

Allergy to bovine β -lactoglobulin: specificity of human IgE to tryptic peptides

I. SÉLO, G. CLÉMENT, H. BERNARD, J.-M. CHATEL, C. CRÉMINON*, G. PELTRE† and J.-M. WAL

Laboratoire Associé INRA-CEA d'Immuno Allergie Alimentaire, CEA-Saclay, Gif sur Yvette, *CEA, SPI, CEA-Saclay, Gif sur Yvette and †Unité d'Immuno Allergie, Institut Pasteur, Paris, France

Summary

Background Bovine β -lactoglobulin (Blg) is a major cow's milk allergen. It is the main whey protein, without any counterpart in human milk. Blg chemical hydrolysates appeared to retain most of the immunoreactivity of the native protein. Allergenicity of Blg has already been shown to be associated with the four peptides derived from cyanogen bromide cleavage of Blg.

Objectives To map the major allergenic epitopes (e.g. regions of the molecule able to bind IgE) on Blg using specific IgE from sera of 46 milk-allergic patients as a probe.

Methods Direct and competitive inhibition enzyme immunoassays involving immobilized native protein or purified peptides derived from Blg tryptic cleavage.

Results Several peptides capable of specifically binding human IgEs were identified and were classified according to the intensity and frequency of the responses. The major epitopes appeared to be fragments (41–60), (102–124) and (149–162) recognized by 92, 97 and 89% of sera, respectively, whilst a second group which contained the fragments (1–8) and (25–40) was recognized by 58 and 72% of the population. A third group, comprising peptides (9–14), (84–91) and (92–100), was still detected by more than 40% of sera.

Conclusion Three peptides were identified as major epitopes, recognized by a large majority of human IgE antibodies. Numerous other epitopes are scattered all along the Blg sequence.

Keywords: bovine β -lactoglobulin, cow's milk allergy, ELISA, IgE binding fragments, tryptic peptides

Clinical and Experimental Allergy, Vol. 29, pp. 1055–1063. Submitted 3 September 1998; revised 18 January 1999; accepted 3 February 1999.

Introduction

Cow's milk allergy/intolerance was diagnosed in 1.9–2.8% of general populations of infants in the first two years of life in different countries of Northern Europe [1,2].

Sensitivities to the various milk proteins have been proved to be widely distributed, the major allergens being caseins [3] and β -lactoglobulin (Blg). Indeed specific anti-Blg IgE have been evidenced in 60–80% of cow's milk-allergic patients [4–6]. Blg is the major whey protein. It accounts for 10 and 50% of the protein fraction of milk and of lactoserum, respectively, and it is normally absent from

human breast milk. Blg presents a dimeric structure at physiological pH, each monomer being an 18-kDa globular protein of 162 amino acids [7] containing five cysteine residues, four of which are involved in intrachain disulphide bridges. Blg may be present as two major isoforms (e.g. variants A and B) differing at two locations: Gly⁶⁴ and Ala¹¹⁸ in β -lactoglobulin variant B are replaced by Asp and Val in variant A [8]. The tertiary structure is known. It belongs to the lipocalin family and is considered as a retinol-binding protein [9]. The spatial conformation has recently been fully elucidated [10].

Although hydrolysates of whey proteins are currently used in hypoallergenic formulas, very few studies on epitope analysis have been reported with human sera from

Correspondence: J. M. Wal, INRA-CEA, SPI Bat 136, CEA-Saclay, 91191 Gif sur Yvette Cedex, France.

allergic patients. These studies only determined the specificity of either one serum against a few fragments [11,12] or of a limited number of sera against a single characterized peptide [13,14]. The IgE binding capacity of peptides derived from cyanogen bromide cleavage of Blg was recently reported. Most sera recognized several peptides and the human IgE response appeared to be very variable and heterogenous [15]. Haddad *et al.* have detected serum IgE from allergic patients using RAST tests with a total tryptic hydrolysate of Blg even when no IgE response could be detected with native Blg. Interestingly, they thus demonstrated several years ago that tryptic hydrolysis of Blg may unmask hidden epitopes [16].

We have prepared and isolated purified peptides covering the entire sequence of Blg so as to specify the incidence of each region in the immunoreactivity of the whole molecule. Tryptic hydrolysis appeared to be appropriate for this purpose because this enzyme contributes to the processing of Blg during the natural digestion of food and generates relatively small fragments thus allowing precise analyses.

Materials and methods

Materials

Human serum albumin (HSA) was obtained from the Centre National de Transfusion Sanguine (Les Ulis, France). Unless otherwise stated, all chemicals were of analytical grade and purchased from Sigma (St Louis, MO, USA).

The enzyme immunoassay (EIA) equipment (LabSystem, Helsinki, Finland) consisted of an automatic microplate washer, an automatic dispenser autodrop and a multiskan spectrophotometer set at 414 nm. Microtitre plates were 96F immunoplates from Nunc (Roskilde, Denmark). The Fast Protein Liquid Chromatography (FPLC) system was from Pharmacia (Uppsala, Sweden). High-performance liquid chromatography (HPLC) was performed using a Waters Millennium System (Milford, MI, USA). Collected fractions were dried under vacuum using a Speedvac apparatus (Savant, Farmingdale, NY, USA).

Preparation and purification of Blg

Preparation of Blg from the milk of a cow homozygous for the variant B of Blg was performed as described in [6]. Complete purification was achieved using immunoaffinity chromatography on an antibovine IgG column, then anion exchange chromatography (Q-Sepharose Fast Flow, Pharmacia) to remove residual traces of immunoglobulins and α -lactalbumin, respectively. This preparation thus appeared to be homogenous using HPLC and SDS-PAGE analyses. Additional EIA experiments confirmed the absence of contamination by other milk proteins.

Preparation and purification of peptides derived from tryptic cleavage of Blg

Blg was digested by trypsin (E.C. 3.4.21.4 TPCK treated) overnight at 37 °C using an enzyme/substrate ratio of 2.5% M/M, in 0.1 mol/L TRIS buffer pH 9 (10 mg Blg/mL). The reaction was stopped by freezing at –80 °C.

The tryptic hydrolysate was first purified by anion exchange chromatography. Hydrolysates (350 mg) were applied to a Q-Sepharose fast flow column (150 mL) previously equilibrated with 20 mmol/L TRIS buffer at pH 7 (Buffer A). Elution was achieved at a 8-mL/min flow rate using a three-step procedure (30-min linear step at 0% buffer B, then a linear gradient from 0 to 18% buffer B in 90 min followed by 18–100% buffer B in 40 min). Buffer B = Buffer A + 1 mol/L NaCl. Elution of the different peptides was monitored at 280 nm. The collected fractions were concentrated on C18 Sep-pack cartridges, eluted by a mixture of acetonitrile-water (60:40; v/v). After drying under vacuum, the fractions were further purified by HPLC using a Nucleosil C18 column (7.8 × 300 mm, Shandon, France) heated at 40 °C and eluted at a 3-mL/min flow rate. Five to 10 mg of each fraction were purified using conditions previously described [17] with a three-step linear gradient (0–22.5% solvent B in 9 min, 22.5–50% solvent B in 34 min and 50–60% solvent B in 7 min). Solvent A: TFA, 0.115% in water; solvent B: acetonitrile-TFA 0.1% in water (60:40; v/v). Elution was monitored at 214, 260 and 280 nm. The collected peaks were dried under vacuum and stored at –20 °C.

To purify the (102–124) peptide, which contains a free cysteine in addition to a disulphide bridge, the Blg was first mono-alkylated as described in [18] except that the dialysis buffer was TRIS 50 mmol/L pH 7.4 to prevent Blg precipitation.

Peptide identification

Identification of each of the isolated pure peptide was achieved by analyses of amino acid composition using a Pico-Tag system (Waters), which allowed assignment in the Blg sequence.

Human sera

Sera from patients allergic to milk were obtained from banks of sera from paediatric and allergy departments of different hospitals. Forty-six sera were selected according to the clinical history of patients and to the positive IgE responses to milk proteins and particularly to Blg. Specific IgE were determined as described below. Four sera from nonallergic patients were used as the control.

Enzyme immunoassays

On each individual serum, anti-Blg and antipeptides specific IgE were quantified by the two-site immunometric assay previously described [6]. Briefly, microtitre plates were coated by passive adsorption with a 5 μ g/mL solution of Blg or tryptic peptides. For the shortest peptides, an alternative method of immobilization using biotin–streptavidin interaction was also applied [19]. Human serum albumin was used as saturating agent to avoid non-specific binding. Plates were washed just before use.

Fifty microlitres of individual serum at appropriate dilution were dispensed per well. After incubation overnight at room temperature and extensive washing, 50 μ L of a solution of an antihuman IgE mouse monoclonal antibody (MoAb) (i.e. clone BS17) labelled with acetylcholinesterase (AChE) were added per well. Following an overnight incubation, 200 μ L of Ellman's reagent used as enzyme substrate were dispensed into each well and absorbance was measured at 414 nm.

Four sera from nonallergic patients were used as negative controls. Moreover, non-specific binding was also tested in positive sera by using a synthetic 11-mer non-Blg related peptide as immobilized antigen.

Quantification of specific IgE as IU (i.e. 2.4 ng/mL) was made by comparison with dose–response curves obtained with a total IgE assay performed with known standards of human IgE (Pharmacia Prist test Kit). The corresponding immunometric assays of total and specific IgE were performed under identical conditions. An antihuman IgE MoAb (clone LE27), complementary to the BS17-AChE tracer, was

used as capture antibody for the total IgE assay, instead of the immobilized allergen used for specific IgE determination.

The detection limit of specific IgE, calculated as the dose corresponding with the average non-specific binding plus 3SD was ≈ 0.1 IU/mL. To allow valuable comparisons between the amounts of the different anti-peptide IgE, results were expressed as percentages of the signal measured with immobilized native Blg. No cross-reactivity with serum anti-Blg IgG was observed.

To confirm direct determinations, fragments were also tested by competitive inhibition EIA in a one-step procedure. A 50- μ L fraction of a mixture (v/v; 50/50) of diluted human serum and inhibitor (for peptides, at concentrations ranging from 0.03 to 20 nmol/mL and for Blg from 0.003 to 2 nmol/mL) was added to plates coated with peptides. The procedure was then identical to the direct EIA.

Results

Tryptic peptides

Twelve fractions containing several peptides were separately collected after FPLC analyses (data not shown). Further purification of each fraction was then performed by HPLC allowing separation of the different peptides. Each single peptide thus isolated was checked for its purity by analytical HPLC and was identified by analysis of its amino acid composition (data not shown). Twelve peptides covering almost the totality of the protein were thus obtained (Fig. 1). The peptide (149–162), which contains a free

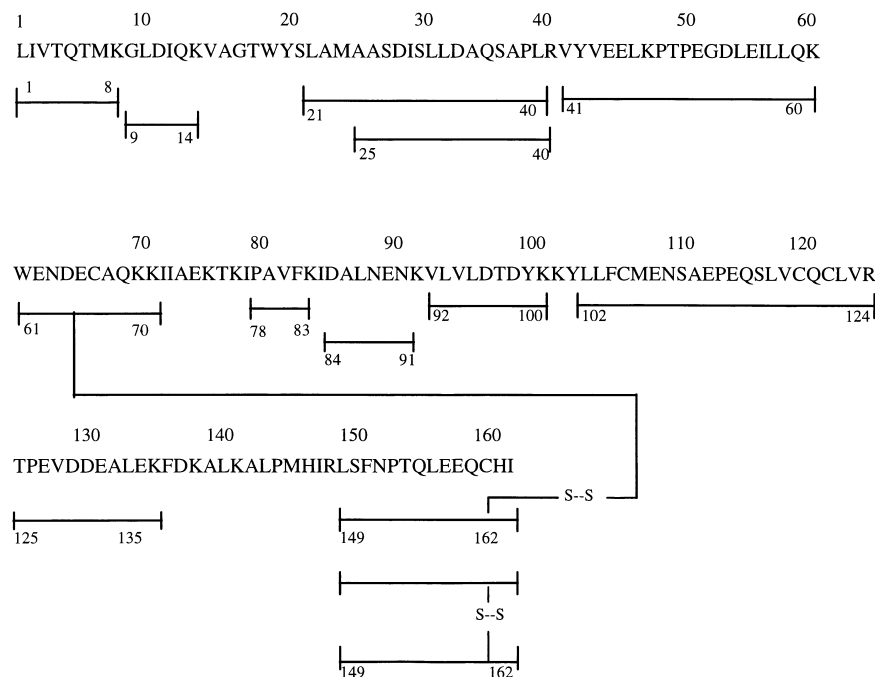


Fig. 1. Location on the Blg sequence of the different peptides derived from tryptic cleavage.

cysteine, was isolated as a homodimer. We also purified a heterodimer from the native Blg corresponding to peptide (149–162) linked by a disulphide bridge to peptide (61–70).

Determination of specific anti-Blg and antipeptide IgE in sera from allergic patients

Specific anti-Blg IgE concentrations ranged from 0.5 to 796 ng/mL. Therefore sera were classified into four groups according to their anti-Blg IgE titre (Table 1). Most of the sera (84%) contained specific IgE to several peptides (e.g. 5–10), no serum appearing monospecific. Comparisons of the IgE binding capacity of the different peptides and of their incidence on the total allergenicity of the whole native Blg are summarized in Tables 1 and 2, and in Fig. 2. For 20 sera out of 46 (45%) more than 50% of the Blg immunoreactivity was accounted for by the sum of immunoreactivities of tryptic peptides. The total recognition of all the peptides was equivalent to that of Blg for six sera and, more surprisingly, with four sera, peptides generated higher total signals than the native Blg. Three peptides: (41–60), (102–124) and (149–162) provided the highest IgE binding capacity in terms of either frequency or mean reaction intensity. These three peptides were recognized by almost all patient sera with frequencies of 94, 96 and 89%, respectively. They accounted for 10–15% of the total IgE

response to the native Blg. Peptides (1–8), (25–40) or (21–40) (both of them have been obtained but gave similar results) and (92–100) were recognized by IgE from 65, 65 and 52% of the sera, respectively, and corresponded with $\approx 4\%$ of the total immunoreactivity of the Blg. Specific IgE to peptides (9–14), (84–91) were found in 40% of the sera, and accounted for $\approx 2\text{--}3\%$ of the total immunoreactivity of the Blg. Peptides (125–135) and (78–83) were recognized by only 28% of sera and accounted for 1–2% of immunoreactivity of native Blg.

At least six fragments thus appear to be recognized by more than 50% of sera in each group of the population of allergic patients to Blg, whatever the amount of specific IgE to native Blg.

Competitive inhibition EIA was performed on plates coated with the peptides and using either native Blg or the peptide itself as competitor at a concentration of 10 nmol/mL. Five peptides were tested on a few sera with regard to the data obtained in direct EIA tests: three peptides highly recognized by human IgE, e.g. (41–60), (102–124) and (149–162), one moderately, and one poorly recognized, e.g. (25–40) and (9–14), respectively. The same observations were made whatever the coated peptide (Table 3). When native Blg was used as competitor, inhibition ranged between 28 and 100%, reaching values above 50% for 60% of the sera. When the peptide was used as competitor,

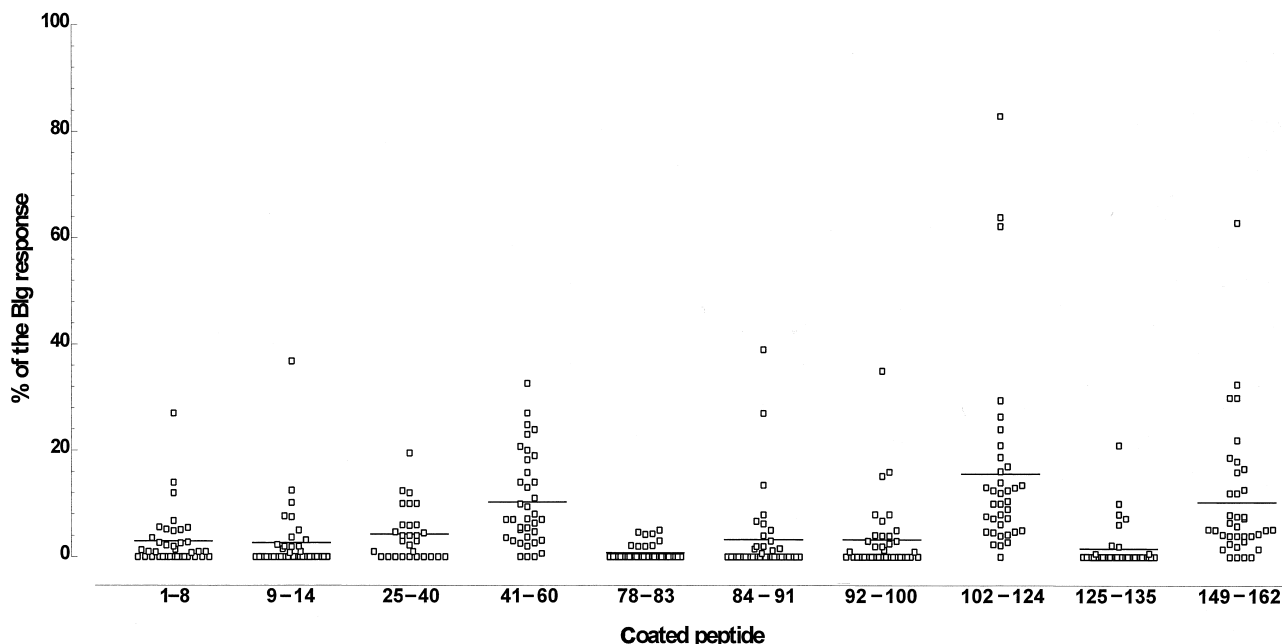


Fig. 2. IgE binding capacity of Blg tryptic peptides. Data represent the specific IgE titre of the different individual sera against each peptide. Measured by direct EIA, they are expressed as a percentage of the IgE responses to the whole native Blg. Using four control sera from nonallergic patients and a control peptide, no signal was detectable (data not shown). The horizontal bars correspond with the mean intensity of the responses.

Table 1. ELISA determination of specific IgE

Very high responders			High responders			Medium responders			Low responders		
Serum	Anti-Blg IgE (IU IgE/mL)	Σ anti-pep IgE/ anti-Blg IgE	Serum	Anti-Blg IgE (IU IgE/mL)	Σ anti-pep IgE/ anti-Blg IgE	Serum	Anti-Blg IgE (IU IgE/mL)	Σ Anti-pep IgE/ anti-Blg IgE	Serum	Anti-Blg IgE (IU IgE/mL)	Σ anti-pep IgE/ anti-Blg IgE
S 1	320	87	S 21	27	15	S 36	10	65	S 51	3	112
S 4	110	44	S 27	25	42	S 38	7	37	S 44	3	35
S 3	108	46	S 32	25	42	S 39	7	23	S 49	3	25
S 16	97	27	S 45	23	20	S 34	7	25	S 50	3	81
S 13	69	49	S 24	20	48	S 33	6	24	S 41	3	41
S 28	60	18	S 26	20	46	S 29	6	88	S 53	2.5	56
S 22	51	37	S 23	16	25	S 52	5	78	S 58	2	212
S 25	40	74	S 31	15	21	S 43	5	32	S 57	2	200
S 35	40	31	S 17	15	22	S 40	4	8	S 54	2	37
			S 30	15	46	S 59	4	16	S 48	2	26
			S 20	13	13	S 37	4	37	S 55	2	44
			S 42	13	34				S 46	2	58
									S 47	1	49
									S 56	0.5	173

The 46 sera have been classified in 4 groups, e.g. very high, high, medium and low responders according to their anti-Blg specific IgE titres. The ratio Σ anti-pep IgE/anti-Blg IgE represents the sum of the specific IgE responses against all of the tryptic peptides expressed as percentage of the IgE response against native Blg. Using four control sera and one 11-mer control peptide, unrelated to Blg, no significant signals were detectable (data not shown).

Table 2. Distributions of IgE response to Blg and to Blg derived tryptic peptides among the total population of allergic patients, and in each of the different subgroups

Blg Peptides	Blg b	1–8	9–14	25–40	41–60	78–83	84–91	92–100	102–124	125–135	149–162
A											
Mean	26.5	3.7	2.3	4.3	10.0	0.9	3.0	3.8	14.2	1.8	9.5
Median	7.0	1.0	0.0	2.0	7.0	0.0	0.0	1.0	9.0	0.0	5.5
SD	52.2	5.7	5.9	6.0	8.0	1.7	7.2	6.5	16.9	4.3	11.4
Frequency	100	65.4	39.1	65.2	93.5	28.3	39.1	52.2	95.7	28.3	89.1
B											
Mean	99.4	2.3	1.6	6.9	10.7	0.4	0.7	3.0	10.1	0.4	10.1
Median	69.0	1.0	1.0	6.0	8.0	0.0	0.0	1.0	8.0	0.0	5.0
SD	87.1	2.2	2.6	5.6	6.3	0.9	1.4	3.6	6.2	0.7	10.3
Frequency (/9)	9	7	5	8	9	2	2	5	9	3	9
C											
Mean	18.9	1.8	2.3	1.8	6.0	0.8	1.8	2.6	9.6	1.0	5.3
Median	18.0	0.0	0.0	0.5	5.5	0.0	0.0	0.0	9.0	0.0	4.5
SD	5.1	3.0	3.9	3.4	3.7	1.4	2.7	5.7	5.7	2.5	3.9
Frequency (/12)	12	2/6	5	6	12	3	5	3	11/11	2	11
D											
Mean	5.9	4.0	1.9	3.6	9.0	0.8	1.1	4.1	11.7	1.5	4.2
Median	6.0	4.5	0.0	2.0	6.0	0.0	0.0	4.0	10.0	0.0	2.0
SD	1.8	3.2	3.1	5.6	6.1	2.1	2.1	4.9	9.1	2.7	4.9
Frequency (/11)	11	3/10	5	8	11	2	4	7	11	3	8
E											
Mean	2.3	6.3	2.8	5.1	13.3	1.3	6.7	5.8	21.3	3.5	15.7
Median	2.0	3.0	0.0	3.0	12.0	0.0	1.0	2.0	10.0	0.0	13.0
SD	0.9	9.3	9.5	7.4	11.0	2.0	11.6	9.6	26.3	6.6	16.1
Frequency (/14)	14	5/8	2	8	11	6	7	8	12	5	7/8

IgE response to Blg is expressed as IU IgE per mL, while IgE response to peptides is expressed as percentage of the IgE response to the whole native Blg which permits relative comparisons. Using four control sera and one control peptide, unrelated to Blg, no significant signals were detectable (data not shown). A: Total population of milk-allergic patients (46 sera), B: Very high responders (9 sera), C: high responders (12 sera), D: medium responders (11 sera), E: low responders (14 sera).

inhibition was generally lower, with values above 50% being observed for only 30% of the sera. For most sera (62%), Blg was a better competitor than the peptide, while the reverse observation was verified for only 14% of the sera. For the remaining ones (24%), inhibiting values observed using Blg or the free peptide were rather similar. Occasionally, in two sera, the binding of specific IgE to the coated peptides (149–162) and (102–124) could not be inhibited using either the free peptide itself or Blg.

Discussion

Twelve fragments from tryptic hydrolysis of Blg were purified and identified unambiguously by their amino acid

composition. They were used to delineate more precisely the immunoreactive regions identified in a previous study using four peptides, e.g. (8–24), (25–107) (108–145) and (146–162) derived from cyanogen bromide chemical cleavage of Blg [15].

Most of the sera from 46 allergic humans recognized a large set of peptides (5–10 peptides). Unlike chemical CNBr cleavage which did not alter immunoreactivity of the native Blg, tryptic hydrolysis largely reduced the immunoreactivity (<50%) for most sera (75%). This suggests the destruction of epitopes by hydrolysis or the alteration of conformation. However, enzymatic hydrolysis did not decrease immunoreactivity for 25% of sera. Furthermore, the IgE response appeared to be enhanced

Table 3. Competitive inhibition EIA of IgE binding to immobilized peptides by Blg or by the corresponding peptide in solution

Coated peptide	Competitor (10 nmol/mL)	Serum			
		S38	S24	S23	S31
(9–14)	Blg	62	44	46	48
	(9–14)	7	38	24	48
(25–40)		S41	S16	S23	S28
	Blg	44	78	100	68
	(25–40)	52	4	16	70
		S41	S24	S36	S46
(41–60)	Blg	91	92	58	83
	(41–60)	28	77	18	70
		S13	S28	S16	
		ϵ	28	42	
(102–124)	Blg	6	14	3	
	(102–124)				
		S46	S13	S17	S28
(149–162)	Blg	59	48	74	40
	(149–162)	67	18	79	ϵ

Results are expressed as percentage inhibition, calculated as $[(1-B/B0) \times 100]$ where B is the signal measured in the presence of 10 nmol/L of inhibitor and B0 corresponds with the signal measured without inhibitor. No inhibition was measured using the control peptide (data not shown). ϵ : non-significant binding inhibition (i.e. $<3 \times$ background signal measured using the control peptide).

for 9% of the sera, in agreement with a previous report [16].

The best recognized peptides, in terms of frequency and intensity of response, were fragments (41–60), (102–124) and (149–162), each of which accounted for 10–15% of whole Blg immunoreactivity. In addition, the most specific sera which recognized two or three peptides contained antibodies directed exclusively against these peptides, which appear clearly as major Blg 'allergenic epitopes' (i.e. fragments with the highest IgE binding capacity). Not surprisingly, two of these peptides (41–60) and (102–124) are part of the most immunoreactive fragments previously determined using Blg CNBr peptides [15]. These results are also in good agreement with reports concerning fragments (97–108), (41–107), and (146–162), described as allergenic epitopes using a limited number of sera from allergic patients [11–14]. It might thus be supposed that a major allergenic epitope of Blg could be more precisely located within or around the common sequence shared by peptides (97–108) and (102–124).

The C-terminus (149–162) peptide forms a turn, it adopts a short structure of α -helix and appears to be quite mobile according to the crystal structure [10]. Peptides (41–60) and

(102–124) form loops stabilized by either hydrogen or disulphide bonds, they are present on the surface of the molecule [10], and hence are accessible to antibodies (Fig. 3a). Kinetic studies of the *in vitro* digestion of Blg by trypsin showed that peptides (41–60) and (149–162) were rapidly released [20]. If a similar time-course occurs during the *in vivo* digestion of Blg, one may consider that rapid proteolysis does not prevent the induction of specific IgE. Less recognized epitopes could be classified into two groups (Fig. 3b,c): the first one comprises peptides (1–8), (25–40) and (92–100) recognized by 52–65% of sera, the second consists of peptides (9–14) and (84–91) recognized by 40% of sera and of fragments (78–83) and (125–135) which were very poorly recognized by human IgE. However, it should be mentioned that the hydrophilicity profile of Blg suggests the antigenicity of sequence (124–134) which is on the surface of the protein and was described as very immunoreactive by Adams *et al.* using the corresponding synthetic peptide and the serum of two allergic patients [13]. Peptide (84–91), which was recognized by approximately 40% of human sera and does not appear to be on the surface of the intact Blg molecule, was described as a minor epitope by Ball *et al.* [14]. The two other peptides, i.e. (9–14) and (92–100) have not been described as molecular targets for IgE of allergic patients yet. These peptides are well protected against esterification and are not particularly exposed at the surface of the molecule [10], which could be indirect explanations for their relative minor importance in terms of immunoreactivity.

It can be noticed that reducing the length of the CNBr peptide (8–24) to the sequence (9–14) fragment led to a drastic decrease of the specific IgE binding. This suggests a predominant role of sequence (15–24) on conformation and epitope presentation. The further hydrolysis of the long (25–107) CNBr fragment into smaller peptides, e.g. fragments (25–40), (41–60), (78–83), (84–91) and (92–100) had no effect on the total IgE binding capacity in 66% of sera. This suggests that at least five different linear epitopes were present on this sequence but that a tryptic cleavage may occur within (a) particular epitope(s) accounting for the slight decrease of immunoreactivity observed in the remaining 33% of sera. The immunoreactivity of the tryptic peptides (102–124) and (125–135) issued from the CNBr (108–145) fragment was lower than that of this peptide, probably for the same reason. IgE binding capacities observed with tryptic peptide (149–162) and CNBr fragment (146–162) were similar.

By using a large number of individual sera this study allowed us to get a general view of the diversity of allergenic structures even if no correlation could be established between these data and clinical data. The identification of short linear immunoreactive sequences within the entire structure of the protein may be of great help for the

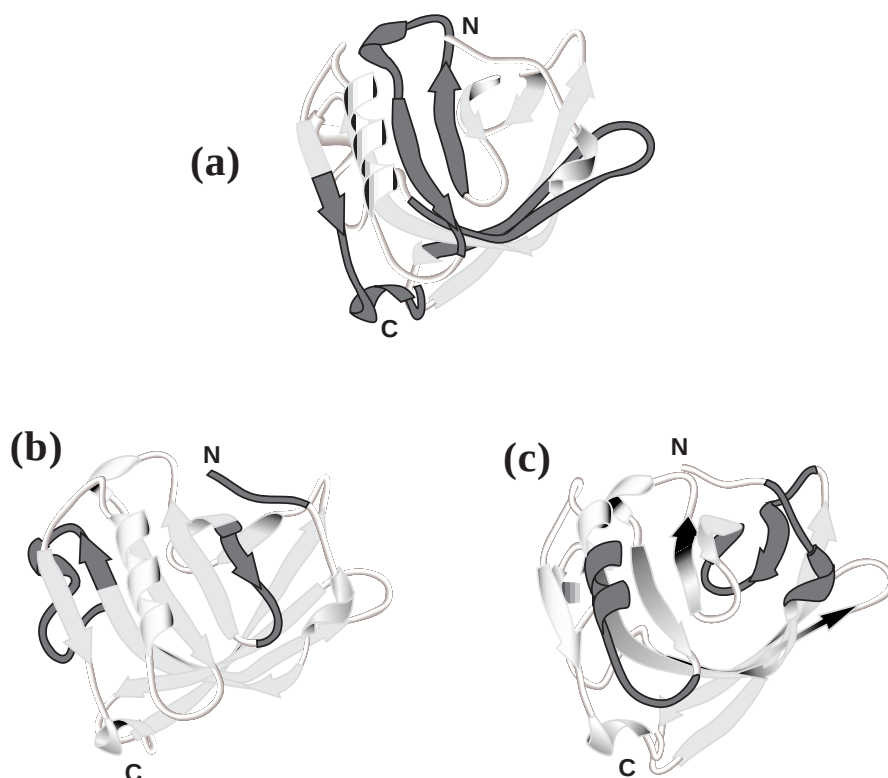


Fig. 3. Location of immunoreactive regions in three-dimensional structure of Blg (native molecule), according to [10]. (a) First group of major allergenic epitopes (e.g. tryptic fragments capable of binding serum IgE antibodies in more than 50% of a population of 46 allergic humans to Blg): f(41–60), f(102–124), and f(149–162) were recognized by anti-Blg specific IgE in 90–100% of the allergic patients population and accounted for 10–15% of the total allergenicity of whole native Blg. (b) Second group of major epitopes: f(1–8), f(25–40) and f(92–100) were recognized by anti-Blg specific IgE in 50–65% of the allergic patients population, and each accounted for 3–5% of the total allergenicity of whole native Blg. (c) Minor epitopes f(9–14), f(78–83), f(84–91) and f(125–135), were recognized with lower frequency (<40%) and intensity (<3% of the allergenicity of the whole Blg).

conception of hypoallergenic foods. However, it appears that no single particular structure can account for a major part of Blg allergenicity. Epitopes are numerous and widely spread along the Blg molecule. They may be located in hydrophobic parts of the molecule, inaccessible for IgE antibodies in the native conformation of the protein but become bio-available after digestive processes. Three-dimensional structure is an important feature in Blg allergenicity but besides the conformational epitopes, IgE binding studies also showed the presence of linear epitopes. Peptides as short as ≈ 12 –14 amino acid residues have been characterized to account for a significant part of the allergenicity of the whole molecule in some patients. This observation justifies the need of being careful before using peptides for hyposensitization or proposing any whey proteins hydrolysate in diet for highly allergic children.

Acknowledgements

This work received financial support from the European Union (Programme FAIR CT94–0970). Dr Klara Miller, co-ordinator of this Programme, is thanked for useful discussions during the course of the Programme and for the preparation of the manuscript. Professor Jean-Yves

Couraoud is also gratefully acknowledged for his constant interest and help. Finally, we thank Dr David Marsh for correcting the manuscript.

References

- 1 Jacobsson I, Lindberg TA. A prospective study of cow's milk intolerance in Swedish infants. *Acta Paediatr Scand* 1979; 68:853–9.
- 2 Host A, Halken SA. A prospective study of cow's milk allergy in Danish infants during the first 3 years of life. Clinical course in relation to clinical and immunological type of hypersensitivity reaction. *Allergy* 1990; 45:587–96.
- 3 Stöger P, Wüthrich B. Type I allergy to cow milk proteins in adults. A retrospective study of 34 adult milk- and cheese-allergic patients. *Int Arch Allergy Immunol* 1993; 102:399–407.
- 4 Gjesing B, Osterballe O, Schwartz RH, Wahn U, Lowenstein H. Allergen-specific IgE antibodies against antigenic components in cow milk and milk substitutes. *Allergy* 1986; 41:51–6.
- 5 Bahna SL, Gandhi MD. Milk hypersensitivity. I Pathogenesis and symptomatology. *Ann Allergy* 1983; 50:218–23.
- 6 Wal JM, Bernard H, Yvon M et al. Enzyme immunoassay of specific human IgE to purified cow's milk allergens. *Food Agric Immunol* 1995; 7:175–87.
- 7 Godovac-Zimmermann J, Braunitzer G. Modern aspects of the

- primary structure and function of β -lactoglobulins. *Milchwissenschaft* 1987; 42:294–7.
- 8 Ebeler SE, Philips LG, Kinsella JE. Purification of β -lactoglobulin: Isolation of genetic variants and influence of purification method on secondary structure. *Milchwissenschaft* 1990; 45:694–8.
- 9 Papiz MZ, Sawyer L, Eliopoulos EE et al. The structure of β -lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* 1986; 324:383–5.
- 10 Brownlow S, Morais Cabral JH, Cooper R et al. Bovine β -lactoglobulin at 1.8 resolution – still an enigmatic lipocalin. *Structure* 1997; 5:481–95.
- 11 Otani H, Dong XY, Hara T, Kobayashi M, Kayahara H, Hosono A. Specificities to cow milk proteins of human serum antibodies from clinical patients. *Milchwissenschaft* 1989; 44:267–70.
- 12 Tokita F. Some reading on antigenic determinants of bovine milk proteins. *Japan J Dairy Sci* 1985; 34:229–35.
- 13 Adams SL, Barnett D, Walsh BJ, Pearce RJ, Hill DJ, Howden MEH. Human IgE-binding synthetic peptides of bovine β -lactoglobulin and α -lactalbumin. In vitro cross-reactivity of the allergens. *Immunol Cell Biol* 1991; 69:191–7.
- 14 Ball G, Shelton MJ, Walsh BJ, Hill DJ, Hosking CS, Howden MEH. A major continuous epitope of bovine β -lactoglobulin recognized by human IgE binding. *Clin Exp Allergy* 1994; 24:758–64.
- 15 Selo I, Negroni L, Creminon C, Yvon M, Peltre G, Wal JM. Allergy to bovine β -lactoglobulin: specificity of human IgE using CNBr derived peptides. *Int Archs Allergy Immunol* 1998; 117:20–8.
- 16 Haddad ZH, Kalra V, Verma S. IgE antibodies to peptic and peptic-tryptic digests of betalactoglobulin: significance in food hypersensitivity. *Ann Allergy* 1979; 42:368–71.
- 17 Chobert JM, Dalgalarondo M, Dufour E, Bertrand-Harb C, Haertlé T. Influence of pH on the structural changes of β -lactoglobulin studied by tryptic hydrolysis. *Biochem Biophys Acta* 1991; 1077:31–4.
- 18 McKenzie HA, Ralston GB, Shaw DC. Location of sulphhydryl and disulfide groups in bovine β -lactoglobulins and effects of urea. *Biochemistry* 1972; 11:4539–47.
- 19 Selo I, Negroni L, Creminon C, Grassi J, Wal JM. Preferential labelling of α -amino N terminus groups in peptides by biotin: an application for coating procedures in enzyme immunoassays. *J Immunol Methods* 1996; 199:127–38.
- 20 Dalgalarondo M, Chobert JM, Dufour E, Bertrand-Harb C, Dumont JP, Haertlé T. Characterization of bovine β -lactoglobulin B tryptic peptides by reverse-phase high performance liquid chromatography. *Milchwissenschaft* 1990; 45: 212–6.