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Effects of Enzymatic Hydrolysis on the Allergenicity of Whey Protein Concentrates

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ABSTRACT

Cow's milk whey consists of many protein components and some of them are antigens to human and known to modulate immune responses. Enzymatic hydrolysis is a useful method to modify proteins with allergenicity. The objective of this study was to identify whether the *in vitro* enzymatic hydrolysis could reduce the allergenicity of whey protein concentrates (WPC).

In this study, WPC were hydrolyzed by trypsin and twenty-four BALB/c mice were divided into three groups and fed with WPC formula and WPC hydrolysates formula, while the control mice received milk-free diet.

The results revealed that there was no significant difference between the body weights among all groups. WPC-fed mice produced an elevated spleen lymphocyte proliferation level than WPC hydrolysates-fed mice and also produced higher levels of WPC-specific IgE in intestinal tract and serum in comparison to WPC hydrolysates-fed mice and control group. Significant up-regulation of plasma histamine levels were also observed and showed the same trend with IgE. The secretions of IL-4 and IL-5 were significantly enhanced by WPC. WPC significantly suppressed the secretion of IFN- γ while hydrolysates of WPC significantly increased the secretion of IFN- γ compared to control group.

These results suggest that hydrolysis may play a role to reduce the allergenicity of WPC.

Keywords: Hydrolysis; Hypersensitivity; Mouse; Whey protein

INTRODUCTION

Whey is the by-product of cheese production and has been widely used as it is not only easy to digest, but also has high biological value, high protein efficiency

rate and high utilization. In addition, it contains most of the essential amino acids needed by human body, while the composition model of these essential amino acids is almost the same with skeletal muscle, thus it is extremely easy to be absorbed by human body. Furthermore, whey protein is an excellent source of branch chained amino acids.¹ Therefore, it is usually used as the best source to develop cow's milk protein-based infant formula and can meet the needs of high concentration of whey protein similar to breast milk.

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Whey protein contains many protein components that are considered antigenic and capable of inducing immune responses. Studies carried out on many allergic patients have indicated that the most abundant proteins in whey especially lactoglobulins (Ig) are the major allergens; however, some kinds of proteins which are present in low contents, such as bovine serum albumin, lactoferrin and immunoglobulins have shown to be of great importance in inducing milk allergies.² As a result, many researchers have developed a variety of methods to reduce the sensitization of whey protein or its certain components including, heat treatment, glycation, enzymatic hydrolysis, etc.³⁻⁵ Heat treatment is the most common method to reduce pathogens but it remains controversial whether this method reduces the risk of allergies,⁶ while glycation is one of the most frequent chemical modifications during industrial processing.⁷ Hydrolysis also is the most effective method to modify proteins, which uses some digestive enzymes to change the immunoreactivity of protein allergens⁸ and has been frequently applied to modify specific milk components in recent years.

Formulae containing hydrolyzed proteins have been used to feed infants with allergy or food intolerance, and have been advocated for prevention of allergy and food intolerance in infants.⁹ At present, a variety of protein hydrolysate-based infant formulae have been developed as hypoallergenic.^{10,11} Differences in hydrolysates come from the protein sources used for the production of those hydrolysed formulae. They can indeed be based either on partially and exclusively casein or whey proteins or on a mixture of whey and casein (in a ratio 60/40 similar to the one found in breast milk) or an amino acid-based formula¹² or even on a soy protein or a mixture of soy protein and bovine collagen.¹³ However, whey protein is well digested and easily absorbed and can result in a rapid increase in the blood level of amino acids.⁴ In addition, it has been shown that ingestion of carbohydrates with whey protein accelerates glucose uptake by increasing glycoregulatory enzyme activity in muscle, and the effect is greater compared with that of dietary casein or soy.^{5,14} Therefore, partially and exclusively hydrolyzed whey protein infant formulae are very useful for infants with a high risk for development of allergic diseases, including asthma, hayfever, atopic dermatitis, allergic urticaria, etc.

In this paper, trypsin was used to hydrolyze WPC, and a series of immunologic indicators were examined

in groups of mice including spleen cell proliferation level, WPC-specific serum and intestinal tract IgE antibody level, plasma histamine level, 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) to identify whether the hydrolysis could reduce the allergenicity of WPC.

MATERIALS AND METHODS

Experimental Materials and Hydrolyzing Conditions

WPC and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The enzymatic conditions 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 at 90°C for 10 min, then cooling immediately.

Measurement of Degree of Hydrolysis

Degree of hydrolysis (DH) is defined as the percentage of free amino groups cleaved from protein, which was calculated from ratio of α -amino nitrogen (AN) and total nitrogen (TN). The AN was determined by a modified formol titration method. Ten milliliter of hydrolysates of WPC sample was added with an equal amount of distilled water. The mixture was adjusted to pH 7.0 using 0.1 M NaOH. Then 10 ml of 38% (v/v) formaldehyde solution was added into the mixture and titration was continued to the end point at pH 9.5 with 0.2 M standard NaOH solutions. TN was determined by Kjeldahl method.¹⁵

Mice and Diets

Twenty-four male BALB/c mice aged 6-7 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. These mice were bred in a controlled environment with 12 hours light and 12 hours dark cycle and room temperature approximately 22°C with free access to food and water. Control mice were fed with a standard milk-free mouse chow (provided by the Laboratory Animal Center of Tumour Hospital of Harbin Medical University) comprising 18% wt/wt total protein of the dry weight diet (protein source derived from fish, meat bone, and soybean

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cakes). Test mice were fed with a diet containing approximately 25% wt/wt WPC or its hydrolysates (NZMP, Wellington, New Zealand) as the sole protein source. Carbohydrate/fiber and fat were provided by grain and soybean, as detailed in Table 1. It provided an overall protein level of 19.2% w/w in the diet, and starch, cellulose and corn oil were included as additional sources of carbohydrate/fiber and fat, as shown in Table 1.

All groups of 8 mice were fed with WPC-based or WPC hydrolysate-based diets ad libitum for periods of 6 weeks (control mice were maintained on standard mouse chow pellets over the same period). At the end of 6 weeks, all groups of mice were orally immunized with cholera toxin (CT). Each immunization comprised 10 µg CT per mouse (Sigma, St. Louis, MO, USA).

All animal procedures were approved by Institutional Animal Care and Use Committee of Northeast Agricultural University, number SCXK(H) 2006-008, and conducted in compliance with local guidelines regulating laboratory animal care and housing.

Lymphocyte Proliferation Assay

Splenocytes were individually prepared from mice (at the end of 6 weeks) under aseptic conditions. Erythrocytes were removed by lysis buffer. The remaining spleen lymphocyte suspensions were washed twice in complete RPMI-1640 medium, supplemented with 100 U/ml Penicillin, 100 µg/ml Streptomycin and 10% fetal bovine serum, and adjusted to 1×10^6 viable cells per well in complete RPMI-1640 medium and cultured with WPC or its hydrolysates at certain

concentrations (100 µg/ml) in the presence or absence of the Concanavalin A (ConA), 2.5 µg/ml (Sigma). Control cells were cultured with complete RPMI-1640 medium in the presence of ConA. After cells were cultured for 48 hours at 37°C (5% CO₂), lymphocyte proliferation was tested by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega Corporation, USA) assay. The results were expressed as a stimulation index (SI) as following revised equations:¹⁶

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

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

Determination of WPC and Its Hydrolysates-specific IgE Levels

The blood samples were collected by taking the eyeball for blood and separating the serum. Serum was obtained by centrifuging (1000xg for 10 min, 4°C), collected and frozen at -80°C, and intestinal tracts were also collected. Levels of WPC-specific IgE were determined by an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with 1 µg/ml of WPC 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 with 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, washed 3 times again.

Table 1. Diet ingredient composition

Ingredients	Chow pellets (control diet) (%)	WPC-containing test diet (%)	WPC hydrolysates- containing test diet (%)
Meat and bone meal	5	WPC	25
Fish meal	6		
Chinese sorghum	12.5	Starch	55
Wheat bran	12		
Wheat	16		
Corn	30	Corn oil	9
Soybean cake	18	Cellulose	1
Total protein	18	Total protein	19.2
Total fat	7	Total fat	8.3
Vitamin mix	0.15	Vitamin mix	5
Mineral mix	0.35	Mineral mix	5

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 horseradish peroxidases (HRP, AbD serotec, England) was added for 1 hour at 37°C, then washed 4 times. Staining was performed with 3, 3'-5, 5'-tetramethyl-benzidine (TMB) (Sigma) for 30 min at 25°C in dark, stopped with 2 M H₂SO₄, and plates were read by enzyme microplate reader (Bio-RAD Model 680, 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 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 duplicates.

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 (Usnlife Science & Technology CO., LTD, China), as described by the manufacturer.

Measurement of Cytokine Secretions in Lymphocyte Supernatants

The effect of WPC and its hydrolysates on cytokine production was determined by adding WPC or its hydrolysates to cell culture in vitro at a set concentration. Lymphocyte culture was conducted as described above in the presence of WPC and hydrolysates of WPC and in the absence and presence of ConA (2.5 µg/ml). After the cells were cultured for

48h at 37°C, the supernatants were collected and the cytokines IFN-γ, IL-4, IL-5 and IL-10 in the supernatants were determined by enzyme immunoassay kits (R&D Systems, USA) according to the manufacturer's instructions.

Statistical Analysis

Results are expressed as mean±SEM. Statistical significance ($p<0.05$) was determined by one-way analysis of variance (ANOVA). SPSS 15.0 software (SPSS Inc, Chicago, USA) was used to analyze the significance level.

RESULTS

Determination of Degree of Hydrolysis

The DH of WPC proceeded at high rates during the initial 150 min, and slowed down thereafter (Figure 1), which indicated that the maximum cleavage of peptides occurred within 150 min of hydrolysis. The highest DH of WPC was 9.5% which was obtained at 240 min. The trend and curve shape of the hydrolysis were similar to those reported by other studies for various protein sources.^{17,18}

Change of Body Weight

Each test group contained 8 mice with even body weights. The body weights of mice were measured at weekly intervals. The results showed that feeding WPC and hydrolysates of WPC did not affect body weight gain during the six weeks of study (Figure 2). The body weight of mice fed with standard chows was slightly higher than that of the mice fed with WPC and hydrolysates of WPC, but the body weight of mice fed

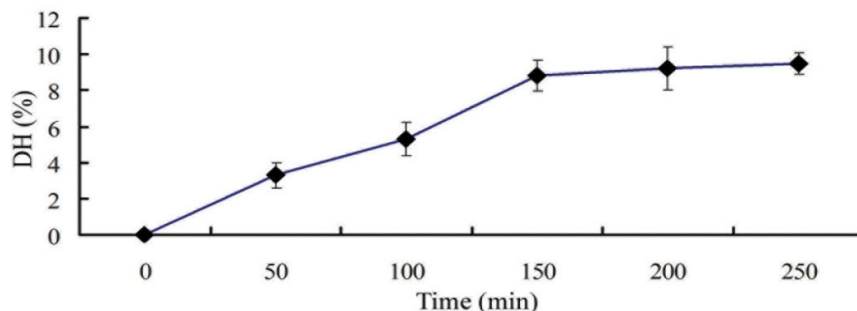


Figure 1. Degree of hydrolysis (DH, %) measured during the hydrolysis of WPC using trypsin (enzyme: substrate ratio of 1:200 (w/w) at temperature 42°C). Bars represent means and standard deviations from triplicate determinations

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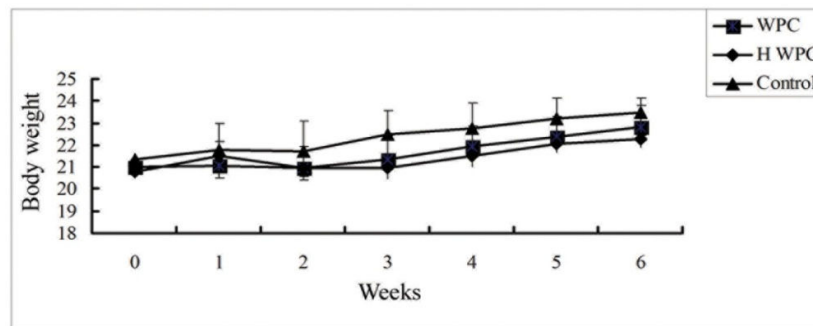


Figure 2. Evaluation of the body weight in BALB/c mice of three dietary groups in six weeks

with WPC was similar to that of mice fed with hydrolysates of WPC ($22.8 \pm 0.75\text{g}$, $22.28 \pm 0.66\text{g}$, respectively).

Effects of WPC or its Hydrolysates on Lymphocyte Proliferation

The immunomodulating effects of WPC and its hydrolysates on lymphocytes derived from spleen cell suspensions were investigated *in vitro*. The results were expressed as SI and evaluated with and without suboptimal concentration ($2.5 \mu\text{g/ml}$) of ConA (Figure 3). Both WPC and its hydrolysates stimulated lymphocyte proliferation by dietary treatment. The SI values were lower in groups fed with hydrolysates of

WPC than native WPC, indicating that the hydrolysis could suppress the level of lymphocyte proliferation. The group in the absence of ConA showed significantly lower SI value than the group in the presence of ConA.

Effects of WPC and its Hydrolysates on IgE Secretion Levels in Serum and Intestinal Tracts

To investigate the IgE production developed by systemic and mucosal antibody responses, the IgE levels in serum and intestinal tracts were monitored by ELISA. The serum IgE level of mice fed with WPC formula generated a significantly high level of antibody response compared to that of fed with hydrolysates of WPC and control groups. However, there was no

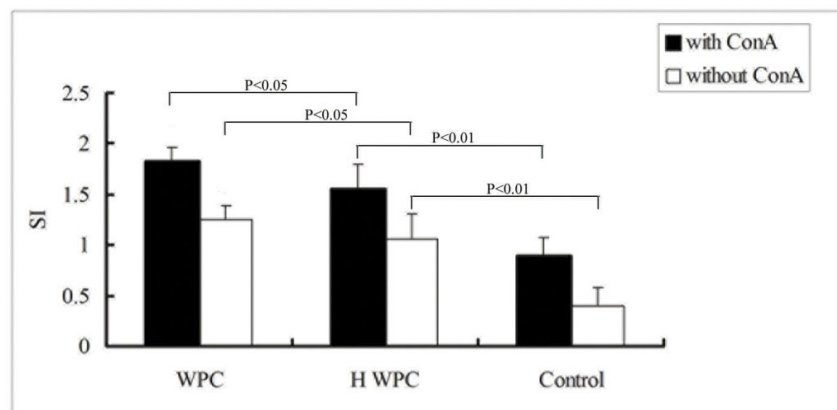


Figure 3. Effect of WPC- or hydrolysates of WPC-based diets on lymphocyte proliferative responses *in vitro*. All groups of mice were fed with diets containing WPC, hydrolysates of WPC or a control standard mouse chow pellet diet. Spleen was removed from each mouse ($n=8$ per group) immediately after killing, erythrocytes were then lysed and spleen-derived lymphocytes from each mouse were cultured in the presence or absence of ConA ($2.5 \mu\text{g/ml}$). Control cells were cultured in complete RPMI-1640 medium with ConA. After cells were cultured for 48 hours, lymphocyte proliferation was tested by MTS assay. The absorbance of each well was read at 490nm and the results were expressed as $\text{SI} \pm \text{SEM}$ as described in the material and method. ANOVA was used to determine statistical significance. WPC represented WPC, H WPC represented hydrolysates of WPC, Control represented control mice.

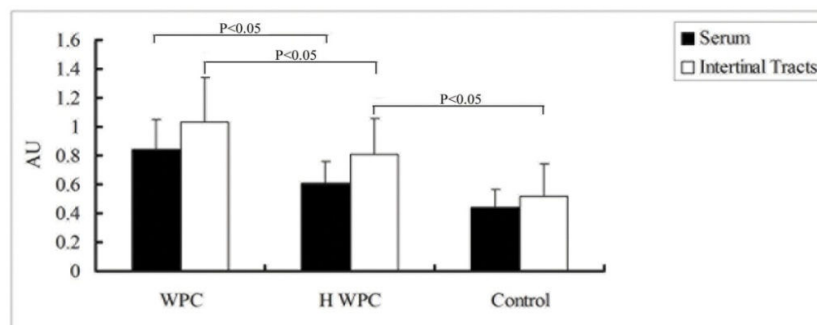


Figure 4. Effects of WPC and hydrolysates of WPC on systemic (serum) and mucosal (intestinal tracts) antibody responses. Serum and intestinal tracts from all mice (n=8 per group) were collected after killing the mice. WPC or its hydrolysates specific-IgE level was determined by ELISA. Values were expressed as arbitrary units (AU) deduced from the optical densities of the reference serum curve with a high level of antibodies after subtracting the blanks. ANOVA was used to determine statistical significance. WPC represented WPC, H WPC represented hydrolysates of WPC, Control represented control mice.

significant difference between hydrolysates of WPC and control group (Figure 4). ELISA was also used to detect the IgE level in intestinal tracts that showed a significantly higher trend than that in serum for all three groups which was only significant in WPC group.

Effects of WPC and its Hydrolysates on Secretion of Histamine in Plasma

Histamine levels in plasma were determined by an ELISA kit and the results indicated that the histamine level of mice fed with WPC was significantly higher than that fed with hydrolysates of WPC and control group. The histamine level of mice fed with WPC was significantly higher than that fed with standard chows. These results indicated that histamine was one of the major mediators involved in the anaphylaxis in this experiment (Figure 5).

Effects of WPC or its Hydrolysates on Cytokines Secretions in Vitro

The cytokines were examined in lymphocyte supernatants which were the indicators of the systemic responses to anaphylactic reaction. The effects of WPC and hydrolysates of WPC on cytokines secretions of the supernatants of spleen lymphocytes in vitro were shown in Table 2 (with or without ConA, with a final concentration of ConA at 2.5 $\mu\text{g/ml}$). In cytokine secretion test in the presence of ConA, WPC and its hydrolysates showed almost no stimulation role on the secretions of IL-10 in supernatants of spleen cells. WPC significantly enhanced the secretions of IL-4, IL-5 while its hydrolysates had no significant effects on these two cytokines in the presence of ConA compared to control group. Furthermore, WPC significantly suppressed the secretion of IFN- γ and

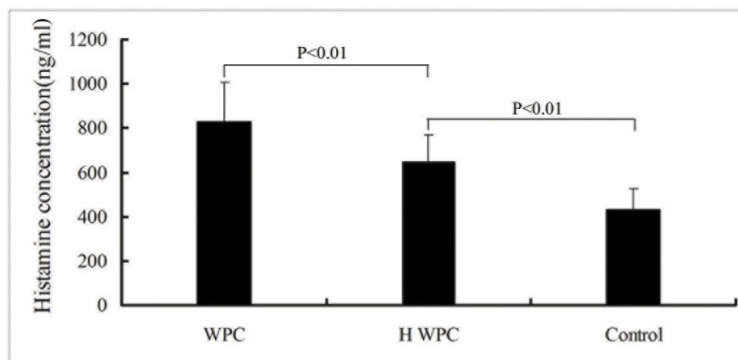


Figure 5. Effects of WPC and hydrolysates of WPC on plasma histamine level. Plasma from all mice (n=8 per group) were collected after killing mice. Histamine concentration was determined by ELISA. Results are expressed as concentration (ng/ml) \pm SEM. WPC represented WPC, H WPC represented hydrolysates of WPC, Control represented control group.

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Table 2. Cytokine secretion measured in the supernatants of cultured murine splenocytes by ELISA

Cytokine (pg/ml)	Without ConA			With ConA		
	Control	WPC	Hydrolysates of WPC	Control	WPC	Hydrolysates of WPC
IL-4	9.32±1.22	56.24±5.54*	18.68±2.15	12.65±1.27	75.07±7.02*	21.13±3.26
IL-5	8.87±0.99	51.43±7.27*	15.23±1.32	14.16±2.08	64±6.43*	19.21±2.03
IL-10	118±9.94	132±12.34	123±3.35	105±7.26	129±7.68	131±12.66
IFN- γ	87.34±3.68	24.32±5.15*	164.13±8.21*	92.21±4.56	32.12±3.56*	189.7±3.9*

* Indicates a significant difference ($p < 0.05$) between the samples and the control cells.

hydrolysates of WPC significantly increased the secretion of IFN- γ compared to control group. In addition, in cytokine secretion test in the absence of ConA, the secretion levels of IL-4, IL-5, IL-10 and IFN- γ showed similar trend with that in the presence of ConA.

DISCUSSION

Hydrolysis has been proven to be the most effective method to modify proteins, which uses some digestive enzymes to change the immunoreactivity of protein allergens.⁸ In this study, we used animal model designed by previous report¹⁹ to confirm whether the hydrolysis could reduce the allergenicity of WPC. Although we can not provide further insight from this study into either the possible mechanism of action or the individual components of WPC which may have immunological activity, these results are still important as they identify the health potential of a readily available food-quality protein source for infant formula with lower sensitization.

Our study showed that the body weight gain was not significantly modified in mice fed with WPC, hydrolysates of WPC and standard chows, and this result confirmed previous report, i.e. energy expenditure, body weight gain, and body composition was not significantly modified in C57Bl/6 mice exposed for 14 weeks to adequate protein diet supplemented with leucine or high-protein diets that were approximately 3 times higher in leucine concentration when compared to the adequate protein control diet.²⁰

In addition, the results in this study indicated that the enhancement of humoral immune responses in this model was frequently accompanied by elevated histamine level. Although the origin of plasma histamine has not been determined, it has been

suggested that plasma histamine was released from infiltrating inflammatory cells in human patients with atopic dermatitis.²¹ When protein allergens penetrated mucosal barriers and contacted with IgE antibodies bound to mast cells or basophils, the histamine and other mediators which induce symptoms of immediate hypersensitivity were released. Therefore, significantly increased plasma histamine indicated that histamine release was involved in the induction of systemic anaphylaxis. This result is probably analogous to what occurred in individuals allergic to food. Kadoya et al. used cat as an animal model to investigate the changes of histamine levels in cats with allergic dermatitis and they found that plasma histamine levels were markedly elevated in five of the 15 cats with allergic dermatitis.²¹ Similar findings have also been reported that plasma histamine levels were elevated in some patients with atopic dermatitis.^{22,23}

In the present study, we sought to understand the effects of hydrolysis on WPC and its hydrolysates of WPC through investigating its roles on systemic and intestinal tract antigen-specific antibody production. For this purpose, BALB/c mice were sensitized to WPC and its hydrolysates by feeding. It has been identified that IgE antibodies play a crucial role in mediating type I hypersensitivity responses in humans¹⁹ and an elevated IgE antibody level was detected in the mice fed with WPC compared to that in each of the test groups. Our results indicated that the humoral immune responses of mice fed with WPC could be significantly increased while the humoral immune responses of mice fed with hydrolysates of WPC could be significantly decreased. One study also confirmed that orally delivered WPC could significantly increase humoral immune responses,²⁴ and further identified that the kinetics and magnitude of WPC-mediated immune enhancement were similar whether dietary treatment began prior to or, at the same time, as commencement

of an immunization regime. Thus, although previous reports have demonstrated that feeding WPC to mice could significantly enhance systemic-level cellular and humoral immune responses,^{24,25} the present study provided evidence that WPC could also up-regulate immune responses in the intestinal tract and serum, when used as a whole dietary protein.

One of the most important steps in specific immune reactivity is clonal expansion (proliferation) to produce a pool of antigen-reactive lymphocytes. For experimental purposes, *in vivo* cellular proliferation is usually simulated *in vitro* by many researchers via the addition to cell cultures of lectin mitogens, which are targeted toward B or T lymphocytes.²⁶ Our result showed that hydrolysis could significantly reduce the stimulation of WPC to lymphocyte proliferation *in vitro*. However, a previous study has shown that WPC suppressed lymphoproliferative responses when directly added to cultures of murine splenic lymphocytes,¹⁶ resulting in no significant influences on lymphocyte proliferation after 4 or 8 weeks of feeding the mice, and this result was not impossible entirely because *in vitro* lymphocyte proliferation studies by Otani and Hata²⁷ have clearly demonstrated that patterns of immunomodulation by milk proteins could be radically altered by exposing to gut-digestive enzymes, indicating that dietary treatment and *in vivo* studies are necessary to demonstrate the true immunomodulatory potential of protein sources.

Cytokines have been demonstrated to play an important role in regulating various allergic diseases, including asthma, and the effect of WPC on cytokine expression profiles was investigated in this study. As we all knew, the decrease of IFN- γ level and increase of IL-4 level represented a stimulation for IgE antibody responses. In this study, the secretion of IFN- γ was suppressed in the group sensitized by WPC but elevated in the group sensitized by hydrolysates of WPC, and the corresponding IgE antibody responses were verified in the effects of WPC and hydrolysates of WPC on IgE levels in serum and intestinal tracts. It has been also reported that a modified WPC rich in glycomacropeptide suppressed the secretion of IFN- γ in a ConA-induced murine splenic lymphocyte culture and that the effect was partly abolished following enzymatic digestion of the extract with pepsin and pancreatin.²⁷ Similarly, hydrolysates of WPC enhanced the secretion of IFN- γ , which indicated that the IgE antibody response was inhibited, and this presumption

was in line with the results showed in Figure 4. The results were consistent with a study which showed that IL-4 and IL-5 levels were significantly increased in cow's milk protein-stimulated cultures when compared with unstimulated cells.¹⁹

It could be concluded that the hydrolysis reduced the immunogenicity of WPC from the results of *in vivo* test, but the results of *in vitro* test were not consistent with the *in vivo* test. Therefore, we needed to further conduct dietary treatment and *in vivo* studies to demonstrate the true immunomodulatory potential of protein sources. However, there is no evidence to support feeding with a hydrolysed formula for the prevention of allergy compared to exclusive breast feeding. In high risk infants who are unable to be completely breast fed, there is limited evidence that prolonged feeding with a hydrolysed formula compared to a cow's milk formula reduces infant and childhood allergy and infant cow's milk allergy.¹⁹ Nevertheless, this study provides certain theoretical evidence for other researchers to further study the method to develop infant formula with low-sensitization.

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