Milk allergy prevention and treatment

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(54) Title: MILK ALLERGY PREVENTION AND TREATMENT

(57) Abstract: The invention provides a new strategy for achieving desensitisation or induction of tolerance to milk protein allergens, e.g. BLG, in humans or animals, comprising formulating and using a composition comprising a purified intact expressed milk protein together with one or more purified peptides from said intact milk protein.

MILK ALLERGY PREVENTION AND TREATMENT

FIELD OF THE INVENTION

The present invention relates to food allergy and compositions with reduced allergenicity for use in desensitisation or induction of tolerance to food proteins. In particular, the invention relates to allergy against milk proteins, primarily beta-lactoglobulin.

BACKGROUND TO THE INVENTION

Food allergy is defined as an immune mediated adverse reaction to otherwise harmless proteins in the food and is thought to be caused by a failure of the immune system to develop tolerance after exposure to these proteins. Food allergy is a major health problem in the Western countries, where it affects around 5-8% of young children and 2-4% of adults and appears to be an increasing problem. At the moment no cure exists for food allergy and avoidance of the offending food is presently the only reliable management of food allergy [Nowak-Wegrzyn & Sampson (2011). J. Allergy Clin. Immunol. 127:558-573; Berin & Sicherer (2011). Curr. Opin. Immunol. 23:794-800]. Food allergy has a huge impact on the quality of life of allergic patients and their families living in daily fear of fatal reactions. The need for efficient preventive and treatment strategies are therefore evident.

There is currently no reliable method to identify who will be at risk of developing food allergy, and there is no general accepted strategy for prevention of food allergy. However, for infants with family members who have an atopic disease, who are not exclusively breastfed, specific hypoallergenic infant formulas are suggested in order to prevent cow's milk allergy and the so-called allergic march [Muraro *et al*. (2014). Allergy. 69:1008-1025; de Silva *et al*. (2014). Allergy. 69:581-589; Weinberg *et al*. (2010). CME. 28:64-68].

Hypoallergenic infant formulas are based on hydrolysed cow's milk whey or casein. The idea behind a preventive effect of the hypoallergenic infant formulas is that if the cow's milk proteins are hydrolysed to such an extent that the sensitising capacity is minimised, and at the same time peptides with sizes large enough to be recognised by the immune system are maintained, the ability to induce tolerance will be preserved [Crittenden & Bennett (2005). J. Am. Coll. Nutr. 24:582S-591S; Bahna (2008). Ann. Allergy Asthma Immunol. 101:453-459]. Although there might be benefits of this alternative to traditional cow's milk formulas for infants with a high risk of developing food allergy, the preventive efficacy of

hypoallergenic infant formulas remains uncertain, as well as does the ideal design of the hydrolysates [Crittenden & Bennett (2005). J Am. Coll. Nutr. 24:582S-591S; Muraro *et al*. (2014). Allergy. 69:1008-1025; de Silva *et al*. (2014). Allergy. 69:581-589; Adrian *et al*. (2011). J. Allergy Clin. Immunol. 128:360-365].

Currently different therapeutic strategies targeting food allergy are under investigation. Immunotherapeutic products are on the market for treatment of e.g. inhalant allergy, but for food allergy the therapeutic approaches are still in the research phase. Immunotherapeutic strategies for food allergy have included allergen-specific as well as allergen non-specific approaches based on oral, sublingual, subcutaneous, nasal, intramuscular, intradermal, epicutaneous or rectal route of administration [Nowak-Wegrzyn & Sampson (2011). J. Allergy Clin. Immunol. 127:558-573; Berin & Sicherer (2011). Curr. Opin. Immunol. 23:794-800; Virkud & Vickery (2012). Discov. Med. 14:159-165].

For allergen-specific immunotherapy, approaches have been based on either the native allergens (whole foods, protein extracts, purified intact allergens or extensively heated foods) or modified versions of the offending allergens (heat-killed E.coli expressing modified proteins or in mixture with modified food allergens, peptides, plasmid DNA, conjugates of immune stimulatory molecules and allergens, human Fc-Fc fusion protein, sugar-modified allergens or denatured proteins) with or without use of different adjuvants. Different immunotherapeutic strategies for food allergy are in various stages of development ranging from animal models to clinical trials [Nowak-Wegrzyn & Sampson (2011). J. Allergy Clin. Immunol. 127:558-573; Berin & Sicherer (2011). Curr. Opin. Immunol. 23:794-800; Virkud & Vickery (2012). Discov. Med. 14:159-165].

Due to the high rate of unpredictable severe adverse reactions upon food allergy immunotherapy the oral route (oral immunotherapy and sublingual immunotherapy) has been regarded as the safest and are the most intensively studied strategies. Even though oral immunotherapy and sublingual immunotherapy have shown reduced risk compared to injection immunotherapies, also here adverse reaction are very common, and often patients are forced to drop out of the clinical trial. Immunotherapy by the oral route has proven successful in desensitisation of food allergic patients but no studies have been able to fully demonstrate development of tolerance. Further a major problem in food allergy immunotherapy is the fact that not all patients seem to respond to food allergy immunotherapy [Nowak-Wegrzyn & Sampson (2011). J. Allergy Clin. Immunol. 127:558-573; Berin & Sicherer (2011). Curr. Opin. Immunol. 23:794-800; Virkud & Vickery (2012). Discov. Med. 14:159-165].

WO 2008/088472 discloses milk protein hydrolysates comprising small peptides for use in foods and non-foods with reduced immunogenicity.

The strategy when developing allergy preventive or treatment approaches is to develop products that do not induce an allergic response but maintain the capacity to induce an immune response. An active immune response is required to induce *de novo* tolerance or change the immune response from allergy to tolerance. Further the ultimate goal of food allergy therapy is a permanent state of tolerance, which it said to be established when food can be ingested without allergic symptoms despite prolonged periods without administration of the prophylactic or therapeutic product [Nowak-Wegrzyn & Sampson (2011). J. Allergy Clin. Immunol. 127:558-573]. By far most studies have been examining the tolerance inducing capacity in subjects with allergy, but some approaches have likewise shown promise in prophylactic effect against food allergy.

In a recent study, the present inventors have shown that administering of partially digested beta-lactoglobulin (BLG) from cow's milk to rats, could significantly reduce inducement of IgG and IgE compared to administering BLG alone [Bøgh *et al*. (2013). Int. Arch. Allergy Immunol. 161:21-36]. The partially digested BLG was obtained in an *in vitro* model simulating the gastro-duodenal digestion process and consisted of a mixture of about 85 % digested BLG partly organized in smaller and larger complexes and 15% un-digested BLG . The mixture of complexed peptides, free peptides and intact BLG resulting from the partial digestion of BLG G was used in the model without any prior fractionation and/or mixing of the intact BLG and BLG-derived peptides and complexes. Partial hydrolysis and complex formation is dependent on many parameters and therefore not exactly reproducible and thus of potential risk if administered to a sensitive patient. The roles of the different parts of the mixture in managing allergy are not disclosed and thus remain speculative. . Järvinen [Järvinen (2013). Int. Arch. Allergy Immunol. 161:195-196] and Olivier *et al.* [Olivier et al. (2013). J. Allergy Ther. 4:148] have discussed the relevance of the study by Bøgh *et al*. to the clinical disease and development of tolerance in humans.

The major challenge of current preventive and treatment approaches is to be efficient without at the same time inducing allergic reactions which may be fatal. In order to be a future prophylactic and therapeutic strategy, there is a need for identifying means for the production of a reliable, reproducible and secure product for the benefit of affected patients with milk allergy and for prophylactic treatment, i.e. induction of tolerance to milk proteins.

SUMMARY OF THE INVENTION

The present invention addresses the need for a new strategy for prevention and treatment of food allergy induced by milk proteins. A solution to this need is presented in the following and concerns, in a first aspect, a composition comprising the intact milk protein and one or more peptides from the milk protein in isolated form comprising a first amino acid sequence from said milk protein. The "first amino acid sequence" is selected from a group of distinct amino acid sequences taken from said milk protein which, when comprised in a peptide and co-administered with the intact milk protein, induces reduced allergenicity towards the milk protein. In a second aspect of the present invention, the composition comprising the intact milk protein and one or more peptides comprising said "first amino acid sequence" is devoid of any peptides comprising a second amino acid sequence from said milk protein which can induce allergenicity on its own if present, and therefore, when comprised in a peptide and co-administered with the intact milk protein and the one or more peptides comprising said "first amino acid sequence", abolishes the otherwise induced reduced allergenicity towards the milk protein. In another aspect of the present invention, the one or more peptides is/are produced synthetically. In a further aspect of the present invention the one or more peptides is/are isolated from a milk protein hydrolysate. A preferred example of milk protein allergen is beta-lactoglobulin (BLG).

The BLG protein and its amino acid sequence is known from different lactating species including the domestic cow. Many variants of the BLG amino acid sequence have been reported from different species and even from the same species. BLG is not found in human breast milk and therefore seen by the human body as a foreign agent and a potential allergenic threat, especially to young children receiving cow's milk or infant formulas. The present invention relates to BLG secreted in milk from cow (Bos Taurus) and related lactating animals such as wild yak (Bos mutus), domestic water buffalo (Bubalus bubalis), goat (Capra hircus), sheep (Ovis aries), muflon (Ovis orientalis) and reindeer (Rangifer tarandus). Figure 1 shows the amino acid sequences of the full length BLG from these species, including variants from the same species and including the signal peptides. Signal peptides are shown by transparent boxes, and differences in amino acids (aa) sequence are shown by gray boxes. Uniprot accession numbers are also given in the figure as well as in Table 1 for each individual sequence (e.g. http://www.uniprot.org/uniprot/**P02754**).

The invention relates in particular to the use of intact, expressed BLG secreted in milk, i.e. a protein having the sequences given in Figure 1 without the signal peptides (transparent

boxes) and peptides derived therefrom. Expressed, secreted BLG is used synonymously with "intact BLG" throughout the present description.

In a particular embodiment, the present invention relates to intact BLG from cow (bos d5) with SEQ ID NO. 1:

LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENGECAQK KIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKAL KALPMHIRLSFNPTQLEEQCHI

Alternatively, the invention relates to an amino acid sequence with at least 90% identity to SEQ ID NO 1, such as at least 93%, 95%, 97% or 99 % identity to SEQ ID NO 1, including BLG from related species.

In the context of the present invention, the term "amino acid sequence .. identity" relates to a quantitative measure of the degree of identity between two amino acid sequences of equal length. If the two sequences to be compared are not of equal length they must be aligned to the best possible fit. The sequence identity can be calculated as

(Nref-Ndif)*100)/(Nref),

wherein Ndif is the total number of non-identical residues in the two sequences when aligned, and wherein Nref is the number of residues in one of the sequences. Sequence identity can for example be calculated using appropriate BLAST-programs, such as the BLASTp-algorithm provided by National Center for Biotechnology Information (NCBI), USA.

According to the present invention, one or more peptides is or are provided for co-administration together with intact BLG for use in desensitisation or tolerance induction to milk proteins in humans or animals. The peptide(s) may be produced synthetically or isolated from or present in a fraction or mixture isolated from hydrolysed BLG.

It has been shown by the present work that peptides of a certain minimum length, from different parts of the intact BLG protein, are able to induce a reduced allergenicity when co-administered with intact BLG. Further, it has been shown in a preferred embodiment of the invention that the presence of peptides comprising a certain stretch of amino acids from the BLG when administered together with the peptides otherwise inducing reduced allergenicity can hinder or block the reduction of allergenicity. Such "poisonous" peptides should be avoided or omitted from the composition.

In order to investigate whether the results seen in the studies reported by Bøgh et al., *supra*, were linked to the use of the specific sample of partially hydrolysed BLG containing

intact BLG and aggregated complexes of peptides, the aggregated peptides or specific peptides in the aggregated complexes being used, new studies were set up focusing on whether specific peptides could play the same role as the complexes in the previous study. If that would be the case, it would be possible to control the treatment of humans and animal by use of fully characterised, defined and reproducible compositions of intact BLG and peptides.

As a start, it was examined whether the size of the peptides, i.e. the number of amino acids in the BLG peptides, plays a role when used in combination with intact BLG. For that purpose a small set of BLG peptides of different length were synthesised. Three pools of synthetic peptides were created, each pool containing peptides of a certain length which together covered the entire BLG (see Figure 2). In order to reduce the number of peptides to be tested, it was decided to synthesise peptides that had no overlaps between the peptides in each pool, thus however, running the risk of missing out positive results linked to certain stretches not being represented in the pools. The first pool consisted of peptides having 14 or 15 consecutive amino acids from the intact BLG, the second pool consisted of peptides having 23 or 24 consecutive amino acids from the intact BLG, and the third pool consisted of peptides having 32 or 33 consecutive amino acids from the intact BLG (Figure 2). The length and starting position of the peptides were arbitrary chosen and resulted in three pools of 11, 7 and 5 different specific peptides respectively (Figure 2), which gave a total of 23 peptides that were tested individually and in combinations alone or together with intact BLG. The number of synthesised and tested peptides is only a very small selection from the enormous number of peptides that can be generated from BLG, but it was decided as a first step to see whether these three pools of peptides could be used to establish a minimum length of the peptides from BLG for these to be capable of inducing reduced allergenicity. Further, it was hoped that the synthesised peptides could be used to identify essential and less essential sequences, or stretches of the protein to be present in selected peptides for use alone, in combination and/or in combination with intact BLG. The first two pools of peptides, i.e. the shortest peptides, did not induce any reduction of the allergenicity toward intact BLG when used alone, in combination, or in combination with intact BLG. The third pool of peptides, however, contained peptides which showed a reduced allergenicity towards intact BLG when administered in combination with intact BLG.

In one embodiment, the invention relates to the use of one or more peptides consisting of between 24 and 75 consecutive amino acids, such as between 24 and 70, for example between 24 and 64 or 62 or 60 or 58 or 56 or 54 or 52 or 50 or 48 or 46 or 44 or 42 or 40 or 38 or 36 or 34 consecutive amino acids from intact BLG with sequence SEQ ID NO 1 or from a sequence with at least 90% identity to SEQ ID NO 1, such as at least 93%, 95%,

97% or 99 % identity to SEQ ID NO 1. In one embodiment the invention relates to peptides consisting of between 24 and 33 consecutive amino acids, for example 25, 26, 27, 28, 29, 30, 31, 32 or 33 consecutive amino acids from sequence SEQ ID NO 1 or a sequence with at least 90% identity to SEQ ID NO 1, such as at least 93%, 95%, 97% or 99 % identity to SEQ ID NO 1.

In a preferred embodiment, the one or more peptides consist(s) of between 24 and 75 consecutive amino acids from BLG which comprise(s) at least 24 consecutive amino acids selected from one of the amino acid sequences:

a. LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL (SEQ ID NO. 10),

b. DAQSAPLRVYVEELKPTPEGDLEILLQKWENG (SEQ ID NO. 14),

c. TDYKKYLLFCMENSAEPEQSLACQCLVRTPEVD (SEQ ID NO. 19),

d. DEALEKFDKALKALPMHIRLSFNPTQLEEQCHI (SEQ ID NO. 22), or

e. a sequence equivalent with one of the sequences a, b, c or d, taken from an amino acid sequence with at least 90 %, preferable 93, 95, 97 or 99 % identity to SEQ ID NO. 1.

The term "a sequence equivalent with .. sequence .. taken from an amino acid sequence with at least 90 %, preferable 93, 95, 97 or 99 % identity to SEQ ID NO. 1" used throughout this text means "the same" sequence, i.e. same starting and ending amino acid position and almost identical amino acid sequence, if it had been taken from an amino acid sequence with at least 90, 93, 95, 97 or 99 % identity to SEQ ID NO. 1.

The peptides for use in a composition according to the present invention may belong to one or more of the above groups a-d and each peptide may consist of between 24 and 75 amino acids, or between 24 and less than 75 amino acids, as discussed above.

In a preferred embodiment of the invention, the one or more peptides in the composition consist of at least 24, such as between 24 and 64 consecutive amino acids from the sequence:
LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENG, which is SEQ ID NO. 10 plus SEQ ID NO. 14, or from a sequence equivalent to this sequence taken from a sequence with at least 90 %, preferably 93, 95, 97 or 99 % identity to SEQ ID NO 1.

In another preferred embodiment of the invention, the one or more peptides for use in the composition consist of at least 24, such as between 24 and 32, such as 24, 25, 26, 27, 28,

29, 30, 31, or 32 consecutive amino acids from the sequence LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL (Peptide 19; SEQ ID NO. 10) and/or from the sequence DAQSAPLRVYVEELKPTPEGDLEILLQKWENG (peptide 20; SEQ ID NO. 14), or from a sequence equivalent to one of these sequences taken from a sequence with at least 90 %, preferably 93, 95, 97 or 99 % identity to SEQ ID NO 1.

When rats were co-administered with intact BLG and different synthetically produced peptides, it was seen that some of the peptides, especially peptide 19 and peptide 20 could induce reduced amounts of both IgG and IgE antibodies against intact BLG. When the peptides 21, 22 or 23 were administered together with intact BLG, no significant reduction in allergenicity was observed. The present studies, however, surprisingly showed that co-administering of peptide 21 together with intact BLG induced IgE antibodies not only against intact BLG and peptide 21, but also against the peptides 19, 20, 22 and 23. None of the peptides 19, 20, 22 and 23 showed such adverse allergenicity-inducing properties when co-administered with intact BLG. Peptide 21 was thus shown to represent an allergenic part or linear stretch of intact BLG, which in addition to its own allergenicity also enhances the allergenicity of other individual BLG peptides. Hence, it appears that at least one linear stretch of consecutive amino acids (a "second amino acid sequence") comprised in peptide 21, when being present in at least one peptide and co-administered with intact BLG, results in IgE antibodies being induced not only against intact BLG but also against other peptides derived from intact BLG. It was concluded that at least a part of the sequence in peptide 21 (SEQ ID NO. 17), when present in a peptide being administered together with intact BLG, inheres an allergenic capacity to induce IgE antibodies toward linear stretches of BLG and/or peptides derived from BLG and that such peptides preferably should be excluded in a composition according to the present invention for use desensitisation or induction of tolerance to BLG.

Accordingly, in a preferred embodiment of the invention, the composition is devoid of peptides from said intact BLG comprising at least 8, for example between 8 and 32, such as 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31 or 32 consecutive amino acids from the sequence ECAQKKIIAEKTKIPAVFKIDALNENKVLVLD (peptide 21; SEQ ID NO. 17) or from an equivalent sequence from a BLG sequence having at least 90 % (or 93, 95, 97 or 99 %) identity to SEQ ID NO. 1. SEQ ID NOs. 5, 6, 7 and 15 show further sequences which may be comprised in peptides to be avoided.

In a further embodiment, the invention relates to intact BLG as defined above in combination with peptides as defined above for use in desensitisation or induction of tolerance to BLG in a human or animal.

Other examples of peptides for use in a composition of the invention are peptides containing between 24 and 33 amino acids, such as 24, 25, 26, 27, 28, 29, 30, 31, 32 or 33 consecutive amino acids spanning two adjacent sequences according to SEQ ID NO. 10 – SEQ ID NO. 27, such as for example a peptide spanning SEQ ID NO. 10 and SEQ ID NO. 14.

Further examples of peptides for use in a composition of the invention are peptides containing more than 33 consecutive amino acids spanning two or more adjacent sequences according to SEQ ID NO. 10 – SEQ ID NO. 27, such as for example a peptide spanning SEQ ID NOs. 10, 14 and 19.

"More than 33 consecutive amino acids" means between 34 and 75, 70, 65, 64, 60, 55, 50, 45 or 40 consecutive amino acids from BLG, such as 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54,55, 56, 57, 58, 59, 60, 61, 62, 63, 64, etc. consecutive amino acids from BLG. Preferably, the peptide(s) contain less than 75 amino acids, less than 65 amino acids, less than 60, 55, 50, 45 or 40 amino acids.

The preferred peptides contain between 24 and 75 amino acids, or between 24 and 70, 65, 60, 55, 50, 45 or 40 amino acids.

More examples of peptides for use in a composition of the invention are peptides wherein one or more of the amino acids in the natural BLG sequence has or have been substituted by conservative amino acid(s).

In another embodiment of the invention, particular amino acids, for example up to 6 amino acids, such as 1, 2, 3, 4 or 5 amino acids are added to the N- and/or C-terminal end(s) of synthetically produced peptides for increasing the solubility of one or more of the peptide(s) in solution, such as in a physiologically acceptable solution for administration to the affected individual. Preferably, amino acid(s) added to the N- and/or C-terminal end(s) is/are lysine or glutamic acid.

In the present description and claims, these amino acids, not being part of the native BLG and only added to some of the synthetically produced peptides for solubility purposes, are shown in bold or in parentheses and not counted in as part of the length of the peptide. For example, peptide 19 (SEQ ID NO. 46) with the sequence: ***KKK***LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL is counted as a peptide having 32 amino acids and considered equal to LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL, i.e. SEQ ID NO. 10.

In a further embodiment of the invention, the peptide comprises 1, 2, 3 or 4 amino acid substitutions for increasing the solubility of the peptide in solution.

In one aspect of the present invention, the peptide(s) is/are synthetic peptide(s). In another aspect of the present invention, the peptide(s) is/are isolated after hydrolysis of intact BLG.

A another embodiment of the invention, the composition for desensitisation or induction of tolerance to milk protein in a human or animal comprises one or more peptide(s) according to the invention. The composition may comprise 1, 2, 3, 4, 5 or more than 5 different peptides according to the invention. If more than one peptide is used, the peptides are different and may cover the same or different stretches of consecutive amino acids and all or different parts of the intact BLG.

BLG peptide(s) for co-administration with intact BLG is/are, according to one aspect of the present invention, produced by isolation of one or more mixtures or fractions of peptides with a certain size after hydrolysis of intact BLG by use of for example one or more enzymes, such as one or more proteases. Peptides comprising a certain amino acid sequence may be isolated from the mixture or fraction as single peptides of sub-fraction(s) if desired. Any product containing or consisting of intact BLG, such as for example whey, whey protein concentrates (WPC) and whey protein isolates (WPI), may be hydrolysis or used as a source for intact BLG. A peptide fraction is typically obtained by separation of the hydrolysed mixture into different fractions according to size (peptide length) by filtration in a first step. Such a fraction would normally comprise two or more different peptides of a defined size (length/number of amino acids), such as peptides above a certain size, for example defined by the cut-off value of a filter or separated in a HPLC column or by similar measures. A further separation to isolate a sub-fraction, e.g. one specific peptide or a mixture of peptides with a more specific size or a common amino acid stretch, may be added to the isolation process in additional steps. Alternatively, a specific peptide or mixture of peptides with a common specific amino acid stretch may be removed from the hydrolysate or isolated in an additional or alternative hydrolysis process or in an additional separation or purification step. The fraction or mixture of one or more peptides is essentially free of intact BLG and any other intact milk proteins. "Essentially free" means less than 10%, preferably less than 8%, mere preferably less than 5% and most preferably less than 2%.

In one embodiment of the invention, the composition of the present invention may comprise a small amount of peptides smaller than 24 amino acids, as long as these peptides do not comprise a part of the amino acids stretch from BLG defined by SEQ ID NO.: 17 (peptide 21), SEQ ID NO. 32 (peptide 5), SEQ ID NO. 33 (peptide 6), SEQ ID NO. 34 (peptide 7) or SEQ ID NO. 42 (peptide 15). Peptides derived from the stretch of BLG defined by SEQ ID NO.: 17, 32, 33, 34 or 42 may, however, be tolerated if being small enough not to influence

the properties of the composition, for example smaller than 10, 9, 8, 7, 6, 5, 4, 3 or 2 amino acids. Especially hydrolysates of intact BLG may comprise "smaller" peptides due to the limitation on the fractionation and purification techniques. The presence of such smaller peptides in the final product will not affect the medical properties directly but may influence the effective load/content of one or more peptide in the final composition. Accordingly, the one or more peptides co-administered with intact BLG in a composition according to the present invention is substantially free of peptides with less than 24 amino acids, such as comprising less than 10%, 5%, 2% or 1% peptides with less than 24 amino acids. The composition is free or substantially free of aggregated peptides.

The weight ratio between intact BLG and BLG peptides in the composition is preferably 1:99, 2:98, 5:95, 10:90, 15:85, 20:80, 25:75 or 30:70. A preferred ratio is between 5:95 and 25:75 (w/w) or between 5:95 and 20:80 (w/w).

In further embodiment of the invention, the composition is comprised in an immunotherapeutic product, for example as a vaccine, which may further comprise an adjuvant.

The immunotherapeutic product may be for oral, sublingual, intramuscular, intradermal, epidermal, subcutaneous, intranasal, intraperitoneal, intravenous or intralymphatic administration. It is surprising that oral administration of the present composition, contrary to intact BLG alone or milk products comprising intact BLG, survives the gastrointestinal tract to such a degree that a tolerance inducing capacity to BLG can be seen.

An immunotherapeutic product for use for in sublingual, intramuscular, intradermal, epidermal, subcutaneous, intranasal intravenous or intralymphatic administration may contain the composition according to the present invention and further comprising one or more pharmaceutically acceptable excipients.

In yet another embodiment of the invention, the composition according to the invention may be comprised in food and nutritional products such as infant formulas, beverages such as sport drinks, dairy products, protein supplement, nutritional supplements, or the like. A food and/or nutritional product, e.g. an infant formula, according to the present invention contains the present composition as the essential only source of BLG protein and BLG-derived peptides. Such products may further comprise fat, carbohydrate, vitamins and/or minerals and the like, and/or be low in lactose or lactose-free.

When designing new strategies for prevention or treatment of food allergy, the overall aim is to design a product with a low allergenicity concurrent with a high tolerogenicity. Co-

administration of intact BLG and peptides of a certain size representing either the entire or part of the whole BLG sequence, has with the current presented studies demonstrated to fulfilled this. Co-administration of intact BLG with peptides resulted in a reduced allergenicity of BLG compared to administration of the intact BLG alone in the i.p. sensitisation studies. At the same time co-administration of peptides with intact BLG resulted in a stronger tolerance inducing capacity compared to administration of the peptides alone in the oral tolerance studies. Further it can be concluded from the presented studies that both allergenicity and tolerogenicity studies are needed in order to identify the most prominent combination of intact BLG and peptide(s). From the present studies it were shown that a peptide corresponding to the synthetic peptide 21 contained a high allergenicity and therefore is preferred not to have in the mixture of intact BLG and peptide(s), while peptides corresponding to the synthetic peptides 19 and 20 contained both an allergy reducing capacity. A peptide corresponding to the synthetic peptide 20 was shown to contain an allergy reducing capacity together with a tolerance inducing capacity when administered together with intact BLG and therefore is preferred to have in the mixture.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Beta-lactoglobulin sequences from different species taken from Uniprot.

Figure 2: Design of synthetic peptides. Three different designs of peptides representing the entire length of the primary sequences of intact BLG molecule (SEQ ID NO. 1) were chosen for synthesis: 1) 11 peptides of 14/15 amino acids residues 2) seven peptides of 23/24 amino acids residues, and 3) five peptides of 32/33 amino acid residues. Separation of the BLG molecules into peptides are represented by a solid line for the 14/15 amino acids peptides, by a dashed line for the 23/23 amino acids peptides, and by a dotted line for the 32/33 amino acid peptides.

Figure 3: BLG-specific IgG1 and IgE response for animal experiment 1. The specific IgG1 (A) and IgE (B) response against intact BLG was evaluated by means of ELISA. Each symbol represents the specific antibody response for an individual rat and horizontal bars indicate median value for each group of rats.

Figure 4: BLG-specific IgG1 and IgE response for animal experiment 2. The specific IgG1 (A) and IgE (B) response against intact BLG was evaluated by means of ELISA. Each symbol represents the specific antibody response for an individual rat and horizontal bars indicate median value for each group of rats. Statistically significant differences between groups were determined by the non-parametric one-way ANOVA, Kruskal-Wallis test, followed by Dunn's multiple comparison test. Asterisks indicate a statistically significant difference between the given group and the group of animals immunised with intact BLG alone.

Figure 5 (5-1 and 5-2): Peptide-specific IgE responses for animal experiment 2. The specific IgE response against synthetic peptide 19 (A), 20 (B), 21 (C), 22 (D) and 23 (E) was evaluated by means of ELISA. Each symbol represents the specific antibody response for an individual rat and horizontal bars indicate median value for each group of rats. Notice that the X-axis intersects the Y-axis in 0 and not 2 as in the former figures and that peptide 19 and 20 was used in an eight times higher amount than peptide 21, 22 and 23 in the ELISA to detect a specific IgE response.

Figure 6 (6-1 and 6-2): BLG-specific IgG1 response for animal experiment 3. The specific IgG1 response against intact BLG was evaluated by means of ELISA, after oral administration (no post-immunisation (A), 2. post-immunisation (B), 3. post-immunisation (C), 4. post-immunisation (D) and 5. post-immunisation (E). Each symbol represents the specific antibody response for an individual rat and horizontal bars indicate median value for each group of rats. Asterisks indicate a statistically significant difference between the given group and the group of animals administered with water.

Figure 7 (7-1 and 7-2): BLG-specific IgE response for animal experiment 3. The specific IgE response against intact BLG was evaluated by means of ELISA, after oral administration (no post-immunisation (A), 2. post-immunisation (B), 3. post-immunisation (C), 4. post-immunisation (D) and 5. post-immunisation (E). Each symbol represents the specific antibody response for an individual rat and horizontal bars indicate median value for each group of rats. Asterisks indicate a statistically significant difference between the given group and the group of animals administered with water.

Figure 8: Match of MS/MS identified peptides to the primary BLG sequence. Peptides from the fraction of large peptides were analysed by MS/MS sequencing. The part of the expressed BLG primary structure (here, amino acid residue 17-178) to which peptides could be aligned are shown in bold underline.

Figure 9: BLG-specific IgG1 and IgE response for animal experiment 4. The specific IgG1 (A) and IgE (B) response against intact BLG was evaluated by means of ELISA. Each symbol represents the specific antibody response for an individual rat and horizontal bars indicate median value for each group of rats.

Figure 10 (10-1 and 10-2): BLG-specific IgG1 response for animal experiment 5. The specific IgG1 response against intact BLG was evaluated by means of ELISA, after oral administration (no post-immunisation (A), 1. post-immunisation (B), 2. post-immunisation (C), 3. post-immunisation (D) and 4. post-immunisation (E). Each symbol represents the specific antibody response for an individual rat and horizontal bars indicate median value for each group of rats.

Figure 11 (11-1 and 11-2): BLG-specific IgE response for animal experiment 5. The specific IgE response against intact BLG was evaluated by means of ELISA, after oral administration (no post-immunisation (A), 1. post-immunisation (B), 2. post-immunisation (C), 3. post-immunisation (D) and 4. post-immunisation (E). Each symbol represents the specific antibody response for an individual rat and horizontal bars indicate median value for each group of rats. Asterisks indicate a statistically significant difference between the given group and the group of animals administered with water.

Figure 12: Overlaid chromatograms of a partial hydrolysate before fractionation (black line) and a synthetic peptide of BLG consisting of 24 amino acid residues (grey line).

Figure 13: Overlaid chromatograms of an UF permeate fraction from a fractionation on a KOCH HFK-131 6338 using 400 % dia-water (black line) and a synthetic peptide of BLG consisting of 24 amino acid residues (grey line).

DETAILED DESCRIPTION OF THE INVENTION

In the present invention, peptides derived from the sequence of intact (expressed) milk proteins, such as BLG, are used in a formulation together with the intact milk protein allergen to formulate a composition that can be used in treatment or prevention of milk allergy caused by milk proteins, in particular by BLG.

Definitions

"Milk proteins" include caseins; the whey proteins beta-lactoglobulin, alfa-lactalbumin, bovine serum albumin and the immunoglobulins; milk fat globule membrane proteins and minor proteins such as serum transferrin.

By the term "expressed beta-lactoglobulin" is meant expressed BLG secreted in milk from a lactating animal. Examples of BLG are shown in Figure 1. "Expressed BLG" is BLG without the signal peptide.

By the term "intact beta-lactoglobulin" is meant secreted expressed BLG, i.e. BLG as secreted and present in untreated milk. BLG exists in its natural form as a dimer.

By the term "peptide" is meant a chain of amino acids, normally up to 75 amino acids long. As used herein, "peptide" is to be understood as a fragment of the "intact BLG" which can be synthesised or obtained by hydrolysis of intact or partially intact BLG. The "peptide" is not the entire intact BLG. By the term "one peptide" is meant any number of peptides having the same amino acid sequence. The term "more peptides" means any number of two or more different peptides where the different peptides have different amino acid sequences and "each peptide" has the same amino acid sequence. The different peptides may or may not have overlapping amino acid sequences.

By the terms "contain", "contains" and "containing" used herein is meant "consisting of", i.e. does not comprise more, such as additional amino acids.

By the term "comprising" is meant that additional subject-matter, such as additional amino acids, may be present. For example, when it is stated that a peptide consisting of between 24 and 75 consecutive amino acids from BLG comprises at least 24 consecutive amino acids selected from the amino acid sequence: LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL, it means that the peptide consists of between 24 and 75 consecutive amino acids from BLG and includes a stretch of any 24 or more (maximum 32) consecutive amino acids taken from the sequence LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL.

By the terms "induction of tolerance" and "tolerance induction" are meant a permanent immunologic unresponsiveness to the given offending protein. By the term "tolerogenic capacity" is meant an inherent capacity of the composition to induce tolerance or desensitisation, which may be shown as a reduction in the allergic response, and not necessarily a complete abrogation of the allergic response.

By the term "desensitisation" is meant a temporary loss of responsiveness due to continuous exposure to the given offending protein.

By the term "sequences spanning two adjacent sequences" is meant an amino acid sequence comprising some or all amino acids from two naturally adjacent sequences as defined herein. If the peptide spans three adjacent sequences as defined herein, it means that all or parts of the N- and C-terminal sequences in three naturally adjacent sequences are included and all of the middle sequence is included.

"Uniprot accession number" is the number defining a particular protein/peptide in www.uniprot.org.

By the term "conservative amino acid" is meant an amino acid that can be replaced with another amino acid that is similar in size and chemical properties. The term is known to the person skilled in the art.

By the terms "synthetic peptide" and "synthetically produced peptide" are meant a molecule with multiple amino acids linked by peptide bonds that are created chemically outside a living cell. Opposite to normal protein biosynthesis amino acids are added to the C-terminal.

By the terms "fraction of a hydrolysate" is meant a separated and isolated part of the total amount of peptides present in or as a mixture after hydrolysis of a protein. The fraction may be obtained by separation and isolation of a mixture of peptides according to the size of the peptides, i.e. the amino acid length of the peptides. Separation preferably includes filtration, such as ultrafiltration and/or gelfiltration. The protein may be hydrolysed by use of acids, enzymes, or the like, preferably by use of an enzyme or a combination of enzymes. Enzymes and especially proteases are preferred due to their reproducible specificity which is necessary for securing reproducible mixtures of peptides. Specific stretches of the amino acid sequence to be avoided in peptide fraction may be broken by site-specific hydrolysis.

By the term "adjuvant" is meant any agent (substance), inorganic or organic chemicals, macromolecules or entire cells of e.g. killed bacteria that may modify the effect of the composition.

By the term "immunotherapy" is meant any prevention or treatment of a disease that acts by affecting the immune system by an induction, enhancing or suppression of the immune response.

Beta-lactoglobulin protein (BLG)

In the present invention one or more peptides from intact BLG are used to control immune reactions towards the presence of BLG in food products such as milk, cheese, whey nutritional formulas, e.g. infant formulas, beverages, foods, etc., and other products

comprising BLG. Intact BLG is a secreted form of BLG found in milk from cows and related hoofed milk producing animals. A list of animals and related BLG selected from the Uniprot database is given in Table 1 together with the Uniprot accession numbers. The percentage identity is in relation to a reference BLG from cow (P02754). The corresponding amino acid sequences are given in Figure 1.

Protein	Organism	Uniprot Accession number	Accession name	% identity to P02754 in the expressed seq.	SEQ ID NO.
Beta-lactoglobulin	Bos taurus (Bovine)	P02754	LACB_BOVIN	100	1
Uncharacterized protein	Bos taurus (Bovine)	G5E5H7	G5E5H7_BOVIN	99	3
Beta-lactoglobulin	Bos mutus grunniens	L8J1Z0	L8J1Z0_9CETA	99	4
Major allergen beta-lactoglobulin	Bos taurus (Bovine)	B5B0D4	B5B0D4_BOVIN	99	2
Beta-lactoglobulin	Bubalus bubalis (Domestic water buffalo)	C3W955	C3W955_BUBBU	99	5
Beta-lactoglobulin	Bubalus bubalis (Domestic water buffalo)	P02755	LACB_BUBBU	99	5
Beta-lactoglobulin	Capra hircus (Goat)	P02756	LACB_CAPHI	96	7
Beta-lactoglobulin-1/B	Ovis aries (Sheep)	P67976	LACB_SHEEP	96	8
Beta-lactoglobulin	Bubalus bubalis (Domestic water buffalo)	Q0PNH9	Q0PNH9_BUBBU	94	6
Beta-lactoglobulin	Rangifer tarandus tarandus	Q00P86	Q00P86_RANTA	96	9
Beta-lactoglobulin	Capra hircus (Goat)	A5JSR7	A5JSR7_CAPHI	97 (91)*	Aa 1-151 of 7*
Beta-lactoglobulin	Ovis orientalis musimon (Mouflon)	P67975	LACB_OVIMU	96	8

Table 1. List of sequenced beta-lactoglobulins reported in Uniprot. * lacking 11 C-terminal amino acids compared to SEQ ID NO. 7; 97% identity for the given aa sequence.

Intact BLG with the expressed amino acid sequence from P02754 was used as starting point for investigating the potential of polypeptides derived therefrom in emolliating milk allergy. The amino acid sequence for intact BLG from P02754 is given below as SEQ ID NO. 1:

LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENGECAQK
KIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKAL
KALPMHIRLSFNPTQLEEQCHI.

Other expressed BLG sequences within the invention are (Table 1 and Figure 1):

From Bos Taurus: SEQ ID NO. 2 (B5B0D4) and SEQ ID NO 3 (G5E5H7):

SEQ ID NO. 2:
LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENDECAQK
KIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPEVDDEALEKFDKAL
KALPMHIRLSFNPTQLEEQCHI.

SEQ ID NO 3:
LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENGECAQK
KIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPEVDDEALEKFDKAL
KALPMHIRLSFNPTQLEEQCHI.

From Bos mutus: SEQ ID NO. 4 (L8J1Z0):

LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENGECAQK
KIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKAL
KALPMHIRLSFNPTQLEGQCHI.

From Bubalus bubalis: SEQ ID NO. 5 (P02755 and C3W955) and SEQ ID NO. 6 (Q0PNH9):

SEQ ID NO. 5:
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENGECAQKK
IIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKALK
ALPMHIRLSFNPTQLEEQCHV.

SEQ ID NO. 6:
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENGECAQKK
IIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEKAWPASAWVRTPEVDDEALEKFDKAL
KALPMHIRLSFNPTQLEEQCHI.

From Capra hircus: SEQ ID NO. 7 (P02756 and A5JSR7 (aa 1-151)):

IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKK
IIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDKALK
ALPMHIRLAFNPTQLEGQCHV.

From Ovis aries (P67976) and Ovis orientalis (P67975): SEQ ID NO. 8:

IIVTQTMKGLDIQKVAGTWHSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQK KIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDNEALEKFDKAL KALPMHIRLAFNPTQLEGQCHV.

From Rangifer tarandus: SEQ ID NO. 9 (Q00P86):

IIVTQTMKDLDVQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPGGDLEILLQKWENGKCAQK KIIAEKTEIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDDEAMEKFDKAL KALPMHIRLSFNPTQLEEQCRV.

The amino acids letter code is given below.

A	ALA	Alanine
R	ARG	Arginine
D	ASP	Aspartic acid
N	ASN	Asparagine
C	CYS	Cysteine
E	GLU	Glutamic acid
Q	GLN	Glutamine
G	GLY	Glycine
H	HIS	Histidine
I	ILE	Isoleucine
L	LEU	Leucine
K	LYS	Lysine
M	MET	Methionine
F	PHE	Phenylalanine
P	PRO	Proline
S	SER	Serine
T	THR	Threonine
W	TRP	Tryptophan
Y	TYR	Tyrosine
V	VAL	Valine

Synthetic peptides derived from BLG

At the start of the work that led to the present invention, it was not known whether specific peptides or peptides of a certain size and/or comprising certain linear stretches could contain tolerogenic capacity when administered alone or together with intact BLG. From the inventors own earlier investigations (Bøgh *et al*. (2013), *supra*), it was shown that a partially digested BLG comprising complexed (aggregated) peptides together with a small amount of remaining intact BLG could induce tolerance mechanisms to intact BLG in rats. However, as most of the peptides resulting from the simulated gastric and intestinal degradation were complexed in different aggregates they were unavailable for further characterisation and studies of properties of the individual peptides and their possible

involvement in the observed effect. Thus, as a first approach to further explore properties of peptides obtainable from intact BLG, three different designs of peptides representing the entire length of the primary sequences of the intact BLG molecule (SEQ ID NO. 1) were chosen for further investigation (Table 4; Figure 2): 1) 11 peptides of 14/15 amino acids residues from SEQ ID NO. 1; 2) seven peptides of 23/24 amino acids residues from SEQ ID NO. 1; and 3) five peptides of 32/33 amino acid residues from SEQ ID NO. 1.

The first pool of peptides for use in the test design to study anti-allergenic properties of BLG peptides comprised synthetic peptides No. 1-11 (SEQ ID NO: 28-38) containing 14 or 15 amino acids from intact BLG, the second pool comprised synthetic peptides No. 12-18 (SEQ ID NO: 39-45) containing 23 or 24 amino acids from intact BLG, and the third pool comprised synthetic peptides No. 19-23 (SEQ ID NO: 10/46, 14, 17, 19 and 22) containing 32 or 33 amino acids from intact BLG. Three lysine residues have been added to 6 of the synthesised peptides for solubility reasons.

Alternative peptides to the peptides in Table 3 and Figure 2 may be derived in a similar way from SEQ ID NOs. 2-9 or from a sequence with at least 90 %, preferably at least 93, 95, 97 or 99 % identity to SEQ ID NO. 1.

By testing all peptides from the three pools of synthesised peptides, it turned out that peptides from the first two pools with the shortest peptides, contrary to peptides from the third pool with the longest peptides, were not able to reduce the allergenicity/sensitising capacity of BLG in the test design (Example 1). Following this, it was concluded that only peptides of a certain minimum size (> 23/24 amino acids) are able to induce the desired effect of inducing desensitisation or tolerance to BLG.

Accordingly, the invention concerns the use of one or more peptides having a certain minimum length of more than 23 amino acids, i.e. comprising at least 24 consecutive amino acids from intact BLG with SEQ ID NO. 1:

LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENGECAQK
KIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKAL
KALPMHIRLSFNPTQLEEQCHI,

or from a sequence with at least 90%, preferably at least 93, 95, 97 or 99 % identity to SEQ ID NO. 1 in a composition for desensitisation or induction of tolerance to milk BLG in a human or animal.

Illustrative of peptides for use in the present invention taken from SEQ ID NO. 1 are the following which have been produced synthetically for this study (see Figure 2):

LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL (SEQ ID NO. 10; 32 amino acids),
DAQSAPLRVYVEELKPTPEGDLEILLQKWENG (SEQ ID NO. 14; 32 amino acids),
TDYKKYLLFCMENSAEPEQSLACQCLVRTPEVD (SEQ ID NO. 19; 33 amino acids) and
DEALEKFDKALKALPMHIRLSFNPTQLEEQCHI (SEQ ID NO. 22; 33 amino acids).

The peptide or peptides for use in the invention may contain 24, 25, 26, 27, 28, 29, 30, 31, 32 or 33 consecutive amino acids from one of the sequences according to BLG SEQ ID NOs. 10, 14, 19, 22 or a sequence containing 24, 25, 26, 27, 28, 29, 30, 31, 32 or 33 consecutive amino acids from an equivalent sequence taken from a BLG sequence with at least 90%, preferably at least 93, 95, 97 or 99 % identity to SEQ ID NO. 1.

Alternatively, the peptide or peptides for use in the invention may contain 24, 25, 26, 27, 28, 29, 30, 31, 32 or 33 consecutive amino acids spanning two adjacent sequences selected from SEQ ID NOs. 10, 14, 17, 19 and 22.

More alternatively, the peptide or peptides for use in the invention may contain more than 33 consecutive amino acids spanning two or more adjacent sequences selected from SEQ ID NOs. 10, 14, 19 and 22.

The peptide or peptides for use in the invention may contain between 24 and 75 consecutive amino acids from BLG, such as 24, 25, 26, 27, 28, 29, 30, 31, 32 or 33 amino acids or more than 33 amino acids, such as 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 59, 59, 60, 61, 62, 63, 64, 65, 70 or 75 consecutive amino acids sequences selected from BLG SEQ ID NOs. 1.

It is to be understood that by the term "the peptide or peptides for use in the invention may contain 24, 25, .. etc. consecutive amino acids sequences selected from .." is meant that the peptide containing 24, 25, .. etc. consecutive amino acids may start from any one of the first 138 amino acids in SEQ ID NO. 1:
LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENGECAQK
KIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKAL
KALPMHIRLSFNPTQLEEQCHI,

or from a sequence with at least 90%, preferably at least 93, 95, 97 or 99 % identity to SEQ ID NO. 1.

The present studies including the synthetic peptides have shown that peptide 19 with the sequence (KKK)LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL (SEQ ID NO. 10/46) and peptide 20 (DAQSAPLRVYVEELKPTPEGDLEILLQKWENG; SEQ ID NO. 14) possess particularly beneficial properties in the form of being able to reduce specific IgE responses against intact BLG when used together with intact BLG. The smaller synthetic peptides embedded in SEQ ID NO. 10, i.e. LIVTQTMKGLDIQK (Peptide 1; SEQ ID NO. 28), (KKK)VAGTWYSLAMAASDA (peptide 2; SEQ ID NO. 29) and (KKK)LIVTQTMKGLDIQKVAGTWYSLA (peptide 12; SEQ ID NO. 39) did not show similar beneficial properties when tested alone or together with intact BLG. Peptide 22 (TDYKKYLLFCMENSAEPEQSLACQCLVRTPEVD; SEQ ID NO. 19) and peptide 23 (DEALEKFDKALKALPMHIRLSFNPTQLEEQCHI; SEQ ID NO. 23) showed minor reductions in specific IgE responses against intact BLG when used together with intact BLG and optionally peptide 19 and/or peptide 20, but not when used alone.

When peptide 21 with the sequence ECAQKKIIAEKTKIPAVFKIDALNENKVLVLD (SEQ ID NO. 17) was co-administered with intact BLG, IgE antibodies were induced against intact BLG and all the tested long peptides, thus suggesting that peptide 21 and peptides comprising peptide 21 or parts of the peptide 21 sequence inhere an allergenic capacity to induce specific IgE responses against different parts of intact BLG, both present in intact BLG and in certain fragments/peptides derived from intact BLG. None of the other tested long peptides seem to have a similar peptide-specific IgE inducing effect as peptide 21. Peptide 21 thus presents or includes a sequence of consecutive amino acids from BLG which comprises a stretch of amino acids involved in the development of sensitivity toward BLG and thus in developing allergic reactions upon intake of milk, milk products or other products containing BLG. It is clear that in order to possess the IgE inducing properties observed for peptide 21, fragments of peptide 21, i.e. peptides derived from intact BLG comprising said "second sequence" from peptide 21 having the adverse allergenicity-inducing properties, need to have a certain minimum size, such as at least 8 amino acids, for example at least 8, 9, 10, 11, 12, 13, 14, 15 or 16 amino acids taken from peptide 21. Peptides 6 and 15 are examples of fragments from peptide 21, while peptides 5 and 7 are examples of peptides that comprise fragments from peptide 21. Thus, in a preferred embodiment of the present invention, the composition is devoid of any peptides consisting of from 8 to 32 consecutive amino acids from peptide 21, or comprising from 8-32 consecutive amino acids from peptide 21.

Accordingly, in a preferred embodiment, the composition according to the present invention does not contain any peptides comprising the sequence ECAQKKIIAEKTKIPAVFKIDALNENKVLVLD (SEQ ID NO. 17) or a sub-sequence thereof or a

peptide comprising the sequence or a sub-sequence thereof having adverse allergenicity-inducing properties as discussed above.

Accordingly, preferred peptides for use in the present invention taken from SEQ ID NO. 1 are selected from the following (see Figure 2):

LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL (SEQ ID NO. 10),
DAQSAPLRVYVEELKPTPEGDLEILLQKWENG (SEQ ID NO. 14),
TDYKKYLLFCMENSAEPEQSLACQCLVRTPEVD (SEQ ID NO. 19) and
DEALEKFDKALKALPMHIRLSFNPTQLEEQCHI (SEQ ID NO. 22),

or any peptide(s) with a sub-sequence thereof having more than 23 amino acids, or any longer peptide including all or a part of any of these sequences as explained above.

More preferred peptides for use in the present invention taken from SEQ ID NO. 1 are selected from the following two peptides (see Figure 2):

LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL (SEQ ID NO. 10) and
DAQSAPLRVYVEELKPTPEGDLEILLQKWENG (SEQ ID NO. 14),

or any peptide with a sub-sequence thereof having more than 23 amino acids, or any longer peptide including all or a part of any of these sequences as explained above.

As part of the invention, SEQ ID NO. 1 can be substituted by any one of SEQ ID NOs. 2 to 9; SEQ ID NO. 10 can be substituted by any one of SEQ ID NOs. 1-13; SEQ ID NO. 14 can be substituted by SEQ ID NOs. 15 or 16; SEQ ID NO. 17 can be substituted by SEQ ID NO. 18; SEQ ID NO. 19 can be substituted by SEQ ID NOs. 20 or 21; and SEQ ID NO. 22 can be substituted by any one of SEQ ID NOs. 23-27, in all of the embodiments concerning SEQ ID NO. 1 and peptides derived therefrom as disclosed above.

IIVTQTMKGLDIQKVAGTWYSLAMAASDISLL (SEQ ID NO. 11),
IIVTQTMKGLDIQKVAGTWHSLAMAASDISLL (SEQ ID NO. 12),
IIVTQTMKDLDVQKVAGTWYSLAMAASDISLL (SEQ ID NO. 13)

DAQSAPLRVYVEELKPTPEGNLEILLQKWENG (SEQ ID NO. 15),
DAQSAPLRVYVEELKPTPGGDLEILLQKWENG (SEQ ID NO. 16),

KCAQKKIIAEKTEIPAVFKIDALNENKVLVLD (SEQ ID NO. 18),

TDYKKYLLFCMENSAEPEQSLVCQCLVRTPEVD (SEQ ID NO. 20),
TDYKKYLLFCMENSAEPEKAWPASAWVRTPEVD (SEQ ID NO. 21),

DEALEKFDKALKALPMHIRLSFNPTQLEGQCHI (SEQ ID NO. 23)
DEALEKFDKALKALPMHIRLSFNPTQLEEQCHV (SEQ ID NO. 24)
KEALEKFDKALKALPMHIRLAFNPTQLEGQCHV (SEQ ID NO. 25),
NEALEKFDKALKALPMHIRLAFNPTQLEGQCHV (SEQ ID NO. 26),
DEAMEKFDKALKALPMHIRLSFNPTQLEEQCRV (SEQ ID NO. 27).

In a preferred embodiment of the present invention, the composition for desensitisation or induction of tolerance to the milk protein beta-lactoglobulin (BLG) in a human or animal comprises a mixture of isolated intact BLG and one or more peptides derived from said intact BLG, wherein

a) the intact BLG has the amino acid sequence SEQ ID NO. 1:

LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENGECAQK KIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKAL KALPMHIRLSFNPTQLEEQCHI, or a sequence with at least 90 %, preferably 93, 95, 97 or 99 % identity to SEQ ID NO. 1,

b) the one or more peptides consist(s) of between 24 and 75, such as between 24 and 32 consecutive amino acids from the intact BLG and comprises as least 24 consecutive amino acids selected from the sequences:

a. LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL (SEQ ID NO. 10),

b. DAQSAPLRVYVEELKPTPEGDLEILLQKWENG (SEQ ID NO. 14), and

c. a sequence equivalent to sequence a or b taken from an amino acid sequence with at least 90 %, preferable 93, 95, 97 or 99 % identity to SEQ ID NO. 1., and wherein

c) said composition is devoid of any peptides comprising at least 8, such as at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 consecutive amino acids from the amino acid sequence ECAQKKIIAEKTKIPAVFKIDALNENKVLVLD (SEQ ID NO. 17) or from a sequence equivalent with SEQ ID NO. 17 taken from an amino acid sequence with at least 90 %, preferable 93, 95, 97 or 99 % identity to SEQ ID NO. 1.

The invention further comprises the use of peptides as defined above wherein one or more of the amino acids is or are substituted by conservative amino acid(s).

By a conservative substitution is meant that one amino acid is replaced with another which is similar in size and chemical properties (see Table 3). Such conservative amino acid

substitutions may have minor effects on protein structure and can thus be tolerated without compromising function.

Residue	Conservative Substitutions	Residue	Conservative Substitutions
Ala	Ser	Leu	Ile; Val
Arg	Lys	Lys	Arg; Gln
Asn	Gln; His	Met	Leu; Ile
Asp	Glu	Phe	Met; Leu; Tyr
Gln	Asn	Ser	Thr; Gly
Cys	Ser	Thr	Ser; Val
Glu	Asp	Trp	Tyr
Gly	Pro	Tyr	Trp; Phe
His	Asn; Val	Val	Ile; Leu
Ile	Leu; Val		

Table 2. Conservative amino acid substitutions

The peptide(s) for use in a composition according to the first aspect of the invention are one or more synthetic peptides.

In the second aspect of the invention, the peptides for use in a composition according to the invention are one or more peptides, such as a mixture or fraction containing two or more peptides isolated from a controlled hydrolysis of isolated BLG, or mixture of milk proteins, e.g. whey proteins, such as WPC or WPI. It is preferred to use synthetic peptides in treatment of patients with milk allergy due a very low risk of the composition comprising other or remaining unwanted milk proteins which may present a serious risk when treating already affected and allergic patients. Peptides or mixed peptide isolates obtained from hydrolysis of BLG are less expensive to produce than synthetic peptides and do not present a serious risk for humans or animals in preventive treatment, i.e. in inducing tolerance to subjects not being allergic to the BLG. Peptides isolated from hydrolysed milk proteins, e.g. BLG, are thus ideal for use in infant formulas, nutritional foods, beverages, white milk, etc. It is surprising that the components of the present composition can "survive" an oral administration to allow treatment or prevention of milk allergy.

Peptide synthesis

Synthetic peptides for use in the invention may be synthesised in any suitable way for the present purpose, as the synthesis is not critical for the intended use. The skilled person is aware of different routes for synthesis of the peptides as discussed herein. As an example, the peptides may be synthesised by coupling the carboxyl group or C-terminus of one amino acid to the amino group or N-terminus of another. Due to the possibility of unintended reactions, protecting groups are usually necessary. Alternatively, desirable peptides may be produced recombinantly.

Options to Improve Solubility

A potential problem in formulating peptide compositions, e.g. peptide vaccines, is the peptide's solubility in appropriate solvents, most preferably in aqueous solutions (e.g. pure water or physiologically acceptable buffers). The chemical and physical properties of peptides are dictated by their amino acid composition. Although some peptides easily dissolve in aqueous solutions, a common problem encountered is very low solubility or even insolubility, especially for peptides with long sequences of hydrophobic amino acids.

Peptide solubility	
Hydrophilic	D, E, H, K, N, Q, R, S, T,
Intermediate	C, G
Hydrophobic	A, F, I, L, M, P, V, W, Y
Peptide Charge	
Positive	K, R, H, peptide N-terminus
Negative	D, E, Y, peptide C-terminus

Table 3. Physical properties of amino acids.

Hydrophilic peptides containing >25% charged residues (e.g., D, K, R, H and E) and 25% hydrophobic amino acids are usually soluble in water or aqueous buffers.

Peptides containing 50% and more hydrophobic residues might be insoluble or only partly soluble in aqueous solutions. In such cases, organic solvents may be used. Alternatively, for some peptides, it is possible to add a set of polar residues to improve solubility in an aqueous solution or pure water.

Accordingly, the invention also relates to the use of peptides as discussed above, further comprising 1, 2, 3, 4 or 5 amino acids added to the N- and/or C-terminal end for increasing the solubility of the peptide in solution. For some peptides, it is possible to arbitrarily add a set of polar residues to improve solubility. For acidic peptides, 1 to 4 glutamic acid (E) residues may be added to the N or C terminus and for basic peptides 1 to 4 lysine (K) residues may be added to the N or C terminus. For this reason, the synthetic peptides Nos. 2 (SEQ ID NO. 29), 3 (SEQ ID NO. 30), 9 (SEQ ID NO. 36), 12 (SEQ ID NO: 39), 14 (SEQ ID NO. 41), and 19 (SEQ ID NO. 46) all comprise 3 lysine residues at the N-terminal end of the respective peptides from SEQ ID NO. 1. Alternative residues for addition to one of the ends are known in the art. Other peptides for use in the invention may need addition of different amino acids. Obviously if the N and C termini cannot be altered, this approach should not be applied.

Peptide solubility may also be improved by changing one or more residues within the sequence. Often, a single replacement can dramatically improve solubility and that change may be relatively conservative; for example replacing alanine with glycine. Accordingly, the peptides of the invention may comprise 1, 2, 3 or 4 amino acid substitutions for increasing the solubility of the peptide in solution.

For sequences that contain a large number of hydrophobic residues such as Trp, Phe, Val, Ile, Leu, Met, Tyr and Ala solubility problems may be seen in peptides where >50% of the residues are these hydrophobic amino acids. In order to increase the polarity of the peptide, it may be useful to lengthen the sequence, provided the added amino acids increase peptide polarity. Alternatively, the sequence may be shortened to eliminate hydrophobic residues and hence increase peptide polarity. The more polar the peptide, the more likely it is to be soluble in aqueous solutions.

Peptides from BLG obtained by hydrolysis

Peptides for use in the composition of the present invention may be obtained by isolation of single peptides or mixtures or fractions of peptides after hydrolysis and fractionation of BLG purified from milk products, such as intact BLG purified from a whey protein concentrate (WPC) or a whey protein isolate (WPI). Alternatively, the peptides may be obtained by isolation of single peptides or mixtures or fractions of peptides after hydrolysis and fractionation of proteins in a milk product, such as for example by hydrolysis and fractionation of WPC or WPI. Whey proteins typically consist of about 65% BLG, about 25% alpha-lactalbumin, about 8% bovine serum albumin and about 2% other proteins, including immunoglobulins, lactoferrin, transferrin and membrane proteins. A typical WPC obtained

from sweet whey, for example WPC80, comprises about 50% BLG, about 18% alpha-lactalbumin, about 18% caseinomacropeptide, about 5% bovine serum albumin, about 3% immunoglobulins and about 6% other proteins. WPC from acid whey contains no caseinomacropeptide, but is otherwise similar to WPC from sweet whey, with the content of the different proteins being proportional larger. Whey and WPC can be fractionated into WPI with no fat and depending on the process comprise from 50-90% BLG.

For the hydrolysis step, preferably one or more suitable enzyme(s), i.e. more or more proteases, is/are used. The enzyme(s) may be specific or unspecific and be selected in accordance with the desired result to be achieved, for example a desired length of the resulting peptides and/or conservation or breaking of a specific stretch or stretches of amino acids in the protein. Proteases are involved in digesting long protein chains into shorter fragments by splitting the peptide bonds that link amino acid residues. Some detach the terminal amino acids from the protein chain (exopeptidases, such as aminopeptidases, carboxypeptidase A); others attack internal peptide bonds of a protein (endopeptidases, such as trypsin, chymotrypsin, pepsin, papain, elastase). Suitable proteases may be selected among different classes, such as serine proteases, threonine proteases, cysteine proteases, aspartate proteases, glutamic acid proteases and metalloproteases. Many proteases are commercially available, such as for example Alcalase™, a protease from *Bacillus licheniformis*, and Neutrase™, a protease from *Bacillus amyloliquefaciens*, both being available from Novozymes, Denmark. A combination of two or more enzymes may be beneficial in obtaining peptides of a specific length and/or including or eliminating a specific stretch of the protein in the resulting peptide(s). Suitable proteases may be selected in accordance with a desired cleavage site or sites present in the protein. In a preferred embodiment, the enzyme(s) and conditions are selected in such a way to facilitate hydrolysis peptides comprising the undesirable stretch present in peptide 21 as discussed above.

Hydrolysis condition, including time and temperature may be varied in order to achieve a desired rate of hydrolysis, for example if full hydrolysis or partial hydrolysis is desired. Selection of useful enzyme(s) and hydrolysis conditions vis-à-vis hydrolysis of a protein with a known amino acid sequence are known in the art.

After hydrolysis, the mixture of resulting different peptides, remaining intact BLG and eventually other whey proteins can be separated in order to isolate one or more peptides or fractions containing peptides with the desired size and properties for use in the present invention, i.e. peptides of a certain minimum length and or peptide with or without a certain amino acid stretch. Separation methods such as filtration, e.g. ultrafiltration and diafiltration

may be applied. Cut-off values for the selected membrane are selected according to the size (length) of the desired peptides. Membrane material may influence the separation efficiency of the peptide mixture and should therefore be taken into consideration, as the different peptides may have different properties apart from length, such as hydrophobicity, charge and degree of aggregation. Selection of useful separation methods, including ultrafiltration and selection of membrane, vis-à-vis separation of hydrolysed BLG protein are within the knowledge of the skilled person and known in the art. Alternative or in addition, gel-filtration and/or HPLC separation may be applied.

An example illustrating the hydrolyzation of BLG from WPI raw material is shown in Example 4. The fraction obtained in example 4 comprised the following peptides:

- LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY
- LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLR
- LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPL
- LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQ
- LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL
- TMKGLDIQKVAGTWYSLAMAASDISLL
- DIQKVAGTWYSLAMAASDISLL
- DTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDDE
- DTDYKKYLLFCMENSAEPEQS
- DTDYKKYLLFCMENSAEPE
- NSAEPEQSLACQCLVRTPEVDDE.

In a preferred method, an ultrafiltration (UF)-retentate containing large peptides (> 24 aa) is obtained as a mixture of large peptides, complexes of smaller peptides and/or larger peptides and intact whey protein, including intact BLG. This mixture may be used as it is, concentrated or dried and used in for example in infant formulas. However, an analysis of the content or ratio of large peptides to intact BLG in the mixture need to be made in order to secure a desirable and reproducible amount of large BLG-derived peptides and intact BLG. Amounts of large BLG-derived peptides or intact BLG may be added to the mixture in order to obtain the desired amount and/or ratio. The retentate can also be subjected to further treatment steps in order to remove some or all of the intact BLG and/or smaller peptides from complexes and/or complexes. Complexes may be removed or suitable additives may be used to break the complexes followed by filtration according to size to concentrate the larger BLG-derived Peptides with less or no small BLG-derived peptides present. Additional filtering and/or different chromatography methods, including ion-, exclusion-, etc. may be used in order to purify the fraction of large peptides.

The skilled person would know how to vary the starting material and different steps and ingredient based on the example and common general knowledge.

Composition

The peptide or peptides as disclosed above is or are solubilised in a composition that may be used directly or formulated together with additional ingredient, for desensitisation or tolerance induction to the milk protein BLG in a human or animal, for prevention or treatment of milk protein allergy.

The composition comprises 1, 2, 3, 4, 5 or more than 5 different peptides on an individual basis or as a mixture of peptides selected according to size (length) and/or presence or absence of a particular amino acid sequence as disclosed above. The peptides may be synthetic or in the form of a hydrolysis fraction as disclosed above. The peptides may cover all or part(s) of the intact BLG. The composition will further comprise intact BLG which may be isolated and/or purified from milk, e.g. bovine milk, before being mixed with the peptides. The intact BLG may preferably be added to and mixed with the peptide or mixture of peptides. If the peptides isolated from hydrolysis of BLG for use in mixing with intact BLG do not contain any intact whey proteins, including intact BLG, alpha-lactalbumin, bovine serum albumin or immunoglobulin, which have survived hydrolysis, the composition according to the present invention does not contain any other intact milk proteins than the added intact BLG. The content (amount and identity of the peptides or fraction) of both peptide(s) and intact BLG may therefore be controlled in the composition. If the peptides isolated from hydrolysis of BLG for use in mixing with intact BLG contain remaining intact whey proteins, including intact BLG and optionally intact alpha-lactalbumin, bovine serum albumin and/or immunoglobulin, which have survived hydrolysis, the composition according to the present invention will contain such intact milk proteins. Pure intact BLG may be added if desired to obtain a predetermined amount. The content (amount and identity of the peptides or fraction) of both peptide(s) and intact BLG may therefore be controlled in the composition in different ways.

The composition wherein the total amount of BLG-derived peptide(s) consisting of between 24 and 75 consecutive amino acids from said intact BLG constitutes more than than 40 wt%, 45 wt%, 50 wt%, 60 wt%, 70 wt%, 80 wt% or more than 90 wt% of the total amount of BLG-derived peptides in the composition

The weight ratio between intact BLG and BLG-derived peptides is preferably 1:99, 2:98, 5:95, 10:90, 15:85, 20:80, 25:75 or 30:70. A preferred ratio is between 5:95 and 25:75 (w/w) or between 5:95 and 20:80 (w/w). In one specific example, the ratio is between 10:90 and 15:85. However, optimal ratios may be dependent on the peptide(s) used in the composition, leading to variations in general ratio. Additives used in the compositions may also influence the optimal ration between intact BLG and BLG-derived peptides.

For injective application of the composition according to the present invention, an adjuvant may be used, such as inorganic compounds, mineral oil, bacterial products, nonbacterial organics, delivery systems, cytokines, nucleotide sequences, sugars or combinations thereof. The skilled person would know how to optimise any use of an adjuvant. Adjuvants are known in the art.

Yet an aspect of the invention pertains to a protein/peptide mixture comprising at total amount of intact BLG and BLG-derived fragments having a length of at least 24 consecutive amino acids of at least 25% (w/w) relative to the total amount of protein and peptide in the mixture, preferably at least 50% (w/w) and even more preferably at least 70% (w/w). Other proteins and peptides may for example be casein, plant proteins and the like and peptides thereof.

For example, the protein/peptide mixture may comprise at total amount of intact BLG and BLG-related fragments having a length of at least 24 consecutive amino acids of at least 80% (w/w) relative to the total amount of protein and peptide, preferably at least 90% (w/w) and even more preferably at least 90% (w/w).

The protein/peptide mixture preferably comprise a total amount of protein and peptide of at least 50% (w/w dry weight), preferably at least 70% (w/w dry weight), and even more preferably at least 80% (w/w dry weight). For example, the protein/peptide mixture may comprise a total amount of protein and peptide of at least 85% (w/w dry weight), preferably at least 90% (w/w dry weight), and even more preferably at least 95% (w/w dry weight).

Food products:

Another aspect of the invention pertains to a food product comprising a total amount of intact BLG and BLG-derived fragments having a length of at least 24 consecutive amino acids of at least 0.01% (w/w), preferably at least 0.1% (w/w) and even more preferably at least 1% (w/w). The food product should contain the composition according to the present invention as the only sources of intact BLG.

For example, the food product may comprise a total amount of intact BLG and BLG-derived fragments having a length of at least 24 consecutive amino acids in the range of 0.01-10% (w/w), preferably in the range of 0.1-5% (w/w), and even more preferably at least 1-4% (w/w).

Alternatively, the food product may comprise a total amount of intact BLG and BLG-derived fragments having a length of at least 24 consecutive amino acids in the range of 0.01-2% (w/w), preferably in the range of 0.05-1% (w/w), and even more preferably at least 0.1-0.5% (w/w).

The food product may for example be a dairy product. In some preferred embodiments of the invention the food product is a dairy product, such as e.g. a liquid white milk, a liquid flavoured milk, or a milk powder. In some pepi the dairy product is an acidified dairy product such as e.g. a yoghurt-types product.

In the context of the present invention, the term "liquid white milk" pertains to a liquid having the visual appearance, and preferably also substantially the same sensory characteristics, of a bovine liquid milk having a fat content of 0.01-5% (w/w), such as e.g. skim milk, semi-skim milk, or whole milk . For example, the liquid white milk may have the same chemical composition as bovine liquid milk having a fat content of 0.01-5% (w/w) except for a reduced level of intact BLG and the presence of one or more BLG-derived fragments as described herein.

The liquid white milk may for example comprise at most 5% w/w milk fat. An example of such a liquid white milk is full fat milk or whole milk which typically contains 2-4% w/w milk fat, and preferably approx. 3% w/w milk fat.

Alternatively, the liquid white milk may comprise at most 2% w/w milk fat. An example of such a liquid white milk is semi-skim milk which typically contains 0.7-2% w/w milk fat, and preferably 1-1.5% w/w milk fat.

For example, the liquid white milk may comprise at most 0.7% w/w milk fat. An example of such a liquid white milk is skim milk which normally contains 0.01-0.7% w/w milk fat, and preferably 0.1-0.6% w/w milk fat, such as e.g. approx. 0.1-0.2% w/w milk fat.

In some preferred embodiments of the invention, the liquid white milk is a lactose-reduced milk.

In the context of the present invention, the term "lactose-reduced milk" relates to a liquid milk comprising at most 0.5 g lactose per kg milk. It may even be preferred that the liquid

white milk is lactose-free. In the context of the present invention, the term "lactose-free milk" relates to a milk comprising at most 0.05 g lactose per kg milk.

In some preferred embodiments of the invention, the liquid white milk comprises 1-6% w/w casein, 0.1-5% w/w milk serum protein, and 0.01-5% w/w milk fat. For example, the liquid white milk may comprise 2-5% w/w casein, 0.2-4% w/w milk serum protein, and 0.1-1% w/w milk fat.

In some preferred embodiments of the invention the food product is a nutritional composition.

Typically the nutritional composition comprises carbohydrate, protein, and fat, e.g. provided by a carbohydrate source, a protein source and/or protein equivalent source, and a fat source.

In certain embodiments, the nutritional composition is a pediatric nutritional composition, such as an infant formula or a growing-up milk, an adult nutritional composition, or a nutritional supplement.

"Nutritional composition" means a substance or formulation that satisfies at least a portion of a subject's nutrient requirements. The terms "nutritional(s)", "nutritional formula(s)", "enteral nutritional(s)", and "nutritional supplement(s)" are used as non-limiting examples of nutritional composition(s) throughout the present disclosure. Moreover, "nutritional composition(s)" may refer to liquids, powders, gels, pastes, solids, concentrates, suspensions, or ready-to-use forms of enteral formulas, oral formulas, formulas for infants, formulas for pediatric subjects, formulas for children, growing-up milks and/or formulas for adults.

The term "enteral" means deliverable through or within the gastrointestinal, or digestive, tract. "Enteral administration" includes oral feeding, intragastric feeding, transpyloric administration, or any other administration into the digestive tract.

"Administration" is broader than "enteral administration" and includes parenteral administration or any other route of administration by which a substance is taken into a subject's body.

The term "medical food" refers enteral compositions that are formulated or intended for the dietary management of a disease or disorder. A medical food may be a food for oral ingestion or tube feeding (nasogastric tube), may be labeled for the dietary management of

a specific medical disorder, disease or condition for which there are distinctive nutritional requirements, and may be intended to be used under medical supervision.

The term "protein equivalent" or "protein equivalent source" includes any protein source, such as soy, egg, whey, or casein, as well as non-protein sources, such as peptides or amino acids. Further, the protein equivalent source can be any used in the art, e.g., nonfat milk, whey protein, casein, soy protein, hydrolysed protein, peptides, amino acids, and the like. Bovine milk protein sources useful in practicing the present disclosure include, but are not limited to, milk protein powders, milk protein concentrates, milk protein isolates, nonfat milk solids, nonfat milk, nonfat dry milk, whey protein, whey protein isolates, whey protein concentrates, sweet whey, acid whey, casein, acid casein, caseinate (e.g. sodium caseinate, sodium calcium caseinate, calcium caseinate), soy bean proteins, and any combinations thereof. The protein equivalent source can, in some embodiments comprise hydrolysed protein, including partially hydrolysed protein and extensively hydrolysed protein. The protein equivalent source may, in some embodiments, include intact protein. The composition of the present invention should however be the only source of intact BLG.

The term "protein equivalent source" also encompasses free amino acids. In some embodiments, the amino acids may comprise, but are not limited to, histidine, isoleucine, leucine, lysine, methionine, cysteine, phenylalanine, tyrosine, threonine, tryptophan, valine, alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, proline, serine, carnitine, taurine and mixtures thereof. In some embodiments, the amino acids may be branched chain amino acids. In certain other embodiments, small amino acid peptides may be included as the protein component of the nutritional composition. Such small amino acid peptides may be naturally occurring or synthesised.

In some preferred embodiments of the invention, the nutritional composition, such as e.g. an infant formula, comprises a total amount of intact BLG and BLG-derived fragments having a length of at least 24 consecutive amino acids of at least 0.01% (w/w), preferably at least 0.1% (w/w) and even more preferably at least 1% (w/w).

For example, the nutritional composition may comprise a total amount of intact BLG and BLG-derived fragments having a length of at least 24 consecutive amino acids in the range of 0.01-10% (w/w), preferably in the range of 0.1-5% (w/w), and even more preferably at least 1-4% (w/w).

Alternatively, the nutritional composition may comprise a total amount of intact BLG and BLG-derived fragments having a length of at least 24 consecutive amino acids in the range

of 0.01-2% (w/w), preferably in the range of 0.05-1% (w/w), and even more preferably at least 0.1-0.5% (w/w).

"Pediatric subject" means a human less than 13 years of age. In some embodiments, a pediatric subject refers to a human subject that is between birth and 8 years old. In other embodiments, a pediatric subject refers to a human subject between 1 and 6 years of age. In still further embodiments, a pediatric subject refers to a human subject between 6 and 12 years of age. The term "pediatric subject" may refer to infants (preterm or full term) and/or children, as described below.

"Infant" means a human subject ranging in age from birth to not more than 1 year and includes infants from 0 to 12 months corrected age. The phrase "corrected age" means an infant's chronological age minus the amount of time that the infant was born premature. Therefore, the corrected age is the age of the infant if it had been carried to full term. The term infant includes low birth weight infants, very low birth weight infants, and preterm infants. "Preterm" means an infant born before the end of the 37^{th} week of gestation. "Full term" means an infant born after the end of the 37^{th} week of gestation.

"Child" means a subject ranging in age from 12 months to about 13 years. In some embodiments, a child is a subject between the ages of 1 and 12 years old. In other embodiments, the terms "children" or "child" refer to subjects that are between 1 and about 6 years old, or between about seven and about 12 years old. In other embodiments, the terms "children" or "child" refer to any range of ages between 12 months and about 13 years.

"Children's nutritional product" refers to a composition that satisfies at least a portion of the nutrient requirements of a child. A growing-up milk is an example of a children's nutritional product.

"Infant formula" means a composition that satisfies at least a portion of the nutrient requirements of an infant. Infant formulas typically contains macronutrient, vitamins, minerals, and other ingredient levels in an effort to simulate the nutritional and other properties of human breast milk.

The term "growing-up milk" refers to a broad category of nutritional compositions intended to be used as a part of a diverse diet in order to support the normal growth and development of a child between the ages of about 1 and about 6 years of age.

"Nutritionally complete" means a composition that may be used as the sole source of nutrition, which would supply essentially all of the required daily amounts of vitamins,

minerals, and/or trace elements in combination with proteins, carbohydrates, and lipids. Indeed, "nutritionally complete" describes a nutritional composition that provides adequate amounts of carbohydrates, lipids, essential fatty acids, proteins, essential amino acids, conditionally essential amino acids, vitamins, minerals and energy required to support normal growth and development of a subject.

Therefore, a nutritional composition that is "nutritionally complete" for a preterm infant will, by definition, provide qualitatively and quantitatively adequate amounts of carbohydrates, lipids, essential fatty acids, proteins, essential amino acids, conditionally essential amino acids, vitamins, minerals, and energy required for growth of the preterm infant.

A nutritional composition that is "nutritionally complete" for a full term infant will, by definition, provide qualitatively and quantitatively adequate amounts of all carbohydrates, lipids, essential fatty acids, proteins, essential amino acids, conditionally essential amino acids, vitamins, minerals, and energy required for growth of the full term infant.

A nutritional composition that is "nutritionally complete" for a child will, by definition, provide qualitatively and quantitatively adequate amounts of all carbohydrates, lipids, essential fatty acids, proteins, essential amino acids, conditionally essential amino acids, vitamins, minerals, and energy required for growth of a child.

As applied to nutrients, the term "essential" refers to any nutrient that cannot be synthesised by the body in amounts sufficient for normal growth and to maintain health and that, therefore, must be supplied by the diet. The term "conditionally essential" as applied to nutrients means that the nutrient must be supplied by the diet under conditions when adequate amounts of the precursor compound is unavailable to the body for endogenous synthesis to occur.

In some embodiments, the nutritional composition comprises between about 1 g and about 7 g of a protein equivalent source per 100 kcal. In other embodiments, the nutritional composition comprises between about 3.5 g and about 4.5 g of protein equivalent source per 100 kcal.

Additionally, the protein equivalent source including the peptide component may be added or incorporated into the nutritional composition by any method well known in the art. In some embodiments, the peptide component may be added to a nutritional composition to supplement the nutritional composition. For example, in one embodiment, the peptide component may be added to a commercially available infant formula. For example, Enfalac, Enfamil®, Enfamil® Premature Formula, Enfamil® with Iron, Enfamil® LIPIL®, Lactofree®,

Nutramigen®, Pregestimil®, and ProSobee® (available from Mead Johnson & Company, Evansville, IN, U.S.A.) may be supplemented with suitable levels of the peptide component, and used in practice of the present disclosure.

In some embodiments, the nutritional composition comprises at least one carbohydrate source. The carbohydrate source can be any used in the art, e.g., lactose, glucose, fructose, corn syrup solids, maltodextrins, sucrose, starch, rice syrup solids, and the like. The amount of the carbohydrate component in the nutritional composition typically can vary from between about 5 g/100 kcal and about 25 g/100 kcal. In some embodiments, the amount of carbohydrate is between about 6 g/100 kcal and about 22 g/100 kcal. In other embodiments, the amount of carbohydrate is between about 12 g/100 kcal and about 14 g/100 kcal. In some embodiments, corn syrup solids are preferred. Moreover, hydrolysed, partially hydrolysed, and/or extensively hydrolysed carbohydrates may be desirable for inclusion in the nutritional composition due to their easy digestibility. Specifically, hydrolysed carbohydrates are less likely to contain allergenic epitopes.

Non-limiting examples of carbohydrate materials suitable for use herein include hydrolysed or intact, naturally or chemically modified, starches sourced from corn, tapioca, rice or potato, in waxy or non-waxy forms. Non-limiting examples of suitable carbohydrates include various hydrolysed starches characterized as hydrolysed cornstarch, maltodextrin, maltose, corn syrup, dextrose, corn syrup solids, glucose, and various other glucose polymers and combinations thereof. Non- limiting examples of other suitable carbohydrates include those often referred to as sucrose, lactose, fructose, high fructose corn syrup, indigestible oligosaccharides such as fructooligosaccharides and combinations thereof.

The nutritional composition may also comprise a fat source. The term "fat" would be interpreted broadly and also include non-triglyceride lipids, such as phospholipids. Suitable fat sources for the nutritional composition of the present disclosure may be any known or used in the art, including but not limited to, animal sources, e.g., milk fat, butter, butter fat, egg yolk lipid; marine sources, such as fish oils, marine oils, single cell oils; vegetable and plant oils, such as corn oil, canola oil, sunflower oil, soybean oil, palm olein oil, coconut oil, high oleic sunflower oil, evening primrose oil, rapeseed oil, olive oil, flaxseed (linseed) oil, cottonseed oil, high oleic safflower oil, palm stearin, palm kernel oil, wheat germ oil; medium chain triglyceride oils and emulsions and esters of fatty acids; and any combinations thereof.

In some embodiment the nutritional composition comprises between about 1.3 g/100 kcal to about 7.2 g/100 kcal of a fat source. In other embodiments the fat source may be

present in an amount from about 2.5 g/100 kcal to about 6.0 g/100 kcal. In still other embodiments, the fat source may be present in the nutritional composition in an amount from about 3.0 g/100 kcal to about 4.0 g/100 kcal.

The nutritional composition of the present disclosure may also contain a source of long chain polyunsaturated fatty acids ("LCPUFAs"). Suitable LCPUFAs include, but are not limited to DHA, eicosapentaenoic acid ("EPA"), ARA, linoleic (18:2 n-6), γ-linolenic (18:3 n-6), dihomo- γ-linolenic (20:3 n-6) acids in the n-6 pathway, a-linolenic (18:3 n-3), stearidonic (18:4 n-3), eicosatetraenoic (20:4 n-3), eicosapentaenoic (20:5 n-3), and docosapentaenoic (22:6 n-3). [0098] The amount of LCPUFA in the nutritional composition is at least about 5 mg/100 kcal, and may vary from about 5 mg/100 kcal to about 100 mg/100 kcal, more preferably from about 10 mg/100 kcal to about 50 mg/100 kcal.

Sources of LCPUFAs include dairy products like butterfat; eggs; marine oils, such as cod, menhaden, sardine, tuna and many other fish; certain animal fats, lard, tallow and microbial oils such as fungal and algal oils, or from any other resource fortified or not, form which LCPUFAs could be obtained and used in a nutritional composition. The LCPUFA could be part of a complex mixture obtained by separation technology known in the art aimed at enrichment of LCPUFAs and the derivatives or precursors of LCPUFAs in such mixtures.

The LCPUFAs may be provided in the nutritional composition in the form of esters of free fatty acids; mono- di- and tri-glycerides; phosphor-glycerides, including lecithins; and/or mixtures thereof. Additionally, LCPUFA may be provided in the nutritional composition in the form of phospholipids, especially phosphatidylcholine.

In an embodiment, especially if the nutritional composition is an infant formula, the nutritional composition is supplemented with both DHA and ARA. In this embodiment, the weight ratio of ARA:DHA may be between about 1 :3 and about 9:1. In a particular embodiment, the weight ratio of ARA:DHA is from about 1 :2 to about 4:1.

DHA is advantageously present in the nutritional composition, in some embodiments, from at least about 17 mg/100 kcal, and may vary from about 5 mg/100 kcal to about 75 mg/100 kcal. In some embodiments, DHA is present from about 10 mg/100 kcal to about 50 mg/100 kcal.

The nutritional composition may be supplemented with oils containing DHA and/or ARA using standard techniques known in the art. For example, DHA and ARA may be added to the composition by replacing an equivalent amount of an oil, such as high oleic sunflower oil, normally present in the composition. As another example, the oils containing DHA and

ARA may be added to the composition by replacing an equivalent amount of the rest of the overall fat blend normally present in the composition without DHA and ARA.

If utilized, the source of DHA and/or ARA may be any source known in the art such as marine oil, fish oil, single cell oil, egg yolk lipid, and brain lipid. The DHA and ARA can be in natural form, provided that the remainder of the LCPUFA source does not result in any substantial deleterious effect on the infant. Alternatively, the DHA and ARA can be used in refined form.

Furthermore, some embodiments of the nutritional composition may mimic certain characteristics of human breast milk. However, to fulfill the specific nutrient requirements of some subjects, the nutritional composition may comprise a higher amount of some nutritional components than does human milk. For example, the nutritional composition may comprise a greater amount of DHA than does human breast milk. The enhanced level of DHA of the nutritional composition may compensate for an existing nutritional DHA deficit.

The nutritional composition may also contain one or more prebiotics (also referred to as a prebiotic source) in certain embodiments. Prebiotics can stimulate the growth and/or activity of ingested probiotic microorganisms, selectively reduce pathogens found in the gut, and favorably influence the short chain fatty acid profile of the gut. Such prebiotics may be naturally-occurring, synthetic, or developed through the genetic manipulation of organisms and/or plants, whether such new source is now known or developed later. Prebiotics useful in the present disclosure may include oligosaccharides, polysaccharides, and other prebiotics that contain fructose, xylose, soya, galactose, glucose and mannose.

The disclosed nutritional composition(s) may be provided in any form known in the art, such as a powder, a gel, a suspension, a paste, a solid, a liquid, a liquid concentrate, a reconstituteable powdered milk substitute or a ready-to-use product. The nutritional composition may, in certain embodiments, comprise a nutritional supplement, children's nutritional product, infant formula, human milk fortifier, growing-up milk or any other nutritional composition designed for an infant or a pediatric subject. Nutritional compositions of the present disclosure include, for example, orally-ingestible, health-promoting substances including, for example, foods, beverages, tablets, capsules and powders. Moreover, the nutritional composition of the present disclosure may be standardized to a specific caloric content, it may be provided as a ready-to-use product, or it may be provided in a concentrated form. In some embodiments, the nutritional

composition is in powder form with a particle size in the range of 5 micron to 1500 micron, such as in the range of 10 micron to 300 micron.

If the nutritional composition is in the form of a ready-to-use product, the osmolality of the nutritional composition may e.g. be between about 100 and about 1 100 mOsm/kg water, such as e.g. typically about 200 to about 700 mOsm/kg water.

In certain preferred embodiments, the nutritional composition is hypoallergenic. The nutritional composition may also be lactose-free. WO 2012/056106 discloses milk products comprising hydrolysed protein and being low in lactose.

In some preferred embodiments, e.g. in relating to infant formulas, the nutritional composition is nutritionally complete, containing suitable types and amounts of lipids, carbohydrates, proteins, vitamins and minerals to be a subject's sole source of nutrition. Indeed, the nutritional composition may optionally include any number of proteins, peptides, amino acids, fatty acids, probiotics and/or their metabolic by-products, prebiotics, carbohydrates and any other nutrient or other compound that may provide many nutritional and physiological benefits to a subject. Further, the nutritional composition of the present disclosure may comprise flavors, flavor enhancers, sweeteners, pigments, vitamins, minerals, therapeutic ingredients, functional food ingredients, food ingredients, processing ingredients or combinations thereof.

The nutritional composition of the present disclosure may be standardized to a specific caloric content, it may be provided as a ready-to-use product, or it may be provided in a concentrated form.

In some embodiments, the nutritional composition of the present disclosure is a growing-up milk. Growing-up milks are fortified milk-based beverages intended for children over 1 year of age (typically from 1 -3 years of age, from 4-6 years of age or from 1 -6 years of age). They are not medical foods and are not intended as a meal replacement or a supplement to address a particular nutritional deficiency. Instead, growing-up milks are designed with the intent to serve as a complement to a diverse diet to provide additional insurance that a child achieves continual, daily intake of all essential vitamins and minerals, macronutrients plus additional functional dietary components, such as non-essential nutrients that have purported health-promoting properties.

The exact composition of a nutritional composition according to the present disclosure can vary from market-to-market, depending on local regulations and dietary intake information of the population of interest. In some embodiments, nutritional compositions according to

the disclosure consist of a milk protein source, such as whole or skim milk, plus added sugar and sweeteners to achieve desired sensory properties, and added vitamins and minerals. The fat composition is typically derived from the milk raw materials. Total protein can be targeted to match that of human milk, cow milk or a lower value. Total carbohydrate is usually targeted to provide as little added sugar, such as sucrose or fructose, as possible to achieve an acceptable taste. Typically, Vitamin A, calcium and Vitamin D are added at levels to match the nutrient contribution of regional cow milk. Otherwise, in some embodiments, vitamins and minerals can be added at levels that provide approximately 20% of the dietary reference intake (DRI) or 20% of the Daily Value (DV) per serving. Moreover, nutrient values can vary between markets depending on the identified nutritional needs of the intended population, raw material contributions and regional regulations.

One or more vitamins and/or minerals may also be added in to the nutritional composition in amounts sufficient to supply the daily nutritional requirements of a subject. It is to be understood by one of ordinary skill in the art that vitamin and mineral requirements will vary, for example, based on the age of the child. For instance, an infant may have different vitamin and mineral requirements than a child between the ages of one and thirteen years. Thus, the embodiments are not intended to limit the nutritional composition to a particular age group but, rather, to provide a range of acceptable vitamin and mineral components.

In some preferred embodiments of the invention, the nutritional composition, e.g. an infant formula, comprises:

- between about 1 g and about 7 g of protein source or protein equivalent source per 100 kcal
- between about 5 g and about 25 g of carbohydrate component per 100 kcal
- between about 1.3 g to about 7.2 g of a fat source per 100 kcal,
- vitamins and minerals, and
- and a total amount of intact BLG and BLG-derived fragments having a length of at least 24 consecutive amino acids in the range of 0.01-10% (w/w),

wherein the weight ratio between intact BLG and BLG-derived fragments having a length of at least 24 consecutive amino acids of the nutritional composition is in the range of 1:99-30:70, such as 1:99, 2:98, 5:95, 10:90, 15:85, 20:80, 25:75 or 30:70. A preferred ratio is between 5:95 and 25:75 (w/w) or between 5:95 and 20:80 (w/w).

In some preferred embodiments of the invention, the nutritional composition is nutritionally complete for an infant.

Testing (Animal experiments)

Studying the induction of allergy is not possible in humans for ethical reasons. Therefore animal models are needed. The Brown Norway (BN) rat is a high IgE responder strain, and therefore to a certain degree resembles atopic humans in their predisposition to develop IgE-mediated allergy (type I allergy). Induction of allergy results in the generation of IgE antibodies specific to the offending protein(s). The level of specific IgE antibodies is therefore an indicator for the level of allergy. The BN rats may also be used to study the induction of tolerance and desensitisation. Here a reduction in the specific IgE antibody response is an indicator of tolerogenic capacity.

The compositions and product of the invention are for desensitisation of a patient suffering from milk allergy or for inducing tolerance to BLG in a human or animal. Thus, the compositions and product as disclosed herein are for treatment and/or prevention of milk allergy caused by the presence BLG in foods ingested by humans or animals.

Accordingly, the compositions and/or product of the invention are for oral, sublingual, intralymphatic, intramuscular, intradermal, epicutaneous, subcutaneous, intranasal, intraperitoneal or intravenous administration.

Formulation for oral administration may comprise any product normally comprising milk proteins, such as infant nutritional formulas, sport drink, protein supplement, nutritional supplement and other product for oral consumption. Preferably, the present compositions replace other milk protein components in such products.

EXAMPLES

Example 1 – I.p. immunisation of intact BLG together with different mixtures of synthetic peptides

Introduction

I.p. immunisation is the most common administration route used in order to study the inherent sensitising (allergy inducing) capacity of proteins and derivatives hereof. In this study, the sensitising capacity of intact BLG administered together with synthetic peptides representing the entire primary sequence of BLG was investigated. This was done by comparing the specific antibody responses induced by intact BLG alone with the specific antibody response induced upon administering the intact BLG together with different

mixtures of synthetic peptides. A reduced specific IgE antibody response to intact BLG, when co-administrating BLG with synthetic peptides compared to administrating intact BLG alone is an indicator of tolerance.

Methods

Intact BLG

BLG was from a pilot batch of purified BLG kindly delivered by Arla Foods Ingredients (Videbæk, Denmark).

Design of synthetic peptides

The appropriate size of peptides that may contain tolerogenic capacity, when administered together with intact allergen, is not known and may differ from protein to protein. Therefore, three different designs of peptides representing the entire primary structure of the expressed intact BLG molecule were chosen: 1) 11 peptides of 14/15 amino acids, 2) seven peptides of 23/24 amino acids, and 3) five peptides of 32/33 amino acids (Figure 2). Peptides were obtained from CASLO ApS (Kgs. Lyngby, Denmark) and in order to make all peptides soluble in buffers suitable for animal immunisation, three consecutive lysine (K) were coupled to the N-terminal of certain peptides (Table 4). This resulted in: 1) 11 peptides of 14 to 18 amino acid residues with a MW spanning from approximately 1,570 Da to 1,920 Da, 2) seven peptides of 23 to 26 amino acid residues with a MW spanning from approximately 2,450 Da to 3,000 Da, and 3) five peptides of 32 to 35 amino acid residues with a MW spanning from approximately 3,400 Da to 3,850 Da.

Synthetic peptide no. (SEQ ID NO.)	Peptide sequence[a]	Amino acid number in expressed β-lactoglobulin[b]	Number of amino acid residues
1 (28)	LIVTQTMKGLDIQK	1-14	14
2 (29)	***KKK***VAGTWYSLAMAASD*	15-28	3+14
3 (30)	***KKK***ISLLDAQSAPLRVY*	29-42	3+14
4 (31)	VEELKPTPEGDLEIL	43-57	15
5 (32)	LQKWENGECAQKKII	58-72	15
6 (33)	AEKTKIPAVFKIDAL	73-87	15

7 (34)	NENKVLVLDTDYKKY	88-102	15
8 (35)	LLFCMENSAEPEQSL	103-117	15
9 (36)	***KKK***ACQCLVRTPEVDDEA*	118-132	3+15
10 (37)	LEKFDKALKALPMHI	133-147	15
11 (38)	RLSFNPTQLEEQCHI	148-162	15
12 (39)	***KKK***LIVTQTMKGLDIQKVAGTWYSLA*	1-23	3+23
13 (40)	MAASDISLLDAQSAPLRVYVEEL	24-46	23
14 (41)	***KKK***KPTPEGDLEILLQKWENGECAQK*	47-69	3+23
15 (42)	KIIAEKTKIPAVFKIDALNENKV	70-92	23
16 (43)	LVLDTDYKKYLLFCMENSAEPEQ	93-115	23
17 (44)	SLACQCLVRTPEVDDEALEKFDK	116-138	23
18 (45)	ALKALPMHIRLSFNPTQLEEQCHI	139-162	24
19 (46)	***KKK***LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL*	1-32	3+32
20 (14)	DAQSAPLRVYVEELKPTPEGDLEILLQKWENG	33-64	32
21 (17)	ECAQKKIIAEKTKIPAVFKIDALNENKVLVLD	65-96	32
22 (19)	TDYKKYLLFCMENSAEPEQSLACQCLVRTPEVD	97-129	33
23 (22)	DEALEKFDKALKALPMHIRLSFNPTQLEEQCHI	130-162	33

Table 4. Design of synthetic peptides

[a] Peptide sequences to which three lysines (K) have been added are indicated with an asterisk.

[b] Cow's' milk beta-lactoglobulin is synthesised as a 178 amino acid protein, of which the first 16 amino acid residues correspond to a signal peptide not existing in the 162 amino acid expressed beta-lactoglobulin.

Animals

BN rats, a high IgE responder strain that to a certain degree resembles humans predisposed to develop allergy, were from an in-house breeding colony at the National Food Institute (Technical University of Denmark, Denmark), weaned at three weeks of age and then housed in macrolon cages (three per cage) with light:dark cycle, at 22 ± 1 °C and 55 ± 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 against cow's milk protein. The rat diet was produced in-house and based on rice flour, potato protein and fish meal as protein sources. Diet and acidified water were given *ad*

libitum. 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.

Animal experimental design 1

To study the ability of co-administration of intact BLG and synthetic peptides of different lengths representing the entire primary amino acid sequence of BLG, to reduce or abolish the allergy inducing capacity of BLG, an animal experiment was conducted. BN rats, 4-7 weeks of age, were allocated into nine groups. Each group consisting of 6 rats (three per. sex) were immunised i.p. without any use of adjuvant, at day 0, 14 and 28, according to Table 5. Rats were sacrificed day 35 by exsanguination using carbon dioxide inhalation as anesthesia.

Group no.	Amount of intact BLG (µg)	Amount of 14/15 amino acid long peptide mixture. Peptide no. 1-11. (µg)	Amount of 23/24 amino acid long peptide mixture. Peptide no. 12-18. (µg)	Amount of 32/33 amino acid long peptide mixture. Peptide no. 19-23. (µg)
1	-	-	-	-
2	50	-	-	-
3	50	450	-	-
4	50	-	450	-
5	50	-	-	450
6	50	225	225	-
7	50	225	-	225
8	50	-	225	225
9	50	150	150	150

Table 5. Design of animal experiment 1

Enzyme-linked immunosorbent assay (ELISA) for detection of BLG-specific IgG1

For detection of the BLG-specific IgG1 response raised against intact BLG, ELISAs were performed. Plates (96 well, microtitre, Maxisorp, Nunc, Roskilde, Denmark) were coated with 100 µL/well of 10 µg/mL antigen (intact expressed BLG) solution in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated overnight at 4 °C. Between each step, plates were washed five times in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2) 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 µL/well, were added and incubated for 1 hour at RT. For detection, 50 µL/well of HRP-labelled mouse-α-rat IgG1 (3060-05, Southern Biotech,

Birmingham, AL, USA) diluted 1:20,000 (v/v) in PBS-T was added to each well and incubated for 1 hour at RT. Reaction was visualised by adding 100 µL/well of 3,3',5,5-tetramethylbenzidine (TMB)-one substrate (Kem-En-Tec, Taastrup, Denmark) for approximately 12 min and stopped with 100 µL/well of 0.2 M H_2SO_4. 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 are expressed as the Log_2 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.

Antibody-capture ELISA for detection of BLG-specific IgE

For detection of BLG-specific IgE, antibody-capture ELISA was performed, where plates (96 well, Maxisorp, Nunc) were coated with 100 µL/well of 0.5 µg/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 hour at 37 °C in 200 µL/well of 3% (v/v) rabbit sera. Two-fold serial dilution of serum (starting at 1:8, v:v) in PBS-T, 50 µL/well, were added and incubated for 1 hour at RT. Subsequently, plates were incubated with 50 µL/well of 0.2 µg/mL of digoxigenin-coupled BLG (10:1) in 3% (v:v) rabbit sera in PBS-T for 1 hour at RT. Plates were then incubated with 50 µL/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 3% rabbit sera in PBS-T for 1 hour at RT. Reaction was visualised by adding 100 µL/well of TMB-one substrate (Kem-En-Tec) for approximately 12 min and stopped with 100 µL/well of 0.2 M H_2SO_4. Absorbance was measured at 450 nm with a reference wavelength of 630 nm, using a microtitre reader (Gen5, BioTek Instruments). IgE antibody titres are expressed as the Log_2 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.

Curve calculations and Statistical analyses

Statistical calculations were made using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, 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. 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 group of animals immunised with intact BLG alone. 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

Immunisation of BN rats with intact BLG resulted in the induction of specific IgG1 (immunogenicity) as well as IgE (allergenicity) antibodies (Figure 3). Administration of the intact BLG together with different mixtures of synthetic peptides representing the entire length of intact BLG resulted in various outcomes. Most interesting, co-administration of the intact BLG together with a mixture of the largest peptides (synthetic peptide 19 to 23) resulted in a great reduction of the allergenicity of the intact BLG (Figure 3B). This showed that a mixture of intact BLG together with five peptides of around 33 amino acids representing the entire amino acid sequence of the intact BLG molecule contained a reduced (5-fold) allergenicity compared to the same amount of intact BLG alone.

From the BLG-specific IgE response (Figure 3B) it is evident, that even though administering intact BLG together with the peptides of largest sizes (peptide 19 to 23) irrespective of the presence of smaller peptides had an effect on reducing the allergy inducing capacity of intact BLG, the group of rats which received the intact BLG together with only the peptide 19-23, had the greatest effect in reducing the BLG-specific IgE antibody response. These results clearly demonstrated the effectiveness of administrating the intact BLG together with the peptides of larges size (peptide 19 to 23), at the same time emphasising the importance of administrating the intact BLG together with only peptides of the larger sizes, as the presence of smaller peptides significantly reduced the effect of the larger peptides.

Discussion

Co-administration of intact BLG with synthetic peptides of an amino acid length of 32-33 representing the whole amino acid sequence of the intact BLG molecule, clearly shows a reduced allergenicity compared to the intact BLG alone. The present study demonstrates that synthetic peptides may indeed contain the capacity to reduce the allergenicity (IgE) of intact BLG, and that this is dependent on the length of these synthetic peptides.

Example 2 – I.p. immunisation of intact BLG together with individual synthetic peptides

Introduction

In order to investigate whether the individual synthetic peptides from the mixture of 32/33 amino acid long peptides (synthetic peptide 19-23) reduces the sensitising capacity of intact BLG when administered together with the intact allergen, a new animal study was set up. Here animals were administered with intact BLG together with the individual synthetic

peptides representing only a small part of the entire intact BLG primary sequence. A reduced specific IgE antibody response to intact BLG, when co-administrating BLG with a certain synthetic peptide compared to administrating intact BLG alone, is an indicator of tolerance.

Methods

Intact BLG

As described in example 1.

Design of synthetic peptides

As described in example 1, with the exception of only synthetic peptide 19-23 being used.

Animals

As described in example 1.

Animal experimental design 2

To study the ability of co-administration of intact BLG and individual synthetic peptides of 32/33 amino acid residue length representing 1/5 of the entire expressed amino acid sequence of BLG to reduce or abolish the allergy inducing capacity of BLG, an animal experiment was conducted. BN rats, 5-7 weeks of age, were allocated into eight groups. Each group consisting of 6 rats (three per. sex) were immunised i.p. without any use of adjuvant, at day 0, 14 and 28, according to Table 6. Rats were sacrificed at day 35 by exsanguination using carbon dioxide inhalation as anesthesia.

Group no.	Amount of intact BLG (µg)	Amount of peptide 19 (µg)	Amount of peptide 20 (µg)	Amount of peptide 21 (µg)	Amount of peptide 22	Amount of peptide 23 (µg)
1	-	-	-	-	-	-
2	50	-	-	-	-	-
3	50	90	90	90	90	90
4	50	450	-	-	-	-
5	50	-	450	-	-	-
6	50	-	-	450	-	-
7	50	-	-	-	450	-
8	50	-	-	-	-	450

Table 6. Design of animal experiment 2

ELISA for detection of BLG-specific IgG1

As described for example 1.

Antibody-capture ELISA for detection of BLG-specific IgE

As described for example 1.

Antibody-capture ELISA for detection of synthetic peptide-specific IgE

For detection of synthetic peptide-specific IgE, antibody-capture ELISAs were performed, where plates (96 well, Maxisorp, Nunc) were coated with 100 µL/well of 0.5 µg/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. For peptide 19-22 plates were blocked for 1 h at 37 °C in 200 µL/well of 1% (v:v) rabbit sera while for peptide 23 plates were blocked in 5% (v:v) rabbit sera. Two-fold serial dilution of serum (starting at 1:2, v:v) in PBS-T, 50 µL/well, were added and incubated for 1 hour at RT. Subsequently, plates were incubated with 50 µL/well of 2 µg/mL of biotin-coupled peptide 19 or 20 in PBS-T, with 50 µL/well of 0.25 µg/mL of biotin-coupled peptide 21 or 22 in 1% (v:v) rabbit sera in PBS-T, or with 50 µL/well of 0.25 µg/mL of biotin-coupled peptide 23 in 5% (v:v) rabbit sera in PBS-T for 1 hour at RT. Plates were then incubated with 50 µL/well of HRP-labelled NeutrAvidin (Pierce® High Sensitive NeutrAvidin®, 31030, Rockford, IL, USA) diluted 1:5,000 (v:v) in PBS-T for peptide 19 to 22 or in 5% (v:v) rabbit sera in PBS-T for peptide 23 for 1 h at RT. Reaction was visualised by adding 100 µL/well of TMB-one substrate (Kem-En-Tec) for approximately 12 min and stopped with 100 µL/well of 0.2 M H_2SO_4. 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 Log_2 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.

Curve calculations and Statistical analyses

As described in example 1. Asterisks indicate a statistically significant difference between the given group and the group of animals immunised with intact BLG alone.

Results

Immunisation of BN rats with intact BLG resulted in the induction of specific IgG1 (immunogenicity) as well as IgE (allergenicity) antibodies (Figure 4). Administration of the intact BLG together with the different synthetic peptides of around 33 amino acid length representing only a small part of the intact BLG primary sequence or with a mixture of these peptides representing the entire primary length of the intact BLG molecule resulted in various outcomes. As previously shown in example 1, co-administration of the intact BLG together with a mixture of the peptides (synthetic peptide 19 to 23) resulted in a reduction of the allergenicity of the intact BLG (Figure 4B). However, results showed that administering the intact BLG together with the synthetic peptide 19 were even more efficient in reducing the allergenicity of intact BLG and that administering the intact BLG together with the synthetic peptide no. 20 resulted in approximately equal efficiency in reducing the allergenicity of intact BLG. Despite the use of only few animals in each group a statistically significant difference in the BLG-specific IgE response were evident between rats immunised with BLG alone compared to administering the same amount of BLG together with peptide 19. Furthermore, for a single rat the BLG-specific IgE response was completely abolished. Though synthetic peptide 19 was the most effective peptide for reduction of the allergy inducing capacity of intact BLG, all five peptides had the ability to reduce the BLG-specific IgE antibody response when administered together with intact BLG compared to administration of intact BLG alone.

In order to investigate whether or not the synthetic peptides could induce an additional peptide specific IgE response, peptide-specific IgE ELISAs were made. From figure 5A-E, it is evident that the peptides, with the exception of the synthetic peptide 21, do not induce an additional IgE response, and that the IgE reactivity against the peptides may be a result of IgE antibodies raised against the intact BLG molecule. Co-immunisation with the synthetic peptide no. 21 did not only induce a higher IgE response towards all peptides but was also the peptide with the highest IgE antibody reactivity. On the other hand, the synthetic peptide no. 20 induced the overall lowest IgE reactivity towards the peptides.

Discussion

Results presented in this example confirmed the results presented in example 1, demonstrating that co-administration of intact BLG with a mixture of synthetic peptides representing the entire amino acid sequence of the BLG molecule, could strongly reduce the allergenicity of the intact BLG. Further results showed that a single peptide could reduce the allergenicity (IgE) of BLG, though the effect was dependent on the given peptide. For one peptide (synthetic peptide 19) the effectiveness was even more pronounced than the mixture of synthetic peptides and this to a significant level. This indicates that the entire

primary sequence of BLG is not needed to induce tolerance to BLG. The fact that it only takes a single peptide to induce a reduced allergenicity of intact BLG makes this strategy more controllable and potentially more cost-effective than with a mixture of peptides.

On the other hand, the peptide-specific IgE antibody responses showed that not all individual peptides may be suited for administration together with intact BLG, since one of the peptides (synthetic peptide 21) resulted in an enhanced peptide-specific IgE reactivity.

The potential application of this new approach in therapeutic options in food allergy may be several-fold. It gives the possibility to reduce the side effects seen during oral delivery, giving the possibility to provide larger amount of foods without causing severe allergic reaction or even the possibility to for administration of intact protein. Further as these studies are conducted in animals with no previous sensitisation to the allergen of interest, it could potentially be a strategy for primary allergy prevention, where intact protein could be administered without induction of allergy.

Example 3 – Oral administration of intact BLG and synthetic peptides

Introduction

In this study, the tolerance inducing capacity of intact BLG and individual synthetic peptides administered alone or in combination is being investigated. In order to confirm an enhanced tolerance inducing capacity of intact BLG administered together with synthetic peptides in comparison to synthetic peptide alone, as well as to investigate the impact of the number of individual synthetic peptides administered together with intact BLG, the oral tolerance inducing capacity of the synthetic peptides alone is being compared with the oral tolerance inducing capacity of co-administration of the synthetic peptides and intact BLG. This is done by administering the different products or combinations of products *ad libitum* in BN rat drinking bottles for three weeks, followed by i.p. post-immunisations with intact BLG. A reduced specific (IgE) antibody response of animals administered with a product after post-immunisations compared to animals administered with water is a proof of tolerance inducing capacity of the given product. The lower the IgE response as well as the longer the duration of the reduced IgE response, the larger the tolerogenic capacity of the product. The synthetic peptides 19 and 20 were chosen for this study due to their allergy reducing capacities.

Methods

Intact BLG

As described in example 1.

Design of synthetic peptides

Based on results from example 2, the synthetic peptides 19 and 20 were selected as model peptides. See Table 4 and Figure 2.

Animals

As described in example 1.

Animal experimental design 3

In order to study the oral tolerance inducing capacity of intact BLG and synthetic peptides either alone or in combinations as well as to investigate the impact of the number of synthetic peptides, an animal experiment is being conducted. BN rats, 5 weeks of age, are allocated into seven groups. Each group consisting of 6 rats (three per. sex) are administered *ad libitum* in the drinking bottles with the different products and combinations of these for three weeks (day 1-21), according to Table 7. One week after last *ad libitum* administration the post-immunisation procedure is started. For post-immunisation rats were immunised i.p. with 100 µg/rat/day of intact BLG at day 28, 35, 42, 49 and 56. Rats were sacrificed day 63 by exsanguination using carbon dioxide inhalation as anesthesia.

Group no.	Amount of intact BLG in drinking bottles (g/L)	Amount of synthetic peptide 19 in each drinking bottles (g/L)	Amount of synthetic peptide 20 in drinking bottles (g/L)
1	-	-	-
2	0.4	-	-
3	4.0	-	-
4	0.4	3.6	
5	0.4	-	3.6
6	0.4	1.8	1.8
7	-	4.0	-

Table 7. Design of animal experiment 3.

Enzyme-linked immunosorbent assay (ELISA) for detection of BLG-specific IgG1

As described in example 1.

Antibody-capture ELISA for detection of BLG-specific IgE

As described in example 4.

Antibody-capture ELISA for detection of BLG-specific IgE

As described in example 1, except for blocking solution being 5% horse serum instead of 3% rabbit serum and digoxigenin-coupled BLG concentration being 0.05 µg/mL instead of 0.2 µg/mL.

Curve calculations and Statistical analyses

As described in example 1. Asterisks indicate a statistically significant difference between the given group and the group of animals administered with water.

Results

Oral administration of intact BLG and individual synthetic peptides either alone or in combination, resulted in various outcomes (Figure 6 and 7). From Figure 7A it is evident that none of the products and combinations hereof did induce specific IgE antibodies against the intact BLG upon oral administration. From the Figure 7B it is evident that already after 2 post-immunisations a clear specific IgE response is induced against BLG for BN rats administered with either water (as control) or the synthetic peptide no. 19, irrespectively of the synthetic peptide no. 19 was administered alone, together with BLG or together with both the BLG and the synthetic peptide no.20. The same picture is evident after 3. post-immunisation (Figure 7C). This indicates that BLG and BLG together with the synthetic peptide no. 20 contain the highest tolerance inducing capacity. Further this indicates that the synthetic peptide no. 19 inhibits some of the tolerance inducing capacity of intact BLG administered either alone or in the combination with the synthetic peptide no. 20. After the 4. and 5. post-immunisation (Figure 7D) results clearly shows that the tolerance inducing capacity differed between products administered with and without intact BLG, irrespectively of the specific peptide. This demonstrates that the tolerance inducing capacity is strongly dependent on the presence of intact BLG.

Discussion

This study demonstrated that co-administration of intact BLG with specific synthetic peptides resulted in a much stronger tolerance inducing capacity compared to administration of peptide(s) alone and that the strength of this tolerance inducing capacity is dependent on the specific peptide administered together with the intact BLG.

Example 4 – I.p. immunisation of intact BLG together with different fractions of hydrolysates

Introduction

In this study, the sensitising capacity of intact BLG administered together with different fractions of a cow's milk whey hydrolysates was investigated. In order to examine the impact of the peptide sizes on the capacity to reduce the sensitising capacity of intact BLG, two fractions containing peptides of either small or large sizes, were studied. This was done by comparing the specific antibody response induced by intact BLG alone with the specific antibody response induced upon administering the intact BLG together with either the fraction of small peptides or the fraction of large peptides. A reduced specific IgE antibody response, when administering intact BLG together with a hydrolysate fraction compared to that of intact BLG alone is an indicator of tolerance.

Methods

Intact BLG

As described in example 1.

Design of fraction of hydrolysates

A whey protein hydrolysate and an ultrafiltration retentate were produced from a raw material, Lacprodan® DI-9224K, a whey protein isolate from Arla Foods Ingredients (Videbæk, Denmark) with a protein concentration of 90.8% (Table 8). DI-9224K (55 kg) was reconstituted into 500 kg water to a protein concentration of 9%. The solution was stored at 10 °C for about 20 hours, heated to 53 °C using a plate heater and transferred to a 1200 L hydrolysis tank at a protein concentration of 8%. Next, pH is adjusted from 6.1 to 7.0 at 53 °C using a saturated solution of 10% $Ca(OH)_2$. Hydrolysis was initiated by adding 3463 AU-A Alcalase BG followed by 748 AU-N Neutrase BG (both enzymes from Novozymes A/S) 5 minutes later. The enzymes were added as 10% solutions in water. Hydrolysis was continued for 6 hours and the product was inactivated by passage through a plate heater. The product was immediately cooled to 10 °C. The product is ultra-filtrated through two KOCH-HFK131 6338 30D membranes at 10 °C. A water volume of 380% of the ultrafiltration feed was used to dia-filtrate over the membrane. The ultrafiltration process gave a total permeate volume of 2400 L. About 20% of the protein was retained as UF retentate. The ultrafiltration retentate was concentrated on an Alfa Laval HSRO 48D reversed osmosis membrane to 6% protein as is and spray dried on an Anhydro MS 750-1 spray drying tower with in and out temperatures of 180 °C and 85 °C, respectively to yield a final dry matter protein concentration of 85%. The ultrafiltration permeate was nano-

filtrated using six Dow NF245 30 membranes at 10 °C to a protein concentration of 20%. Activated carbon (0.610 g) was added to the nano-filtration retentate and the slurry was stirred for 30 minutes after which the product was micro-filtered through three 0.2 m2 50 nm SCT Membralox membranes. The microfiltration permeate was dried on an Anhydro MS 750-1 spray drying tower with in and out temperatures of 180 °C and 85 °C, respectively. The protein concentration in the final whey protein hydrolysate was 96%.

Constituents and process data	
Water (kg)	500
Lacprodan DI-9224K WPIAK-1 (90.8%) (kg)	55
Protein as is in reconstituted solution (%)	9
Protein as is in heated solution (%)	8
pH before adjustment with 10% $Ca(OH)_2$	6.1
pH after adjustment with 10% $Ca(OH)_2$	7.0
Alcalase BG (AU-A)	3463
Neutrase BG (AU-N)	748
Inactivation	6 seconds at 120 °C
Ultrafiltration membranes	KOCH-HFK131 6338 30D
Ultrafiltration permeate volume	2400 L
Ultrafiltration retentate volume	70 L
Reversed osmosis	Alfa Laval HSRO 48D
Protein as is in concentrated ultrafiltration retentate (%)	6
Protein dry matter in dried ultrafiltration retentate (%)	85
Protein as is in nano-filtration retentate (%)	20
Nanofiltration retentate volume (liter)	110
Activated carbon – PicaPure HP 120N	0.610 g
Final product dry matter protein content (%)	96

Table 8. Constituents and process data of fraction of hydrolysates.

Protein-chemical characterisation

The fraction of small peptides as well as the fraction of large peptides were analysed for degree of hydrolysis (DH) and peptide size distribution profile by GPC, and the fraction of large peptides was analysed for the BLG peptide profile by LC-MS/MS.

Animals

As described in example 1.

Animal experimental design 4

In this study the sensitising capacity of intact BLG, either immunised alone or in combination with a fraction or small peptides or a fraction of large peptides was investigated. BN rats, 6-9 weeks of age, were allocated into three groups. Each group consisting of 6 rats (three per. sex) were immunised i.p. without any use of adjuvant, at day 0, 14 and 28, according to Table 9. Rats were sacrificed day 35 by exsanguination using carbon dioxide inhalation as anesthesia.

Group no.	Amount of intact BLG (µg)	Amount of fraction of small peptides (µg)	Amount of fraction of large peptides (µg)
1	50	-	-
2	50	450	-
3	50	-	450

Table 9. Design of animal experiment 4

Enzyme-linked immunosorbent assay (ELISA) for detection of BLG-specific IgG1

As described in example 1.

Antibody-capture ELISA for detection of BLG-specific IgE

As described in example 1.

Curve calculations and Statistical analyses

As described in example 1. Asterisks indicate a statistically significant difference between the given group and the group of animals immunised with intact BLG alone.

Results

Protein-chemical analyses showed that the hydrolysate was fractionised into two fractions with very different peptide size profiles, as shown in Table 10.

	Fraction of small peptides	Fraction of large peptides
Degree of hydrolysis (DH)[a]	25.4%	2.9%
Peptide size distribution profile in weight: Da (no. of amino acids)		
<375 (1-3)	19.8%	2.5%
375-750 (4-6)	34.7%	4.9%
750-1250 (7-10)	25.2%	9.4%
1250-2500 (11-20)	18.4%	38.4%
>2500 (>20)	2.0%	44.9%

Table 10. DH and peptides size distribution profiles of the two fractions. [a] DH is a measure of the percentage of peptide bond which have been cleaved.

MS/MS analysis of the fraction of large peptides revealed that parts of the intact BLG molecule were represented by peptides (Figure 8 and Table 11). However, the analyses also showed that there were parts of the intact BLG molecule that were not represented by peptides in this fraction. In a comparison with the synthetic peptides the MS/MS analysis showed that the entire part of peptide 19 stemming from BLG (SEQ ID NO.:46 without KKK = SEQ ID NO.:10) and the entire peptide 22 (SEQ ID NO.:19) were represented by peptides, while parts of peptide 20 (SEQ ID NO::14) and peptide 23 (SEQ ID NO.:22) were represented. On the other hand the peptide 21 (SEQ ID NO.:17) was not represented by peptides in the fraction of large peptides. Peptide 19 without KKK (SEQ ID NO.:10) was represented in the fraction in its total length as well as was the exact peptide with the sequence: LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL (Table 11).

Start	**End**	**Sequence**
17	48	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL
17	51	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQ
17	55	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPL
17	56	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLR
17	58	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY

22	51	TMKGLDIQKVAGTWYSLAMAASDISLLDAQ
22	55	TMKGLDIQKVAGTWYSLAMAASDISLLDAQSALP
22	56	TMKGLDIQKVAGTWYSLAMAASDISLLDAQSALPR
22	58	TMKGLDIQKVAGTWYSLAMAASDISLLDAQSALPRVY
27	48	DIQKVAGTWYSLAMAASDISLL
59	67	VEELKPTPE
59	71	VEELKPTPEGDLE
61	71	ELKPTPEGDLE
112	119	DTDYKKYL
112	121	DTDYKKYLLF
112	124	DTDYKKYLLFCME
112	130	DTDYKKYLLFCMENSAEPE
112	132	DTDYKKYLLFCMENSAEPEQS
112	147	DTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDDE
125	147	NSAEPEQSLACQCLVRTPEVDDE
133	147	LACQCLVRTPEVDDE
137	143	CLVRTPE
137	147	CLVRTPEVDDE
138	147	LVRTPEVDDE
139	147	VRTPEVDDE
139	150	VRTPEVDDEALE
165	178	LSFNPTQLEEQCHI

Table 11. Identification of peptides present in the fraction of large peptides. Smaller peptides identified in this fraction are likely to have been complexed during fractionation.

Immunisation of BN rats with intact BLG resulted in the induction of specific IgG1 (immunogenicity) as well as IgE (allergenicity) antibodies (Figure 9). Administration of the intact BLG together with the fraction of small peptides or the fraction of large peptides resulted in various outcomes. Co-administration of the intact BLG together with only the fraction of large peptides resulted in a great reduction of the sensitising capacity of the intact BLG (Figure 9). This showed that combining intact BLG with a fraction of a hydrolysate comprising peptides where nearly 50% had a MW of more than 2.5 kDa and with a DH of 2.9% contained a reduced (>3-fold) allergenicity compared to administration of the same amount of intact BLG alone. In contrast, combining intact BLG with a fraction of a hydrolysate comprising peptides where only 2% had a MW of more than 2.5 kDa and with a

DH of 25.4%, showed no reduction in allergenicity compared to administration of the same amount of intact BLG alone.
These results clearly demonstrated the effectiveness of administrating the intact BLG together with peptides above a certain size, in reducing the allergenicity of intact BLG.

Discussion

Co-administration of intact BLG with the hydrolysate fraction of large peptides representing a DH of 2.9% clearly showed a reduced allergenicity compared to administration of the intact BLG alone. In comparison the large synthetic peptides represented a DH of 2.5%. The present study demonstrated that hydrolysates may indeed contain the capacity to reduce the allergenicity (IgE) of intact BLG, and that this is dependent on the length of peptides comprising the hydrolysate, probably in combination with the presence of specific peptides.

Example 5 – Oral administration of intact BLG and different fractions of hydrolysates

Introduction

In this study, the tolerance inducing capacity of intact BLG and fractions of peptides administered alone or in combination was investigated. In order to investigate the impact of the peptide sizes as well as the impact of the presence of intact BLG on the tolerogenic capacity of a product, the oral tolerance inducing capacity of the two fractions containing peptides of either small or large sizes as well as intact BLG alone was compared with the oral tolerance inducing capacity of co-administration of the fractions and intact BLG. This was done by administering the different products or combinations of products *ad libitum* in drinking bottles for three weeks, followed by i.p. post-immunisations with intact BLG. A reduced specific (IgE) antibody response of animals administered with a product after post-immunisations compared to animals administered with water is a proof of tolerance inducing capacity of the given product. The lower the IgE response as well as the longer the duration of the reduced IgE response, the larger the tolerogenic capacity of the product.

Methods

Intact BLG

As described in example 1.

Design of fraction of hydrolysates

As described in example 4.

Protein-chemical characterisation

As described in example 4.

Animals

As described in example 1.

Animal experimental design 5

In order to study the oral tolerance inducing capacity of intact BLG, the fraction of small peptides, and the fraction of large peptides and to compare it with the oral tolerance inducing capacity of the fraction of small peptides and the fraction of large peptides in combination with intact BLG, an animal experiment was conducted. BN rats, 4-6 weeks of age, were allocated into six groups. Each group consisting of 6 rats (three per. sex) were administered *ad libitum* in the drinking bottles with the different products and combinations of these for three weeks (day 1-21), according to Table 12. One week after last *ad libitum* administration the post-immunisation procedure was started. For post-immunisation rats were immunised i.p. with 100 µg/rat/day of intact BLG at day 28, 35, 42, 49 and 56. Rats were sacrificed day 63 by exsanguination using carbon dioxide inhalation as anesthesia.

Group no.	Amount of intact BLG in drinking bottle (g/L)	Amount of fraction of small peptides in drinking bottle (g/L)	Amount of fraction of large peptides in drinking bottle (g/L)
1	-	-	-
2		12.5	-
3	1.25	11.25	-
4	-	-	12.50
5	1.25	-	11.25
6	1.25	-	-

Table 12. Design of animal experiment 5.

Enzyme-linked immunosorbent assay (ELISA) for detection of BLG-specific IgG1

As described in example 1.

Antibody-capture ELISA for detection of BLG-specific IgE

As described in example 4.

Curve calculations and Statistical analyses

As described in example 1. Asterisks indicate a statistically significant difference between the given group and the group of animals administered with water.

Results

Protein-chemical analyses were as described in example 4.

Oral administration of intact BLG and a fraction of small peptides or large peptides either alone or in combination, resulted in various outcomes (Figure 10 and 11). From Figure 11A it is evident that none of the products and combinations hereof did induce specific IgE antibodies against the intact BLG upon oral administration. After the 2. post-immunisation (Figure 11C) it is clearly shown that specific IgE antibodies are induced against BLG for BN rats administered with either water (as control) or the fraction of small peptides, which indicate no or only a low tolerance inducing capacity, respectively. Products containing either the fraction of large peptides or intact BLG were in contrast able to inhibit the induction of specific IgE antibodies, an evidence of tolerance inducing capacity. The same picture is evident after 3. post-immunisation (Figure 11D). After the 4. post-immunisation (Figure 11E) results clearly showed that the tolerance inducing capacity differs between products administered with and without intact BLG, irrespectively of the fraction of the hydrolysate. This demonstrates that the tolerance inducing capacity of the fraction of large peptide alone is lower than in combination with the intact BLG molecule, with an IgE response being inhibited in only one out of six animals when the fraction of large peptide were administered alone compared to four out of six animals when the fraction of large peptides were administered together with intact BLG. Such results are also reflected by the IgG1 results (Figure 10).

Discussion

This study demonstrated that co-administration of intact BLG with either of the hydrolysate fractions resulted in a much stronger tolerance inducing capacity compared to administration of the fractions alone.

EXAMPLE 6 - Production of a partial hydrolysate containing more than 40 % of its peptides above 24 aa.

Whey protein concentrate (WPC) with a protein concentration of 79.5 % (45-50 % of the protein is BLG) derived from sweet whey from a standard cheese production process was used as raw material for the hydrolysis reaction.

The WPC was hydrated in 10 °C polished water at a protein concentration of 9 %. The hydrated WPC was heated to 50 °C using a plate heat exchanger and adjusted to pH 7.0 prior to enzyme addition at a protein concentration of 8 % using a 10 % NaOH/KOH (61:39) basemix. The hydrolysis was initiated by the addition of Alcalase Conc BG (1.52 AU/kg protein), Protamex (6.08 AU/kg protein) and Flavorpro 750P (83.6 AU/kg protein) with 10 minutes in between each enzyme. The enzyme was hydrated in water prior to addition (10 % total solids). pH was controlled using a 10 % NaOH/KOH (61:39) basemix during the hydrolysis. After 6 hours of hydrolysis the reaction mixture was diluted to 4 % protein prior to heat inactivation of the enzymes using a plate heat exchanger. The inactivated product was immediately cooled to 5 °C.

The inactivated hydrolysate is fractionated on an ultrafiltration setup using a KOCH HFK-131 6338 type membrane with a 30 mill spacer with a feed pressure of 3-6 bar at 10 °C. A water volume of up to 400 % of the ultrafiltration feed is used to dia-filtrate over the membrane. The ultrafiltration retentate is concentrated by reverse osmosis on an Alpha Laval HSRO type membrane with a 48 mill spacer to approx. 8 % protein as is before spray drying.

Constituents and process data	
Lacprodan DI-8590 (79.5 % protein as is)	
Protein as is in reconstituted solution (%)	9
Protein as is in heated solution (%)	8
Temperature (°C)	50
pH after adjustment with 10 % NaOH/KOH (39.5:60.5)	7.0
Alcalase Conc BG (AU-N/kg protein)	1.56
Protamex (AU/kg protein)	6.08
Flavorpro 750P (AU/kg protein)	83.6
Protein as is before heat inactivation	4 %
Ultrafiltration membranes	KOCH-HFK131 6338 30D

Ultrafiltration permeate volume (% of feed volume)	400
Reversed osmosis	Alfa Laval HSRO 48D
Protein as is in concentrated ultrafiltration retentate (%)	8

Protein-chemical characterisation

The peptide size distribution was conducted using gel permeations chromatography on a 3xTSKgel G2000SWXL; 5µm, 7.8 x 300mm column. Prior to analysis filtration of the sample was conducted and to quantify the retained fraction the insolubility at 62,000 x g was evaluated as a measurement of this fraction in the inactivated hydrolysate. A synthetic peptide of BLG consisting of 24 amino acid residues was used as size marker (One letter code of the peptide is ALKALPMHIRLSFNPTQLEEQCHI).

The hydrolysis has been conducted so that 56.35 % of the inactivated product is peptides being smaller than the 24 amino acid residue marker (Figure 12), which result in 44 % of the product being larger than the 24 amino acid residue marker before fractionation.

Analysing the UF permeate from a KOCH HFK-131 membrane filtration demonstrate that solely peptides being smaller than 24 amino acid residues penetrates the membrane (Figure 13), hence, UF filtration on a KOCH HFK-131 membrane will further enrich the fraction above 24 amino acid residues in the inactivated hydrolysate. Diafiltration with up to 400 % of the feed volume will remove up to 95 % of the fraction allowed to penetrate the membrane.

Hydrolysate composition before and after fractionation			
	Feed	UF permeate*	UF retentate**
Protein	100 %	51 %	49 %
Peptides >24 AA	44 %	<1 %	94 %
Peptides <24 AA	56 %	>99 %	6 %

Table 13 * fraction of small peptides; ** fraction of large peptides

The UF-retentate contains the large peptides as a mixture of large peptides, complexes of smaller peptides and/or larger peptides and intact whey protein, including intact BLG. The retentate may be use as such in e.g. infant formulas or further treatment steps may be applied in order to remove e.g. intact BLG and/or smaller peptides from complexes. Complexes may be removed or suitable additives be used to break the complexes followed

by filtration to remove small peptides. Additional filtering and/or different chromatography methods, including ion-, exclusion-, etc. may be used in order to purify the fraction of large peptides further.

EXAMPLE 7 - Production of a partial hydrolysate containing more than 40 % of its peptides above 24 aa.

Whey protein concentrate (WPC) with a protein concentration of 84.5 % (45-50 % of the protein is BLG) derived from sweet whey from a standard cheese production process is the raw material for the hydrolysis reaction.

The WPC is hydrated in 10 °C polished water at a protein concentration of 10 %. The hydrated WPC is heated to 50 °C using a plate heat exchanger and adjusted to pH 7.0 prior to enzyme addition at a protein concentration of 9 % using a 10 % NaOH/KOH (39.5:60.5) basemix. The hydrolysis is initiated by the addition of Neutrase Conc BG (0.448 AU/kg protein). The enzyme is hydrated in water prior to addition (10 % total solids). After two hours of hydrolysis the temperature is raised to 60 °C and the reaction mixture is diluted to 6 % protein. At this temperature pH is adjusted to 8.5 using a 10 % NaOH/KOH (39.5:60.5) basemix. Novozym 12066 (1.0 % of protein) is added and 10 minutes later Alcalase Conc BG (0.662 % of protein) is added. Both enzymes are hydrated in water prior to addition (10 % total solids). The hydrolysis reaction is continued for 4 hours. After 4 hours of hydrolysis pH is adjusted to 7.0 using a 10 % NaOH/KOH (39.5:60.5) basemix prior to heat inactivation of the enzymes using a plate heat exchanger. The inactivated product is immediately cooled to 5 °C.

The inactivated hydrolysate is fractionated on an ultrafiltration setup using a KOCH HFK-131 6338 type membrane with a 30 mill spacer with a feed pressure of 3-6 bar at 10 °C. A water volume of up to 400 % of the ultrafiltration feed is used to dia-filtrate over the membrane. The ultrafiltration retentate is concentrated by reverse osmosis on an Alpha Laval HSRO type membrane with a 48 mill spacer to approx. 8 % protein as is before spray drying.

Constituents and process data	
Lacprodan DI-8790 (84.5 %)	
Protein as is in reconstituted solution (%)	10
Protein as is in heated solution (%)	9

Temperature (°C)	50
pH after adjustment with 10 % NaOH/KOH (39.5:60.5)	7.0
Neutrase Conc BG (AU-N/kg protein)	0.895
Protein as is after two hours of hydrolysis (%)	6
Temperature after two hours of hydrolysis (°C)	60
pH after adjustment with 10 % NaOH/KOH (39.5:60.5)	8.5
Novozym 12066 (% of protein)	1.0
Alcalase Conc BG (% of protein)	1.324
Ultrafiltration membranes	KOCH-HFK131 6338 30D
Ultrafiltration permeate volume (% of feed volume)	400
Reversed osmosis	Alfa Laval HSRO 48D
Protein as is in concentrated ultrafiltration retentate (%)	8

Protein-chemical characterisation

The peptide size distribution is conducted using gel permeations chromatography on a 3xTSKgel G2000SWXL; 5µm, 7.8 x 300mm column. Prior to analysis filtration of the sample is conducted and to quantify the retained fraction the insolubility at 62,000 x g is evaluated as a measurement of this fraction in the inactivated hydrolysate. A synthetic peptide of BLG consisting of 24 amino acid residues is used as size marker (One letter code of the peptide is ALKALPMHIRLSFNPTQLEEQCHI).

The hydrolysis is conducted so that 30 % of the inactivated product is peptides being smaller than the 24 amino acid residue marker, which result in 70 % of the product is larger than the 24 amino acid residue marker before fractionation.

Analysing the UF permeate from a KOCH HFK-131 membrane filtration demonstrate that solely peptides being smaller than 24 amino acid residues penetrates the membrane (Figure 2), hence, UF filtration on a KOCH HFK-131 membrane will further enrich the fraction above 24 amino acid residues in the inactivated hydrolysate. Diafiltration with up to 400 % of the feed volume will remove up to 95 % of the fraction allowed to penetrate the membrane.

Hydrolysate composition before and after fractionation			
	Feed	UF permeate*	UF retentate**
Protein	100 %	66 %	44 %

Peptides >24 AA	30 %	<1 %	90 %
Peptides <24 AA	70 %	>99 %	10 %

Table 14 * fraction of small peptides; ** fraction of large peptides

The UF-retentate contains the large peptides as a mixture of large peptides, complexes of smaller peptides and/or larger peptides and intact whey protein, including intact BLG. The retentate may be use as such in e.g. infant formulas or further treatment steps may be applied in order to remove e.g. intact BLG and/or smaller peptides from complexes. Complexes may be removed or suitable additives be used to break the complexes followed by filtration to remove small peptides. Additional filtering and/or different chromatography methods, including ion-, exclusion-, etc. may be used in order to purify the fraction of large peptides further.

CLAIMS

1. A composition with reduced beta-lactoglobulin (BLG) allergenicity, comprising a mixture of:

a) intact BLG, and

b) one or more BLG-derived peptides each consisting of between 24 and 75 consecutive amino acids from said intact BLG.

2. A composition according to claim 1, wherein the total amount of BLG-derived peptides consisting of between 24 and 75 consecutive amino acids from said intact BLG constitutes more than 40 wt%, 45 wt%, 50 wt%, 60 wt%, 70 wt%, 80 wt% or more than 90 wt% of the total amount of BLG-derived peptides in the composition.

3. A composition according to claim 2 wherein the intact BLG is isolated and/or purified from bovine milk and mixed with said one or more isolated and/or purified BLG-derived peptides consisting of between 24 and 75 consecutive amino acids from said intact BLG.

4. A composition according to any one of claims 1-3, wherein the BLG-derived peptides is a mixture of different peptides each consisting of between 24 and 75 consecutive amino acids from said intact BLG.

5. A composition according to any one of claims 1-4, wherein said intact BLG has the amino acid sequence SEQ ID NO. 1:
LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENGECA
QKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDDEALEK
FDKALKALPMHIRLSFNPTQLEEQCHI,

or a sequence with at least 90 %, preferably at least 93, 95, 97 or 99 %, identity to SEQ ID NO. 1.

6. A composition according to any one of claims 1-5, wherein the one or more BLG-derived peptides is/are selected from the group a-e of amino acid sequences:

a. LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL (SEQ ID NO. 10),
b. DAQSAPLRVYVEELKPTPEGDLEILLQKWENG (SEQ ID NO. 14),
c. TDYKKYLLFCMENSAEPEQSLACQCLVRTPEVD (SEQ ID NO. 19),
d. DEALEKFDKALKALPMHIRLSFNPTQLEEQCHI (SEQ ID NO. 22), or

e. a sequence equivalent with one of the sequences a, b, c or d, taken from an amino acid sequence with at least 90 %, preferable 93, 95, 97 or 99 % identity to SEQ ID NO. 1.

7. A composition according to any one of claims 1-6, wherein the one or more BLG-derived peptides consist of between 24 and 64 consecutive amino acids from the sequence LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENG (SEQ ID NO. 10 + SEQ ID NO. 14) or from a sequence equivalent to this sequence taken from an amino acid sequence with at least 90 %, preferable 93, 95, 97 or 99 % identity to SEQ ID NO 1.

8. A composition according to any one of claims 1-7, wherein the one or more BLG-derived peptides consist of between 24 and 32 consecutive amino acids selected from the sequences:

a. LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL (SEQ ID NO. 10),

b. DAQSAPLRVYVEELKPTPEGDLEILLQKWENG (SEQ ID NO. 14), or

c. a sequence equivalent to sequence a or b taken from an amino acid sequence with at least 90 %, preferable 93, 95, 97 or 99 %, identity to SEQ ID NO. 1.

9. A composition according to any one of claims 1-7 comprising one or more BLG-derived peptides consisting of between 33 and 75 consecutive amino acids comprising at least 24 consecutive amino acids selected from one of the amino acid sequences:

a. LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL (SEQ ID NO. 10),
b. DAQSAPLRVYVEELKPTPEGDLEILLQKWENG (SEQ ID NO. 14), or
c. a sequence equivalent to sequence a or b taken from a an amino acid sequence with at least 90 %, preferable 93, 95, 97 or 99 %, identity to SEQ ID NO. 1.

10. A composition according to any one of claims 1-9, wherein said composition is devoid of any peptides from said intact BLG comprising between 8 and 32 consecutive amino acids from the sequence ECAQKKIIAEKTKIPAVFKIDALNENKVLVLD (SEQ ID NO. 17) or from a sequence equivalent to SEQ ID NO. 17 taken from an amino acid sequence with at least 90 %, preferably 93, 95, 97 or 99 %, identity to SEQ ID NO. 1.

11. A composition according to any one of claims 1-10, wherein the one or more BLG-derived peptides is/are synthetically or recombinantly produced.

12. A composition according to claim 11 , wherein at least one of the one or more BLG-derived peptides consisting of between 24 and 75 consecutive amino acids from said intact BLG further comprises 1, 2, 3 or 4 amino acid residues added to the N- and/or C-terminal end to enhance solubility and/or to inhibit protease degradation.

13. A composition according to claims 11 or 12, wherein at least one amino acid in at least one peptide of the one and more BLG-derived peptides consisting of between 24 and 75 consecutive amino acids from said intact BLG is substituted by another amino acid, such as in a conservative amino acid substitution or deleted or substituted by one or more amino acids in order to enhance solubility and/or to inhibit protease degradation.

14. A composition according to any one of claims 1-10, wherein the one or more BLG-derived peptides consisting of between 24 and 75 consecutive amino acids from said intact BLG is/are isolated from a hydrolysis mixture resulting from hydrolysis of said intact BLG.

15. A composition according to claim 14, wherein the hydrolysis is an enzymatic hydrolysis.

16. A composition according to claim 14 or 15, wherein the one or more BLG-derived peptide consisting of between 24 and 75 consecutive amino acids from said intact BLG is/are obtained by isolation of one or more fractions/mixtures of peptides according to size/length.

17. A composition according to any one of claims 14-16, wherein the one or more BLG-derived peptides consisting of between 24 and 75 consecutive amino acids from said intact BLG is/are obtained by isolation of a fraction or mixture of peptides devoid of any peptides comprising between 8 and 32consecutive amino acids from the amino acid sequence ECAQKKIIAEKTKIPAVFKIDALNENKVLVLD (SEQ ID NO. 17) or from a sequence equivalent with SEQ ID NO. 17 taken from an amino acid sequence with at least 90 %, preferable 93, 95, 97 or 99 % identity to SEQ ID NO. 1.

18. A composition according to any one of claims 14-17, wherein the one or more BLG-derived peptides consisting of between 24 and 75 consecutive amino acids from said intact BLG is/are obtained from hydrolysed isolated BLG, a hydrolysed BLG-containing product or a hydrolysed whey protein product, such as a whey protein concentrate (WPC).

19. A composition according to any one of claims 16-18, wherein the fraction or mixture is essentially free of intact BLG and any other intact milk proteins.

20. A composition according to any one of claims 1-19, wherein the weight ratio between intact BLG and the one or more BLG-derived peptides consisting of between 24 and 75 consecutive amino acids from said intact BLG is between 1:99 and 30:70, between 5:95 and 25:75, or between 5:95 and 20:80, such as 1:99, 2:98, 5:95, 10:90, 15:85, 20:80, 25:75 or 30:70.

21. A composition according to any one of claims 1-20 for oral, sublingual, intramuscular, intradermal, epidermal, subcutaneous, intranasal, intraperitoneal, intravenous or intralymphatic administration.

22. An infant formula comprising intact BLG and one or more BLG-derived peptides consisting of between 24 and 75 consecutive amino acids from said intact BLG.

23. An infant formula according to claim 22 comprising a composition according to any one of claims 1-21.

24. An infant formula according to claim 23 comprising a composition according to any one of claims 1 to 17 as the essentially only source of BLG protein and BLG-derived peptides.

25. An infant formula according to any one of claim 22-24 comprising the composition according to any one of claims 1 to 21 as the essential only source of protein and peptides.

26. An infant formula according to any one of claims 22-25, further comprising fat, carbohydrate, vitamins and/or minerals.

27. A food product comprising the composition according to any one of claims 1-21 as the essential only source of BLG protein and BLG-derived peptides.

28. A food product according to claim 27 selected from a beverage, a dairy product, a protein supplement and a nutritional supplement, optionally further comprising fat, carbohydrate, vitamins and/or minerals.

29. A method of producing a composition according to any one of claims 14-21, comprising:

a. hydrolysing a BLG-containing protein product,

b. fractionating the hydrolysed proteins according to size/length of the resulting BLG-derived peptides,

c. isolating one or more fractions of BLG-derived peptides with a size/length of at least 24 amino acids,

d. optionally isolating/purifying one or more BLG-derived peptides or peptide fractions from the isolated fraction(s) in step c, and

e. mixing the isolated BLG-derived peptide(s) or fraction(s) from step d with isolated intact BLG in a suitable ratio.

5

1	*MKCLLLA---LALTCGAQA*LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY	58	P02754	Bos taurus
1	*MKCLLLA---LALTCGAQA*LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY	58	G5E5H7	Bos taurus
1	*MKCLLLA---LALTCGAQA*LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY	58	L8J1Z0	Bos mutus
1	*MKCLLLA---LALTCGAQA*LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY	58	B5B0D4	Bos taurus
1	*MKCLLLALGLALACGAQA***I**IVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY	60	C3W955	Bubalus bubalis
1	*MKCLLLALGLALACAAQA***I**IVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY	60	P02755	Bubalus bubalis
1	*MKCLLLALGLALACGIQA***I**IVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY	60	P02756	Capra hircus
1	*MKCLLLALGLALACGVQA***I**IVTQTMKGLDIQKVAGTW**H**SLAMAASDISLLDAQSAPLRVY	60	P67976	Ovis aries
1	*MKCLL----ALALACGAQA***I**IVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY	58	Q0PNH9	Bubalus bubalis
1	*MKCLLITLGLALACGAQA***I**IVTQTMK**D**LD**V**QKVAGTWYSLAMAASDISLLDAQSAPLRVY	60	Q00P86	Rangifertarandus
1	*MKCLLLALGLALACGIQA***I**IVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY	60	A5JSR7	Capra hircus
1	----------**I**IVTQTMKGLDIQKVAGTW**H**SLAMAASDISLLDAQSAPLRVY	42	P67975	Ovis orientalis

59	VEELKPTPEGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY	118	P02754	Bos taurus
59	VEELKPTPEGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY	118	G5E5H7	Bos taurus
59	VEELKPTPEGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY	118	L8J1Z0	Bos mutus
59	VEELKPTPEGDLEILLQKWEN**D**ECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY	118	B5B0D4	Bos taurus
61	VEELKPTPEGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY	120	C3W955	Bubalus bubalis
61	VEELKPTPEGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY	120	P02755	Bubalus bubalis
61	VEELKPTPEG**N**LEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY	120	P02756	Capra hircus
61	VEELKPTPEG**N**LEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY	120	P67976	Ovis aries
59	VEELKPTPEGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY	118	Q0PNH9	Bubalus bubalis
61	VEELKPTP**G**GDLEILLQKWENGKCAQKKIIAEKT**E**IPAVFKIDALNENKVLVLDTDYKKY	120	Q00P86	Rangifer tarandus
61	VEELKPTPEG**N**LEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY	120	A5JSR7	Capra hircus
43	VEELKPTPEG**N**LEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY	102	P67975	Ovis orientalis

119	LLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI	178	P02754	Bos taurus
119	LLFCMENSAEPEQSL**V**CQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI	178	G5E5H7	Bos taurus
119	LLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLE**G**QCHI	178	L8J1Z0	Bos mutus
119	LLFCMENSAEPEQSL**V**CQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI	178	B5B0D4	Bos taurus
121	LLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCH**V**	180	C3W955	Bubalus bubalis
121	LLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCH**V**	180	P02755	Bubalus bubalis
121	LLFCMENSAEPEQSLACQCLVRTPEVD**K**EALEKFDKALKALPMHIRL**A**FNPTQLE**G**QCH**V**	180	P02756	Capra hircus
121	LLFCMENSAEPEQSLACQCLVRTPEVD**N**EALEKFDKALKALPMHIRL**A**FNPTQLE**G**QCH**V**	180	P67976	Ovis aries
119	LLFCMENSAEPE**KAWPASAW**VRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI	178	Q0PNH9 Bubalus bubalis 121	
	LLFCMENSAEPEQSLACQCLVRTPEVDDEA**M**EKFDKALKALPMHIRLSFNPTQLEEQCR**V**	180	Q00P86	Rangifer tarandus
121	LLFCMENSAEPEQSLACQCLVRTPEVD**K**EALEKFDKALKALPMHIRL**A**F----------	169	A5JSR7	Capra hircus
103	LLFCMENSAEPEQSLACQCLVRTPEVD**N**EALEKFDKALKALPMHIRL**A**FNPTQLE**G**QCH**V**	162	P67975	Ovis orientalis

FIGURE 1

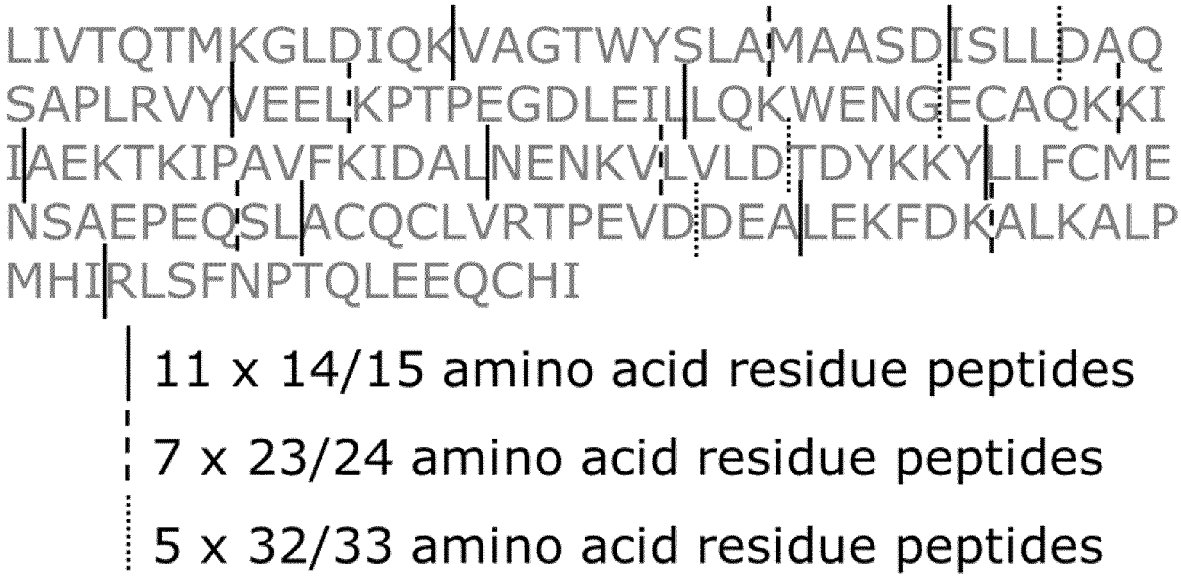
LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQ
SAPLRVYVEELKPTPEGDLEILLQKWENGECAQKKI
IAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCME
NSAEPEQSLACQCLVRTPEVDDEALEKFDKALKALP
MHIRLSFNPTQLEEQCHI
11 x 14/15 amino acid residue peptides
7 x 23/24 amino acid residue peptides
5 x 32/33 amino acid residue peptides

FIGURE 2

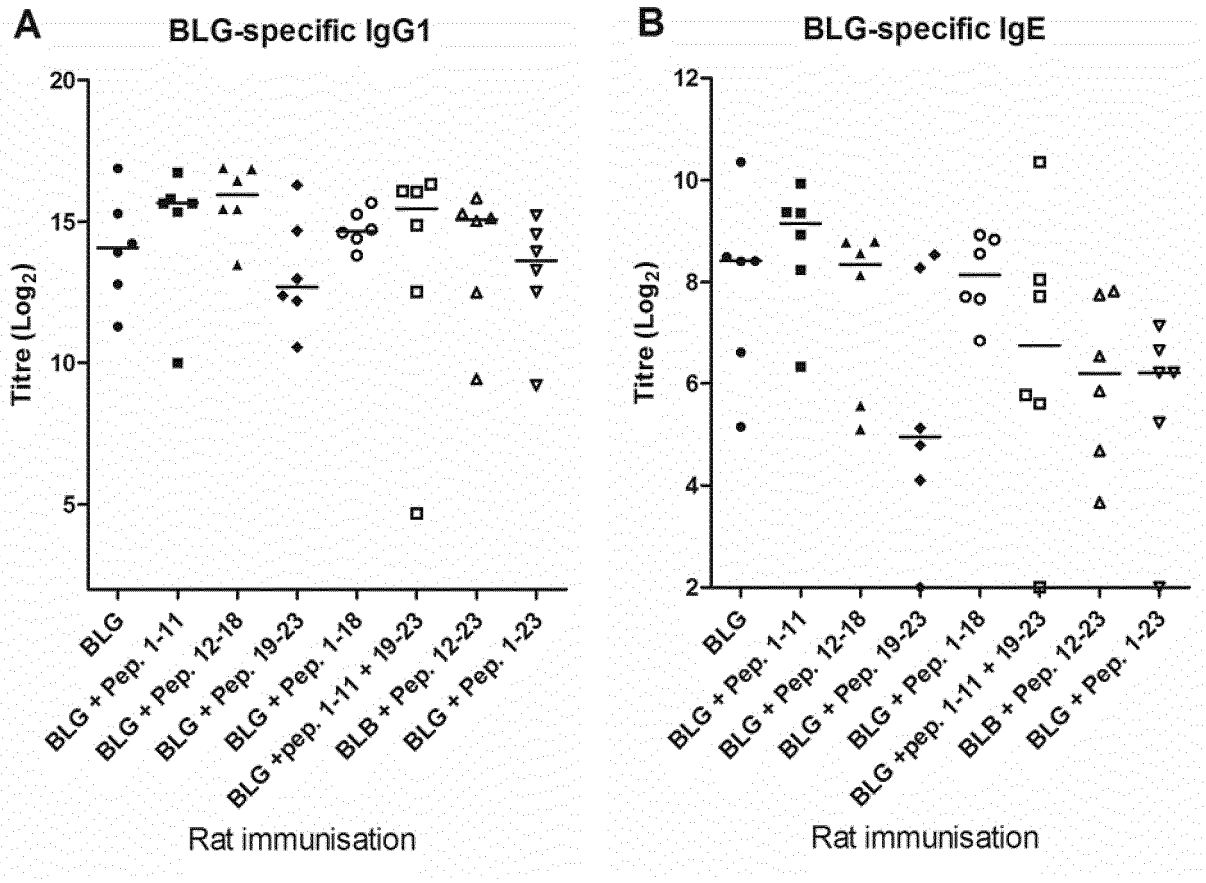
A
BLG-specific IgG1
Titre (Log2)
20
15
10
5
BLG
BLG + Pep. 1-11
BLG + Pep. 12-18
BLG + Pep. 19-23
BLG + Pep. 1-18
BLG +pep. 1-11 + 19-23
BLB + Pep. 12-23
BLG + Pep. 1-23
Rat immunisation
B
BLG-specific IgE
Titre (Log2)
12
10
8
6
4
2
BLG
BLG + Pep. 1-11
BLG + Pep. 12-18
BLG + Pep. 19-23
BLG + Pep. 1-18
BLG +pep. 1-11 + 19-23
BLB + Pep. 12-23
BLG + Pep. 1-23
Rat immunisation

FIGURE 3

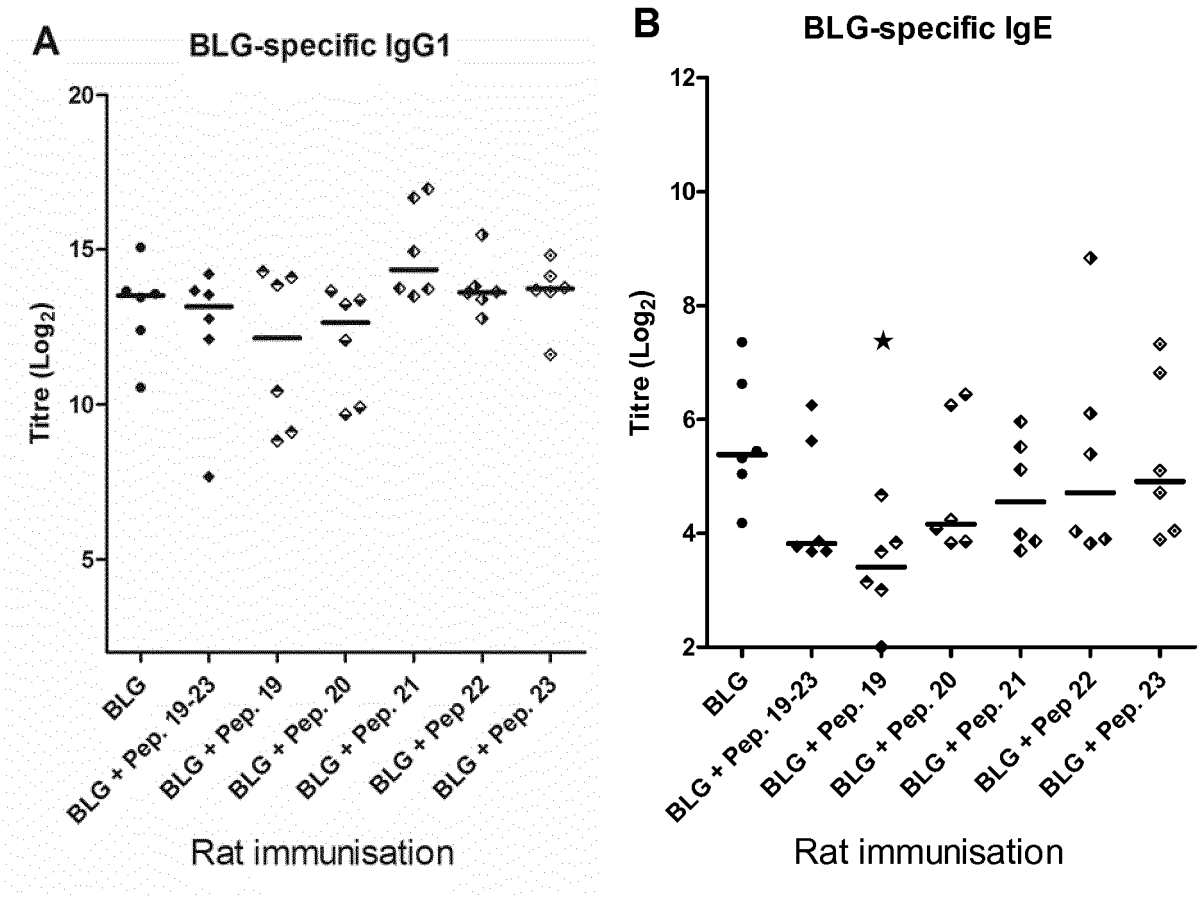
A
BLG-specific IgG1
Titre (Log2)
20
15
10
5
BLG
BLG + Pep. 19-23
BLG + Pep. 19
BLG + Pep. 20
BLG + Pep. 21
BLG + Pep 22
BLG + Pep. 23
Rat immunisation
B
BLG-specific IgE
Titre (Log2)
12
10
8
6
4
2
BLG
BLG + Pep. 19-23
BLG + Pep. 19
BLG + Pep. 20
BLG + Pep. 21
BLG + Pep 22
BLG + Pep. 23
Rat immunisation

FIGURE 4

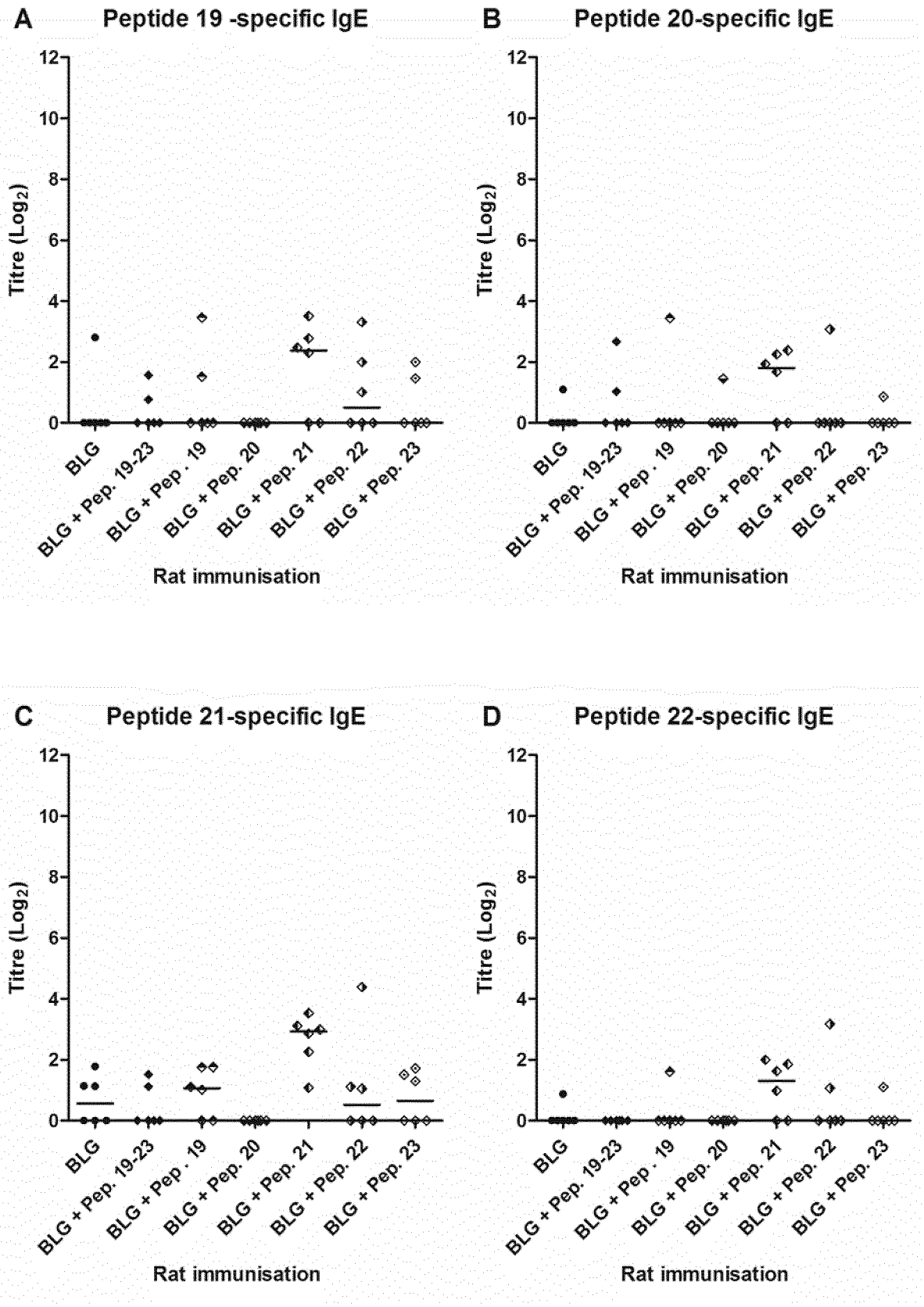
A
Peptide 19 -specific IgE
B
Peptide 20-specific IgE
C
Peptide 21-specific IgE
D
Peptide 22-specific IgE
Titre (Log2)
0
2
4
6
8
10
12
BLG
BLG + Pep. 19-23
BLG + Pep . 19
BLG + Pep. 20
BLG + Pep. 21
BLG + Pep. 22
BLG + Pep. 23
Rat immunisation

FIGURE 5-1

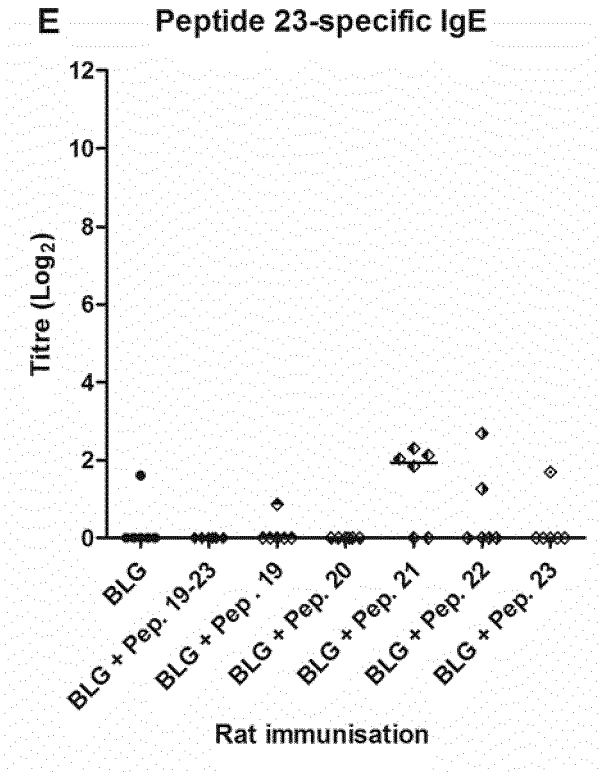
E
Peptide 23-specific IgE
Titre (Log2)
12
10
8
6
4
2
0
BLG
BLG + Pep. 19-23
BLG + Pep . 19
BLG + Pep. 20
BLG + Pep. 21
BLG + Pep. 22
BLG + Pep. 23
Rat immunisation

FIGURE 5-2

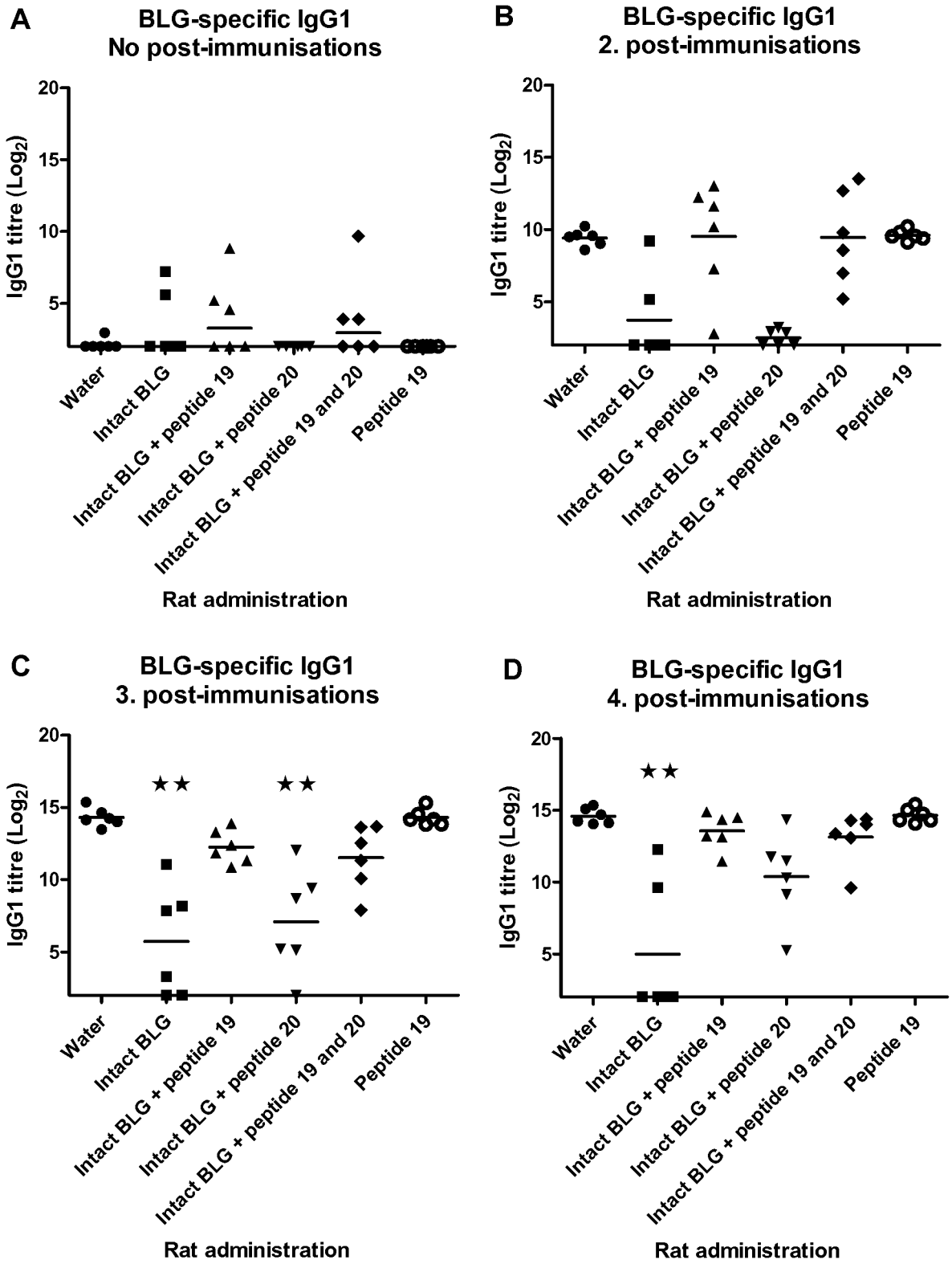
A
BLG-specific IgG1
No post-immunisations
IgG1 titre (Log2)
20
15
10
5
Water
Intact BLG
Intact BLG + peptide 19
Intact BLG + peptide 20
Intact BLG + peptide 19 and 20
Peptide 19
Rat administration
B
BLG-specific IgG1
2. post-immunisations
IgG1 titre (Log2)
Rat administration
C
BLG-specific IgG1
3. post-immunisations
IgG1 titre (Log2)
Rat administration
D
BLG-specific IgG1
4. post-immunisations
IgG1 titre (Log2)
Rat administration

FIGURE 6-1

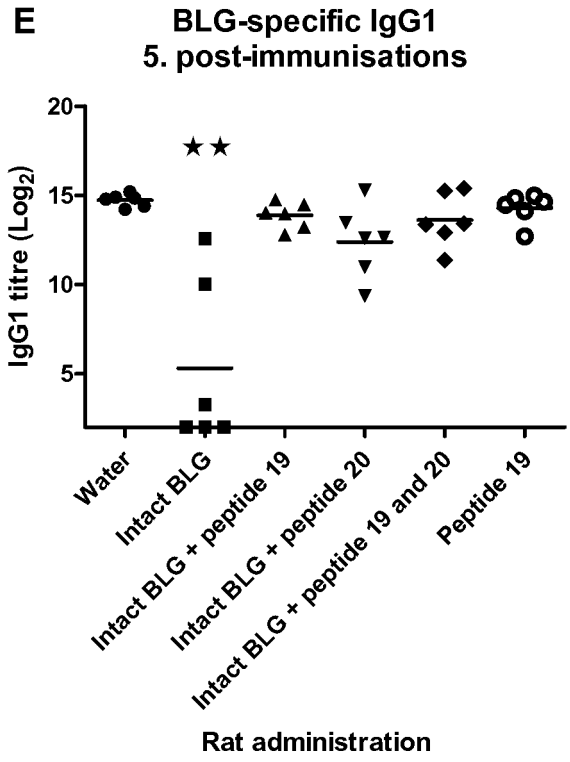
E
BLG-specific IgG1
5. post-immunisations
IgG1 titre (Log2)
20
15
10
5
Water
Intact BLG
Intact BLG + peptide 19
Intact BLG + peptide 20
Intact BLG + peptide 19 and 20
Peptide 19
Rat administration

FIGURE 6-2

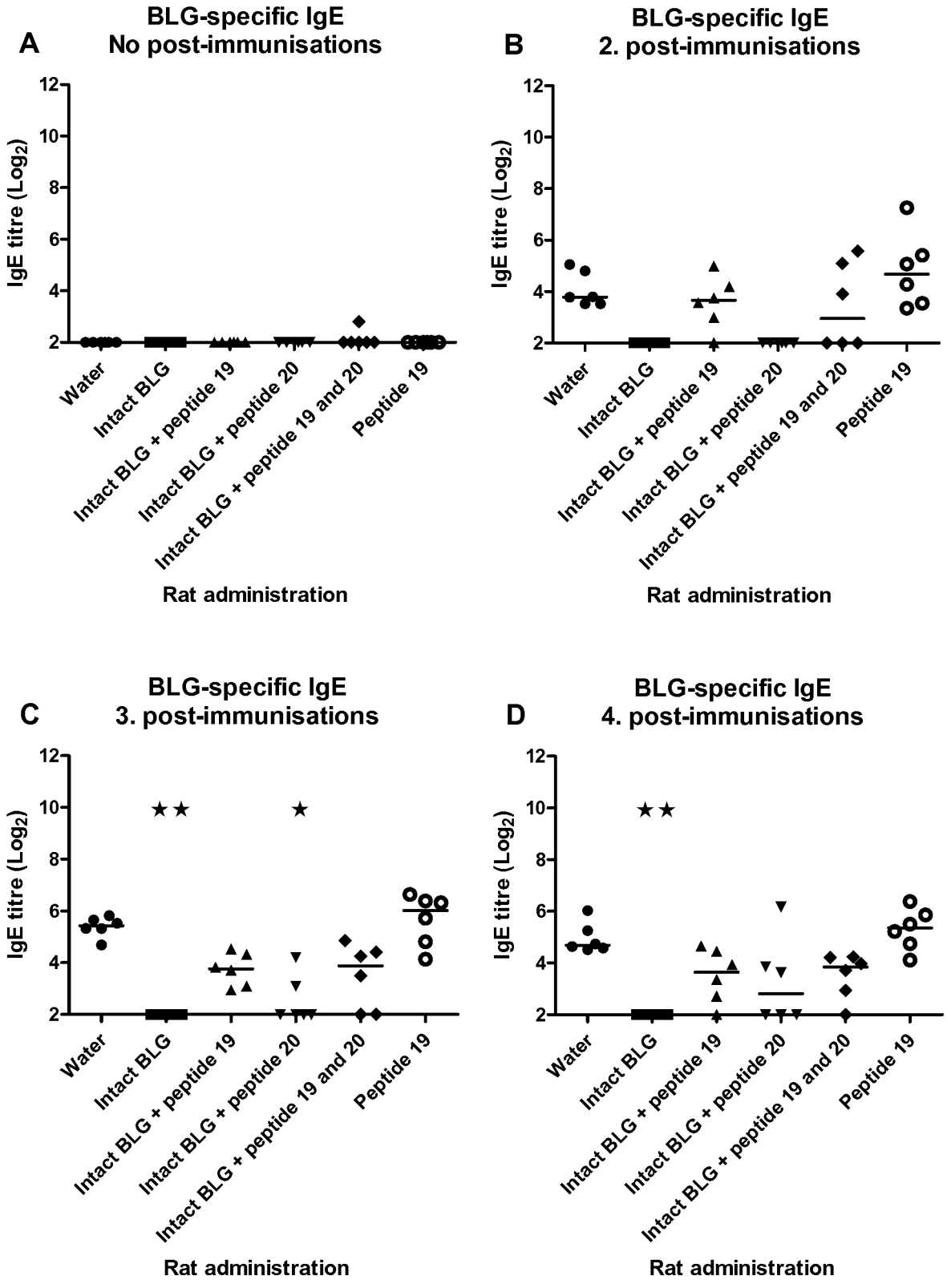
A
BLG-specific IgE
No post-immunisations
B
BLG-specific IgE
2. post-immunisations
C
BLG-specific IgE
3. post-immunisations
D
BLG-specific IgE
4. post-immunisations
IgE titre (Log2)
12
10
8
6
4
2
Water
Intact BLG
Intact BLG + peptide 19
Intact BLG + peptide 20
Intact BLG + peptide 19 and 20
Peptide 19
Rat administration

FIGURE 7-1

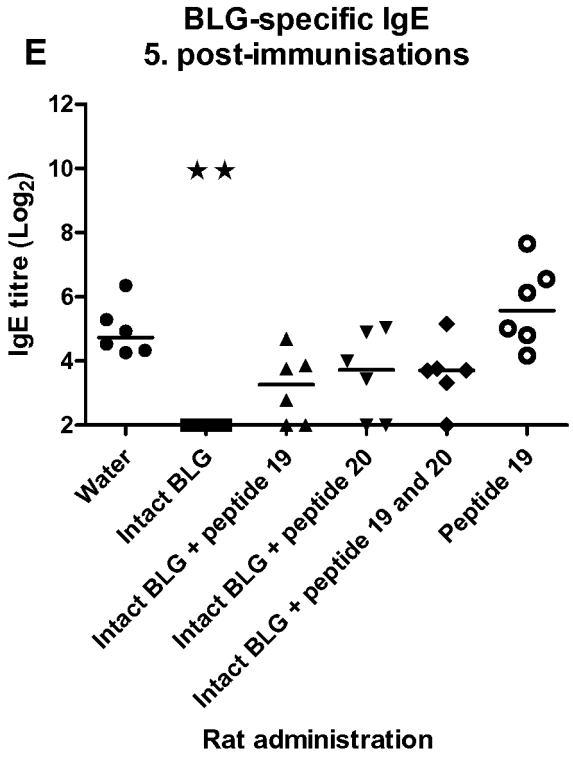
E
BLG-specific IgE
5. post-immunisations
IgE titre (Log2)
12
10
8
6
4
2
Water
Intact BLG
Intact BLG + peptide 19
Intact BLG + peptide 20
Intact BLG + peptide 19 and 20
Peptide 19
Rat administration

FIGURE 7-2

1 MKCLLLALAL TCGAQALIVT QTMKGLDIQK VAGTWYSLAM AASDISLLDA
51 QSAPLRVYVE ELKPTPEGDL EILLQKWENG ECAQKKIIAE KTKIPAVFKI
101 DALNENKVLV LDTDYKKYLL FCMENSAEPE QSLACQCLVR TPEVDDEALE
151 KFDKALKALP MHIRLSFNPT QLEEQCHI

FIGURE 8

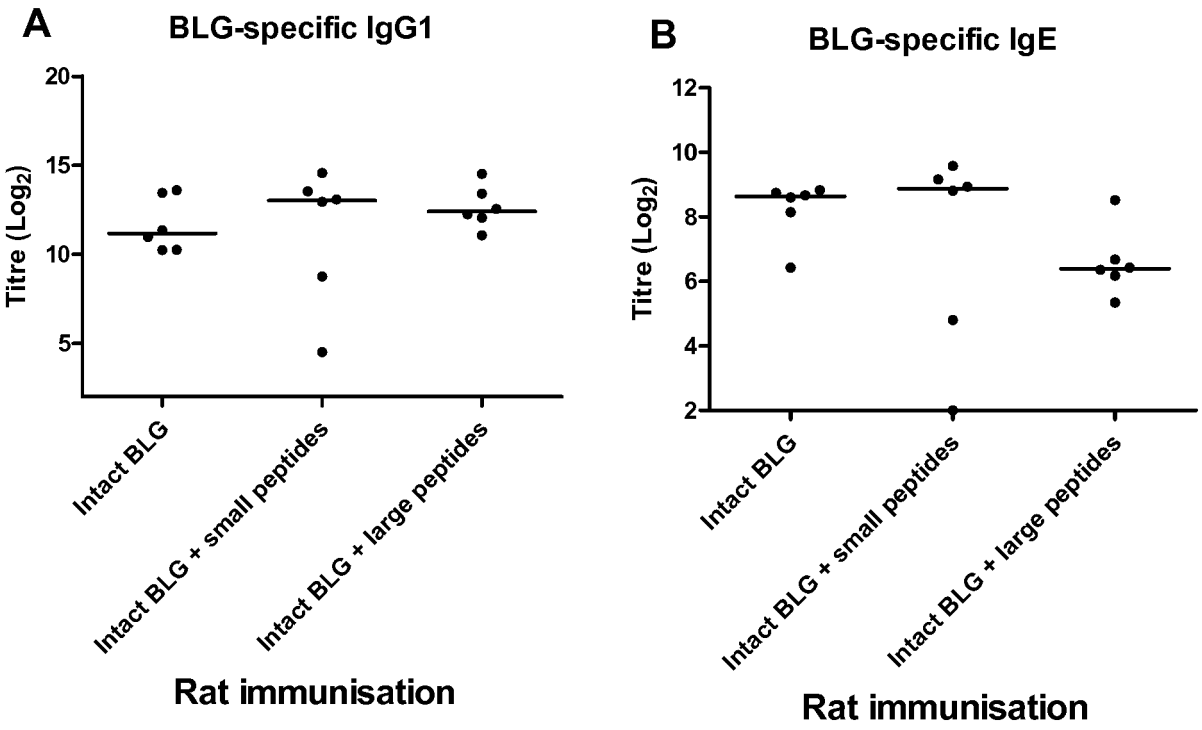
A
BLG-specific IgG1
Titre (Log2)
20
15
10
5
Intact BLG
Intact BLG + small peptides
Intact BLG + large peptides
Rat immunisation
B
BLG-specific IgE
Titre (Log2)
12
10
8
6
4
2
Intact BLG
Intact BLG + small peptides
Intact BLG + large peptides
Rat immunisation

FIGURE 9

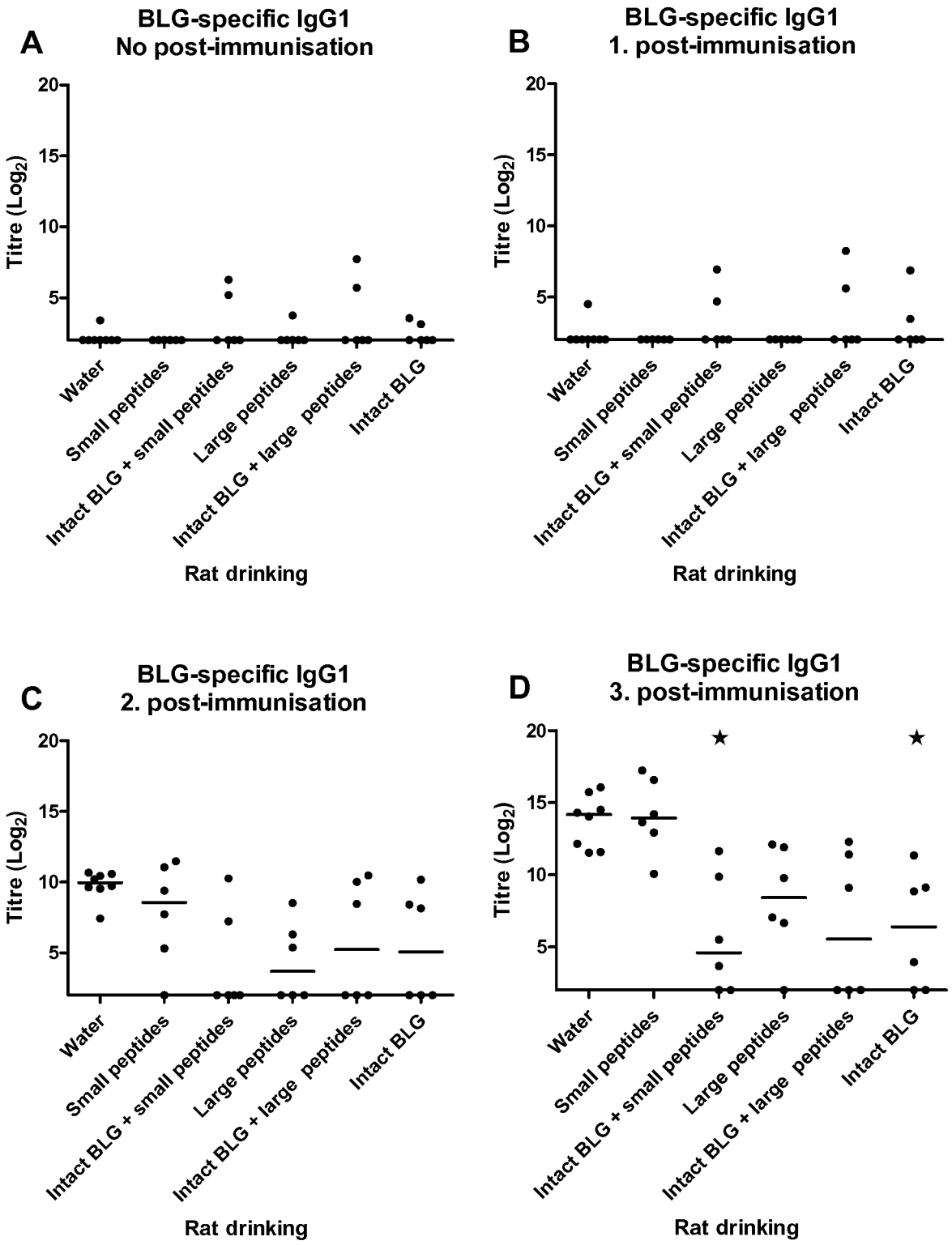
A
BLG-specific IgG1
No post-immunisation
B
BLG-specific IgG1
1. post-immunisation
C
BLG-specific IgG1
2. post-immunisation
D
BLG-specific IgG1
3. post-immunisation
Titre (Log2)
20
15
10
5
Water
Small peptides
Intact BLG + small peptides
Large peptides
Intact BLG + large peptides
Intact BLG
Rat drinking
★

FIGURE 10-1

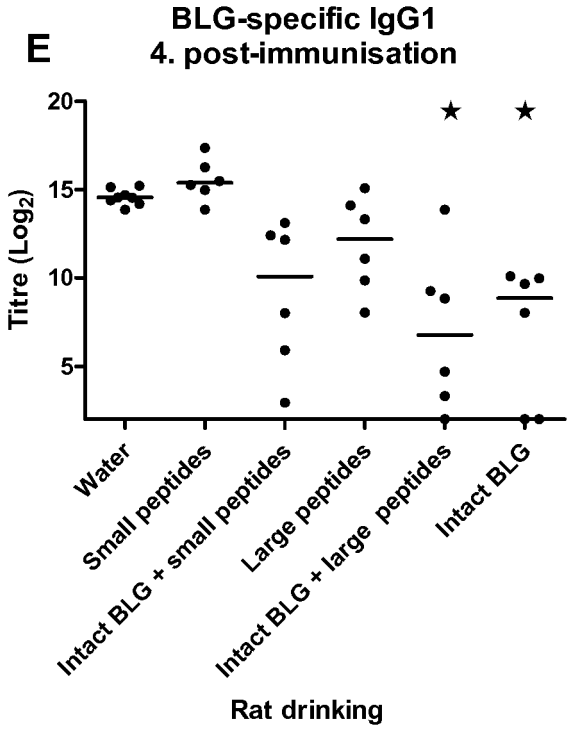
E
BLG-specific IgG1
4. post-immunisation
Titre (Log2)
20
15
10
5
Water
Small peptides
Intact BLG + small peptides
Large peptides
Intact BLG + large peptides
Intact BLG
Rat drinking

FIGURE 10-2

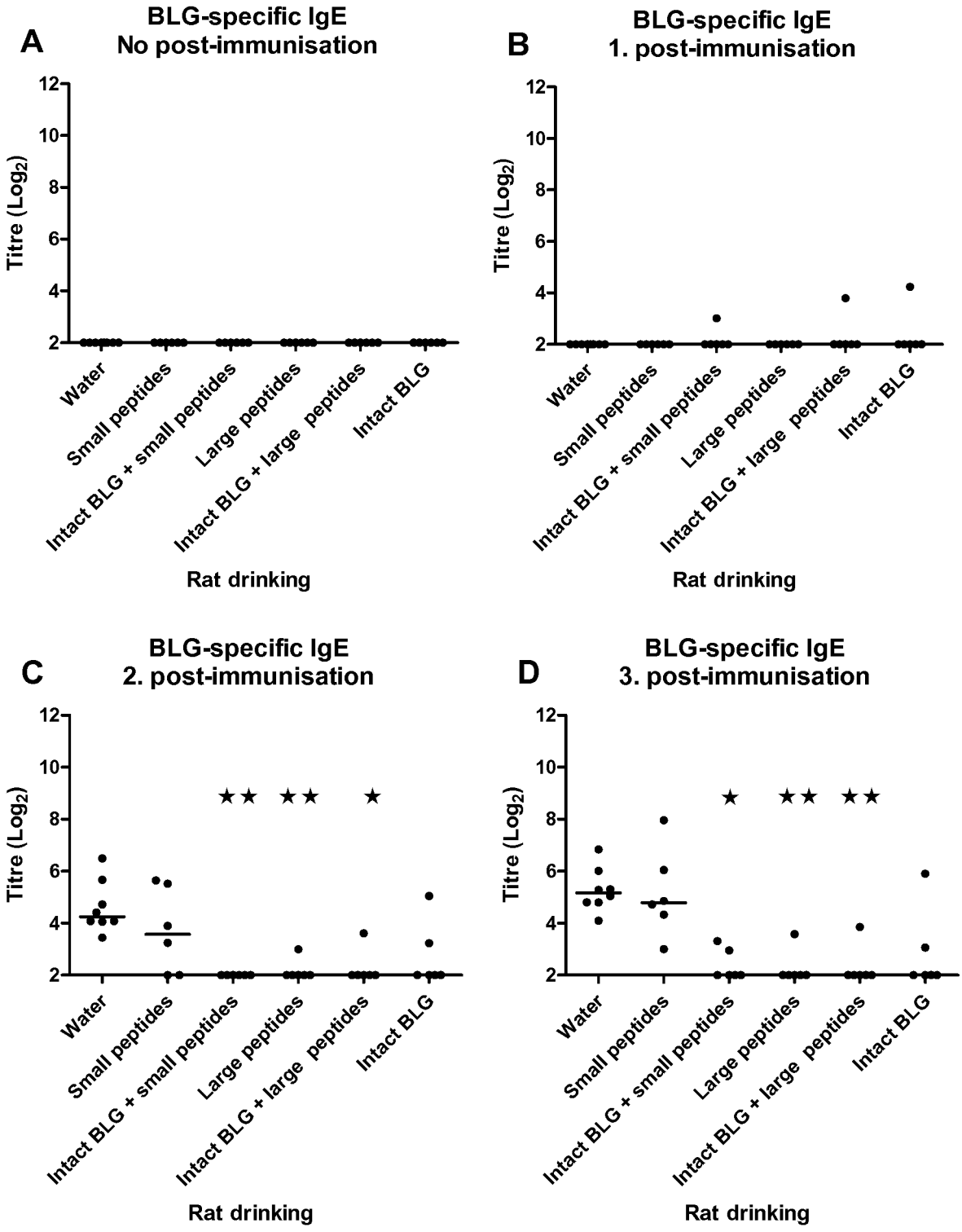
A
BLG-specific IgE
No post-immunisation
B
BLG-specific IgE
1. post-immunisation
C
BLG-specific IgE
2. post-immunisation
D
BLG-specific IgE
3. post-immunisation
Titre (Log2)
12
10
8
6
4
2
Water
Small peptides
Intact BLG + small peptides
Large peptides
Intact BLG + large peptides
Intact BLG
Rat drinking
★★
★★
★
★
★★
★★

FIGURE 11-1

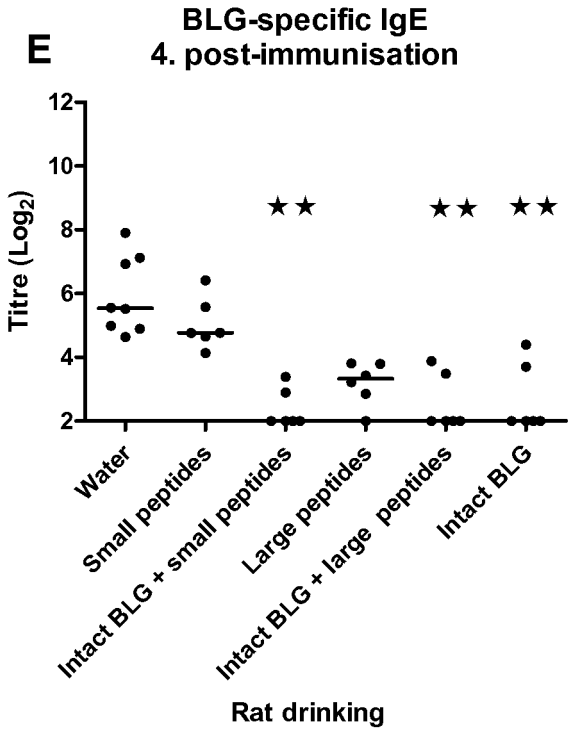
E
BLG-specific IgE
4. post-immunisation
Titre (Log2)
12
10
8
6
4
2
★★
★★
★★
Water
Small peptides
Intact BLG + small peptides
Large peptides
Intact BLG + large peptides
Intact BLG
Rat drinking

FIGURE 11-2

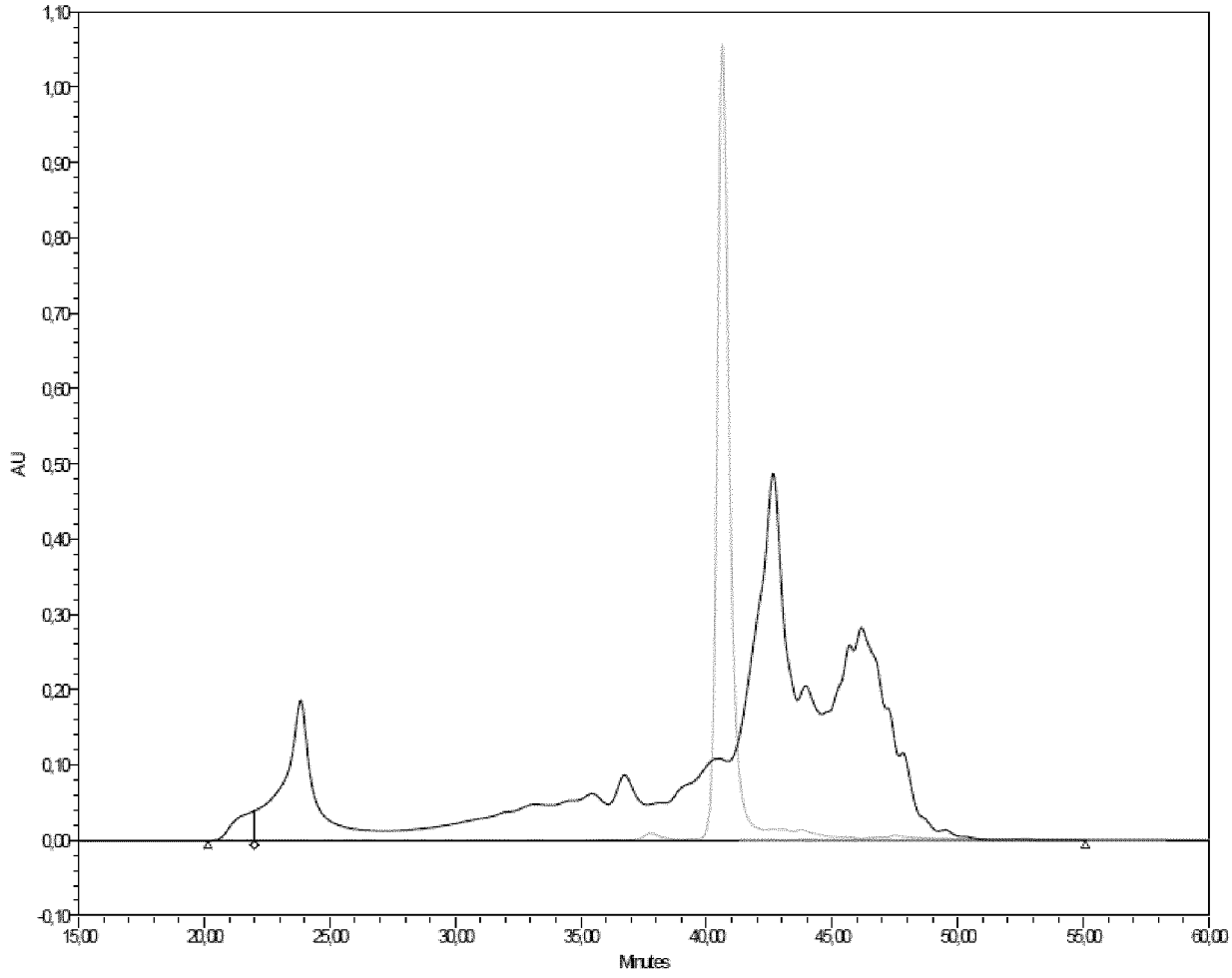
1,10
1,00
0,90
0,80
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
-0,10
AU
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
60,00
Minutes

FIGURE 12

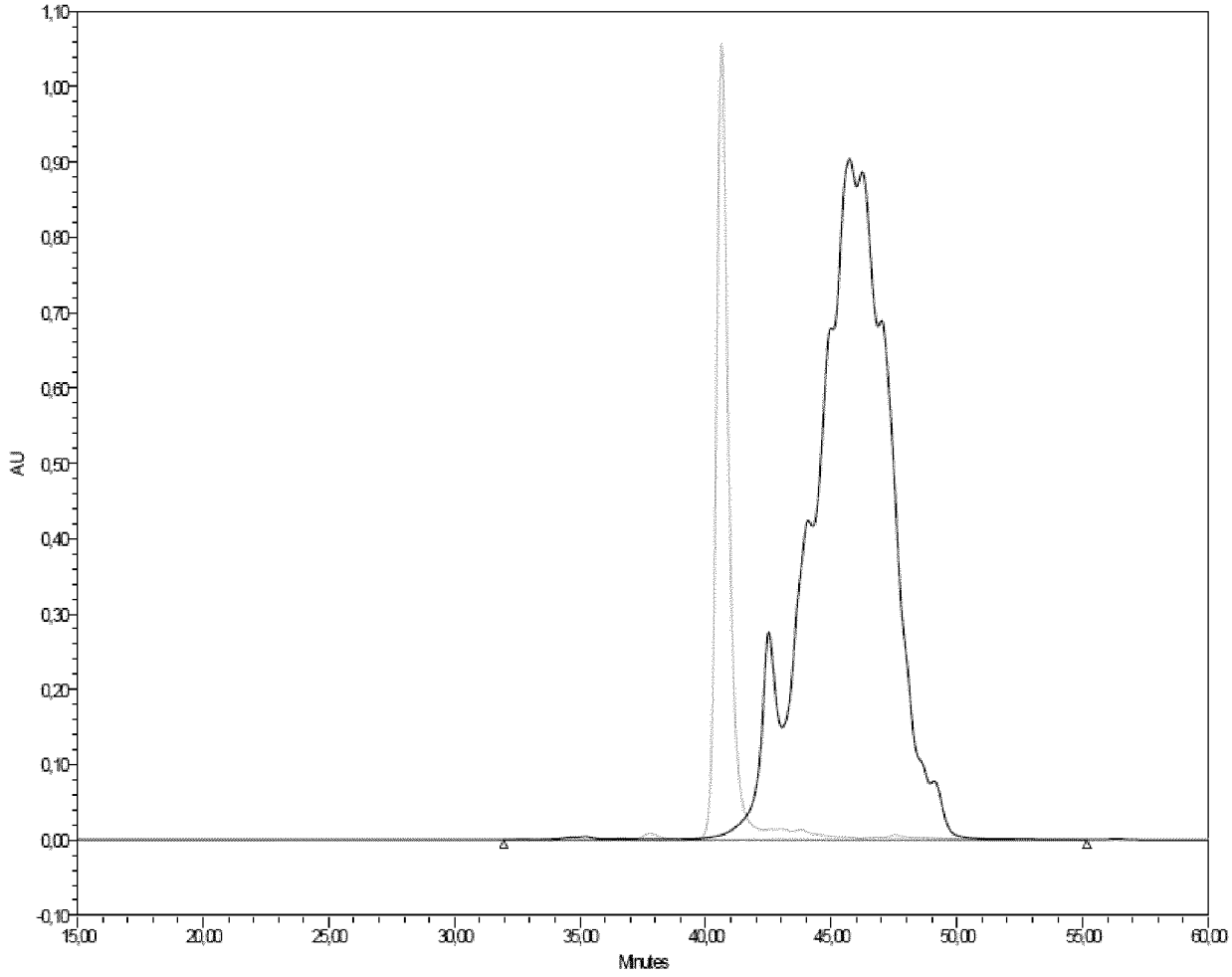
1,10
1,00
0,90
0,80
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
-0,10
AU
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
60,00
Minutes

FIGURE 13

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/054534

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/35 C07K16/18 C07K14/47 G01N33/68 A61K39/00
A23L33/19 A23L33/18
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A23L C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LINDHOLM BOGH K ET AL: "The sensitising capacity of intact [beta]-lactoglobulin is reduced by co-administration with digested [beta]-lactoglobulin", INTERNATIONAL ARCHIVES OF ALLERGY AND IMMUNOLOGY, KARGER AG, CH, vol. 161, no. 1, 1 April 2013 (2013-04-01) , pages 21-36, XP009180558, ISSN: 1018-2438, DOI: 10.1159/000343042 [retrieved on 2012-12-13] page 29, column 1, paragraph 2 - page 31, column 1, paragraph 1; figures 6,7 page 34, column 2, paragraph 2 - page 35, column 2, paragraph 1 abstract page 23 page 31, column 1, paragraph 1	1-29

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
2 May 2017	15/05/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Klee, Barbara

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JARVINEN K M: "Allergy prevention via co-administration of intact food allergen and its epitope soup?", INTERNATIONAL ARCHIVES OF ALLERGY AND IMMUNOLOGY, KARGER AG, CH, vol. 161, no. 3, 1 June 2013 (2013-06-01), pages 195-196, XP002730755, ISSN: 1018-2438, DOI: 10.1159/000346869 [retrieved on 2013-03-15] the whole document ----	1,5, 11-16, 18-29
X	WO 2009/082229 A2 (FRIESLAND BRANDS BV [NL]; AKKERMANS CYNTHIA [NL]; SCHOKKER ERIK PETER) 2 July 2009 (2009-07-02) page 6, paragraph 2 - page 8, paragraph 2 UPI0001B38109 or UPI0001B3810A is identical to SEQ ID NO: 10; Sequence 14 comprises 27 consecutive AA of SEQ ID:22 NO:14; Sequence 18 is identical (only 1 AA substitution) with SEQ ID NO:19; page 15, paragraph 1 - page 16, paragraph 1 ----	1-7, 11-16, 18,19, 26-29
X	WO 2013/083140 A1 (NUTRICIA NV [NL]; KNIPPELS LEON MATHIEU JOHANNES [NL]; GARSSEN JOHAN [) 13 June 2013 (2013-06-13) Sequence 10 identical with SEQ ID NO: 1 with 2 AA substituions; consecutive part of Sequence 10 identical with SEQ ID NO: 10 and SEQ ID NO: 14 and SEQ ID 17 and SEQ ID NO: 19 and SEQ ID NO: 22; claims 1-22 ----	1-29

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/054534

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009082229 A2	02-07-2009	EP 2247202 A2	10-11-2010
		WO 2009082229 A2	02-07-2009
WO 2013083140 A1	13-06-2013	CN 104039173 A	10-09-2014
		EP 2787841 A1	15-10-2014
		EP 3135125 A1	01-03-2017
		US 2014314800 A1	23-10-2014
		WO 2013083140 A1	13-06-2013
		WO 2013083691 A1	13-06-2013