

The Sensitising Capacity of Intact β -Lactoglobulin Is Reduced by Co-Administration with Digested β -Lactoglobulin

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Key Words

Milk allergy • β -Lactoglobulin • Digestion • Immunoglobulin E • Sensitisation • Tolerance • Animal model

Abstract

Background: It is generally believed that protein hydrolysis in the gastrointestinal tract decreases the allergenicity of food allergens. However, it remains unknown if specific properties of digestion products determine whether a sensitisation or tolerogenic immune response will develop. We sought to examine the sensitising capacity of the cow's milk allergen β -lactoglobulin (BLG) and digestion products thereof in a Brown Norway (BN) rat model. **Methods:** Intact BLG was digested in an in vitro model simulating the gastro-duodenal digestion process and subsequently fractionated by gel permeation chromatography. BN rats were dosed with either PBS, 200 μ g of intact BLG, 30 μ g of intact BLG, 200 μ g of partially digested BLG, 200 μ g of digested BLG, or with 200 μ g of a fraction of large complexes or a fraction of small complexes. Sera from BN rats were analysed for specific antibodies and avidity was measured. **Results:** BLG partly resisted the digestion process. However, the BLG molecules that did not survive the digestion process were rapidly broken down

to peptides of sizes less than M_r 4,500. Specific antibody responses revealed that both 200 and 30 μ g of intact BLG had immunogenic as well as sensitising capacity, while digested BLG could not induce any specific antibodies. Most importantly, while intact BLG showed a significant sensitising capacity when administered alone, this sensitising capacity was significantly reduced when co-administered with digested BLG. **Conclusions:** Co-immunisation of intact BLG with digested BLG reduces the sensitising capacity of intact BLG, which could result from tolerogenic mechanisms induced by the digestion products.

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Introduction

Cow's milk allergy (CMA) is a general health problem, mostly affecting young children. The prevalence of IgE-mediated CMA is estimated to be around 2.5% for infants [1], whereupon 80–85% of these children outgrow their

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allergy [2], resulting in less than 1% of adults suffering from CMA [3]. Cow's milk contains several proteins able to induce allergic responses. The major cow's milk allergen β -lactoglobulin (BLG), also designated Bos d 5, is the most abundant protein in the milk whey fraction. It belongs to the lipocalin allergen family and contains two disulphide bonds and one free cysteine group [3, 4]. BLG consists of 162 amino acid residues, corresponding to an M_r of 18,300. Two isoforms of BLG exist and in physiological surroundings these appear in dimer forms, which stabilises the structure of this globular protein [3, 4]. Numerous studies have been performed with the aim of identifying BLG-specific epitopes. They indicate that many conformational as well as many linear epitopes exist, covering most of the molecule [5–7].

It is generally believed that dietary proteins must survive the gastrointestinal digestion process as intact proteins or as large peptide fragments to be absorbed and recognised by the intestinal mucosal immune system [8, 9]. This belief may partly be based on a study by Astwood et al. [9] showing that food allergens were more resistant to simulated gastric fluid compared to proteins of no proven allergenicity. Later studies have shown that resistance to digestion is not an absolute characteristic of food allergens [10–12]. BLG is found to be a very stable protein, resisting both simulated gastric as well as gastro-duodenal digestion processes. The resistance of BLG to digestion coupled with its absence from human breast milk has been proposed as a contributing factor to the major allergenicity of BLG [3, 4].

It is generally accepted that degradation of BLG reduces its allergenicity, but a few studies have shown digestion products of BLG to inhere similar IgE reactivity as the intact protein [13] or even greater IgE reactivity than the intact BLG [14]. Such results indicate that the native BLG structure is not a necessity for the allergenicity of BLG and that linear IgE epitopes could be of importance for the reactivity of BLG, and further could suggest that humans may be sensitised to the denatured and digested BLG in addition to the intact protein.

Fortunately, in most people the intestinal immune system recognises the milk proteins as harmless and develops oral tolerance. This immunological active mechanism of unresponsiveness is believed to be achieved through deletion or anergy of the allergen-specific T cells or by the generation of regulatory T cells [15]. The mechanisms of oral tolerance induction versus sensitisation to dietary proteins remain generally unknown, but CMA is believed to form as a result of either failure or breakdown of tolerogenic processes [3].

In the current study we sought to examine the sensitising capacity of intact BLG as well as different well-characterised digestion products thereof in a Brown Norway (BN) rat model of food allergy.

Materials and Methods

Simulated Gastro-Duodenal Digestion of BLG

A pilot batch of purified BLG was kindly delivered by Arla Food Ingredients (Videbæk, Denmark). The endotoxin content was tested by Lonza endotoxin testing service (Verviers, Belgium) to be <0.01 EU per mg of BLG.

Digestion was performed essentially as described by Bøgh et al. [11], with immobilised enzymes in order to produce enzyme-free digests suitable for animal sensitisation studies. Addition of surfactant (phosphatidylcholine and bile salts) were omitted in order to avoid adverse effects on the sensitisation.

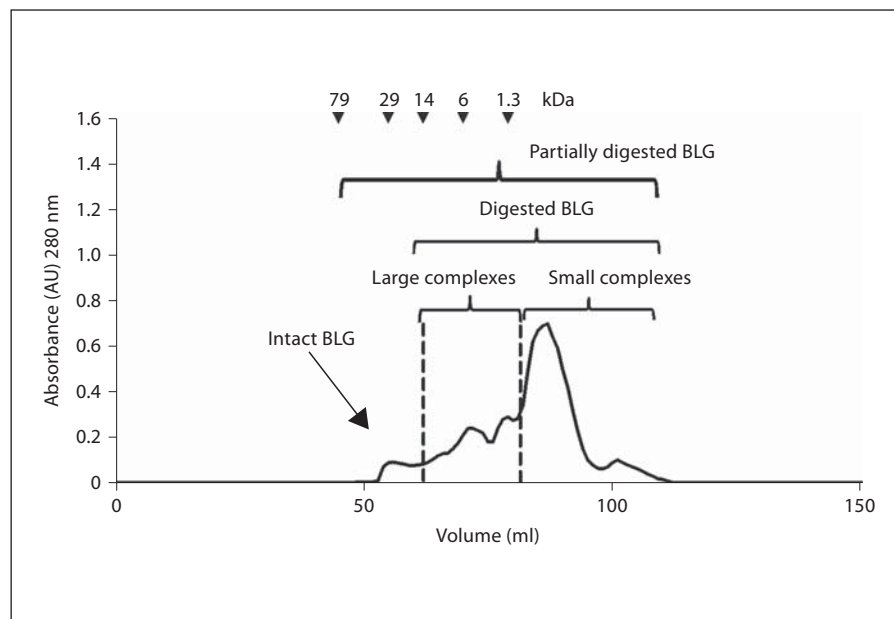
Briefly, for simulated gastric digestion, pepsin immobilised to agarose (P0609, Sigma, St. Louis, Mo., USA) was washed twice in 1 mM HCl (1,000 g, 2 min). Purified BLG (20 mg/ml) in Milli Q water (water drawn from a Milli Q System equipped with an Organex cartridge from Millipore, Bedford, Mass., USA) was adjusted to pH 2.5 with 6 M HCl and was added to the immobilised pepsin to yield a pepsin activity of approximately 180 U per mg of BLG. The solution was placed in a shaking incubator (200 rpm, 37°C) for 120 min. The reaction was stopped by adjusting the pH to 7 with 6 M NaOH, and immobilised pepsin was removed by centrifugation [1,000 g, room temperature (RT), 2 min] followed by filtrating supernatant through a 22- μ m filter (Sterile Syringe Filter, CA 0.2 μ m, Frisette Aps, Knebel, Denmark).

For simulated duodenal digestion, CaCl_2 and Bis-Tris propane was added to the solution of gastric digest of BLG to yield a final concentration of 9.2 mM CaCl_2 and 24.7 mM of Bis-Tris propane. The pH was adjusted to 6.5 with 6 M HCl. Trypsin immobilised to agarose (T1763, Sigma) and chymotrypsin immobilised to agarose (C9134, Sigma) were washed twice in Milli Q water and once in 9.2 mM CaCl_2 , 24.7 mM Bis-Tris propane and added to a quarter of the gastric digest of BLG to yield an activity corresponding to 34.5 U of soluble trypsin per mg of BLG digest and 0.44 U of soluble chymotrypsin per mg of gastric BLG digest (clarified in activity assays using BAPA as trypsin substrate and SUNA as chymotrypsin substrate). The solution was placed in a shaking incubator (200 rpm, 37°C) for 15 min. The reaction was stopped by centrifugation (1,000 g, RT, 2 min) followed by filtrating supernatant through a 0.22- μ m filter (Syringe Filter). To make all gastric digest undergo simulated duodenal digestion, the duodenal digestion phase was repeated three times and BLG digest from the four runs were pooled. Reusability of enzymes was confirmed by comparing the digestion product profiles for the four runs by reverse-phase high-performance liquid chromatography (RP-HPLC; data not shown).

Fractionation of BLG Digests

Fractionation by preparative gel permeation chromatography (GPC) of gastro-duodenal digests of BLG was performed essentially as described in Bøgh et al. [16]. Separated fractions were collected and pooled according to the preparative GPC profile shown in figure 1, resulting in four different pools of BLG digests

Fig. 1. Prep grade GPC of BLG digests. To separate intact BLG from the digestion products generated after 120 min of simulated gastric digestion plus 15 min of simulated duodenal digestion and for fractionation of the digestion products, preparative GPC was performed. Based on the 280 nm absorbance GPC profile, it was decided to make four different pools of BLG digests, indicated with the curly brackets: partially digested BLG, digested BLG, large complexes and small complexes. Partially digested BLG contained all products resulting from the digestion process, including approximately 15% intact BLG, that resisted the digestion. Digested BLG consisted of all digestion products after dissociation from the intact BLG, while the large complexes and small complexes were different fractions thereof, separation based on the sizes of aggregates, together corresponding to the digested BLG.



in the following designation: partially digested BLG, digested BLG, large complexes and small complexes, where partially digested BLG corresponds to the whole pool of BLG digests, containing intact BLG that resisted the digestion process, and where digested BLG corresponds to the combined large and small complexes and thereby the whole pool of digested BLG without any intact BLG.

RP-HPLC Analysis

For analysis of purity and presence of residual intact BLG and for evaluation of chromatography profiles, RP-HPLC was performed as previously described [16].

Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry

For analysis of peptide mass distributions in pools of BLG digests matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) was performed as previously described [16].

Amino Acid Analysis

For determination of concentrations and for examination of amino acid compositions amino acid analysis was performed by ion exchange chromatography after hydrolysis in HCl overnight, as described by Barkholt and Jensen [17].

GPC Analysis

For analysis of the peptide aggregation profiles in the different pools of BLG digests analytical GPC was performed as previously described [16].

Animals

BN rats were from our in-house breeding colony at the National Food Institute (Technical University of Denmark, Den-

mark), weaned at 3 weeks of age and then housed in macrolon cages (2 per cage) with a light:dark cycle, at $22 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ relative humidity. Rats were observed twice daily and clinical signs recorded.

Rats were kept on a diet free of milk for at least three generations to avoid tolerance to BLG, which, like human breast milk, is not a constituent of rat's milk [18, 19]. The rat diet was produced in-house and based on rice flour, potato protein and fish meal as protein sources, as previously described [11], with the exception of maize flakes being substituted with rice flour. Diet and acidified water was given ad libitum.

Experimental Design

For studying the sensitising capacity of intact BLG, partially digested BLG, digested BLG and the two fractions thereof, BN rats 4–9 weeks of age were allocated into seven groups. Each group consisted of 12 rats besides the group immunised with 200 μg of intact BLG, which consisted of 20 rats. Rats were immunised intraperitoneally with either PBS (control), 200 μg of intact BLG, 30 μg of intact BLG, 200 μg of partially digested BLG (which contained 30 μg of intact BLG), 200 μg of digested BLG (without any intact BLG), 200 μg of large complexes or 200 μg of small complexes. Rats were immunised three times without any use of adjuvant, at day 0, 14 and 28, and sacrificed at day 35 by exsanguination using carbon dioxide inhalation as anaesthesia. Blood was collected and converted to sera, which was stored at -20°C until use for analyses. An intraperitoneal sensitisation procedure was chosen for avoidance of in vivo digestion of antigens as well as for the small amount of antigens needed for immunisation.

Positive control sera were produced by intraperitoneal immunisation of BN rats three times, at day 0, 14 and 28 with 200 μg of intact BLG in 2% Alhydrogel in PBS as adjuvant. Rats were sacrificed at day 35 and blood was collected.

Animal experiments were carried out at the National Food Institute (Technical University of Denmark) facilities under conditions approved by the Danish Animal Experiments Inspectorate and the in-house Animal Welfare Committee.

ELISA for Detection of Specific IgG1 and IgG2a

For detection of IgG1 and IgG2a antibodies specific for intact BLG, partially digested BLG, digested BLG as well as the two fractions of digested BLG, ELISA was performed. Plates (96 well, microtitre, Maxisorp, Nunc, Roskilde, Denmark) were coated with 100 μ l/well of 10 μ g/ml antigen solution in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) and incubated overnight at 4°C. Between each step, plates were washed five times in PBS with 0.01% (w/v) Tween 20 (PBS-T). Two-fold serial dilutions of serum (starting at 1:8, v/v) in PBS-T, 50 μ l/well, were added and incubated for 1 h at RT. For detection, 50 μ l/well of either HRP-labelled mouse- α -rat IgG1 (3060-05, Southern Biotech, Birmingham, Ala., USA) diluted 1:20,000 (v/v) in PBS-T or HRP-labelled mouse- α -rat IgG2a (03-9620, Invitrogen, Camarillo, Calif., USA) diluted 1:2,000 (v/v) in PBS-T was added to each well and incubated for 1 h at RT. The reaction was visualised by adding 100 μ l/well of 3,3',5,5'-tetramethylbenzidine (TMB)-one substrate (Kem-En-Tec, Taastrup, Denmark) for approximately 12 min and stopped with 100 μ l/well of 0.2 M H₂SO₄. Absorbance was measured at 450 nm with a reference wavelength of 630 nm using a microtitre reader (Gen5, BioTek Instruments, Winooski, Vt., USA). Antibody titres were expressed as the log₂ titre values and defined as the interpolated dilution of the given serum sample leading to the mean absorbance for the negative control serum +3 SD, correlating to absorbance values of less than OD 0.1 for both IgG1 and IgG2a.

Antibody-Capture ELISA for Detection of Specific IgE

For detection of BLG-specific IgE, antibody-capture ELISA was performed where plates (96 well, Maxisorp, Nunc) were coated with 100 μ l/well of 0.5 μ g/ml mouse- α -rat IgE (HDMAB-123 HybriDomus, Cytotech, Hellebæk, Denmark) in carbonate buffer and incubated overnight at 4°C. Between each step, plates were washed five times in PBS-T. Plates were blocked for 1 h at 37°C in 200 μ l/well of 3% (w/v) rabbit sera. Two-fold serial dilution of serum (starting at 1:8, v/v) in PBS-T, 50 μ l/well, were added and incubated for 1 h at RT. Subsequently, plates were incubated with 50 μ l/well of 0.2 μ g/ml of digoxigenin-coupled BLG (10:1) in 3% rabbit sera in PBS-T for 1 h at RT. Plates were then incubated with 100 μ l/well of HRP-labelled sheep- α -digoxigenin (Anti-Digoxigenin-POD 1 633 716 001, Roche Diagnostics GmbH, Mannheim, Germany) diluted 1:1,000 (v/v) in PBS-T for 1 h at RT. The reaction was visualised by adding 100 μ l/well of TMB-one substrate (Kem-En-Tec) for approximately 12 min and stopped with 100 μ l/well of 0.2 M H₂SO₄. Absorbance was measured at 450 nm with a reference wavelength of 630 nm using a microtitre reader (Gen5, BioTek Instruments). IgE antibody titres were expressed as the log₂ titre values and defined as the interpolated dilution of the given serum sample leading to the mean absorbance for the negative control serum +3 SD.

ELISA for Detection of Specific IgA

Detection of BLG-specific IgA was performed essentially as described for BLG-specific IgG1 and IgG2a, with the exception of using HRP-labelled goat- α -rat IgA (STAR 111P, AbD Serotec, Ox-

ford, UK) diluted 1:5,000 (v/v) for detection of the specific IgA antibodies.

Avidity Measurements

For measurement of the strength of binding between antigens and IgG1 antibodies a thiocyanate inhibition ELISA based on the method described by El-Khouly et al. [20] was conducted. Plates (96 well, Maxisorp, Nunc) were coated with 100 μ l/well of 10 μ g/ml antigen solution in carbonate buffer and incubated overnight at 4°C. Between each step plates were washed five times in PBS-T. Serum samples were diluted in PBS-T to give an OD between 0.5 and 1, and 50 μ l/well were added in six rows of quadruplicates for each serum sample. After incubation for 1 h at RT, 50 μ l/well of potassium thiocyanate (KSCN, Sigma, St. Louis, Mo., USA) diluted in 1% bovine serum albumin in PBS-T (w/v) was added to the plates in increasing concentrations (0, 0.1, 0.2, 0.5, 1 and 2 M) and incubated for 30 min at RT. For detection, 50 μ l/well of HRP-labelled mouse- α -rat IgG1 (3060-05, Southern Biotech) diluted 1:20,000 (v/v) in PBS-T was added to each well and incubated for 1 h at RT. The reaction was visualised by adding 100 μ l/well of TMB-one substrate (Kem-En-Tec) for approximately 12 min and stopped with 100 μ l/well of 0.2 M H₂SO₄. Absorbance was measured at 450 nm with a reference wavelength of 630 nm, using a microtitre reader (Gen5, BioTek Instruments). Under the given assay conditions, it was determined that KSCN did not influence the binding of antigen to the plates.

Avidity results of individual serum samples are expressed as the concentration of KSCN required for inhibition of 50% (IC₅₀) of antigen-antibody binding, defined as a 50% reduction of OD, so that the lower the concentration of KSCN needed for 50% inhibition the lower the avidity of antigen-antibody interactions. Calculations were performed as described by El-Khouly et al. [20]. For further description of the basis for this avidity measurement methodology, see El-Khouly et al. [20] and Pullen et al. [21].

Curve Calculations and Statistical Analyses

Curve calculations (XY analyses) and statistical calculations were made using GraphPad Prism version 5.00 (GraphPad Software, San Diego, Calif., USA). Antibody titres were examined for group differences using the non-parametric one-way ANOVA, Kruskal-Wallis test, followed by Dunn's multiple comparison test for comparison of three or more groups. Curves (KSCN concentration against percentage reduction in absorbance) used for calculation of IC₅₀ were tested for variances by the one-way ANOVA, Bartlett's test for equal variances, followed by Tukey's multiple comparison test. No significant differences between curves appeared wherefore avidity measures expressed as IC₅₀ were examined for group differences using a parametric one-way ANOVA followed by Tukey's multiple comparison test for comparison of three or more groups. Differences between groups of animals were regarded as significant when $p \leq 0.05$. Asterisks indicate a statistically significant difference between the given group and the control group. Asterisks over a horizontal line indicate a statistically significant difference between the two given groups. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Results

Characteristics of Intact BLG and BLG Digests

BLG was digested in an in vitro model simulating the gastric as well as duodenal digestion process and subsequently subjected to prep grade GPC for separation of the digestion products by means of fractionation. From the prep grade GPC profile it was decided to make four different pools of BLG digests according to figure 1.

From analytical RP-HPLC analyses it was evident that BLG is a relatively resistant protein, being virtually completely resistant to proteolysis with pepsin (fig. 2b), while being degraded to a greater extent by trypsin and chymotrypsin (fig. 2c). Intact BLG was eluted at a retention volume of 4.6 ml (fig. 2a), revealing that some residual intact BLG was left after termination of the gastro-duodenal digestion process (fig. 2c). By calculation of the area under the 220-nm RP-HPLC absorbance curve, it was determined that approximately 15% intact BLG survived 2 h of simulated gastric digestion plus 15 min of simulated duodenal digestion.

From RP-HPLC analyses it was evident that removal of residual intact BLG from the digestion products was successful, since no detectable peaks were evident at an elution volume of 4.6 ml corresponding to the elution volume for intact BLG in the chromatography profiles for either digested BLG or any of the fractions (fig. 2a vs. d–f). While the chromatography profiles of the digestion products of partially digested BLG and digested BLG were virtually identical (fig. 2c, d) it was apparent that chromatography profiles changed when digestion products were separated into fractions (fig. 2d–f), suggesting that peptide composition varies between the two fractions of large and small complexes and thereby differs from the peptide composition of the whole pool of digested BLG.

A comparison of the total amino acid composition of intact BLG and the different pools of BLG digests revealed the amino acid distribution to be essentially identical for intact BLG, partially digested BLG and digested BLG (fig. 3a–c), indicating that no peptide fragments generated in the digestion process were lost in the separation of residual intact BLG from digestion products and that digested BLG contains a peptide composition representative of the intact BLG, with hydrophobic amino acids responsible for approximately 45%, the polar for approximately 16% and the charged for approximately 39% of total amino acid residues. In contrast, the amino acid distribution of the two fractions of digested BLG differs from each other and thereby from that of intact BLG,

with most hydrophobic amino acids gathered in the small complexes and most charged amino acids gathered in the large complexes (fig. 3), indicating that the charged peptides are the ones aggregating to the largest complexes. The amino acid analyses confirms the result of RP-HPLC, suggesting that peptide fragments of digested BLG were not distributed equally by the fractionation process and that the peptide profiles of the large complexes and small complexes were not representative of the intact BLG.

For examination of the peptide mass distribution of the three pools of digested BLG without intact protein, MALDI-TOF MS was performed, which demonstrated that the intact BLG which did not escape the digestion process was digested to small peptide fragments with an apparent M_r of up to 4,500, of which approximately 75% had an M_r between 500 and 2,000 (fig. 4). As in the whole pool of digested BLG, the peptides in the large complexes were less than M_r 4,500, while in the small complexes the peptides were less than M_r 3,000. This indicates that while the peptides in the digested BLG and large complexes were composed of up to approximately 38 amino acid residues, the peptides in the small complexes were composed of up to approximately 25 amino acid residues.

To analyse whether the peptides did aggregate and if so to what degree, analytical GPC was performed. Intact BLG was eluted as a single peak corresponding to an M_r of around 36,000, revealing that BLG existed as a dimer (fig. 5a). A peak corresponding to the dimerised BLG is also evident in the analytical GPC profile of partially digested BLG, and from calculation of the area under the 220 nm curve it was revealed that this peak corresponded to approximately 15% of the total area (equivalent to the 15% residual intact BLG revealed from the RP-HPLC absorbance curve), together indicating that the dimerised intact BLG did not aggregate with the peptide fragments generated from the digestion process (fig. 5b). From analyses of the GPC profiles and the area under the 220-nm absorbance curves corresponding to the peptide fragments emerging from the gastro-duodenal digestion, shown in figure 5b–e, it was revealed that a substantial part of the peptides indeed did aggregate to larger complexes. The analytical GPC profiles of the peptide fragments in partially digested BLG and digested BLG appeared essentially identical and for both the partially digested BLG and the digested BLG it was indicated that approximately 43% of the peptide fragments were in aggregated complexes between apparent M_r 28,000 and 4,500 (fig. 5b, c). This indicates that the fractionation procedure when separating intact BLG from the digestion products did not influence the aggregation state of the

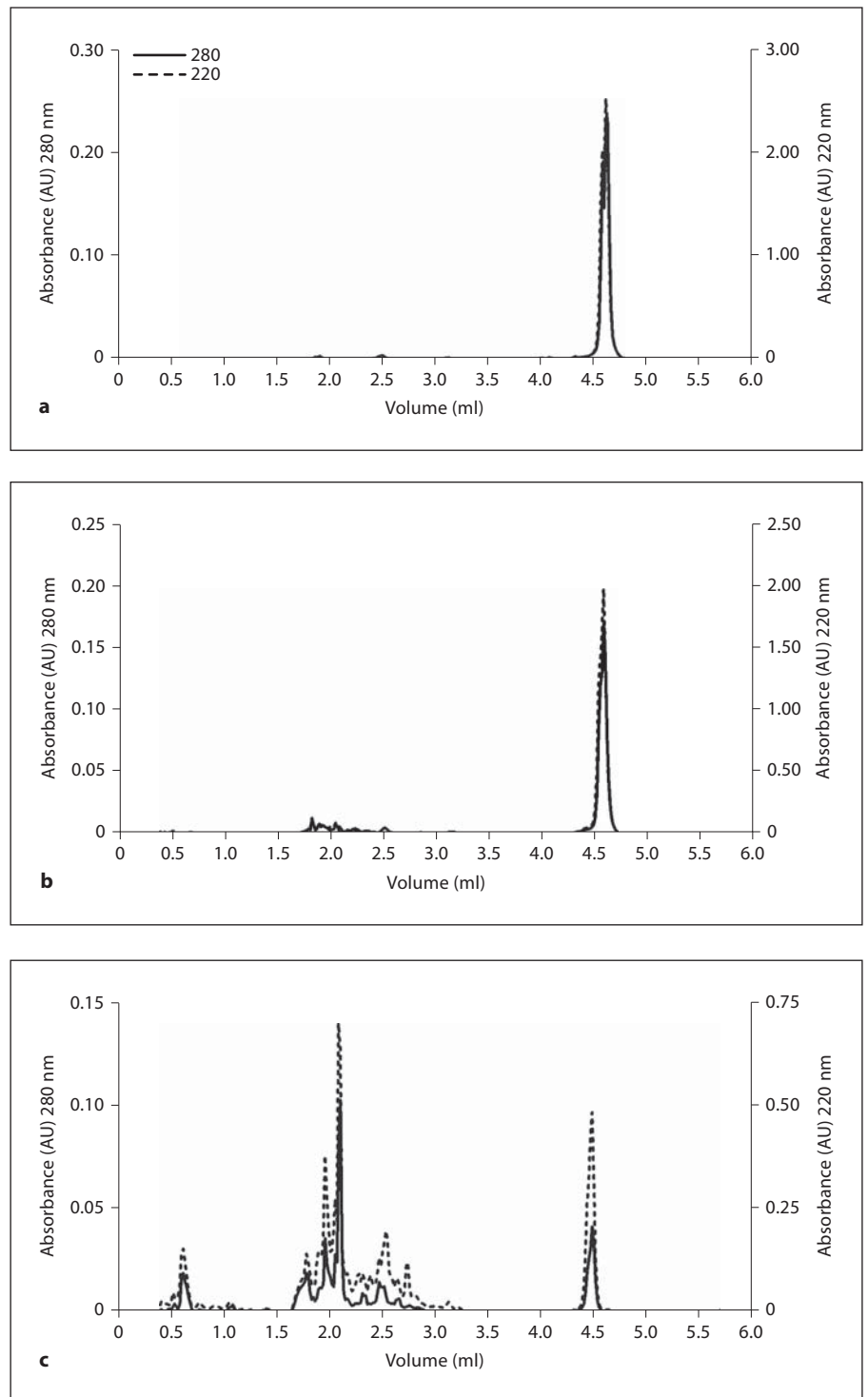


Fig. 2. Analytical RP-HPLC profiles for intact BLG and BLG digests. Comparison of chromatography profiles, shown with absorbance at 220 and 280 nm, for intact BLG (**a**), gastric digests of BLG (**b**), and partially digested BLG after gastro-duodenal digestion (**c**).

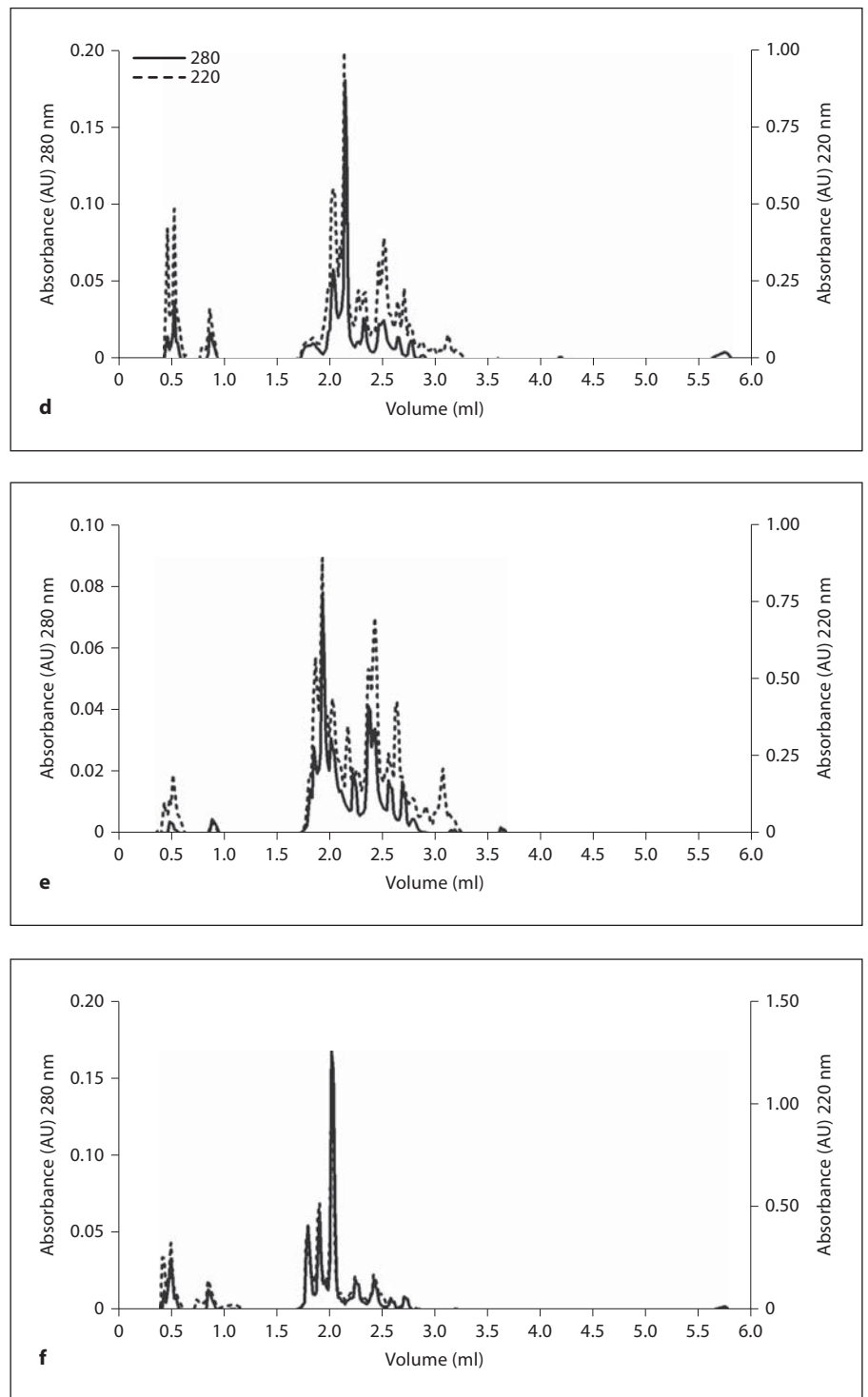


Fig. 2. Analytical RP-HPLC profiles for intact BLG and BLG digests. Comparison of chromatography profiles, shown with absorbance at 220 and 280 nm, for digested BLG (**d**), large complexes (**e**) and small complexes (**f**).

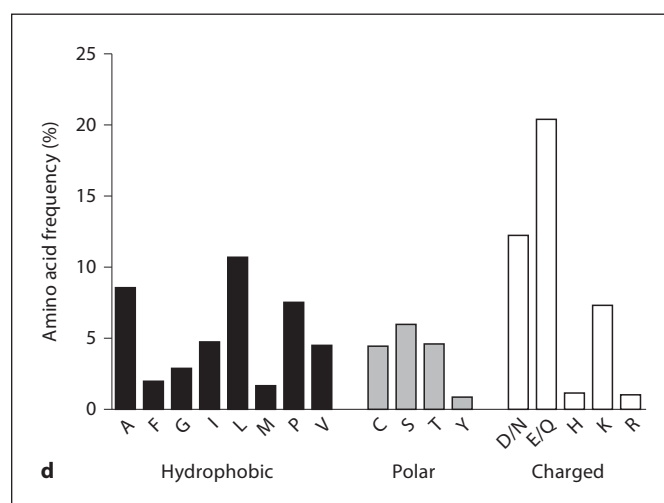
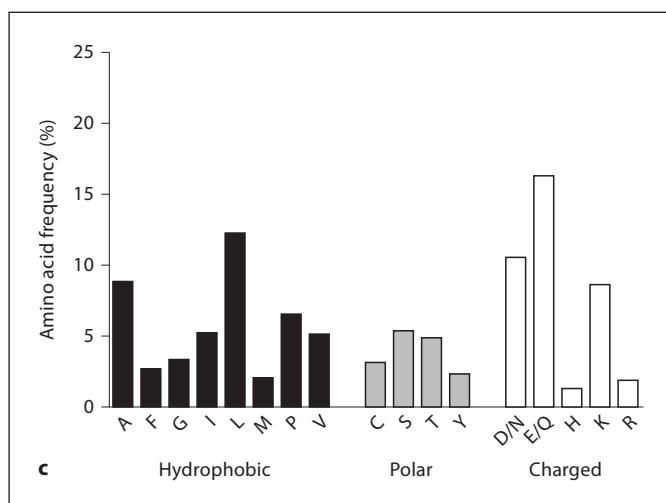
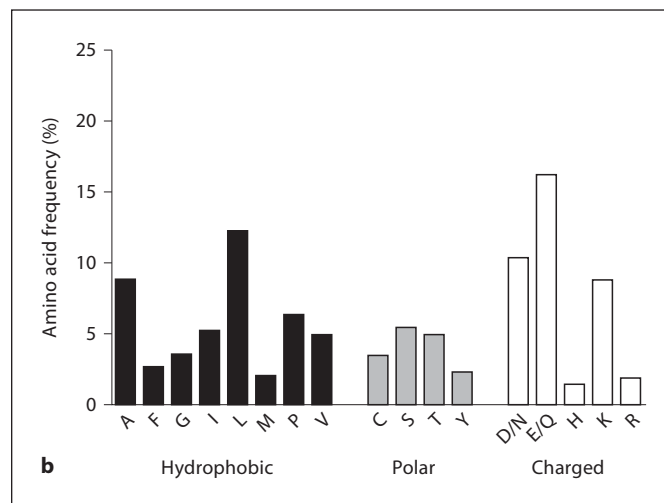
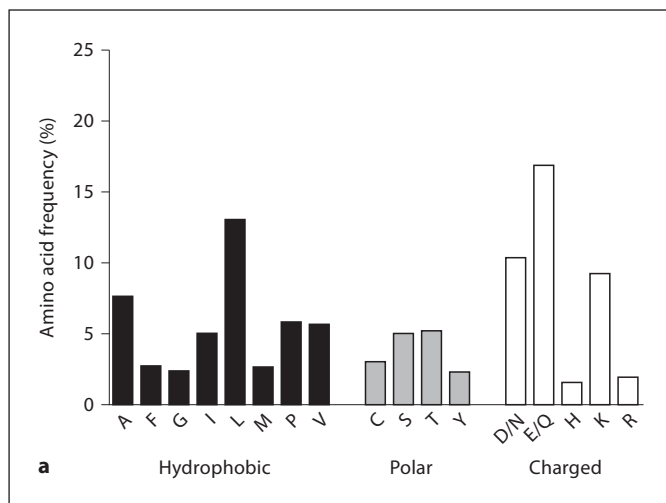
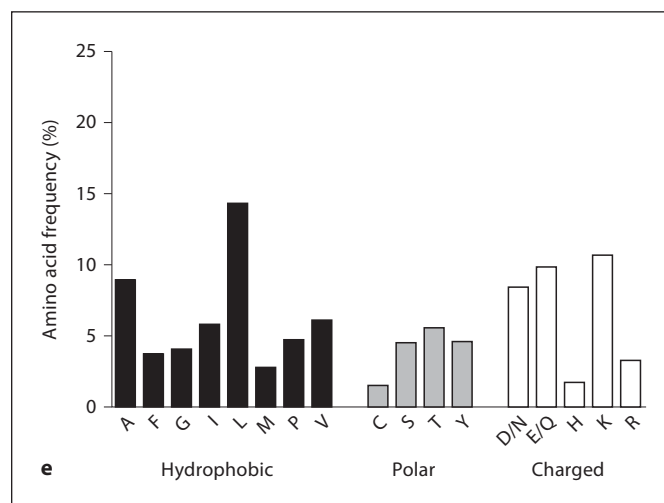
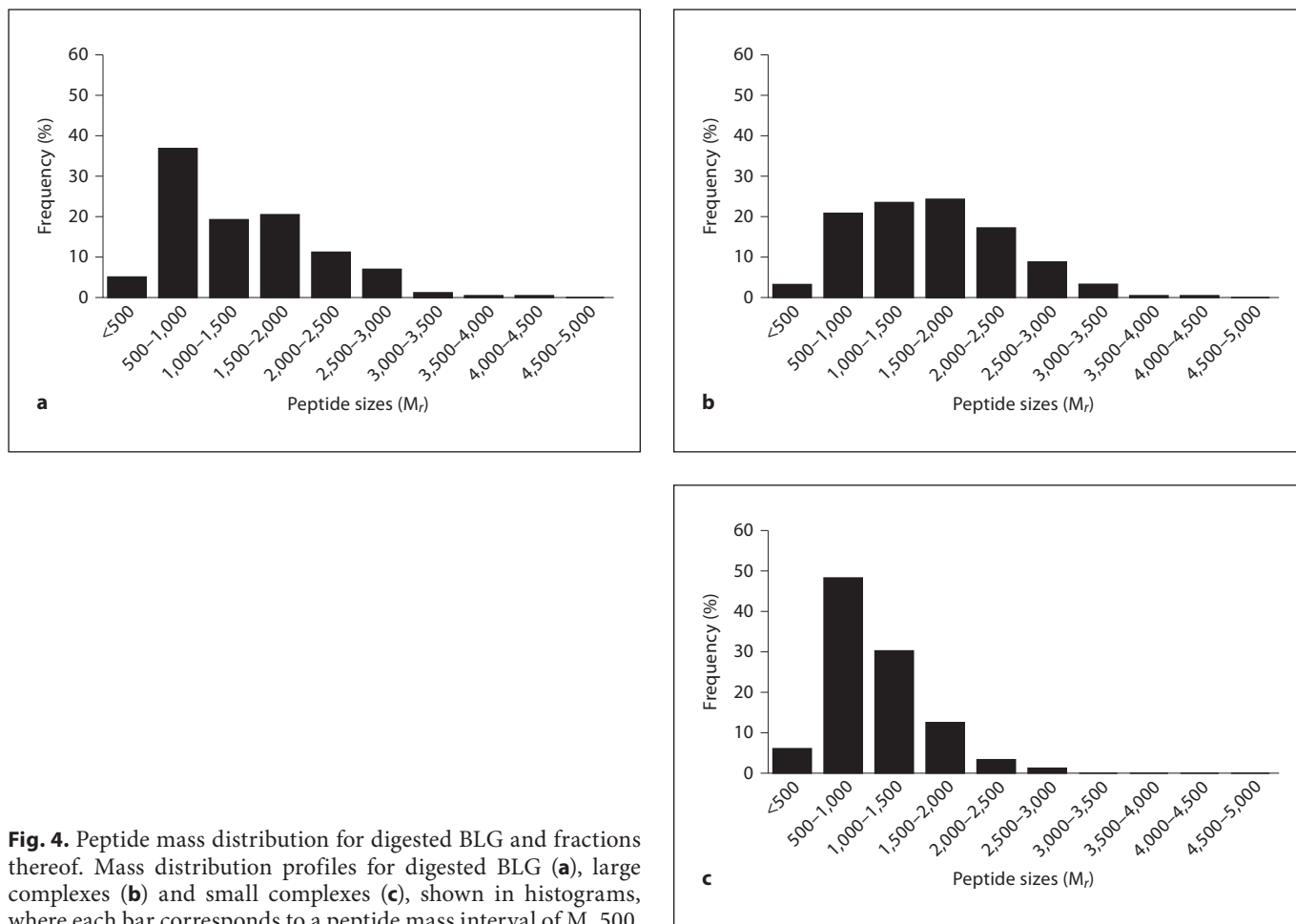


Fig. 3. Amino acid frequency distribution of intact BLG and BLG digests. Comparison of amino acid distribution profiles for intact BLG (a), partially digested BLG (b), digested BLG (c), large complexes (d) and small complexes (e). The bars represent the frequency percentage for each amino acid(s), represented by their one letter code, grouped according to their physicochemical features.





peptides. However, by separating the digested BLG into two fractions, the aggregation state was disturbed, making the complex formation of the peptides change (fig. 5c–e). While approximately 80% of the peptide fragments in the large complexes were aggregated to complexes between M_r 32,500 and 4,500, only approximately 32% of the peptide fragments in the small complexes were aggregated to complexes between M_r 13,250 and 3,000.

Specific Antibody Responses

Sera from BN rats immunised with either PBS (control), 200 μ g of intact BLG, 30 μ g of intact BLG, 200 μ g of partially digested BLG (containing 30 μ g of intact BLG), 200 μ g of digested BLG (without any intact BLG), 200 μ g of large complexes or 200 μ g of small complexes, were evaluated for specific antibody responses against intact BLG as well as the different pools of BLG digests. Analyses of specific IgG1 and IgG2a responses revealed

that while both the high (200 μ g) and the low (30 μ g) amount of intact BLG as well as the partially digested BLG could induce specific IgG antibodies (though only intact BLG alone to a statistically significant degree), neither the digested BLG nor the two fractions thereof could induce any specific IgG response. Specific IgG1 and IgG2a results were very similar, for which reason only the IgG1 results are shown in figure 6. The high and the low immunisation dose of intact BLG induced specific IgG1 reaching equal antibody titre levels in the BN rats that reacted similarly with intact BLG and the partially digested BLG. However, only IgG1 from sera raised in BN rats immunised with the high dose of intact BLG was able to react with digested BLG and the large complexes, indicating differences in IgG1 antibody specificities. From figure 6 it was evident that while digestion of BLG abolished the antibody-inducing capacity of the resulting peptide fragments, both the whole pool of digested BLG

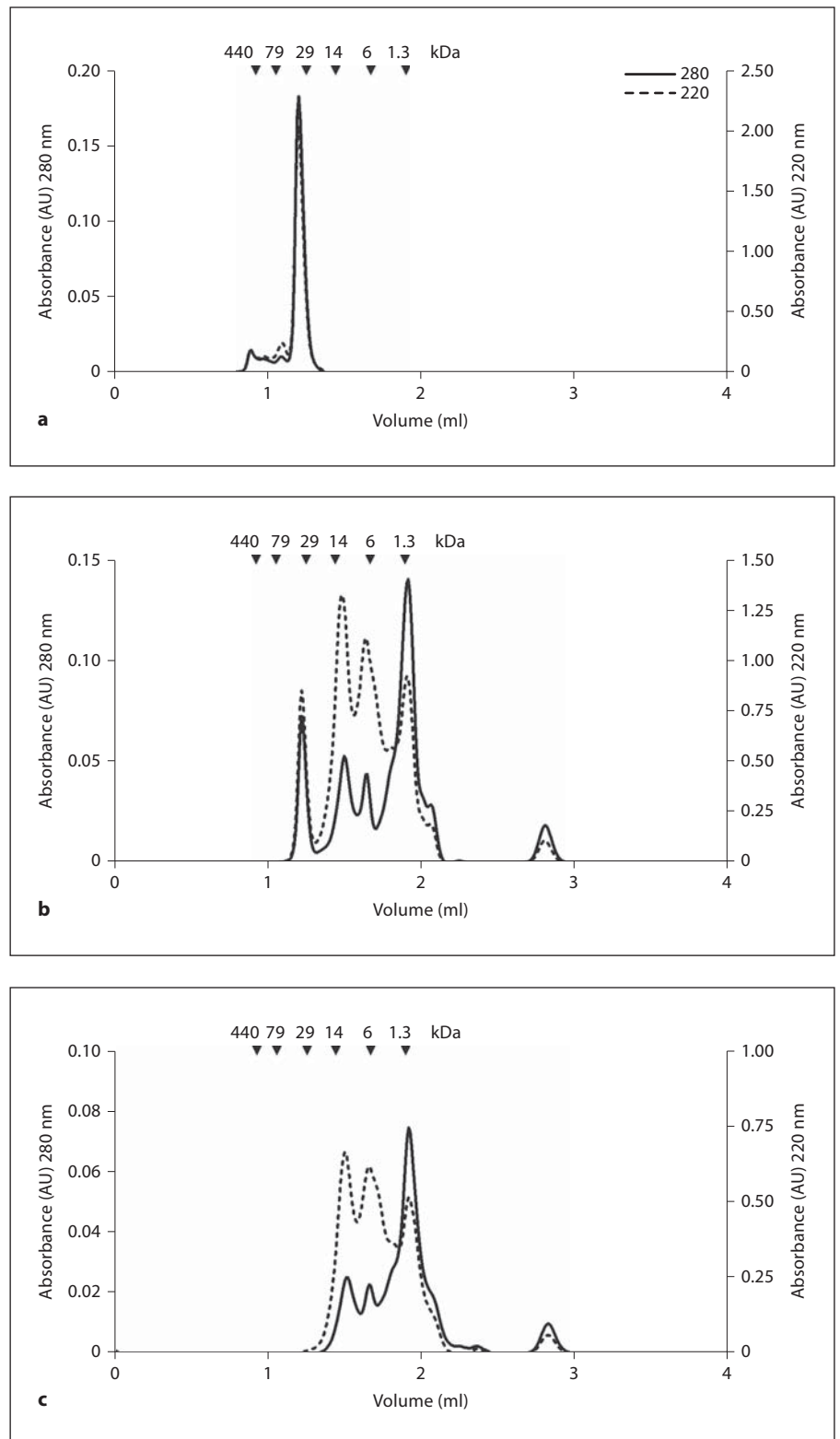


Fig. 5. Analytical GPC for intact BLG and BLG digests. Comparison of chromatography profiles, shown with absorbance at 220 and 280 nm, for intact BLG (**a**), partially digested BLG (**b**), and digested BLG (**c**).

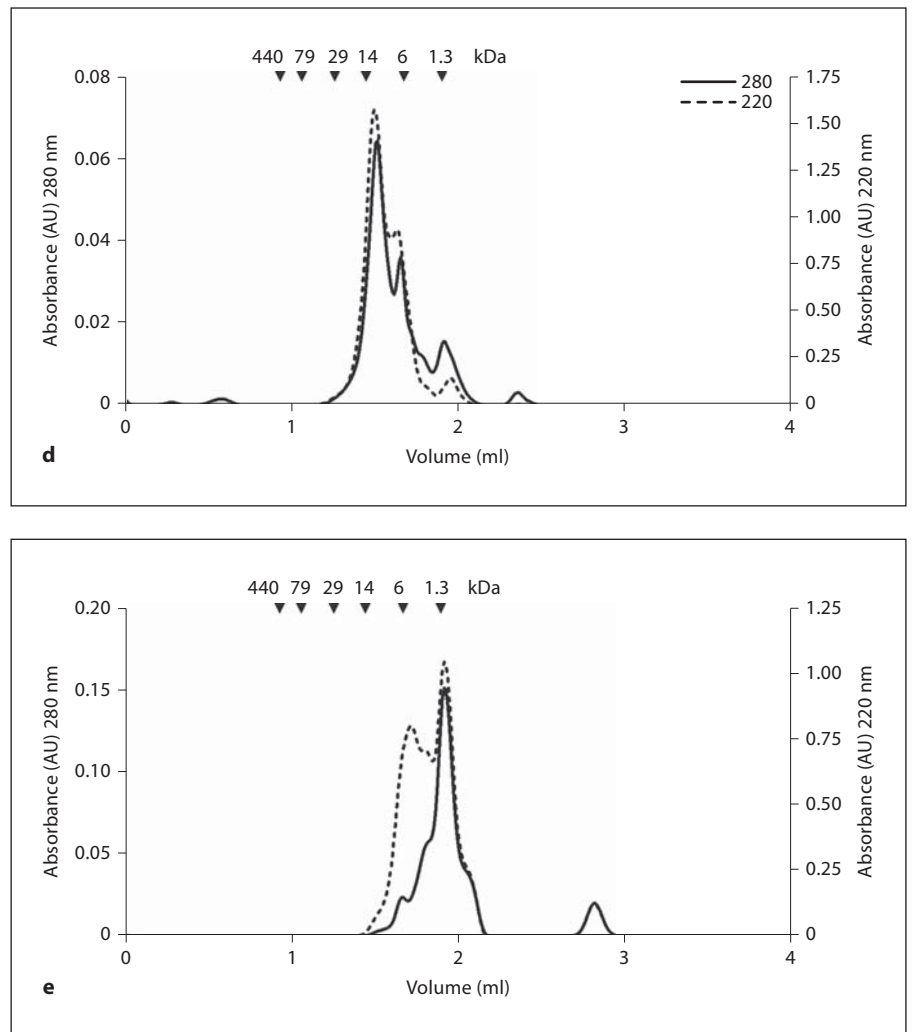


Fig. 5. Analytical GPC for intact BLG and BLG digests. Comparison of chromatography profiles, shown with absorbance at 220 and 280 nm, for large complexes (**d**) and small complexes (**e**).

and the large complexes retained some binding capacity. From the specific IgG1 response analyses it is revealed that by immunising the BN rats with 30 μ g of intact BLG alone a statistically significant antibody response was reached, while immunising the rats with partially digested BLG, containing 30 μ g of intact BLG together with 170 μ g of BLG digestion products, a much lower antibody response was generated. This indicates that the digestion products reduced the antibody response induced by the intact BLG.

From figure 7 it is evident that both the high dose (200 μ g) and the low dose (30 μ g) of intact BLG as well as partially digested BLG had sensitising capacity being capable of inducing specific IgE antibodies, though only the intact BLG alone to a statistically significant level. Conversely, digested BLG and the two fractions thereof had

no sensitising capacity. These results reflect the results of the specific IgG1 responses, indicating that intact BLG have a stronger sensitising capacity when dosed alone, compared to being dosed in combination with peptide fragments from the very same protein.

Analysis of the specific IgA response (fig. 8) shows that only the high (200 μ g) and the low dose (30 μ g) of intact BLG could induce specific IgA antibodies, both to a statistically significant level. These results indicate that the specific IgA response parallels the specific IgG1 and IgE response.

For examination of the avidity between specific antibodies raised in the different groups of rats and the intact BLG as well as the different pools of BLG digests, a KSCN inhibition ELISA assay was performed for those individual sera for which it was possible to obtain an OD of a

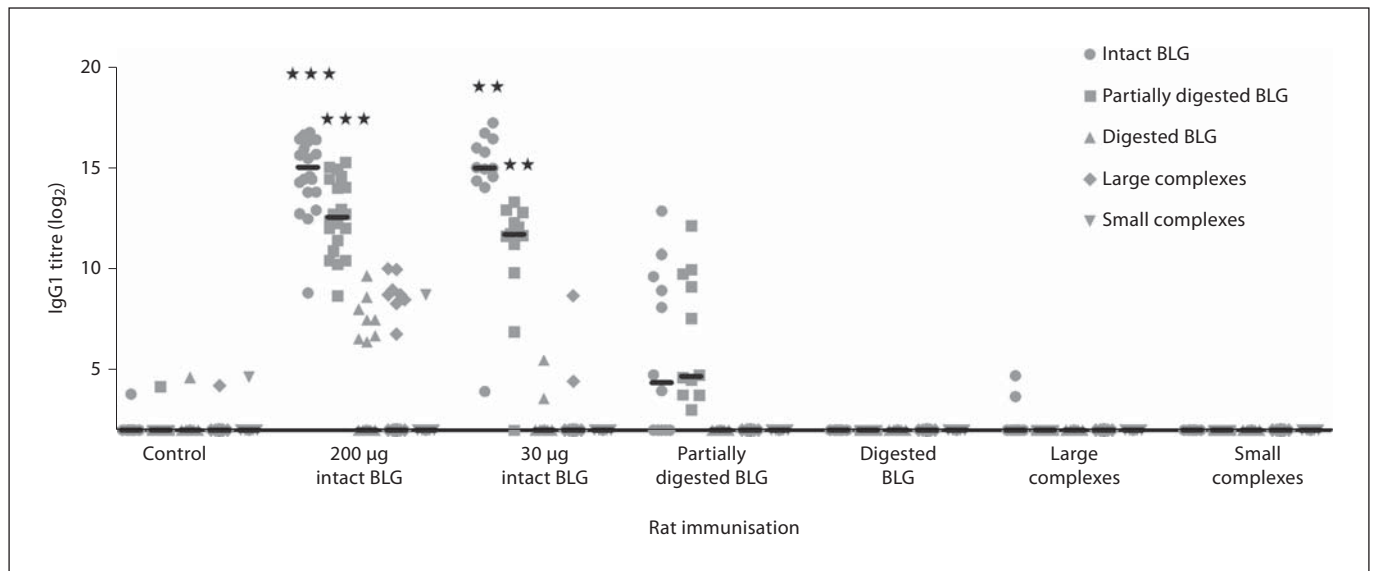


Fig. 6. Specific IgG1 responses. Comparison of specific IgG1 titre ($\log_2$) values for groups of rats immunised with either PBS (control), 200 μg of intact BLG, 30 μg of intact BLG, 200 μg of partially digested BLG (containing 30 μg of intact BLG), 200 μg of digested BLG, 200 μg of large complexes or 200 μg of small complexes. Sera from all rats in each immunisation group were examined for specific IgG1 antibodies against intact BLG, partially digested BLG, digested BLG, large complexes and small com-

plexes, shown with different symbols. Each symbol represents an individual rat. Horizontal bars indicate the median IgG1 titre value for each group of rats towards the given antigen product. Statistically significant differences between groups of rats were determined by use of the Kruskal-Wallis test followed by Dunn's multiple comparison test. Asterisks indicate statistically significant differences of the given group compared to the control group.

minimum of 0.5. No statistically significant differences were observed for the avidity between specific IgG1 and any of the tested antigens (fig. 9). For example, there was no statistically significant difference in the avidity of the binding between specific IgG1 antibodies raised in rats immunised with intact BLG and the antigen solutions of intact BLG, partially digested BLG, digested BLG or large fragments, respectively, though there seems to be a tendency of the antibodies reacting with a lower avidity towards the digestion products compared to the intact BLG. Also, no statistically significant differences in avidity were seen between specific IgG1 raised in the different groups of rats.

Discussion

The present study has confirmed the majority of earlier studies showing that BLG is a protein stable to simulated human digestion, showing that BLG is virtually completely resistant to pepsinolysis, while being more susceptible to digestion by trypsin and chymotrypsin un-

der physiologically relevant conditions [12, 22]. However, once broken down to larger fragments, BLG was rapidly digested to smaller peptide fragments of sizes less than M_r 4,500, of which more than 75% had an apparent M_r between 500 and 2,000. This suggests that when intact BLG is digested to a few larger fragments, these may not retain sufficient structure to allow them to resist further degradation by proteases, rendering these fragments even more susceptible to additional digestion. Peptide fragments generated during the digestion process was shown to aggregate to complexes of larger sizes of up to M_r 28,000. With fractionation of the peptide fragments by means of GPC it was demonstrated that especially the peptides composed of charged amino acid residues tended to form aggregates. This is in accordance with another study showing likewise that peptide fragments resulting from the digestion process of the peanut allergen Ara h 1 formed the largest complexes when composed mainly of charged and least of hydrophobic amino acid residues [16], indicating that non-covalent interactions other than hydrophobic are the main players in the formation of aggregates.

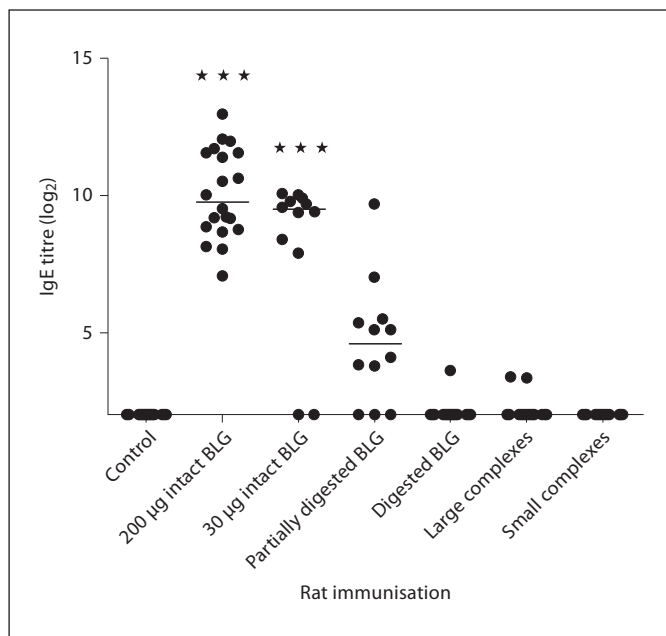


Fig. 7. Specific IgE response against intact BLG. Comparison of BLG-specific IgE titre ($\log_2$) values for groups of rats, immunised with either PBS (control), 200 μg of intact BLG, 30 μg of intact BLG, 200 μg of partially digested BLG (containing 30 μg of intact BLG), 200 μg of digested BLG, 200 μg of large complexes or 200 μg of small complexes. Each symbol represents the IgE titre value for an individual rat. Horizontal bars indicate the median IgE titre value for each group of rats. Statistically significant differences between groups of rats were determined by use of the Kruskal-Wallis test followed by Dunn's multiple comparison test. Asterisks indicate statistically significant differences of the given group compared to the control group.

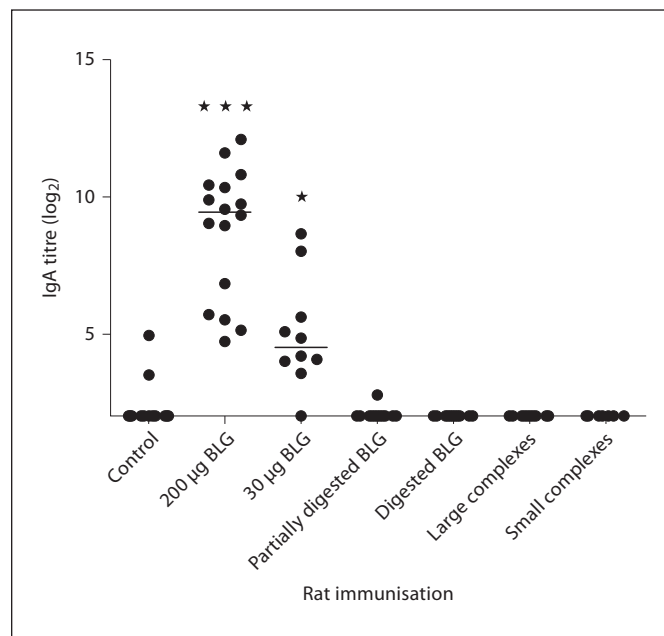


Fig. 8. Specific IgA response against intact BLG. Comparison of BLG-specific IgA titre ($\log_2$) values for groups of rats immunised with either PBS (control), 200 μg of intact BLG, 30 μg of intact BLG, 200 μg of partially digested BLG (containing 30 μg of intact BLG), 200 μg of digested BLG, 200 μg of large complexes or 200 μg of small complexes. Each symbol represents the IgA titre value for an individual rat. Horizontal bars indicate the median IgA titre value for each group of rats. Statistically significant differences between groups of rats were determined by use of the Kruskal-Wallis test followed by Dunn's multiple comparison test. Asterisks indicate statistically significant differences of the given group compared to the control group.

Food allergy develops as a result of impaired formation or breakdown of oral tolerance. The immune system of the BN rats used in this study has never been introduced to BLG and the rats are consequently not tolerant to this protein. The present study showed that only immunisation solutions containing intact BLG could induce specific IgG and IgE responses in the BN rats, and only intact BLG alone to a statistically significant degree. On the other hand, digestion products generated by simulated gastroduodenal digestion could neither induce an immunogenic nor an allergenic response. This demonstrates that the digestion process was successful in abolishing the sensitisation capacity of BLG and demonstrates the importance of a functional digestive system for lowering the risk of developing food allergies. This is in agreement with the studies of Untersmayr et al. [23] and Scholl et al. [24] showing that impairment of digestion enhances the sensi-

tising capacity of caviar and hazelnut proteins in a mouse model and support the inclusion of an assessment of the stability of proteins to in vitro digestion by proteases as a parameter in the weight-of-evidence approach for evaluation of the potential allergenicity of novel proteins in genetically modified food as recommended by the EFSA panel in 2010 [25]. Results from the present study show that digestion of a food allergen completely abolishes its sensitising capability. This is in contrast to our previous study, showing that Ara h 1 retained both the sensitising, IgE binding and eliciting capacity, and thereby acted as a 'complete' allergen, when digested to small peptide fragments of less than M_r 2,000 [11]. Additionally, in another study, we showed that digested Ara h 1 with a peptide distribution profile very similar to that of digested BLG in the present study likewise retained its sensitising capacity [16], proving that sizes of the breakdown products do not alone

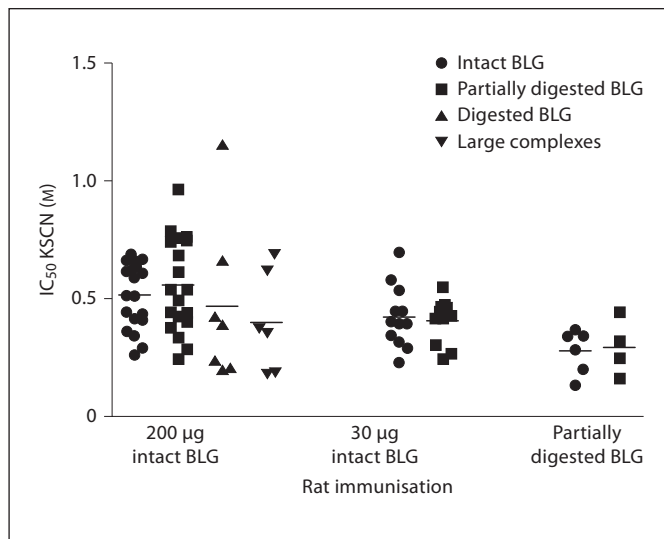


Fig. 9. Avidity measurements of specific IgG1 antibodies. Comparison of specific IgG1 antibody binding avidities towards the given antigen product, expressed as the KSCN concentration needed for inhibition of 50% response, for groups of rats immunised with either 200 µg of intact BLG, 30 µg of intact BLG or 200 µg of partially digested BLG. Sera from all rats mounting a specific IgG1 response of at least OD 0.5 were included in the examination of IgG1 avidity. Each symbol represents the IgG1 avidity for an individual rat. Horizontal bars indicate mean IgG1 avidity for each group of rats. Statistically significant differences between groups of rats were determined by use a parametric one-way ANOVA, followed by Tukey's multiple comparison test for comparison of three or more groups.

determine whether or not the immune system mounts a sensitising response. This also demonstrates the complexity of the immune mechanisms involved in directing the immune response towards sensitisation. While the sensitising capacity of BLG was lost when digested to smaller peptide fragments, both the whole pool of digested BLG as well as the fraction of large complexes retained the binding capacity. Antibodies from BN rats immunised with the high dose of BLG could still react with the digestion products, though with a lower response than towards the intact protein, indicating that some but not all epitopes survived the digestion process, which is in agreement with results of Clement et al. [7]. This suggests that patients sensitised to intact BLG may still recognise the gastro-duodenal digestion products of BLG and that the digestion products may retain the capacity to elicit allergic responses. This may especially be true because of the aggregating tendency of the peptide fragments. Aggregation has earlier been suggested to be the reason for the sustained allergenic capacity of digestion products of allergens, which in

theory were too small to contain two epitopes and thereby not big enough to cross-link two IgE molecules bound to the surface of mast cells or basophils [11, 16, 26]. The finding that IgE epitopes on BLG survive digestion is in accordance with previous studies stating that the major IgE epitopes reported in the literature by Järvinen et al. [6] and Cocco et al. [5] corresponded to areas of BLG where peptides did resist simulated gastro-duodenal digestion [22]. This is in agreement with the current study and could support the presence of major linear B cell epitopes on BLG. Results from the avidity measurements, showing that no significant differences existed for the avidity of IgG1 antibody binding to intact versus digested BLG, further strengthens the likelihood of linear epitopes. On the other hand, if most epitopes of the BLG molecule were conformational, one would expect a significant reduction in the binding strength between antibodies and antigens after the digestion process, because of the reduced possibility of small peptides to sustain sufficient structural integrity to bind antibodies raised against conformational epitopes with a similar avidity. It has been shown that a close relationship exists between the specificity of BLG-specific IgE and IgG epitopes, showing that the major linear IgG epitopes generally co-localised with the major linear IgE epitopes [6]. If our results on IgG avidity are representative for IgE avidity, the present results are in accordance with earlier studies showing the high importance of just linear IgE epitopes on BLG [6, 27]. It may not be possible to generalise data on linear epitopes from BLG to other food allergens, and in contrast to the above assertion, we have previously shown that even small peptide fragments emerging from the digestion of Ara h 1 retained sufficient structural integrity to function as conformational IgE binding epitopes when present in aggregates probably resembling the intact Ara h 1 molecule [16, 28].

The present study has shown that antibody levels in rats immunised with partially digested BLG (containing 30 µg of intact BLG together with 170 µg of BLG digestion products) were strongly reduced compared to the antibody level in rats immunised with the low dose of intact BLG. The amount of intact BLG was the same in the two immunisation solutions, the difference being the additional presence of digestion products in the partially digested BLG. This indicates that the reduced response was induced by the BLG-derived peptide fragments. As we see it, the lower response could be a result of two different situations. The first involves the masking of epitopes implicated in the sensitisation phase by means of the peptide fragments camouflaging the epitopes by an aggregate formation with the intact molecule. The second situation

involves tolerogenic properties of the peptide fragments, which by co-immunisation with the intact protein leads to impaired sensitising capacity of the intact BLG dimeric molecule. The first situation, however, seems unlikely, based on the knowledge from the analytical GPC showing that the dimeric BLG molecules did not form complexes with the peptide fragments present in the partially digested BLG, or at least not to a level which could be detected. This points to the second situation, that the reduced sensitising capacity of partially digested BLG, containing both intact and digested BLG, compared with the intact BLG alone could possibly be a result of some tolerogenic capacity of the digested BLG, leading to antibody responses of significantly lower levels when administering intact BLG together with peptide fragments from the same protein, compared to administering the intact BLG alone. To examine whether the digested BLG induced tolerance mechanisms or whether it was simply ignored by the immune system, specific serum IgA was measured since specific serum IgA have been suggested to be an indicator for development of tolerance, correlating inversely with specific serum IgE in allergen-sensitised patients [29, 30]. However, specific IgA levels in BN rats seem to correlate with the specific IgG1 and IgE responses. These results are therefore not informative about whether the digested BLG inheres tolerogenic potential or whether the digestion products were not recognised by the immune system of the rats. Tolerance may be induced by fundamentally different mechanisms, shown in animal studies of low- versus high-dose induced tolerance [15] and in studies of carrier-specific tolerance induction [31].

Whether or not the peptide fragments were indeed able to induce tolerance mechanisms remains to be fully demonstrated. If this is the case, it also remains to be

demonstrated whether the peptides could induce tolerance mechanisms alone or whether they need to be presented to the immune system together with the native BLG, and in that case, to determine further whether or not the tolerance mechanisms involved then are the same. Peptides may be processed and presented differently from the intact protein, and there is the possibility of presenting a greater variety of different T cell as well as B cell epitopes from the intact compared to the digested protein. Maybe the use of intact protein plus peptides ensures that there will be achieved tolerance against not only those sequences presented by the peptides but also other sequences of the protein, and thereby the entire protein.

Further studies are needed in order to reveal if digested BLG alone has tolerogenic capacity, studying the mechanisms underlying tolerance induction, and to elucidate the general tolerogenic capacity of co-administration of intact allergens with peptide fragments thereof. This would additionally contribute to a better understanding of the underlying mechanisms of sensitisation versus tolerance induction which could be useful in the future for new strategies in the prevention and treatment of allergic diseases. In agreement with the message of Fritsche et al. [32], in such situations animal models would certainly be helpful in prediction of the allergenic versus tolerogenic potential of different hydrolysates and in the understanding of the mechanisms involved.

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