Comparison of sensitization between β-lactoglobulin and its hydrolysates

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Summary

Background: **Bovine β-lactoglobulin is one of the first foreign antigens encountered by some newborn children, and it has been described as the main allergenic protein in cow's milk even when present at low concentration.**

Objective: **Enzymatic hydrolysis has been identified to be a very effective way to reduce the sensitization of β-lg compared to other treatments. The aim of this study was to explore whether enzymatic hydrolysis could reduce the allergenicity of native bovine β-lg.**

Methods: **β-lg was hydrolyzed by trypsin. Twenty four BALB/c mice were divided into three groups and orally sensitized by native bovine β-lg and its hydrolysates five times at weekly intervals.**

Results: **During the sensitization period, many serious systemic anaphylactic symptoms were observed in mice sensitized by native β-lg compared to hydrolysates of β-lg. Mice sensitized by hydrolysates of β-lg showed a significantly lower spleen lymphocyte proliferation level than intact β-lg. The β-lg-specific IgE antibody levels in serum and intestinal fluid samples induced by native β-lg were significantly elevated. Plasma histamine levels were also evaluated and showed the same trend as IgE. Moreover, the hydrolysates of β-lg significantly up-regulated IFN-γ and IL-10 production and down-regulated IL-4 and IL-5 secretions by murine splenocytes.**

Conclusion: **These results suggested that enzymatic hydrolysis could partly reduce the allergenicity of β-lg.** ***(Asian Pac J Allergy Immunol 2012;30:32-9)***

Key words: *Bovine β-lg, hydrolysates of β-lg, enzymatic hydrolysis, allergenicity, mouse*

Introduction

Cow's milk protein allergy, the most prevalent food allergy in early childhood, is estimated to affect about 2%~3% of infants in their first years.[1] It is an adverse reaction to the proteins present in milk. Although immunoglobulin E (IgE) antibodies against all major milk proteins have been found in blood sera of allergenic subjects, β-lactoglobulin (β-lg) is still considered to be the dominant cow's milk allergen.[2] Furthermore, since β-lg is absent from human milk, it is regarded as the most important cow's milk allergen. Indeed, the specific anti-β-lg IgE level is present in 60%–80% of cow's milk allergic patients.[3]

The β-lg monomer is a globular protein with 162 amino acids with a molecular weight of 18.3 kDa. However, bovine β-lg is very stabile against peptic digestion under acidic conditions, which is related to its high allergenicity.[4] As for infants, factors such as low stomach pepsin activity at birth, immature gastric acid generating mechanism as well as malfunction of pancreatic and intestinal enzymes can also allow cow milk proteins to remain intact by limiting their gastric proteolysis.[5] However, bovine milk proteins are not only essential sources of amino acids for neonates, but also the source of biologically active peptides with antimicrobial, opioid, mineral-binding, antihypertensive, antithrombotic, and immunemodulating properties.[6] Therefore, it's not a feasible approach to remove β-lg from infant milk formula and methods to reduce the allergenicity of β-lg should be developed. The hydrolysis is the most effective method to modify proteins, using digestive enzymes to change the immunoreactivity of protein allergens[7] and this method has been frequently

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applied to modify specific milk components in recent years.

Trypsin is a serine protease found in the digestive system of many vertebrates, where it hydrolyses proteins.[8] In the digestive system, trypsin can hydrolyze peptides into their smaller building blocks, namely amino acids. The enzymatic mechanism is similar to that of other serine proteases and these enzymes contain a catalytic triad consisting of histidine-57, aspartate-102, and serine-195,[9] which form a charge relay that serves to make the active site serine nucleophilic.

In this paper, trypsin was used to hydrolyze β-lg and the overall goal of the current study was to identify the effect of hydrolysis on the allergenicity of β-lg. Therefore, a series of immunologic indicators were detected, including β-lg-specific serum IgE antibody level, spleen cell proliferation and plasma histamine levels, secretion levels of Th1 (interferon-gamma, IFN-γ), Th2 cytokines (interleukin-4, IL-4; interleukin-5, IL-5) and Treg cytokines (interleukin-10, IL-10).

Methods

Testing materials and hydrolyzing conditions

β-lactoglobulin and trypsin were purchased from Sigma Chemical Co. (St. Louis. MO, USA). The conditions of the enzymatic process were as follows: pH 8.0, temperature 42°C, ratio enzyme: substrate of 1:200 (w/w). Hydrolysis was performed for 4 hours at constant pH maintained by the addition of 1M NaOH from a burette. Inactivation of the enzyme was achieved by heating to 90°C for 5 min, followed by immediate cooling.

Experimental mice

Twenty-four female BALB/c mice aged 4 weeks were obtained from the Laboratory Animal Center of Tumour Hospital of Harbin Medical University (Harbin, China) and were allocated into three groups with 8 replications. The mice were caged in a controlled environment with 14 hours light and 10 hours dark cycles at room temperature, approximately 22°C, with free access to food and water. All animal procedures were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. One week later, these animals were sensitized intragastrically with 1 mg β-lg/g BW (body weight), 1 mg hydrolysates of β-lg/g BW and PBS as control respectively with 0.3 μg/g of cholera toxin (CT) (Sigma) as an adjuvant for all treatments,[10] and the mice were boosted 5 times at weekly intervals. Oral lavage was performed using a stainless steel blunt feeding needle. Six weeks after the first oral lavage, mice were fasted over night and challenged intragastrically with 2 doses of β-lg given 30 minutes apart.

Evaluation of symptoms

As described in a previous report,[11] the hypersensitivity responses was evaluated, based on a scoring system, at thirty to forty minutes after all mice were challenged intragastrically with PBS, β-lg or hydrolysates of β-lg, respectively, and were scored as follows: 0 = no symptoms; 1 = scratching and rubbing around the nose and head; 2 = puffiness around the eyes and mouth and pilar erecti; 3 = decreased activity with increased respiratory rate; 4 = wheezing and labored respiration; 5 = cyanosis around the mouth and the tail; and 6 = death.

Lymphocyte proliferation assay

Single-cell suspensions were individually prepared from spleens under aseptic conditions. Erythrocytes were removed using a lysis buffer (Beijing Solarbio Science & Technology Co. Ltd., China). The resulting spleen lymphocytes suspensions were washed twice in complete RPMI-1640 medium (HyClone Laboratories, Inc., USA), supplemented with 100 U/ml Penicillin, 100 μg/ml Streptomycin and 10% fetal bovine serum (HyClone), and adjusted to 1×10^6 viable cells per well in complete RPMI-1640 medium.

The effect of the tested materials on lymphocyte proliferation was determined via inclusion cell culture in vitro at a certain concentration (100 μg/ml). Each test concentration of β-lg and hydrolysates of β-lg were added to triplicate wells of 96-well tissue culture plates (Costar, USA) and cultured with or without Concanavalin A (ConA), 0.5 μg/ml (Sigma). Control cells were plated complete on RMPI-1640 medium with ConA. After cells were cultured for 48 hours at 37°C (5% CO_2) and lymphocyte proliferation was tested using the MTS (Promega Corporation, USA) assay. The results were expressed as a stimulation index (SI) using the following equations which were revised according to a previous report:[12]

SI (without ConA) = (OD cells－OD medium)/OD medium)

SI (with ConA) = (OD cells + ConA－OD medium + ConA) / OD medium)

Determination of β-lg-specific IgE level or its hydrolysates specific-IgE level

The blood samples were collected by taking the eyeball for blood and separating the serum. Serum

was obtained by centrifuging (1000×g for 10 min, 4°C), collected and frozen at –80°C, and intestinal tracts were also collected. Levels of β-lg-specific IgE were determined by an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with 1 μg/ml of β-lg or its hydrolysates in coating buffer (0.05 M sodium carbonate-bicarbonate buffer, pH = 9.6±0.2). After overnight incubation at 4°C, the plates were washed 3 times with washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20) and the reactions were blocked with blocking solution (50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin) for 1 hours, then washed 3 times again. Then, serum samples were added to the plates and incubated for 2 hours at 37°C. Plates were washed 4 times and 100 μl of goat anti-mouse IgE antibody conjugated with HRP (horseradish peroxidases, AbD serotec, England) was added for 1 hour at 37°C, then they were washed again 4 times. Staining was performed with 3, 3'-5, 5'-tetramethyl-benzidine (TMB) (Sigma) for 30 min at 25°C in the dark, stopped with 1 M H_2SO_4, and the plates were read by an enzyme micro-plate reader (Bio-RAD Model 680) at 450 nm. Values were expressed as arbitrary units (AU) deduced from the optical densities of the reference serum curve with a high level of antibodies after subtracting the blanks. The reference serum was a separate pool of sera collected for IgE, and its concentration was assigned to be 1 AU. All analyses were performed in duplicate.

Determination of plasma histamine levels

Plasma was separated into chilled tubes containing heparin sodium as anticoagulant. After centrifugation (1713×g) for 10 minutes at 4°C, plasma aliquots were collected and frozen at –80°C. Histamine levels were determined using an enzyme immunoassay kit (Uscnlife Science & Technology CO., LTD, China), as described by the manufacturer.

Measurement of cytokines secretion in lymphocyte supernatant

The effect of native β-lg and hydrolysates of β-lg on cytokine production was determined by inclusion cell culture in vitro at a set concentration, which had been determined from cellular proliferation assays. Lymphocyte culture was conducted as described above in the presence of β-lg and hydrolysates of β-lg and in the absence and presence of ConA (0.5 μg/ml). Cytokines IL-4, IL-5, IL-10 and IFN-γ were determined by enzyme immunoassay kits (R&D Systems, USA) according to the manufacturer's instructions.

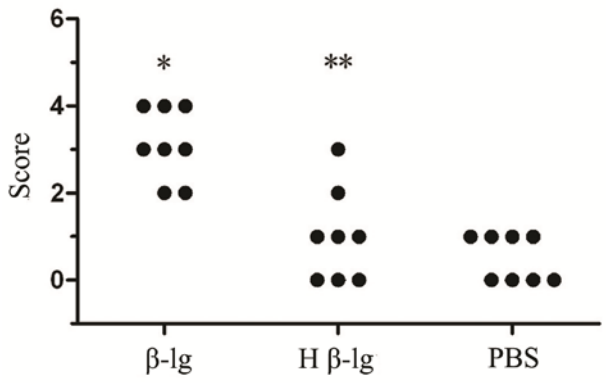

Figure 1. Scores of hypersensitivity symptoms. The black dots represented the individual group's score, β-lg represents β-lg, H β-lg represents hydrolysates of β-lg, PBS represents the control group, CT (cholera toxin) was added to each group as an adjuvant. The Wilcoxon-Mann-Whitney test was performed to evaluate the significance between each group. * P <0.01 vs H β-lg, ** P <0.01 vs PBS.

Statistical analysis

The results were expressed as mean±SEM. Statistical significance (p <0.05 or p <0.01) was determined by Student's t-test, or the Wilcoxon-Mann-Whitney U test. The hypersensitivity symptoms figure was generated by Prism 5 GraphPad software (GraphPad Prism Software, Inc, San Diego, Calif, USA), and other figures were calculated using Microsoft Excell. SPSS 15.0 software (SPSS Inc, Chicago, USA) was used to analyze the significance levels.

Results

Characteristics of hypersensitivity symptoms

After six weeks of sensitization, mice were challenged by gastric lavage with β-lg or hydrolysates of β-lg plus CT. Hypersensitivity symptoms were evident after six weeks of sensitization by lavage with β-lg plus CT within 30 min. The severity of symptom was scored as detailed in the materials and methods section. The mice in the control group showed a mild reaction, which was probably due to weaker allergic responses caused by the CT, diet, environment or other physical factors, and the most severe symptoms were observed in mice challenged with β-lg plus CT. Compared to the β-lg plus CT sensitized

group, mice sensitized by hydrolysates of β-lg plus CT had weaker reactions. The average hypersensitivity score in the PBS group was significantly lower than that observed in β-lg plus CT sensitized group. In contrast, the score for the hydrolysates of β-lg plus CT sensitized group was significantly lower than that for the β-lg plus CT group (Figure 1).

Effect of β-lg or hydrolysates of β-lg on lymphocyte proliferation

Spleen cells were the main lymphocytes and this study investigated the immunomodulating effect of β-lg and hydrolysates of β-lg in vitro by measuring their impact on lymphocyte proliferation which was expressed as SI and evaluated with and without suboptimal concentrations (0.5 μg/ml) of ConA (Figure 2). Both β-lg and hydrolysates of β-lg stimulated lymphocyte proliferation. SI values were lower for hydrolysates of β-lg than for native β-lg, indicating that the hydrolysis could break down the intact β-lg into smaller peptides, so that the epitope structures on the surface of β-lg were destroyed. The group with no ConA had lower SI values than the group with ConA, but the difference was not significant.

β-lg-specific IgE level in serum and intestinal tracts after intragastric β-lg or hydrolysates of β-lg

To investigate the IgE production developed by systemic and mucosal antibody responses, the IgE levels in serum and the intestinal tracts were monitored by ELISA. The serum IgE level of mice challenged intragastrically with β-lg had a significantly higher level of antibody response compared to the hydrolysates of β-lg and PBS groups. However, there was no significant difference between the hydrolysates of β-lg and PBS groups (Figure 3). ELISA was also used to detect the intestinal tract IgE level. IgE from the intestinal tracts showed a significantly higher trend than serum IgE for all three groups that was only significant in the β-lg group (P <0.05).

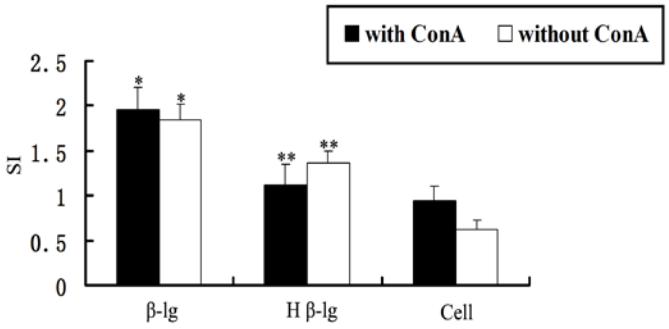

Figure 2. Effects of β-lg and hydrolysates of β-lg on lymphocyte proliferation in vitro. The spleen was removed from each mouse (n = 8 per group) immediately after death, erythrocytes were then lysed and spleen-derived lymphocytes from each mouse were cultured in the presence or absence of β-lg, hydrolysates of β-lg at 100 μg/ml respectively, and cultured with or without ConA (0.5 μg/ml). Control cells were cultured in complete RMPI-1640 medium with ConA. After cells were cultured for 48 hours, lymphocyte proliferation was tested by an MTS assay. The absorbance of each well was read at 490nm and the results were expressed as SI±SEM as described in the materials and methods section. Student's *t*-test was used to determine statistical significance. β-lg represented β-lg, H β-lg represented hydrolysates of β-lg, Cell represented control mice. *P <0.05 vs H β-lg, ** P <0.05 vs Cell.

Elevated plasma histamine level after challenged with β-lg or hydrolysates of β-lg

Plasma histamine levels were determined by an ELISA kit and the results indicated that the histamine levels of mice challenged intragastrically with β-lg were significantly higher than those which received hydrolysates of β-lg or PBS (Figure 4).

Effect of β-lg or hydrolysates of β-lg on cytokines secretion

Cytokines were analyzed in lymphocyte supernatants as indicators of the systemic responses to anaphylactic reactions.

Table 1. Cytokine secretion measured in the supernatants of cultured murine splenocytes by ELISA

Cytokine	Without ConA			With ConA		
(pg/ml)	Control	β-lg	Hydrolysates of β-lg	Control	β-lg	Hydrolysates of β-lg
IL-4	5.43±0.12[a]	2.98±0.31[a]	8.98±0.03[a]	11.25±0.34[a]	1.67±0.05[b]	15.87±0.36[a]
IL-5	0.08±0.001[a]	0.05±0.001[a]	0.07±0.001[a]	2.56±0.08[a]	0.07±0.001[b]	3.01±0.05[a]
IL-10	0.6+0.005[a]	0.8±0.03[a]	0.6±0.002[a]	5.65±0.76[a]	0.87±0.001[b]	8.12±0.76[a]
IFN-γ	80.34+0.86[a]	86.43+1.23[a]	92.28±1.35[a]	121.22±3.45[a]	40.12±1.56[b]	175.87±3.79[b]

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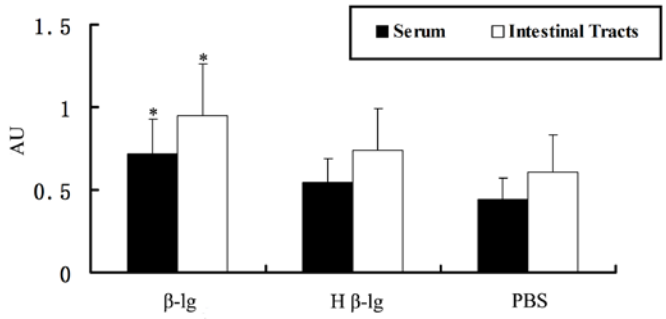

Figure 3. Effects of β-lg and hydrolysates of β-lg on systemic (serum) and mucosal (intestinal tract) antibody responses. Serum and intestinal tracts from all mice (n = 8 per group) were collected 30 min after the last challenge. β-lg specific-IgE levels or its hydrolysates specific-IgE level were determined by ELISA. Values were expressed as arbitrary units (AU) deduced from the optical densities of the reference serum curve with a high level of antibodies after subtracting the blanks. Student's *t*-test was used to determine statistical significance (P <0.05). *P <0.05 vs PBS. β-lg represented β-lg, H β-lg represented hydrolysates of β-lg, PBS represented control group, CT was added to each group as an adjuvant.

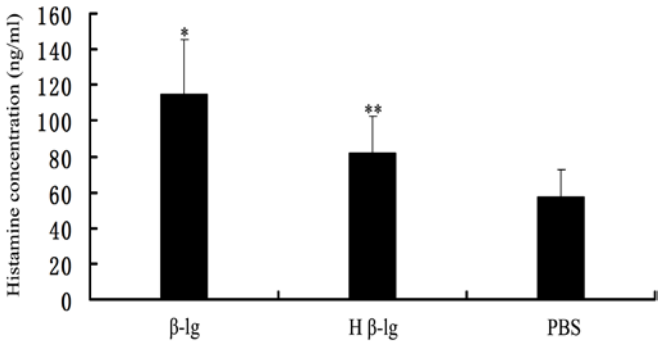

Figure 4. Effects of β-lg and hydrolysates of β-lg on plasma histamine level. Plasmas from all mice (n = 8 per group) were collected 30 min after the last challenge. Histamine concentrations were determined by ELISA. Results are expressed as concentration (ng/ml) ± SEM. Student's *t*-test was used to determine statistical significance (P <0.05). * P <0.05 vs H β-lg, ** P <0.05 vs PBS. β-lg represents β-lg, H β-lg represents hydrolysates of β-lg, PBS represents the control group, CT was added to each group as an adjuvant.

The effect of native β-lg and hydrolysates of β-lg on cytokine secretions of the supernatants of spleen lymphocyte is shown in Table 1 (with or without ConA, with a final concentration of ConA at 0.5 μg/ml). In the cytokine secretion test in the absence of ConA, β-lg and its hydrolysates showed almost no stimulation of secretion of IL-4, IL-5, IL-10 and IFN-γ in supernatants of spleen cells, namely, β-lg and β-lg hydrolysates had insignificant effects on all of these four cytokines without ConA being present, compared to control group. However, when ConA was added to the supernatant of spleen cells, β-lg significantly inhibited the secretion of IL-4, IL-5, IL-10, IFN-γ, while hydrolysates of β-lg significantly stimulated the secretion of IFN-γ.

Discussion

Studies have been carried out to evaluate cow's milk systemic hypersensitivity generated by oral sensitization and challenge using the murine model. The current experiment also used this model to study the immunological effects of hydrolysates of β-lg, as compared to native β-lg. The purpose of this study was to determine whether hydrolysis could play a role in reducing the immunogenicity of native β-lg. Mice were used immediately after weaning, so β-lg was their first contact with external cow's milk protein and no previous sensitization had been produced. This was to ensure that the work was carried out on nonsensitized mice.[11]

The effect of enzymatic hydrolysis on β-lg has been studied by several researchers. The ability of hydrolyzates of β-lg and peptides to induce oral tolerance to native β-lg has been evaluated[13] and the results identified that tolerogenic β-lg peptides had low allergenicity. One study used two steps hydrolysis to reduce the hypersensitivity of whey proteins[14] and the results showed that the hydrolysis of sodium caseinate with Alcalase produced reduction in the material's allergenicity. This study used a one step method of trypsinase to hydrolyze β-lg and the allergenicity of hydrolyzates was evaluated using series of immunological methods.

The immunological properties of hydrolysates of β-lg were evaluated by cell proliferation and immunological methods, including evaluation of symptoms, lymphocyte proliferation assay, cytokine analysis in vitro; serum and intestinal tract IgE levels, and plasma histamine level. These results showed that the hydrolysis decreased lymphocyte stimulation sensitized by native β-lg. Serum and intestinal tracts IgE levels in subjects sensitized by hydrolysates of β-lg were reduced and plasma histamine level was also lower in mice sensitized

with hydrolysates of β-lg compared to native β-lg. Furthermore, hydrolysates of β-lg could regulate the secretion of cytokines.

Lymphocytes play a key role in the development of cow's milk allergy. Proliferation of lymphocytes in the β-lg group was significantly higher than that in hydrolysates of β-lg group, which suggests that hydrolysis could reduce the stimulation of lymphocytes proliferation by β-lg. However, this is not consistent with the results reported by Mercier et al.[15] who showed that β-lg had no impact on cell proliferation. Moreover, ConA-stimulated spleen cells did not show significant stimulation compared to the group with no ConA stimulation.

Cross and Gill used a commercial preparation of a modified whey protein concentrate as a stimulation material to detect the stimulation of splenocytes and their result indicated that the modified whey protein concentrates could suppress lymphocytes proliferation.[16] On the contrary, other workers have observed that 100 and 2000 mg/ml of β-lg had no effect on resting spleen cells[17] and β-lg, α-lactalbumin, glycomacropeptide, and bovine serum albumin (0.5–200 mg/ml) had no effect on the proliferation of resting spleen cells.[15]

It has been demonstrated that IgE antibodies play an important role in mediating type-I hypersensitivity responses in humans.[18] Some studies have investigated the effect of hydrolysates of β-lg-derived tryptic-chymotryptic peptides by *Bifidobacterium lactis* NCC362 enzymes on their allergenicity and they also showed that the IgE binding capacity of acidic tryptic-chymotryptic peptides was reduced by *B. lactis* peptidases-driven hydrolysis.[19] In this study, the IgE level in serum was evaluated by ELISA and the results indicated that β-lg induced mice to generate elevated IgE levels, but the hydrolysates of β-lg inhibited IgE production, which indicated that the hydrolysates of β-lg possessed a lower sensitization ability. The mechanism for this result might be that the hydrolysis destroyed the three-dimensional structure of native β-lg which contained certain antigenic properties.

Furthermore, several studies have demonstrated that the immunisation of animals with β-lg together with adjuvant increased the IgG production in mice serum.[14] Another study reported that a diet supplemented with cow's milk protein suppressed the levels of serum IgG antibodies specific to dietary CT and injected β-lg in mice had no effect on IgM, IgA and IgE antibody responses.[20] However, in this study there was no difference in IgG level among all groups (data not shown).

Although the IgE antibody is the biological mediator of immediate hypersensitivity responses, the development of IgE antibody responses is orchestrated by Th cell subsets.[21] Th cells are polarized into two main phenotypes classified as T-helper type 1 (Th1) and type 2 (Th2) on the basis of their selective cytokine expression patterns. Th1 cells express IFN-γ and Th2 cells produce IL-4, IL-5, IL-10, etc. Th2 cell cytokines collectively facilitate IgE antibody production and participate in eliciting IgE-mediated allergic reactions, whereas the type 1 cytokine IFN-γ inhibits IgE antibody responses.[22]

The regulation of cytokines secretion by various proteins has been studied widely. Cross and Gill showed that a modified whey protein concentrate had no suppressive effect on IL-2-sustained proliferation of motogen-activated T cell blasts, but could significantly stimulate the secretion of IFN-γ.[16] Whey protein isolate inhibited ConA-induced cytokine secretion, whereas enzymatic digests and peptide fractions significantly increased IFN-γ secretion. Acidic or neutral peptide fractions stimulated splenocyte proliferation and cytokine secretion. Prioult et al. studied the allergenicity of acidic peptides from bovine β-lg by hydrolysis with *B. lactis* NCC362 enzymes, and their results indicated that the peptide fragments significantly up-regulated IFN-γ and IL-10 production and down-regulated IL-4 secretion by murine splenocytes.[19]

In this study, in the absence of ConA, β-lg and hydrolysates of β-lg showed no suppression or stimulation role to cytokines IL-4, IL-5, IL-10, IFN-γ, compared to the control group, but when ConA was added, β-lg significantly inhibited the secretion of IL-4, IL-5, IL-10, IFN-γ, while hydrolysates of β-lg significantly stimulated the secretion of IFN-γ, but showed no effect on the secretion of IL-4, IL-5 and IL-10. According to the classification of the two main phenotypes of Th1 cells and Th2 cells, IFN-γ is produced by Th1 cells, while IL-4, IL-5 and IL-10 are generated by Th2 cells. Therefore, the increase of IFN-γ levels represents an inhibition of the IgE antibody responses. Furthermore, the secretion of IFN-γ was inhibited by native β-lg, which indicats that native β-lg could stimulate IgE antibody responses, and this presumption was in line with the results shown in Figure 3. However, in native the β-lg group without ConA, the secretions of IL-4, IL-5 and IL-10 were reduced. It seemed that the IgE

antibody responses should be inhibited, but the results were the reverse, which might indicate that the inhibition role of IFN-γ (Th1) was stronger than the stimulation role of IL-4, IL-5 and IL-10 (Th2).

It has been shown that histamine is a major mediator released during anaphylactic reactions and its level was significantly increased in cow's milk-sensitized/challenged mice.[10] Kadoya et al. used the cat as an animal model to investigate the effect on histamine and they found that plasma histamine levels were markedly elevated in five of the 15 cats with allergic dermatitis.[23] Other workers have reported that plasma histamine levels were elevated in some human patients with atopic dermatitis.[24,25] The results of this study were consistent with the above studies, which demonstrated that the hydrolysates decreased the histamine level in mice plasma compared to intact β-lg. However, the mechanism for reduction of the histamine level was unclear. In the hydrolysates of β-lg group, the hydrolysis could undermine the three-dimensional structure of β-lg, causing reduction in allergenicity, so mice in this group had low levels of histamine.

In conclusion, our results showed that the hydrolysis could partly reduce the immunogenicity of native β-lg and the reason might be that the hydrolysis destroyed the three dimensional structure of β-lg, so some epitopes were removed. This paper may help us to understand the roles of hydrolytic enzyme in reducing hypersensitivity to β-lg. However, the underlying mechanism for reduction of β-lg sensitization needs further study.

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