conjugate (DAKO) used for all the other scans carried out.

PEPSCAN analysis was also performed on the affinity-purified anti-BLG antibodies (from rabbit 2) to check that the mixture of epitopes recognised by the purified antibodies was the same as for antibodies present in the whole antiserum. The same pattern of peaks was apparent (not shown) except for the absence of a minor peak region at PIN numbers 101–109, thus indicating that the purification procedure did not lead to the loss of any major specific antibody population within the polyclonal antiserum.

3.3. PEPSCAN assays of mouse anti-BLG antisera

Mouse anti-BLG PEPSCAN results are shown in Fig. 2. Overall, the scans are simpler than those obtained for the rabbit anti-BLG antisera, i.e., there are fewer peaks and the peaks are narrower. The peaks do, however, overlap with peptide regions identified in the rabbit scans. The scans for the two mouse antisera were not as similar to each other as those observed for the rabbits.

3.4. Screening of pVIII9aa-cys phage display library

Background levels of approximately 10^5 infective phage were recovered from the uncoated polystyrene panning tube as assessed by titrating infective phage at each round of panning. These background results were consistent with previous findings (Folgori et al., 1994). After three rounds of panning with rabbit anti-BLG coated panning tubes approx 500 times more phage than the background level were eluted. This indicated an enrichment of phage clones displaying peptides recognized by rabbit anti-BLG antibodies. Individual phage clones (~200 were screened) from the third round of panning were assessed for their ability to bind purified anti-BLG antibodies by phage ELISA as described in Section

2. Positive phage clones (approx 10% of those screened) were considered to be those that gave ELISA signals, which were four to five times higher than background, i.e., in wells with no phage present or in wells with no added antibody conjugate. The individual phage clones, which gave higher signals, were assessed in experiments that tested the in-

Table 1

Alignment of BLG sequence with deduced nonapeptide sequences of the phage peptides isolated by two separate screens of the phage library

Group 1									
BLG 123	V R T	P E V D D E	A L E K	135	No. sequenced				
1		P W W D D P	Q D I		1(1)				
2	R W W	Q E T D D S			6(1)				
3		E A D D S S	P G R		1(1)				
4	N I Y A	E L D D S			2(1)				
5		E Y S D S G I E V			6(1+2)				
Group 2									
BLG 83	K I D	A L N E N K V L	V 94						
6		A L S E N K L R V			2(1+2)				
7		A Q S E N A R P V			1(1)				
8		H S S E N R S S			3(1+2)				
9	F P S	A F S E N Q			1(1)				
10		L P F S E N R T			2(1)				
11		P T S E N R A H S			2(1+2)				
12	L P S	P L L E N R			5(1+2)				
13		S L Y E N R R V			1(2)				
14	I K N P S	S E N A			1(2)				
Group 3									
BLG 47	K P	T P E G D L E I L L	58						
15		F T P E G D H R P			1(2)				
16	Q F F	P E G D Y L			1(2)				
Group 4									
BLG 26	A S D I S L L D A Q		35						
17	S D I D L L S Q V				1(2)				
18	R Q D L S T L D				1(2)				
19	R A S V D R D L A				1(2)				

Each screen consisted of three rounds of panning with affinity-purified rabbit anti-BLG antibodies. The numbers either side of the BLG sequence denote amino acid sequence numbers. Letters in bold denote exact matches of phage sequences with the BLG sequence and italicised bold letters denote conservative substitutions. Each peptide is flanked by two cysteines which have not been shown in this table. The numbers on the right indicate the number of times the corresponding peptide was isolated and the numbers in brackets refer to the screen at which the peptide was isolated, i.e., the first screen, the second screen or both.

inhibitive effect of intact BLG on the binding of purified anti-BLG antibodies to individual phage clones as shown in Fig. 3a. In the presence of increasing concentrations of intact BLG, dose-dependent inhibition of antibody binding to individual phage clones was observed. An irrelevant competitor (BSA) had very little effect on binding. Phagemid DNA was prepared from positive clones, and DNA sequences in the region encoding the random non-peptide were assessed for homology to the primary sequence of BLG (Table 1). As seen in Table 1, two separate screens of the library resulted in 19 different peptides, which could be divided into groups that had homology with four different regions of BLG. Some peptides were isolated more than once in one screen, e.g., RWWQETDDs which was sequenced six times in the first screen and falls into Group 1 in Table 1. This group of peptides is homologous to residues 126–134 in BLG. Group 2 was made up of peptides, which were found to mimic residues 86–94 in BLG. In this group, Clone 6 showed a striking resemblance to BLG, as its peptide sequence (ALSENKLRV) contained 6 exact matches to the BLG sequence and 1 residue, which was a conservative replacement. Groups 1 and 2 contained phage pep-

tides that were isolated only in the first screen or second screen, as well as some, which were isolated after both screens. The phage peptides that formed Groups 3 and 4 had sequence homology to regions 49–53 and 26–35 in BLG, respectively, and were all isolated in the second screen of the library only. Fig. 3b shows the effect of two synthesised peptides, $^{120}\text{QCLVRTPEVDDEALEK}^{135}$ (Peptide 1) and $\text{C}^{81}\text{FKIDALNENKV}^{91}$ (Peptide 2) on the binding of purified antibodies to two phage clones, clone 5 (EYSDSGIEV) and clone 6 (ALSENKLRV). The presence of Peptide 1 in the phage ELISA resulted in blocking of antibody binding to clone 5, but had no effect on the binding of antibody to clone 6. Peptide 2 was able to inhibit the binding of antibodies to clone 6, but not to clone 5.

3.5. Representation of discovered epitopes on the 3-D structure of BLG

The results from the epitope mapping studies are represented on the monomer form of the Lattice X crystal structure of BLG (Brownlow et al., 1997) as shown in Figs. 4–6. Fig. 4 is a comparison of peptides recognized by PEPSCAN. The BLG struc-

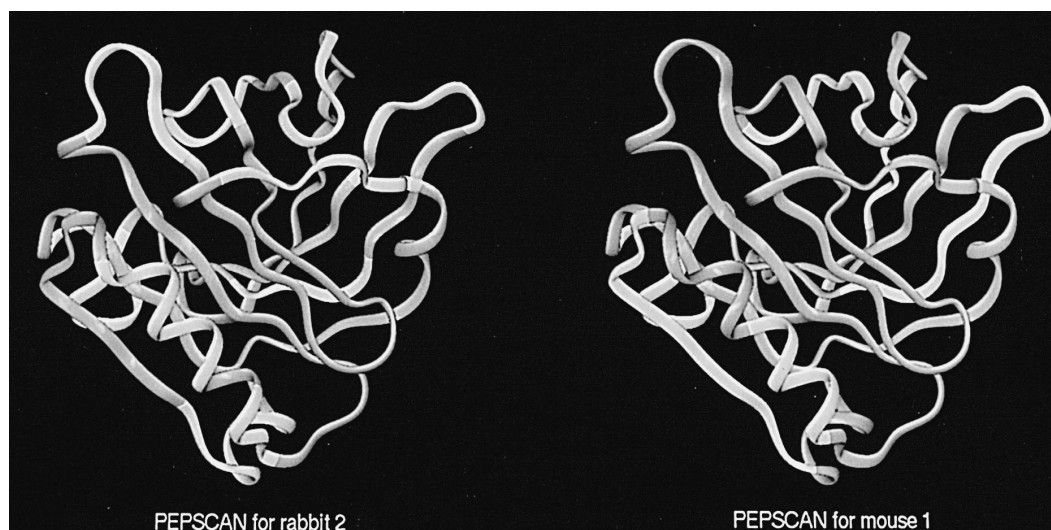


Fig. 4. Representation of epitopes discovered by PEPSCAN for rabbit 2 and mouse 1 on the structure of BLG. Epitope regions are shown in light grey.

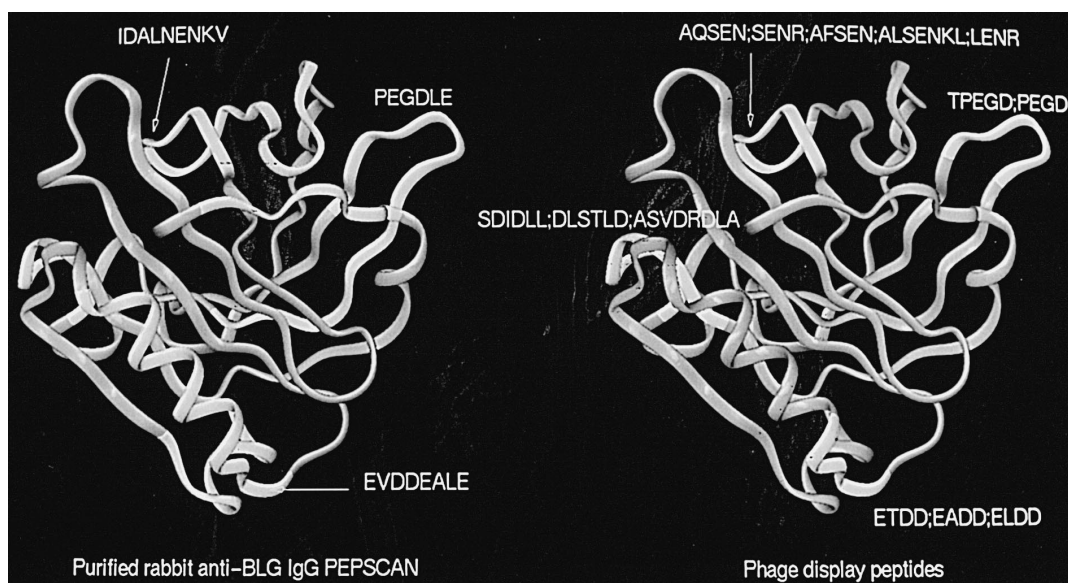


Fig. 5. Representation of epitopes discovered by PEPSCAN analysis of affinity purified anti-BLG antibodies from rabbit 2 (left hand side) compared to regions of the BLG molecule mimicked by the phage peptides isolated from the pVIII9aa-cys nonapeptide library (right hand side). Epitope regions are identified in light grey.

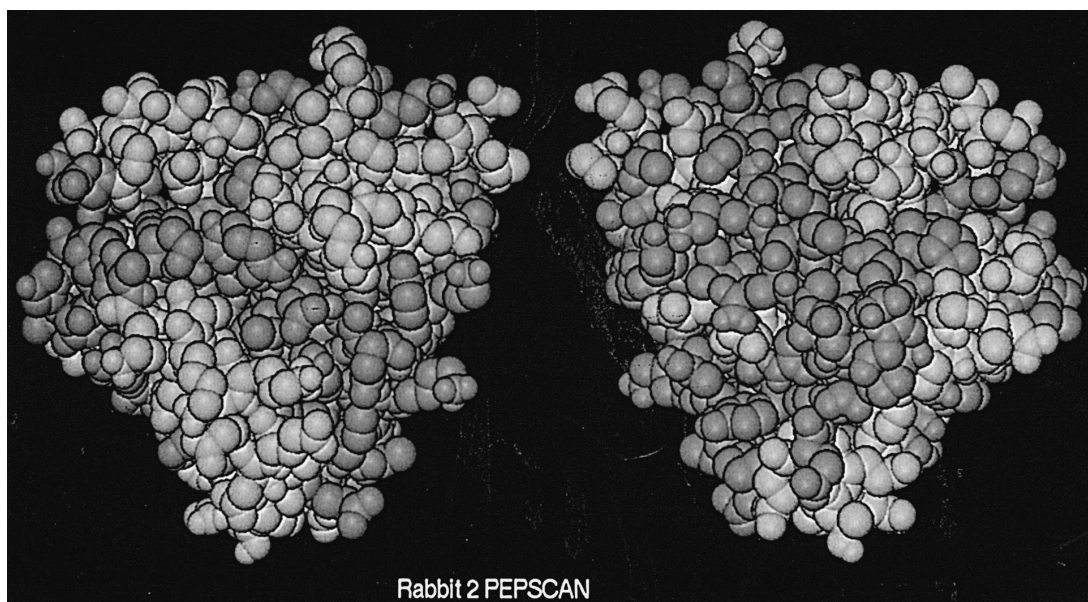


Fig. 6. Surface representation of the BLG molecule with epitopes discovered by PEPSCAN of anti-BLG antisera from rabbit 2 shown in light grey. The molecule is shown in two different orientations so that the molecule on the right is twisted 180° around the y-axis as compared to the molecule on the left.

ture on the left hand side shows linear epitopes recognized by rabbit 2 (see Fig. 1) i.e., the antiserum used for affinity purification of antibodies, and therefore screening of the phage display nonapeptide library. The molecule on the right shows the epitopes recognized by mouse 1 (see Fig. 2). Epitope residues for PEPSCAN analysis were chosen as the amino acid sequences common to all the peptides in a given peak. This was relatively straightforward for rabbit 2 and mouse 1 as the peaks were quite narrow (Figs. 1 and 2) so that common residues in a peak were usually > 5 in number. It is remembered, however, that the regions shown may only represent the core of an epitope region, as different antibodies may recognise epitopes that overlap with the region shown, as well as sequences that extend on either side. Fig. 5 is a comparison of the epitopes discovered by PEPSCAN assay of the affinity-purified anti-BLG, and the regions identified by the phage display peptide library. Fig. 6 shows the surface of BLG with the epitopes identified by PEPSCAN of rabbit 2 shown in lighter grey. It is obvious that the epitopes form clusters on the surface of the native molecule.

3.6. Comparison of epitopes discovered by PEPSCAN and by phage display

In two separate screens of the phage library random peptides were isolated, which had a sequence similarity with four regions within BLG. Two screens of the library provided a larger set of different peptide epitopes than for one screen alone. The PEPSCAN analysis for the same antibody preparation exhibited nine positive regions. There may be several explanations for the fact that the technique of phage display resulted in the isolation of a subset of the epitopes that are recognized by PEPSCAN. It is possible that screening of larger numbers of phage clones would result in the isolation of additional phage peptides with sequence homology to the extra BLG epitope regions found by PEPSCAN, but not represented so far by the phage peptides.

4. Discussion

This study has involved the discovery of epitopes within bovine beta lactoglobulin, which are recog-

nized by IgG antibodies in the rabbit and the mouse. Superimposing epitope regions on the crystal structure of BLG has shown that the majority of epitopes recognised are present on loops and turns in the structure on the exposed surface of the protein. This is as expected, as B cell epitopes are classically defined as being present on the native surface of the antigen (Roitt, 1991). A similar pattern of epitopes within BLG was recognised by each of six different rabbits tested by PEPSCAN (Fig. 1). The two mouse antisera PEPSCANs tested were less similar to one another, but the epitopes recognised were all in regions where rabbit epitopes occurred with the exception of residues 69–76, which were only recognised in the mouse.

Use of the phage display peptide library was also very successful and indicated that four dominant epitope regions were present in BLG, and that these regions formed a subset of the linear epitopes recognised by PEPSCAN. We have also shown that two synthesised linear peptides corresponding to regions of BLG mimicked by individual phage clones were able to inhibit completely the binding of anti-BLG antibodies to these clones (Fig. 3b). Work involving screening of polyclonal antisera with phage display peptide libraries has often involved attempts to screen antibodies from the whole sera of patients where detailed information about the antigen is unknown (Dybwad et al., 1993; Folgori et al., 1994). Only limited work has been carried out to assess the number of different epitope groups that are identified by screening of purified polyclonal antibody preparations (Kay et al., 1993; Roberts et al., 1993). Yao et al. (1995) identified phage peptides mimicking only one region of the antigen used for immunisation. The work presented here, therefore, gives valuable information on the use of phage displayed peptide libraries in screening polyclonal antibodies, where the linear epitopes recognised have also already been characterised by an alternative technique, i.e., PEPSCAN. The library itself may have significant advantages over other libraries, as a proportion of the peptides are displayed in constrained form, so that the conformations which the peptides can adopt are limited (Felici et al., 1991; McLafferty et al., 1993).

It must be remembered that the techniques we have used are suited primarily for the study of linear epitopes. It has been estimated that for any three

mouse monoclonal antibodies, only one will recognise a linear sequence (Meloan et al., 1991). It can also be presumed, therefore, that our polyclonal animal antisera contain a proportion of antibodies directed against epitopes, which are present only in native BLG, i.e., conformational epitopes on which we have no epitope information.

Several studies have investigated antigenic regions within BLG recognised by sera from immunised rabbits and mice (Otani et al., 1983, 1985; Otani and Hosono, 1987; Kaminogawa et al., 1987; Takahashi et al., 1990; Huang et al., 1985). Takahashi et al., 1990 digested BLG with trypsin and stated that the B cell epitopes recognised by a mouse antiserum were contained within four of the tryptic fragments. The four fragments were made up of amino acid residues 21–40, 41–60, 102–124 and 149–162. The first three of these regions were displayed on a schematic representation of the BLG structure adapted from Papiz et al., 1986, and were stated to contain random coil between β -sheets and therefore likely to occur on the surface of BLG. If our PEPSCAN results for the mouse are compared to these findings all of the above regions contain epitopes in at least one of our mice, thus agreeing with these earlier results.

As we are interested in Type I hypersensitivity to bovine milk, it will be interesting to discover whether epitopes recognized by specific IgE antibodies in the sera of patients recognize the same epitopes as those recognised by anti-BLG IgG from immunised rabbits and mice as described in this study. A few studies on milk allergy have involved antibodies of the IgG and IgE classes in the same report (Huang et al., 1985; Adams et al., 1991). Adams et al., 1991 used computer predictions of IgG epitopes (based on hydrophilicity of amino acids in the BLG sequence) to choose potential epitopes for study with IgE antibodies from allergic sera. A peptide that comprised residues 124–134 was shown to bind pooled allergic sera. This sequence RTPEVDDEALE was an important epitope in our study, as it was isolated from both PEPSCAN and phage display. Ball et al., 1994 studied binding of IgE antibodies from allergic patients to overlapping hexamers across the sequence of BLG. Background values in the peptide ELISA appeared high, and a complicated system of subtracting background values was applied. A dominant peptide (re-

sidues 97–108) was recognised in 14 of the 16 peptide sera tested. Interestingly, this peptide is in a region not recognised by any of the rabbit or mouse antisera tested in our study. There have been numerous studies on effects of hydrolysis on the allergenicity of milk (e.g. Haddad et al., 1979; Jost et al., 1991) and these have usually shown a reduction in allergenicity with hydrolysis. Individual fragments of milk proteins have, however, rarely been characterised.

The work presented here has given insight into two important epitope mapping techniques, and applied them to give a large amount of information on linear epitopes within a major milk allergen recognised by antisera from immunised animals. Until now, little work has been carried out, which links discovered epitopes to their position in the structure of the original antigen.

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