ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
February 2014; 13(1):26-32.

Comparison of Immunomodulating Properties of Beta-Lactoglobulin and its Hydrolysates

Cui-cui Duan, Ai-li Li, Li-jie Yang, Rui Zhao, Wen-guang Fan, and Gui-cheng Huo

Key Laboratory of Dairy Science, Ministry of Education,
Northeast Agricultural University, Harbin 150030, China

Received: 28 December 2012; Received in revised form: 26 January 2013; Accepted: 7 February 2013

ABSTRACT

Cow's milk allergy is one of the most common food allergies in childhood. Beta-lactoglobulin (β- Lg) is a dominant allergen in cow's milk. Hydrolysis is known as an effective method to reduce the allergenicity of proteins. Thus, the objective of this study was to compare the allergenicity of β-Lg and its hydrolysates using an animal model.

Twenty four BALB/c mice were divided into three groups and subcutaneously injected with native bovine β-Lg and its hydrolysates on days 0, 7 and 14. During the sensitization period, a number of systemic anaphylactic indicators were observed in mice sensitized by β-Lg compared to those sensitized by hydrolysates of β-Lg.

Mice sensitized by hydrolysates of β-Lg showed a significantly lower spleen lymphocyte proliferation level than that sensitized by intact β-Lg. Antibody levels of β-Lg-specific IgE in serum induced by native β-Lg were significantly high. Plasma histamine levels were also evaluated and showed the same trend as IgE. Moreover, the hydrolysates of β-Lg significantly down-regulated IL-4 and IL-5 secretions in serum.

These results suggested that enzymatic hydrolysis could reduce the allergenicity of β-Lg.

Keywords: Hydrolysis; Hypersensitivity; Lactoglobulins; Milk

INTRODUCTION

Commonly, cow's milk allergy (CMA) is caused by fragments of proteins containing epitopes, which are not hydrolyzed by digestive enzymes, and are able to cross the intestinal barrier and trigger allergic responses.[1] Many studies have been conducted to define and characterize the allergens involved in CMA. As β- lactoglobulin (β-Lg) is absent in human milk, it is considered to be the dominant bovine milk allergen.[2] There are two main isoforms of β-Lg, i.e. genetic variants A and B, which differ only by two point mutations on residues 64 and 118, aspartic acid and valine in β-Lg A, and glycine and alanine in β-Lg B, respectively.[3,4] β-Lg occurs naturally as a mixture of monomers and dimers and there are many allergenic epitopes spread all over the β-Lg structure.

Various food manufacturing processes have been developed and assessed in order to change the allergenicity of cow's milk, such as mechanical,

Corresponding Author: Gui-cheng Huo, PhD;
Key Laboratory of Dairy Science, Ministry of Education, Northeast Agricultural University, Harbin 150030, China. Tel: (86 0451) 5519 1807, Fax: (86 0451) 5519 0340, E-mail: gchuo58@yahoo.com.cn

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

thermal (dry heating, boiling or cooking), biochemical and chemical processes (enzymatic digestion).[5,6] Previous studies have shown that heating and proteolytic treatments can remove conformational epitopes of milk proteins as a result of the loss of tertiary protein structures and therefore lead to a decreased allergenic potential.[7] However, it was found that β-Lg exhibited a complex unfolding mechanism during denaturation as a result of heat treatment, and studies further reported that initial denaturation of β-Lg led to the formation of small well defined clusters with a size independent to concentration, temperature and ionic strength.[5] Furthermore, multiple studies have demonstrated that processing milk, as it is commonly practiced in the food industry, leads to changes in clinical allergenicity.[8-10] Enzymatic hydrolysates were firstly obtained using gastrointestinal enzymes, e.g. trypsin, pepsin, chymotrypsin, in order to potentially mimic physiological digestion and compensate the intestinal and enzymatic systems' immaturities of newborn babies.[11]

In order to identify the effects of enzymatic hydrolysis on the allergenicity of β-Lg, trypsin was used to hydrolyze β-Lg and BALB/c mice were used as an animal model system. Measurements on the animal model included the recording of spleen lymphocyte proliferation levels, β-Lg-specific serum IgE antibody levels, plasma histamine levels, secretion levels of Th1 (interferon-gamma, IFN-γ) and Th2 cytokines (interleukin-4, IL-4; interleukin-5, IL-5) in serum in order to demonstrate the reduction activity of enzymatic hydrolysis on the allergenicity of β-Lg.

MATERIALS AND METHODS

Testing Materials and Hydrolyzing Conditions

β-Lg and trypsin were purchased from Sigma Chemical Co. (St. Louis. MO, USA). The conditions of enzymatic processing 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. Inactivation of the enzyme was achieved by heating samples at 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 randomly allocated into three groups consisting of 8 animals in each group. The mice were kept in a controlled environment with 14 hours light and 10 hours dark cycle and room temperature approximately 22°C. Free access to food and water was provided. All animal procedures were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. After one week in the described environment, the animals in case group were subcutaneously injected with either 100μg β-Lg or 100μg hydrolysates of β-Lg emulsified with 50μg completed Freund's adjuvant (Sigma Chemical Co. St. Louis. MO, USA). The control group was subcutaneously injected with PBS. The mice were immunized on days 0, 7 and 14. Serum and plasma were collected on day 14 after the last immunization.

Lymphocyte Proliferation Assay

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

The effect of β-Lg on lymphocyte proliferation was determined via the inclusion of cell culture in vitro at a specific concentration (100 μg/ml). β-Lg and hydrolysates of β-Lg were added to 96-well culture plates (Corning Incorporated, Cambridge, Massachusetts, USA) in triplicate at set concentrations and cultured with or without Concanavalin A (ConA), 0.5 μg/ml (Sigma Chemical Co. St. Louis. MO, USA). Control cells were treated by addition of complete RMPI-1640 medium with ConA. Following these procedures, cells were cultured for 48 hours at 37°C (5% CO_2) and lymphocyte proliferation was tested by (3-(4,5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) MTS (Promega Corporation, Madison, WI, USA) assay. The results were expressed as a stimulation index (SI) and calculated with following revised equations:[12]

$$SI\ (\text{without ConA}) = (OD_{cells} - OD_{medium})/OD_{medium}$$

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

SI (with ConA)= ($OD_{cells + ConA}$ - $OD_{medium + ConA}$) / OD_{medium})

Determination of β-Lg-specific IgE Level or its Hydrolysates Specific-IgE Level

Blood samples were collected from the eyeball. The serum was isolated by centrifugation (1000×g for 10 min, 4°C), collected and frozen at –80°C. Concentrations 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, plates were washed 3 times by washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20) and reactions were blocked with blocking solution (50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin) for 1 hour, then washed 3 times again. Serum samples were added to the plates and incubated for 2 hours at 37°C. Plates were again washed 4 times, and 100 μl of goat anti-mouse IgE antibody solution conjugated with HRP (horseradish peroxidases, AbD serotec, Oxford, UK) was added and incubated for 1 hour at 37°C. Subsequently, the plates were then washed again for 4 times. Staining was performed by addition of 3, 3'-5, 5'-tetramethyl-benzidine (TMB) (Sigma Chemical Co. St. Louis. MO, USA) for 30 min at 25°C in darkness, stopped with 1 M H_2SO_4, then the plates were read by an enzyme micro-plate reader (Bio-RAD Model 680, Bio-Rad Laboratories, Hercules, CA, USA) 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 blank samples. The reference serum consisted of a separate pool of sera collected for IgE, and its concentration was assigned to be 1 AU. All analyses were performed in duplicates.

Determination of Plasma Histamine Levels

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

Measurement of Cytokine Secretion in Serum

The levels of cytokines IL-4, IL-5 and IFN-γ in mice sensitized by native β-Lg and hydrolysates of β-Lg were determined by enzyme immunoassay kits (R & D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Statistical Analysis

Results are expressed as mean ±SEM. Differences between samples and their respective controls in the spleen cell proliferation (Figure 1), IgE level (Figure 2), histamine level (Figure 3) and cytokine assays (Tables 1) were determined by one-way analysis of variance (ANOVA) using the least significant difference (LSD) test with the significance set at $p < 0.05$. SPSS 15.0 software (SPSS Inc, Chicago, USA) was used to analyze the significance level.

RESULTS

Effect of β-Lg or Hydrolysates of β-Lg on Lymphocyte Proliferation

The effect of β-Lg or hydrolysates of β-Lg on murine splenocyte proliferation was evaluated in the absence and presence of ConA (Figure 1). Both β-Lg and hydrolysates of β-Lg stimulated lymphocyte proliferation. However, compared with native β-Lg (p=0.03), hydrolysates of β-Lg showed a decreased stimulation effect (p=0.108) on murine splenocytes, indicating that the hydrolysis might damage the structure of native β-Lg by removing or hiding epitope structures on the surface of β-Lg. The group of absent ConA generated significantly lower SI than the group with ConA present.

β-Lg or its Hydrolysates-Specific IgE Levels in Serum after Subcutaneous Injection

To investigate the IgE production induced by systemic and mucosal antibody responses, the β-Lg or its hydrolysates-specific IgE levels in serum were detected by ELISA. The mice subcutaneously injected with β-Lg generated a significantly higher level of antibody response compared to that of hydrolysates of β-Lg and Control groups (p=0.002). However, there was no significant difference between hydrolysates of β-Lg and control groups (Figure 2).

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

Elevated Plasma Histamine Level after Subcutaneous Injection of β-Lg or Hydrolysates of β-Lg

Histamine levels in plasma were determined by an ELISA kit and the results indicated that the histamine levels of mice subcutaneously injected with β-Lg were significantly higher than that of mice subcutaneously injected with hydrolysates of β-Lg and PBS (p=0.007) (Figure 3).

Effect of β-Lg or Hydrolysates of β-Lg on Cytokine Secretion

Cytokines, acting as indicators of systemic responses to anaphylactic reaction in serum were analyzed by ELISA.

The effects of native β-Lg and hydrolysates of β-Lg on cytokine secretions in serum are shown in Table 1.

.

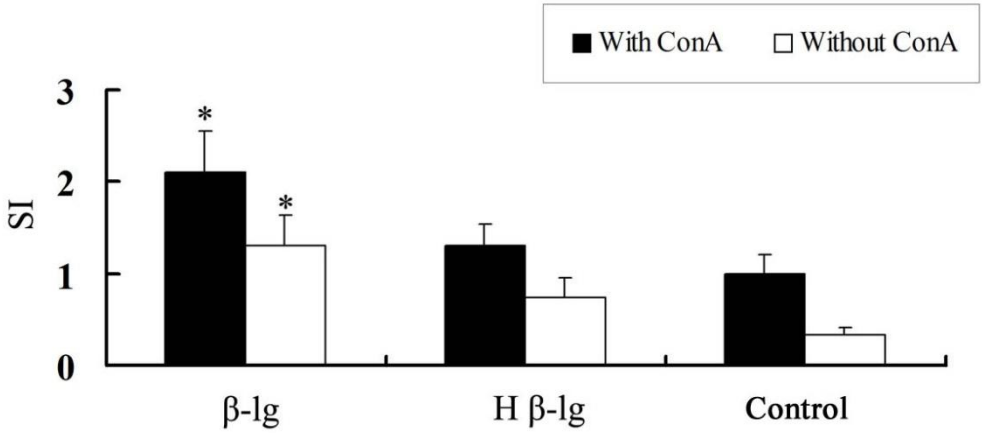

Figure 1. Effects of β-Lg and hydrolysates of β-Lg on lymphocyte proliferation. The spleen was removed from each mouse (n = 8 per group) immediately after death, erythrocytes were then lysed and spleen-derived lymphocytes from each mouse were cultured in the presence or absence of β-Lg, hydrolysates 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. AVONA was used to determine statistical significance. β-Lg represented β-Lg, H β-Lg represented hydrolysates of β-Lg, Control represented control mice. *p<0.05 vs H β-Lg.

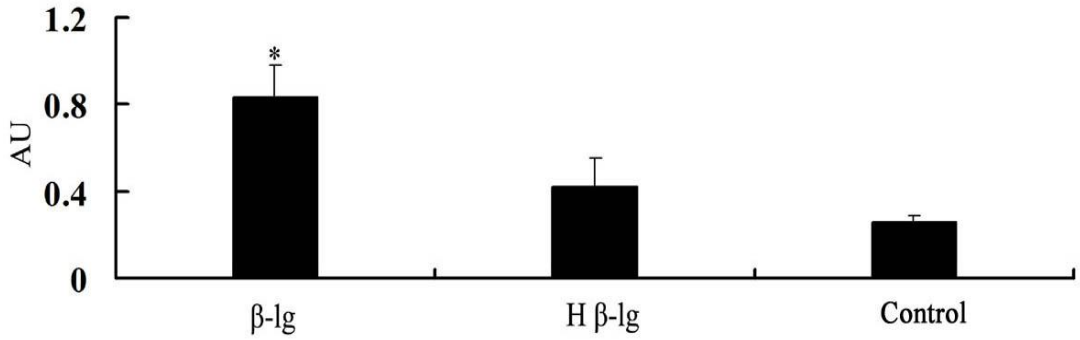

Figure 2. Effects of β-Lg and hydrolysates of β-Lg on systemic (serum) antibody responses. Serum from all mice (n = 8 per group) was collected on day 14 after the last challenge. β-Lg specific-IgE levels or its hydrolysates specific-IgE level were determined by ELISA. Values were expressed as arbitrary units (AU) deduced from the optical densities of the reference serum curve with a high level of antibodies after subtracting the blanks. AVONA was used to determine statistical significance (p<0.01). β-Lg represented β-Lg, H β-Lg represented hydrolysates of β-Lg, Control represented control group. *p<0.01 vs H β-Lg.

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

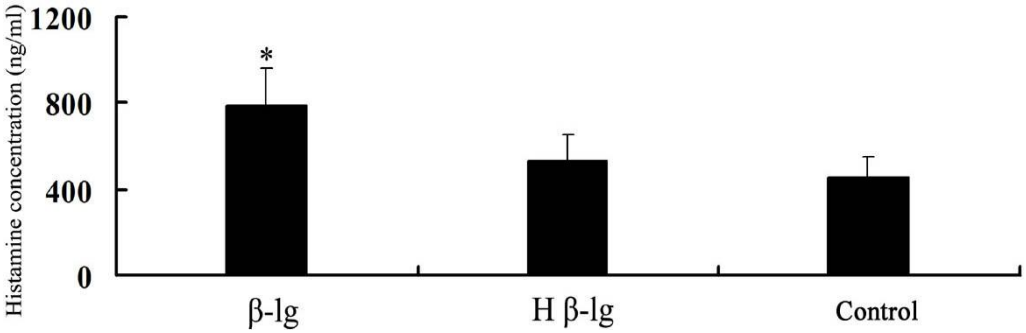

Figure 3. Effects of β-Lg and hydrolysates of β-Lg on plasma histamine level. Plasma from all mice (n = 8 per group) were collected on day 14 after the last challenge. Histamine concentrations were determined by ELISA. Results were expressed as concentration (ng/ml) ± SEM. AVONA was used to determine statistical significance ($p<0.05$). * $P<0.05$ vs H β-Lg. β-Lg represented β-Lg, H β-Lg represented hydrolysates of β-Lg, Control represented control group.

Table 1. Cytokine secretion measured in the serum by ELISA.

Cytokines (pg/ml)	Control	β-Lg	Hydrolysates of β-Lg
IL-4	<1.0	45±12.1 [a]	5±0.8
IL-5	<1.0	67±15.2 [a]	9±1.4
IFN-γ	<4.0	15±3.2	12±2.1

Different superscripts on the top differ significantly ($p<0.05$)

IL-4 and IL-5 levels were significantly elevated in mice subcutaneously injected with β-Lg compared with that in mice subcutaneously injected with hydrolysates of β-Lg and PBS. In contrast, IFN-γ levels in mice subcutaneously injected with hydrolysates of β-Lg and PBS were essentially the same.

DISCUSSION

Many studies have been conducted to evaluate cow's milk systemic hypersensitivity using the murine model. In the present study, a BALB/c model was used to study the immunological changes of hydrolysates of β-Lg compared to native β-Lg. The goal of this study was to identify whether the hydrolysis could reduce the immunogenicity of native β-Lg. Experimental animals were used immediately after weaning to guarantee that β-Lg was their first contact with external cow's milk protein and no previous sensitization had occurred. Therefore, hereby ensure that the work was carried out on nonsensitized mice.[13]

In this study, we aimed to identify the effects of hydrolysis on β-Lg and hydrolysates of β-Lg through investigating its roles on systemic antigen-specific antibody production. For this purpose, BALB/c mice were sensitized with β-Lg and its hydrolysates by subcutaneous injection. It has been identified that IgE antibodies play a crucial role in mediating type I hypersensitivity responses in humans[14] and an elevated IgE antibody level was detected in mice sensitized by β-Lg compared to that in each of the test group. Our results indicated that the humoral immune responses of mice sensitized by β-Lg could be significantly increased while the humoral immune responses of mice sensitized by hydrolysates of β-Lg could be significantly decreased. A relevant study also confirmed that orally delivered whey protein concentrates could significantly increase humoral immune responses.[15]

Consistent with previous results, the results of this study indicated that the enhancement of humoral immune responses in this model was frequently accompanied by an elevated histamine level. Similar findings have been reported that plasma histamine levels were elevated in some human patients with atopic dermatitis.[16-18] It was shown that when food allergens penetrated mucosal barriers and got in contact with IgE antibodies, these bound to mast cells or basophils, which induced symptoms of immediate hypersensitivity after histamine and other mediators

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

were released.[14] Our motivation to conduct the present study was that plasma histamine levels might represent a release from cells localized in affected organs under acute allergic conditions, as seen in food challenge tests in human allergic patients.

One of the most important steps in specific immune reactivity is clonal expansion (proliferation) to produce a pool of antigen-reactive lymphocytes. In this study, spleen proliferation assay was carried out to study their response to different stimulations. Lymphocytes from β-Lg-sensitized mice significantly increased their proliferation ratio when β-Lg was added to the culture medium. In contrast, the proliferation ratio of lymphocytes from hydrolysates of β-Lg-sensitized mice was inhibited. Therefore, the hydrolysis could significantly reduce the stimulation of β-Lg to lymphocyte proliferation in vitro. A similar study indicated that lymphocytes from CM-sensitized mice significantly increased their proliferation ratio when CMP was added to the culture medium.[13] However, relevant study also showed that the hydrolysis had a greater stimulating effect on splenocyte proliferation due to the release of peptides in the process of hydrolyzing.[12]

In order to further confirm the effects of hydrolysis on β-Lg and hydrolysates of β-Lg, cytokines IL-4, IL-5 and IFN-γ were detected. The observed increase of IL-4 and IL-5 levels represented a stimulation of IgE antibody response. Our results showed that the secretions of IL-4 and IL-5 were enhanced in the group sensitized by β-Lg, resulting in an increased IgE antibody response. This result was consistent with that obtained from the β-Lg or its hydrolysates-specific IgE levels in serum after subcutaneous injection. A study also showed that IL-4 and IL-5 levels were significantly increased in cow's milk protein-stimulated cultures when compared with unstimulated cells.[16]

In conclusion, the present results of lymphocyte proliferation, IgE level in serum, histamine level in plasma and cytokine secretion levels in serum indicated that hydrolysis is an effective method to reduce the allergenicity of β-Lg. The reason for this reduced activity might be that hydrolysis destroyed the three dimensional structure of β-Lg, and some of the epitopes were subsequently removed. The present study may aid to understand the role of hydrolytic enzymes on reducing hypersensitivity of β-Lg. However, the more detailed mechanisms of β-Lg sensitization reduction need further studies.

ACKNOWLEDGEMENTS

This work was financially supported by the Innovative Team of Development Science and Technology of Bio-Dairy Products, Northeast Agricultural University (CXT 007) and National Science Foundation of China (31101267).

REFERENCES

1. Justine M, Sophie F, Christian V, Christian S, Luc M. Polysaccharides reduce in vitro IgG/IgE-binding of β-lactoglobulin after hydrolysis. Food Chem 2007; 104(3):1242-9.
2. Prioult G, Pecquet S, Fliss I. Allergenicity of acidic peptides from bovine β-lactoglobulin is reduced by hydrolysis with Bifidobacterium lactis NCC362 enzymes. Int Dairy J 2005; 15(5):439-48.
3. Mecherfi KE-El, Saidi D, Kheroua O, Boudraa G, Touhami M, Rouaud O, et al. Combined microwave and enzymatic treatments for beta-lactoglobulin and bovine whey proteins and their effect on the IgE immunoreactivity. Eur Food Res Technol 2011; 233(5):859-67.
4. Sabadin IS, Villas-Boas MB, Lima Zollner R-de, Netto FM. Effect of combined treatment of hydrolysis and polymerization with transglutaminase on beta-lactoglobulin antigenicity. Eur Food Res Technol 2012; 235 (5):801-9.
5. Monaci L, Tregoat V, van Hengel AJ, Anklam E. Milk allergens, their characteristics and their detection in food: A review article. Eur Food Res Technol 2006; 223(2):149-79.
6. Adel-Patient K, Nutten S, Bernard H, Fritsché R, Ah-Leung S, Meziti N, et al. Immunomodulatory Potential of Partially Hydrolyzed beta-Lactoglobulin and Large Synthetic Peptides. J Agric Food Chem 2012; 60(43):10858-66.
7. Paschke A, Besler M. Stability of bovine allergens during food processing. Ann. Allergy. Asthma Immunol 2002; 89(6 Suppl 1):16-20.
8. Kleber N, Hinrichs J. Antigenic response of beta-lactoglobulin in thermally treated bovine skim milk and sweet whey. Milchwissenschaft 2007; 62(2):121-4.
9. Shriver SK, Yang WW. Thermal and Nonthermal Methods for Food Allergen Control. Food Eng Rev 2011; 3(1):26-43.

10. Fiocchi A, Bouygue GR, Sarratud T, Terracciano L, Martelli A, Restani P. Clinical tolerance of processed foods. Ann Allergy Asthma Immunol 2004; 93(5 Suppl 3):S38-46.
11. Morisawa Y, Kitamura A, Ujihara T, Zushi N, Kuzume K, Shimanouchi Y, et al. Effect of heat treatment and enzymatic digestion on the B cell epitopes of cow's milk proteins. Clin Exp Allergy 2009; 39(6):918-25.
12. Saint-Sauveur D, Gauthier SF, Boutin Y, Montoni A. Immunomodulating properties of a whey protein isolate, its enzymatic digest and peptide fractions. Int Dairy J 2008; 18(3):260-70.
13. Lara-Villoslada F, Olivares M, Xaus J. The balance between caseins and whey proteins in cow's milk determines its allergenicity. J Dairy Sci 2005; 88(5):1654-60.
14. Wong CW, Watson DL. Immunomodulatory effects of dietary whey proteins in mice. J Dairy Res 1995; 62(2):359-68.
15. Imaizumi A, Kawakami T, Murakami F, Soma Y, Mizoguchi M. Effective treatment of pruritus in atopic dermatitis using H1 antihistamines (second-generation antihistamines): changes in blood histamine and tryptase levels. J Dermatol Sci 2003; 33(1):23-9.
16. Li XM, Schofield BH, Huang CK, Kleiner GI, Sampson HA. A murine model of IgE-mediated cow's milk hypersensitivity. J Allergy Clin Immunol 1999; 103(2 Pt 1):206-14.
17. Kimura K, Adachi M, Kubo K, Ikemoto Y. The basal plasma histamine level and eosinophil count in allergic and non-allergic patients. Fukuoka Igaku Zasshi 1999; 90(12):457-63.
18. Kadoya M, Momoi Y, Iwasaki T. Plasma histamine concentration and histamine detection in peripheral blood eosinophils in cats. J Feline Med Surg 2006; 8(5):302-8.