

IgE-Mediated Rat Mast Cell Triggering with Tryptic and Synthetic Peptides of Bovine β -Lactoglobulin

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Key Words

Immunoglobulin E epitopes · β -Lactoglobulin · Mast cell triggering

Abstract

Background: Immunoglobulin E (IgE) epitopes of β -lactoglobulin (β LG) have been identified by ELISA inhibition methods using sera from allergic patients. However, the functional capacity of these epitopes to stimulate mast cells is unknown. It is the goal of the present study to identify bivalent IgE epitopes of β LG able to trigger target mast cells. **Methods:** Peptides were obtained either by purification from tryptic hydrolysates of β LG or by synthesis. They were examined for their triggering activity in vitro on peritoneal ³H-serotonin-labeled rat mast cells passively sensitized with IgE anti- β LG antibodies. In vivo, rats immunized with β LG were administered peptides by gavage for intestinal rat mast cell protease II release. **Results:** Compared with intact β LG, purified or synthetic tryptic-like β LG peptides have a sharply decreased allergenicity. Peptide 149–162 retains the highest bivalent IgE epitope-mediated triggering capacity. **Conclusion:** A functional bivalent IgE epitope was identified at the C terminal end of β LG.

Introduction

Cow's milk allergy is mainly an immunoglobulin E (IgE)-mediated hypersensitivity reaction which usually develops in early infancy. Among cow's milk proteins, β -lactoglobulin (β LG) is a major allergen which is responsible for allergic manifestations in about 60% of cow's milk-allergic patients [1–3]. At physiological pH, β LG presents a dimeric structure, each monomer being a 18-kDa protein of 162 amino acid residues. IgE-binding epitopes of β LG have been identified using sera from experimentally sensitized rats or mice [4–6] and sera from human allergic patients. Either purified tryptic peptides [4, 7, 8], fragments obtained by chemical cleavage using cyanogen bromide [9] or synthetic peptides [10, 11] of β LG were used. These studies showed that a number of sequences on the β LG molecule were able to bind IgE antibodies. Major human IgE epitopes were located in regions 1–8, 25–40, 41–60, 102–124, 149–162 [12], 124–134 [8, 11] and/or 75–86 and 127–140 [11]. Interestingly, regions 21–40, 41–60, 107–117 and 148–162 were also recognized by specific IgE induced in intraperitoneally sensitized rats [4].

All these studies allowed to identify peptide fragments able to bind IgE anti- β LG antibodies, including monovalent IgE epitopes, but provided no information on their

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Table 1. Distribution of the main peptides in the different fractions

Peptides	Fractions														
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15
T6 (84–91)		25.5													
T7 (9–14)						18.7									
T10 (61–68)										11.5		64.3	11.9		
T11 (61–69)											33.9		12.8	15.4	
T12 (125–135)								34.5	26.3						
T15 (78–82)	15.2														
T16 (15–20)				13.3	20.5	12.1									55.1
T17 (92–99) + T18 (125–138)		44.8													
T21 (62–69):S–S:(149–162)							33.2		39.9	48.5	9.7				
T22 (21–40)	13.7														
T23 (41–60)			54.3	74.3											
T24 (149–162)							10.6	26.7							

Values are expressed as percentage of area of peptide/fraction.

capacity to trigger an allergic reaction. Bivalent peptidic fragments, i.e. comprising two distinct linear IgE-binding epitopes within their sequence, with the capacity of IgE-mediated mast cell triggering, have not yet been identified. It is our purpose in the present work to identify such β LG-derived peptides both by using a functional cellular in vitro assay mimicking the elicitation step of type I hypersensitivity [13] and by in vivo triggering of sensitized intestinal mast cells [14].

Methods

Reagents

β LG was from Besnier Bridel Alimentation (Retiers, France; β LG acid batch 751, which contained 95% of total proteins). Trypsin (EC 3.4.21.4, bovine pancreas, 120,200 U/mg benzoyl-arginine ethyl ester, N-tosyl-L-phenylalanine-chloromethyl-ketone) was purchased from Sigma. Reagents for peptide synthesis were from Applied Biosystems (Warrington, UK).

Purification of Peptides Obtained by Tryptic Cleavage of β LG

β LG was digested with N-tosyl-L-phenylalanine-chloromethyl-ketone-treated trypsin using an enzyme:substrate ratio of 1:100 (wt/wt) at 40°C, pH 7.6, during 1 h followed by a 1:50 ratio during 4 h [15]. The reaction was stopped by inactivation of the trypsin at 85°C for 5 min. This total tryptic hydrolysate (TTH) was separated by preparative chromatography on an anion exchange column filled with 1,250 ml of source 15 Q resin (Pharmacia Biotech) connected

to a Biopilot system controlled by a Unicom station (Pharmacia Biotech). Samples were prepared by dissolving 20 g of β LG hydrolysate powder (TTH) in 1,400 ml of double-distilled water. The pH was adjusted to 8 by 1 N sodium hydroxide. After sample injection, the column was washed with 1,200 ml of solvent A (20 mmol/l Tris-hydrochloric acid, pH 8.0). Elution was performed according to the following 2 stages of solvent B (20 mmol/l Tris-hydrochloric acid, pH 8.0, plus 500 mmol/l sodium chloride): 0–40% solvent B by use of 2,400 ml, then 40–100% by use of 3,000 ml. Fifteen fractions (F1–F15) were obtained. All were individually diafiltered to eliminate salts, dialyzed 3 times in 10 volumes of distilled water with SpectraPor membranes with a molecular weight cutoff of 1,000, and then lyophilized. The knowledge of β LG tryptic peptide sequences allowed the characterization of the peptide profile for each of the 15 fractions. Purified fractions of β LG-digested products contained various amounts of tryptic peptides. When analyzed by reversed-phase HPLC (RP-HPLC), enrichment and impoverishment of peptides in each fraction were calculated with the help of the area detected at 214 nm with isonitrogenous injections, considering the total β LG hydrolysate as reference. Table 1 shows the distribution of the main peptides in the different fractions.

Preparation of Synthetic β LG Peptides

Synthetic β LG peptides were synthesized using an ABI433A apparatus (Applied Biosystems, USA) and standard Fmoc chemistry. Each synthesized peptide was purified by RP-HPLC using an AKTA system (Amersham Pharmacia Biotech, Sweden), a 300-Å C18 Vydac column (250 × 22 mm; Shandon, France) and acetonitrile in 0.04% trifluoroacetic acid as elution buffer. Purity and identity were controlled using a second RP-HPLC system, i.e. Source 5RPC column (Amersham Pharmacia Biotech) and elution

Table 2. Sequence and molecular weight of synthetic β LG peptides

Synthetic peptide	Peptide	Amino acid sequence	MW
1–8	I	LIVTQTMK	933.1
21–40	II	SLAMAASDISLLDAQSAPLR	2,030.3
41–60	III	VYVEELKPTPEGDLEILLQK	2,313.6
84–91	IV	IDALNENK	916
92–100	V	VLVLDTDYK	1,065.2
84–100	VI	IDALNENKVLVLDTDYK	1,963.7
102–124	VII	YLLFCMENSAPQSLVCQLVR	2,676.1
125–138	VIII	TPEVDDEALEKFDK	1,635.7
149–162	IX	LSFNPTQLEEQCHI	1,658.8

MW = Molecular weight.

with acetonitrile in 10 mM phosphate buffer, pH 7.4, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry using a Voyager DE RP apparatus (Perspective Biosystems, Framingham, Mass., USA). A single peak corresponding to the expected mass was observed for each purified peptide. Table 2 shows the amino acid sequences of the synthesized peptides

In vitro Rat Mast Cell Triggering Assay

Peritoneal mast cells from normal Sprague-Dawley rats (Iffa-Credo, France) were passively sensitized with rat IgE anti- β LG antibodies, labeled with ^{3} H-serotonin and triggered for mediator release with dilutions of β LG or β LG peptides according to a previously published method [13]. Briefly, peritoneal lavages from three Sprague-Dawley rats were obtained with Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells were washed in this medium and kept overnight at 4°C. After two washes in phosphate HEPES gelatin buffer (PHG), pH 7.0, cells were resuspended at 5×10^5 cells/ml. ^{3} H-serotonin (10 μ Ci/ml) and 1 ml of rat serum pool rich in IgE anti- β LG antibodies were added (immune sera were obtained 14 days after subcutaneous injection of 20 Brown-Norway rats with 100 μ g β LG in the presence of aluminium hydroxide; all sera were pooled). This mixture was then incubated at 37°C for 2 h, washed three times in PHG and resuspended in 2 ml PHG. Triggering for mediator release was set up in microtiter plates by mixing 0.05 ml of β LG or peptide dilution with 0.1 ml of passively sensitized mast cells. Control experiments were done using ^{3} H-serotonin-labeled non-IgE-sensitized mast cells incubated with the same β LG or β LG-derived peptide dilutions. The mixture was incubated for 60 min at 37°C and then centrifuged. An aliquot (0.05 ml) of the supernatant was counted for radioactivity on a Packard β -counter. Samples were analyzed in triplicates and the whole experiment performed three times.

In vivo Intestinal Mast Cell Stimulation Test

Brown-Norway high-IgE responder rats (Iffa-Credo) were injected subcutaneously with 100 μ g of β LG in the presence of 2% AIOH₃. Fourteen days later, the primary IgE response was occurring. For the evaluation of the *in vivo* triggering activity of a product containing β LG fragments which comprise IgE epitopes, groups

of 8 sensitized rats were gavaged with the test product (whole β LG, TTH or its derived peptides, 2 mg in 0.5 ml). Two hours later, rats were bled and the level of rat mast cell protease II (RMCP II) was determined in the serum. RMCP II is released into blood following IgE-mediated triggering of intestinal mast cells. Oral challenge for release of RMCP II is a measure of IgE sensitization at the intestinal mast cell level. RMCP II levels in serum are determined with a commercial ELISA kit (Moredun Animal Health Ltd., Edinburgh, Scotland) based on the sandwich test principle in which the plate coating is made with a monoclonal anti-RMCP II antibody, followed by the addition of test serum and a second sheep anti-RMCP II polyclonal antibody coupled to horseradish peroxidase. The whole experiment was repeated once.

Results

In vitro Mast Cell Triggering with Purified Tryptic β LG Peptides

Purified tryptic β LG fractions were analyzed for their triggering activity on passively sensitized rat mast cells (RMCs). Figure 1a shows dose-dependent ^{3} H-serotonin release curves with all fractions and with TTH. Figure 1b shows their residual β LG allergenicity calculated by comparison with similar amounts of ^{3} H-serotonin released by standard β LG. Values are expressed in microgram β LG/gram of product (β LG fraction or TTH powders). Results show that fraction F7 had the most powerful activity (100 μ g β LG/g product), followed by fractions F8, F9, F11 and F10 to trigger mast cell degranulation and ^{3} H-serotonin release. TTH of β LG for comparison has a residual activity of 35 μ g β LG/g product. Labeled unsensitized mast cells used as negative controls did not release ^{3} H-serotonin upon stimulation with these purified fractions.

As shown in table 1, the most frequent peptides present in the reactive fractions are T11, T12, T21 and T24 which correspond to amino acid sequences 61–68, 125–135 and 149–162. However, fractions F12, F13 and F14, which only contain peptides 61–68 and 61–69, do not show triggering activity, suggesting that this region very likely does not comprise two IgE epitopes necessary for activating IgE-sensitized mast cells and is not responsible for the reactivity of those fractions.

In vitro RMC Triggering with Synthetic Peptides

To further identify bivalent IgE-binding peptides able to stimulate IgE-sensitized mast cells, synthetic 'tryptic-like' peptides (I–IX) were synthesized and then examined for their IgE-mediated mast cell-triggering activity. Figure 2 shows that peptide IX (149–162) and slightly peptides I (1–8), II (21–40) and VII (102–124) induce the

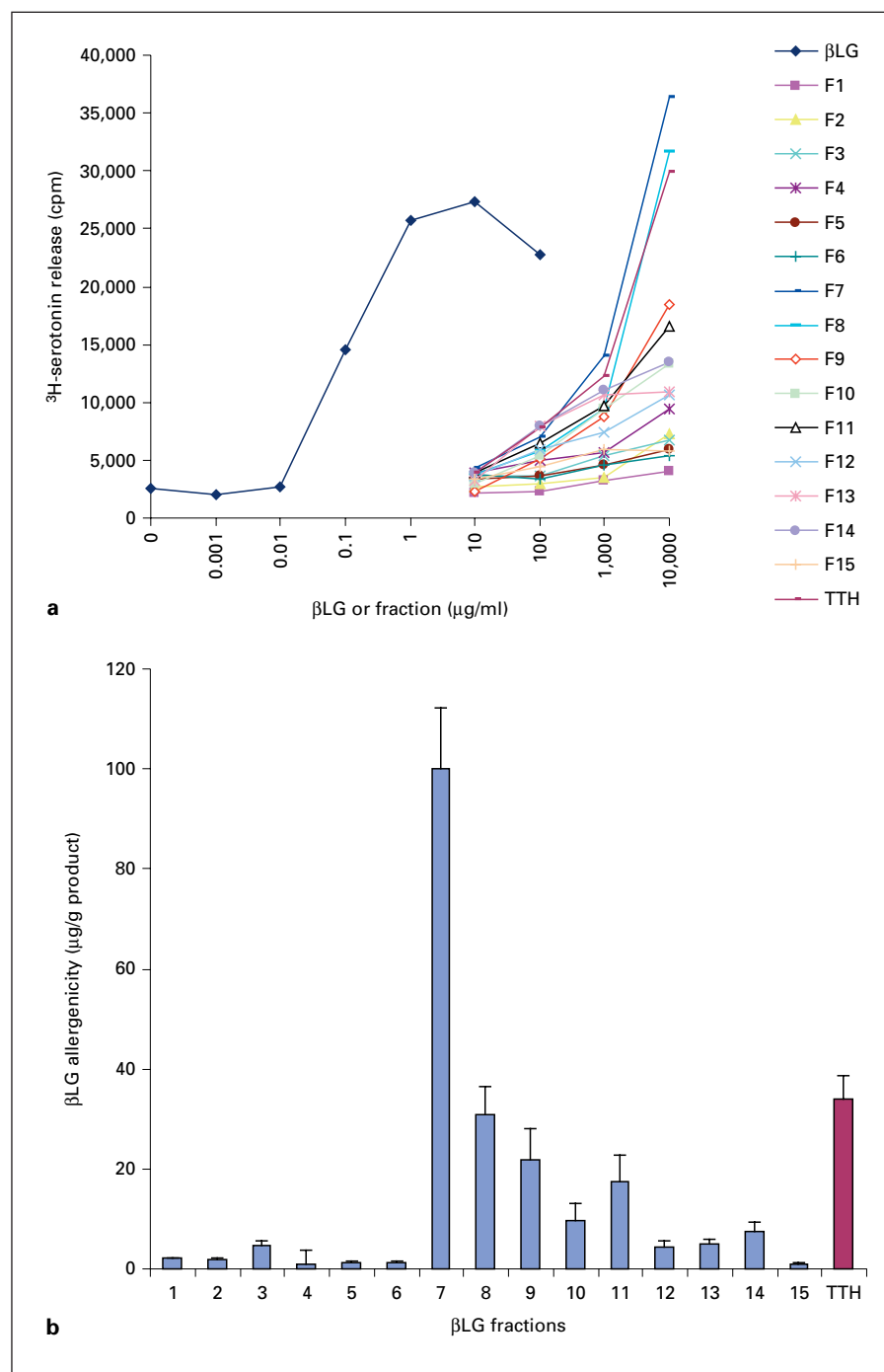


Fig. 1. ^{3} H-serotonin release from IgE anti- β LG-sensitized RMCs triggered with purified fraction of a tryptic hydrolysate of β LG (**a**) and calculated residual β LG allergenicity of these purified β LG tryptic fractions (**b**).

release of serotonin from sensitized mast cells. Other synthetic peptides, including region 125–138, were not active in vitro. All synthetic peptides, except very slightly peptide I that induced a low release (i.e. 2,800 cpm) at a 2-mg/ml concentration, did not trigger ^{3} H-serotonin release from labeled unsensitized mast cells.

In vivo Stimulation of Intestinal Mast Cells

Stimulation of mast cells by synthetic trypsin-like peptides was further studied in vivo. Rats were sensitized by β LG in alum to produce high concentrations of anti- β LG IgE and were further challenged by gavage with whole β LG or with synthetic peptides, at a dose of 2 mg/gavage.

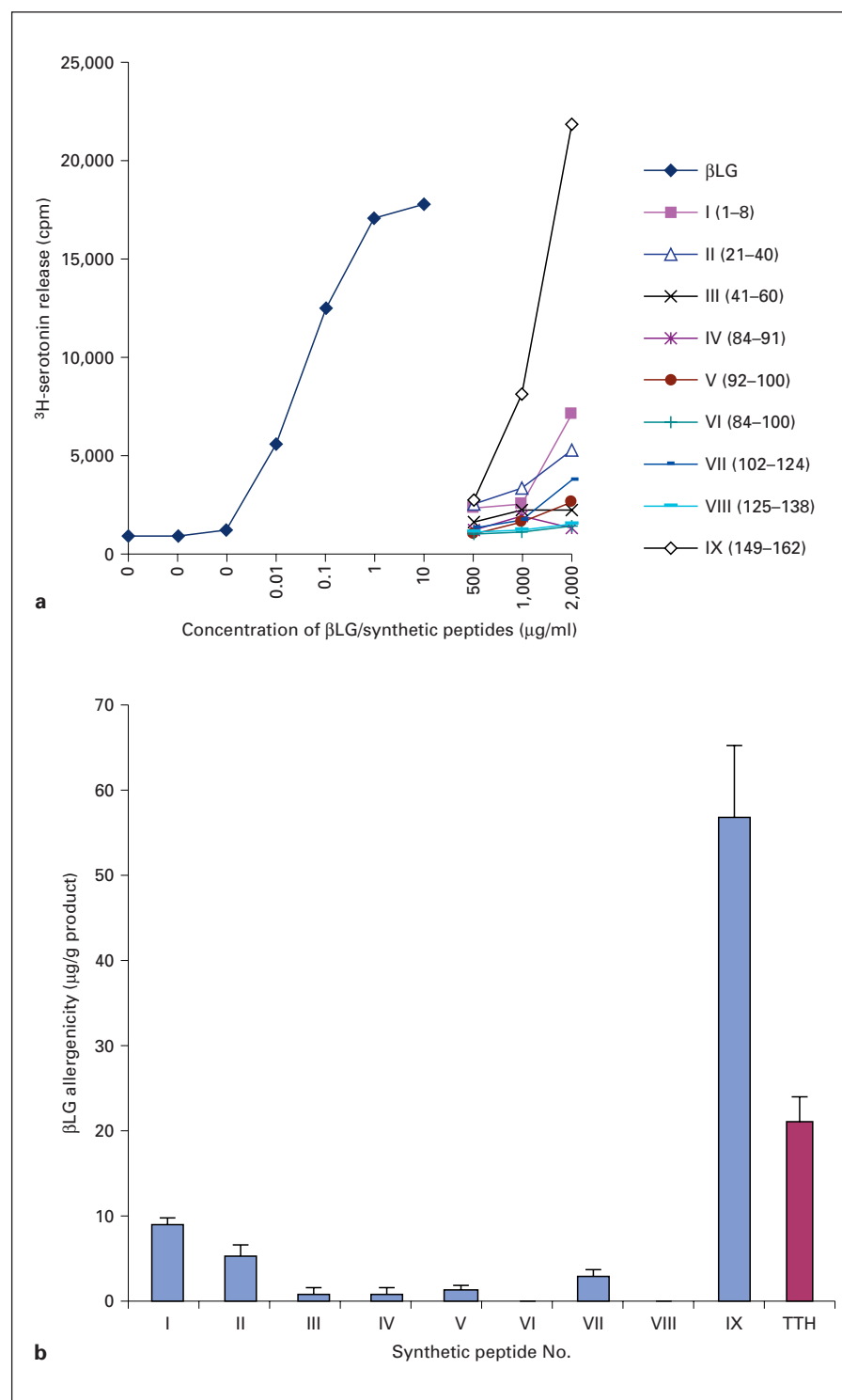


Fig. 2. ^3H -serotonin release from sensitized RMCs with synthetic 'tryptic-like' peptides of βLG (**a**) and allergenicity values (**b**).

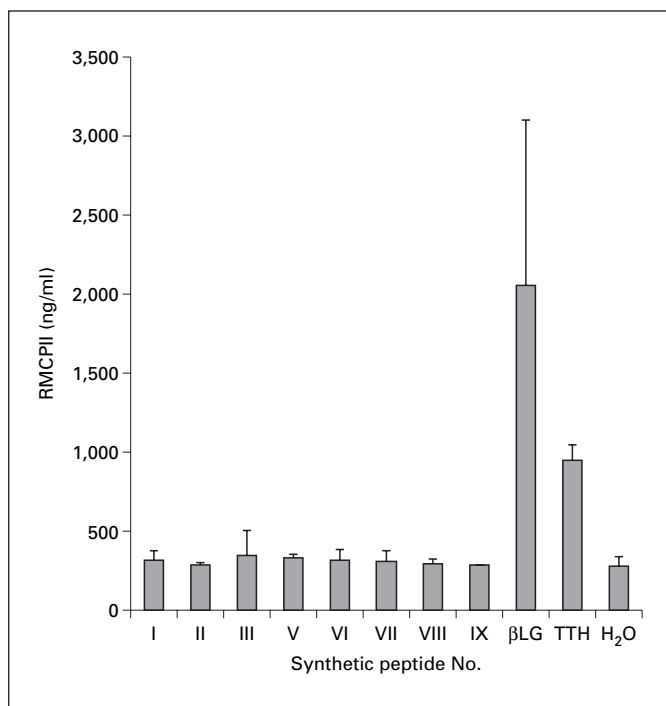


Fig. 3. RMCPII release into sera of sensitized rats gavaged with synthetic 'tryptic-like' peptides of β LG.

Blood RMCPII was then assayed as a measure of IgE-mediated mast cell degranulation at the intestinal level. Figure 3 shows that, compared with whole β LG and to a lesser extent with TTH, synthetic peptides are unable to trigger sensitized intestinal mast cells for RMCPII release.

Discussion

Epitope mapping has allowed to identify regions in the β LG molecule that are recognized by serum IgE of experimentally sensitized animals [4–6] or of allergic humans [7–12], using RAST or ELISA assays. However, the recognition of a tryptic or synthetic peptide by IgE does not demonstrate that this peptide will be functional, i.e. that it will allow the elicitation of the allergic reaction in a sensitized individual. It is generally accepted that such functionality requires 'bivalent' peptides, i.e. comprising two distinct epitopes on the same peptide, allowing cross-linking of two mast cell-bound IgE. Therefore, the aim of the present study was to assess the bivalence of tryptic peptides, using in vitro and in vivo tests.

In a first set of experiments, in vitro tests were performed with tryptic hydrolysate and with purified and characterized tryptic fractions/peptides of β LG. Among the fifteen fractions purified from the tryptic hydrolysate, those enriched with peptides 125–135 and 149–162 were able to trigger RMCs sensitized passively with rat IgE anti-whole β LG antibodies for mediator release.

A similar experiment with synthetic 'tryptic-like' peptides confirmed that region 149–162, containing 14 amino acids, is functional in vitro. Among peptides as long as regions 21–40, 41–60 or 102–124, which were demonstrated to contain major IgE-binding epitopes in rat [4], peptides 21–40 and 102–124 reacted very slightly. Surprisingly, peptide 1–8 reacted weakly as well. It is difficult to imagine that such a small peptide carries two IgE-binding epitopes. The purity of the sample used was checked to confirm the absence of any contaminating longer peptide which could interfere. The weak degranulation observed might thus be due to a slight cytotoxic activity of this peptide observed also with unsensitized mast cells.

Compared with the IgE-mediated mast cell-triggering reactivity of intact β LG, purified or synthetic tryptic-like β LG peptides have a sharply decreased allergenicity. For example, β LG allergenicity of peptide 149–162 was calculated from the ^3H -serotonin release curves to be $56 \mu\text{g } \beta\text{LG/g product}$, namely more than 10,000 times reduced if compared with intact β LG ($10^6 \mu\text{g } \beta\text{LG/g product}$). It is very likely that most IgE-binding peptides analyzed with synthetic tryptic-like peptides certainly are monovalent peptides, i.e. containing only one IgE-binding epitope. β LG has two internal disulphide bonds and considerable secondary structures and a β -barrel tridimensional conformation. Peptides that have been synthesized or prepared after extensive tryptic hydrolysis were then tested after isolation and purification; they might derive from larger fragments ('propeptides') that are formed in physiological conditions and that contain several epitopes. As an example, Sélo et al. [12] have demonstrated that the immunoreactivity of the two isolated tryptic peptides 102–124 and 125–135 derived from the cyanogen bromide 108–145 fragment was lower than that of this peptide. Large fragments might thus comprise two or more IgE epitopes that may be efficiently presented and associated because of the structure and conformation of the fragment, and therefore, are responsible for their higher functionality versus isolated short fragments. Another hypothesis is that IgE affinity for synthetic 'destructured' peptides is too weak to induce activation of sensitized mast cells.

The considerations mentioned above might also explain why intestinal mast cells were not stimulated *in vivo* by any of the synthetic peptides. In addition to structural considerations, it may also be pointed out that the dose used might still have been insufficient and that the administered peptides were not totally bioavailable. EAST inhibition tests carried out with similar high concentrations of peptides, which have no cytotoxic effect *in vitro*, failed to totally suppress the binding of whole β LG to IgE. Moreover, such free peptides were very likely rapidly and at least partially degraded in the gut before they could reach the effector sites of the immune system, unlike β LG, which is much more resistant to digestion by proteases.

However, a short peptide such as the 14-mer 149–162 which has been characterized to account for a significant part of the IgE-binding capacity of the whole β LG [12] has now been demonstrated to be long enough to elicit mast cell degranulation. This work demonstrates that sequential bivalent IgE epitopes exist on β LG. In the future, this observation may help in the analysis and production of hypoallergenic infant formulas designed for cow's milk-allergic infants. Selective technological processes for destroying such mast cell-triggering IgE bivalent epitopes have to be looked for when producing extensively hydrolyzed formulas for allergic infants. Such processes may be based, besides heat and high pressure treatments, on hydrolysis with specific proteolytic enzymes.

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