

ORIGINAL ARTICLE

T-cell epitope-containing hypoallergenic β -lactoglobulin for oral immunotherapy in milk allergy

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Keywords

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Abstract

Background: Optimally hydrolyzed β -Lactoglobulin (β Lg) is a promising milk oral immunotherapy (OIT) candidate with respect to showing reduced B-cell reactivity but retaining the T-cell epitope. To demonstrate that an edible hypoallergenic β Lg hydrolysate containing the T-cell epitope is suitable for OIT. We tested how chymotrypsin affected the retention of the T-cell epitope of β Lg when preparing β Lg hydrolysates using food-grade trypsin.

Methods: We investigated the effect of chymotrypsin activity on the formation of the T-cell epitope-containing peptide of β Lg (β Lg_{102–124}) and prepared an edible β Lg hydrolysate containing β Lg_{102–124} using screened food-grade trypsins. B-cell reactivity was determined using immunoassays in which ELISA was performed with anti- β Lg rabbit IgG and Western blotting was performed with a milk-specific IgE antiserum.

Results: In β Lg hydrolysis performed by varying the activity of trypsin and chymotrypsin, chymotrypsin activity inhibited the formation of β Lg_{102–124} with an increase in hydrolysis time in a dose-dependent manner. β Lg_{102–124} was generated by two of five food-grade trypsins used at a ratio of 1:50 (w/w, enzyme/substrate) for 20 h at 40°C. The edible β Lg hydrolysate retained β Lg_{102–124} and showed a reduction in molecular weight distribution and antigenicity against IgG and IgE.

Conclusions: Chymotrypsin activity inhibited the formation of β Lg_{102–124} in the trypsin hydrolysate of β Lg. This β Lg trypsin hydrolysate is a novel candidate for peptide-based OIT in cow's milk allergy for safely inducing desensitization.

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Cow's milk allergy (CMA) is one of the most common food allergies in young children, affecting 2–3% of the general populations (1). Oral immunotherapy (OIT) has been developed for treating food allergies. In OIT, the allergen is orally

administered at a small dose to induce desensitization and tolerance (2, 3). An approach proposed as an alternative future OIT strategy involves inducing desensitization and tolerance by vaccinating with small peptides that contain the sequences of T-cell epitopes but avoid cross-reaction with IgE. This peptide-based OIT is a promising method for inducing desensitization and tolerance while concomitantly avoiding allergic reactions during allergen-specific immunotherapy (4). Although the main allergen component of cow's milk is casein (Bos d 8) accounting for 80% of milk protein, β -lactoglobulin (β Lg; Bos d 5) is a major whey protein in cow's milk ($\approx$ 3 g/l) with an 18.4 kDa of molecular weight (5). A recent study notes that specific IgE

Abbreviations

CMA, cow's milk allergy; HPSEC, High-performance size-exclusion chromatography; LC, liquid chromatography; MS, mass spectrometry; OIT, oral immunotherapy; TLCK, 1-Chloro-3-tosylamido-7-amino-2-heptanone; TPCK, L-1-tosylamide-2-phenethyl-chloromethyl ketone; USP, United States Pharmacopeia; β Lg, β -Lactoglobulin.

against β Lg is dominant in patients with gastrointestinal CMA (6). The antigenicity of β Lg is differently affected by food-processing steps such as enzymatic hydrolysis and thermal treatment (7). For instance, binding of β Lg-specific IgE from CMA patients is reduced in the enzymatically hydrolyzed β Lg, particularly in a combination with high-pressure treatment (8, 9).

T-cell epitopes are screened using overlapping peptides from the entire β Lg sequence and T-cell lines established from CMA patients (10, 11). In our previous study, β Lg_{97–117} is recognized by the β Lg-specific T-cell lines and clones obtained from patients with CMA (12), and within the β Lg_{97–117} sequence, β Lg_{102–112} was identified as the minimal essential epitope for the T-cell response (13). Therefore, attention must be paid to this essential region when hydrolyzing β Lg in order to reduce its antigenicity but retain its ability to immunostimulate T cells. Trypsin (EC 3.4.21.4) is a pancreatic serine protease that exhibits substrate specificity for C-terminal lysine and arginine, and thus, trypsin should be able to generate a peptide containing the minimal essential epitope β Lg_{102–112} (β Lg_{102–124}; YLLFCMENSAEPEQSLVCQCLVR). However, most industrially available trypsins can be contaminated with chymotrypsin (E.C. 3.4.21.1) activity, which cleaves C-terminal tryptophan, tyrosine, phenylalanine, and leucine (14, 15). Chymotrypsin cleaves the essential region β Lg_{102–112} because of its cleavage site specificity, and therefore, the formation of β Lg_{102–124} would be affected by the residual chymotrypsin activity of trypsin. Previously, we showed that β Lg hydrolysates digested with a mixture of trypsin and chymotrypsin exhibited reduced IgG and IgE reactivity as compared with undigested β Lg, but retained the ability to trigger a proliferative response in peripheral blood mononuclear cells isolated from patients with persistent CMA (16). However, how chymotrypsin activity affects the formation of β Lg_{102–124} in the trypsin hydrolysate of β Lg has remained unclear. We hypothesized that the degree of trypsin hydrolysis could be optimized in order to prevent chymotrypsin activity from influencing the formation of β Lg_{102–124}. This optimized β Lg hydrolysate could serve as a promising candidate for milk OIT for patients with persistent CMA because of exhibiting reduced B-cell reactivity but retaining the T-cell epitope in β Lg.

In this study, we examined the impact of chymotrypsin activity on the formation of β Lg_{102–124} in β Lg hydrolysates prepared using trypsin samples showing varying levels of chymotrypsin activity. We also screened distinct types of food-grade trypsin for their ability to generate β Lg_{102–124}. Based on the results, we identified for the first time an edible β Lg hydrolysate containing T-cell epitope—generated using food-grade trypsin—for use in peptide-based OIT. Furthermore, we investigated the B-cell reactivity of the hydrolysate using antisera obtained from patients with persistent CMA.

Materials and methods

Materials

We obtained analytical-grade β Lg (90%, w/w) from Sigma-Aldrich (St Louis, MO, USA) and edible-grade β Lg (Hiprotal 580; 72%, w/w) from FrieslandCampina Domo

(Amersfoort, The Netherlands). Food-additive-grade trypsin was obtained from Novozymes (Trypsin 4500K, 4500 United States Pharmacopeia (USP) units/mg of trypsin activity, 50 USP units/mg of chymotrypsin activity; PTN 6.0 S, 1250 USP units/mg of trypsin activity, 80 USP units/mg of chymotrypsin activity; Bagsværd, Denmark), Biocon Japan (Trypsin V, 1200 USP units/mg of trypsin activity, 300 USP units/mg of chymotrypsin activity; Trypsin VI, 2400 USP units/mg of trypsin activity, 400 USP units/mg of chymotrypsin activity; Nagoya, Japan), and Higuchi (Trypsin 4.0T; Tokyo, Japan). Analytical-grade trypsin (L-1-tosylamide-2-phenethyl-chloromethyl ketone (TPCK)-treated; 4925 USP units/mg of trypsin activity) and chymotrypsin (1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK)-treated; 1770 USP units/mg of chymotrypsin activity) were purchased from Sigma-Aldrich. Unless otherwise specified, all reagents used were of analytical grade.

Study participants

This study was approved by the Ethics Committees of the Graduate School of Medicine, Gifu University (Gifu, Japan) and was registered with the Japanese University Hospital Medical Information Network Clinical Trials Registry (UMIN000002139). Two CMA patients participated in the study. Each participant provided written informed consent. Total and cow's milk-specific IgE were measured by ImmunoCAP (Phadia AB, Uppsala, Sweden). Patient 1 was 13-month-old boy with CMA and atopic dermatitis, diagnosed by urticaria on milk challenge. IgE levels of patient 1 were 2000.0 IU/ml of total IgE, 60.10 kUA/l of α -lactalbumin, 12.70 kUA/l of β Lg, 86.80 kUA/l of casein, and 93.00 kUA/l of milk. Patient 2 was 10-month-old boy with CMA and atopic dermatitis, diagnosed by angioedema of lips on milk challenge. IgE levels of patient 2 were 13000.0 IU/ml of total IgE, 15.00 kUA/l of α -lactalbumin, 88.00 kUA/l of β Lg, ≥ 100.00 kUA/l of casein, and ≥ 100.00 kUA/l of milk.

Protein digestion

β -Lactoglobulin enzymatic hydrolysates were prepared as follows. In the experiment to determine the effect of chymotrypsin activity on the formation of β Lg_{102–124} using analytical-grade reagents, β Lg (50 μ g/ml) was hydrolyzed at 40°C for 6 h with 5 USP units/ml of TPCK-treated trypsin and 0.02–443 USP units/ml of TLCK-treated chymotrypsin, after which the pH was adjusted to <3.0 with formic acid. For the screening of food-grade trypsins, edible β Lg (50 μ g/ml) was hydrolyzed with 1 and 10 μ g/ml of food-grade trypsin at 40°C for 20 h. In the industrial-scale production, edible β Lg (2.5%, w/w) was hydrolyzed at 40°C for 6 h using Trypsin 4500K at a final enzyme concentration of 0.1 mg/ml. After digestion, the enzymes were inactivated by heating at 120°C for 3 s using a plate-heat exchanger (Sanwa Engineering, Tokyo, Japan). To remove undigested β Lg, the hydrolysate was subjected to ultrafiltration with $<10,000$ -Da molecular weight cutoff filters. The lyophilized hydrolysate was stored at below -20°C until further analysis.

Liquid chromatography–mass spectrometry

β Lg_{102–124} was identified in β Lg hydrolysates by performing liquid chromatography (LC) separation and mass spectrometry (MS) as described in our previous study (17), with minor modifications. Briefly, β Lg hydrolysates were alkylated with iodoacetamide and then subjected to LCMS analysis. The $2H^+$ adducts of alkylated β Lg_{102–124} were used for detection ($m/z = 1424.3$). The peak area of the most intense product ion corresponding to an $[M + H]^+$ ion of β Lg_{113–124} containing cysteine residues alkylated using iodoacetamide ($m/z = 1489.1$) was plotted as a function of the concentration of the β Lg hydrolysates. We also used, as an internal control, β Lg_{102–124} that was ^{13}C - and ^{15}N -labeled at Leu₁₂₂ (NH₂-YLLFCMEN-SAEPEQSLVCQCL*VR-COOH; m/z for the alkylated $[M + 2H]^+ = 1427.8$). Synthetic peptides were obtained from Sigma-Aldrich.

High-performance size-exclusion chromatography (HPSEC)

The molecular weight distribution of the digested peptides was analyzed by performing High-performance size-exclusion chromatography (HPSEC) on a Waters H-Class UPLC system (Waters, Milford, MA, USA) fitted with a corona-charged aerosol detector (Corona Ultra; Thermo Fisher Scientific, Waltham, MA, USA). The samples were applied to a size-exclusion column (TSKgel SuperAW3000; Tosoh, Tokyo, Japan) and eluted with 0.1% (v/v) trifluoroacetic acid and 55% (v/v) acetonitrile in water. Molecular weight calibration of the column was performed using the standard proteins.

IgG inhibition ELISA

IgG inhibition ELISA was performed according to our previous study (16), with minor modifications. Briefly, a 96-well microtiter plate (Maxisorp; Thermo Fisher Scientific) was coated with intact β Lg. After washing, wells were blocked with 0.4% goat serum. The wells were washed and then incubated with anti- β Lg rabbit antisera (1:64,000 dilution; Bethyl Laboratories, Montgomery, TX, USA) and 50 μ l of serially diluted samples (digested and undigested β Lg). After washing, the wells were incubated with a peroxidase-conjugated anti-rabbit IgG goat antibody (1:10,000; MP Biomedicals, Santa Ana, CA, USA), and after washing again, color was developed. The percentage of inhibition was calculated as (total reactivity – remaining reactivity after absorption) $\times$ 100/total reactivity.

SDS-PAGE and IgE Western blotting

SDS-PAGE and IgE Western blotting were performed according to our previous study (16), with minor modifications. Briefly, β Lg (20–80 μ g) and β Lg hydrolysates (50–200 μ g) reduced with 2-mercaptoethanol were electrophoresed on 16.5% Tris–tricine precast gels. A protein molecular weight marker (Polypeptide SDS-PAGE Molecular Weight Standard; Bio-Rad (Hercules, CA, USA), or MagicMark XP

Western Protein Standard; Thermo Fisher Scientific) was included for the appropriate detection system, and proteins and polypeptides were visualized using Coomassie Blue staining or IgE Western blotting. For Western blotting, proteins were transferred to PVDF membranes and reacted with the serum from the two patients with persistent CMA. The blots were incubated with horseradish peroxidase-conjugated human IgE goat antibody (Bethyl Laboratories), and the stained bands were visualized using the Amersham ECL Prime reagent (GE Healthcare, Little Chalfont, United Kingdom).

Results

Effect of chymotrypsin activity on β Lg_{102–124} formation during trypsin hydrolysis of β Lg

The formation of β Lg_{102–124} was investigated in β Lg hydrolyzed using a series of mixtures composed of TPCK-treated trypsin and TLCK-treated chymotrypsin (Fig. 1). At 2 h of hydrolysis, the formation of β Lg_{102–124} was inhibited by chymotrypsin activity in a dose-dependent manner, and little β Lg_{102–124} was detected when the chymotrypsin activity was >55 USP units/ μ l (Fig. 1a). β Lg_{102–124} formation was stable throughout the 6 h of hydrolysis in the absence of chymotrypsin activity; by contrast, β Lg_{102–124} levels decreased with the progression of hydrolysis in the presence of chymotrypsin activity at 28 USP units/mg (Fig. 1b).

Distinct types of commercially available food-grade trypsin were evaluated with respect to β Lg_{102–124} formation (Table 1). At an enzyme-to-substrate ratio of 1/50, two of the five tested trypsin generated β Lg_{102–124} even after 20 h of hydrolysis. By contrast, none of the enzymes generated β Lg_{102–124} when used at a ratio of 1/5 (w/w).

Characterization of an edible β Lg hydrolysate prepared using food-grade trypsin

β Lg_{102–124} formation, molecular weight distribution, and B-cell reactivity were characterized in an edible hydrolysate of β Lg. The hydrolysate was prepared at an industrial scale using β Lg-enriched cow's milk whey and food-grade trypsin. β Lg_{102–124} was detected and its concentration in the hydrolysate was 104 mg/g. The degradation profile of β Lg indicated that the peak corresponding to β Lg was largely hydrolyzed within 1 h (data not shown), and the peaks in the high molecular weight fraction (>10 kDa, eluted before 7.2 min) were degraded at 6 h of hydrolysis (Fig. 2a). By contrast, the low molecular weight fraction (<5 kDa, eluted starting from 7.7 min) was formed after trypsin hydrolysis for 6 h. The antigen-binding capacity of anti- β Lg rabbit IgG antisera was effectively reduced by the hydrolysates to $\approx 1/10$ of the capacity measured in the presence of unhydrolyzed β Lg (Fig. 2b). Lastly, IgE Western blotting revealed that the IgE-binding capacity was completely suppressed toward two patients' antisera in the β Lg hydrolysate, as clearly detected in the Coomassie Blue-stained gel bands (Fig. 2c).

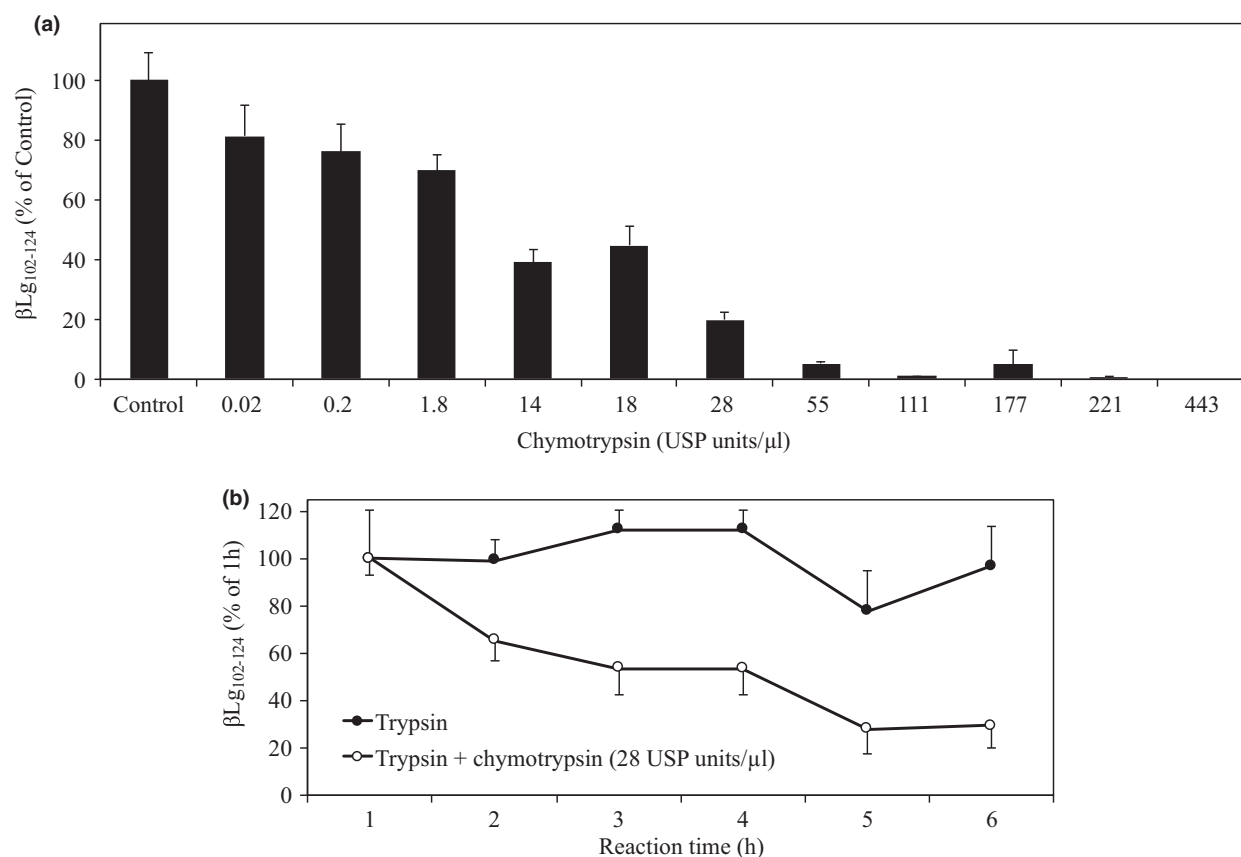


Figure 1 Effect of chymotrypsin activity on T-cell epitope formation. (a) Effect of chymotrypsin activity on the formation of β -lactoglobulin (β Lg)₁₀₂₋₁₂₄. β Lg was hydrolyzed using trypsin and varying chymotrypsin activity. (b) Retention of β Lg₁₀₂₋₁₂₄ during trypsin hydrolysis at 0 (closed) and 28 (open) USP units/ μ l of chymotrypsin.

Table 1 β -Lactoglobulin₁₀₂₋₁₂₄ formation and enzymatic specifications in hydrolysates prepared using food-grade trypsin

Type of trypsin	Trypsin activity (USP units/mg)	Chymotrypsin activity (USP units/mg)	Formation of β Lg ₁₀₂₋₁₂₄ (Enzyme/Substrate, w/w)	
			1/50	1/5
Trypsin 4500K	>4500	<50	+	—
PTN 6.0S	>1250	<80	+	—
Trypsin V	>1200	<300	—	—
Trypsin VI	>2400	<400	—	—
Trypsin 4.0T	N/A	N/A	—	—

N/A, data not available from the manufacturer; β Lg, β -Lactoglobulin.

Discussion

We investigated the impact of chymotrypsin activity on the formation of β Lg₁₀₂₋₁₂₄ in trypsin hydrolysates of β Lg. Our results suggested that the contaminating chymotrypsin hydrolyzed β Lg₁₀₂₋₁₂₄ during trypsin hydrolysis of β Lg (Fig. 2 and Table 1). In the case of analytical-grade enzymes, TPCK and TLCK can be used to inactivate chymotrypsin and trypsin, respectively (18). However, these inactivation techniques cannot be used for food-grade enzymes, and the quality of trypsin

was reported to affect specificity and efficiency even when it was of analytical grade (19). Here, none of the food-grade trypsin samples generated β Lg₁₀₂₋₁₂₄ when used at a 1/5 ratio, but two of the tryptins formed β Lg₁₀₂₋₁₂₄ at 1/50 (Table 1). This result correlated with the dose-dependent effect of chymotrypsin activity on β Lg₁₀₂₋₁₂₄ formation, because the contaminating chymotrypsin hydrolyzed β Lg₁₀₂₋₁₂₄ during trypsin hydrolysis of β Lg (Fig. 1). Thus, the optimized enzyme–substrate ratio must also be considered during trypsin hydrolysis to adequately reduce the effect of chymotrypsin

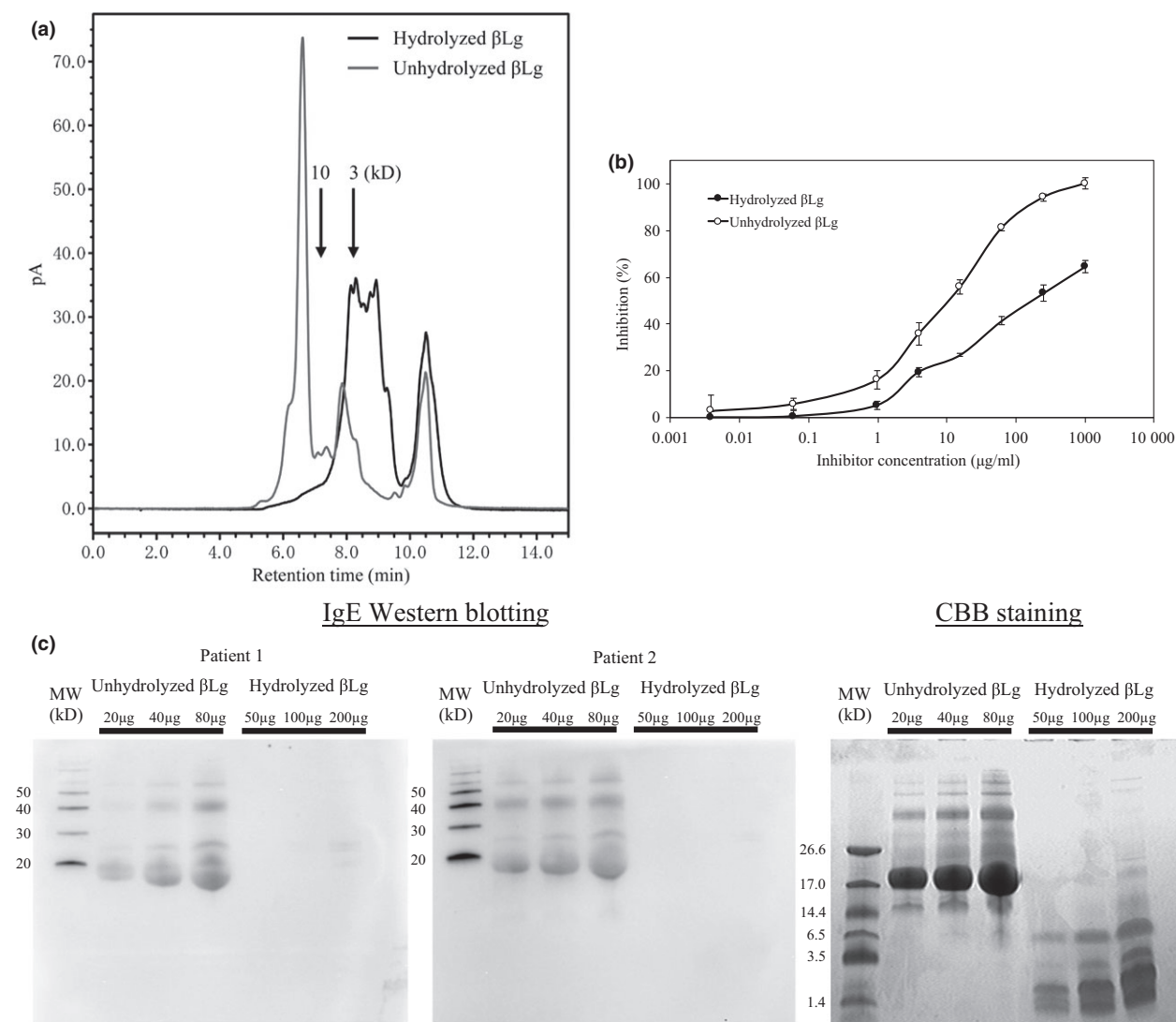


Figure 2 B-cell reactivity of hydrolyzed and unhydrolyzed β -lactoglobulin (β Lg). (a) Molecular weight distribution in hydrolyzed and unhydrolyzed β Lg. (b) Antigenicity of hydrolyzed and unhydrolyzed β Lg toward anti- β Lg IgG. (c) Electrophoretic patterns of hydrolyzed and unhydrolyzed β Lg on a membrane detected for an antigen-bound IgE against β Lg (left, Patient 1; center, Patient 2), and on a gel stained by Coomassie Blue for proteins and peptides (right).

activity when preparing β Lg hydrolysates containing β Lg_{102–124} using commercially available food-grade trypsin. In a comparative MS-based proteomics analysis, trypsin quality was found to affect digestion efficacy and specificity in protein hydrolysates (20); our results are in agreement with the findings of this proteomics study.

Based on the experimental results on the formation of β Lg_{102–124}, we selected Trypsin 4500K because it featured the highest ratio of trypsin activity to chymotrypsin activity. The edible β Lg hydrolysate containing β Lg_{102–124} exhibited a reduction in molecular weight distribution and antigenicity toward IgG and IgE antibodies. The deduced concentration of β Lg_{102–124} was satisfactorily estimated from the proportion of β Lg_{102–124} in β Lg-enriched milk whey. Furthermore, given the similar inhibition efficacy observed in β Lg hydrolysis with

Trypsin V (a mixture of trypsin and chymotrypsin) and chymotrypsin, trypsin hydrolysis could be expected to reduce the antigenicity against the anti- β Lg IgG antibodies to a similar level as the trypsin hydrolysis (16). Although β Lg_{102–124} is reported as an IgE-binding epitope, our results indicated that the impact of β Lg_{102–124} appeared to be limited in the IgE-binding capacity (Fig. 2c). This epitope is not likely to induce IgE-mediated allergic reactions solely in patients with persistent CMA. There are some IgE-binding epitopes but abrogated allergenicity to an allergy mouse model in the β Lg hydrolysate using chymotrypsin (21), supporting that the accumulation of proteolysis product of intermediate size would contribute to the reduced allergenicity. As the presence of casein glycomacropeptide, commonly found in whey, modifies the peptide profiles and reduces IgE binding of β Lg

hydrolysate (22), the purity of β Lg substrate might be a modifiable factor to allergenicity of β Lg hydrolysate. Notably, the safety and effectiveness of our β Lg hydrolysate was demonstrated in a pilot study on 16 CMA patients (threshold amount for β Lg at baseline: 0.2–30 ml of milk equivalent) (23); 8 of 10 patients who completed the pilot protocol showed an increase in the threshold amount for β Lg without anaphylactic reaction throughout the study. The β Lg hydrolysate retained an immunogenic property by lymphocyte stimulation tests using the patients' peripheral blood mononuclear cells (23); the stimulation index was ranging from 0.26 to 3.80 which indicated 10 of 14 patients were more proliferative toward the β Lg hydrolysate than unhydrolyzed β Lg. As the increasing time of hydrolysis in whey reduces allergenicity and immunogenicity in most but not all CMA patients (24), their immunoreactivity is distinctively affected by individuals due to the difference of peptide recognition by T cells. Further investigation should help uncover the

molecular mechanisms underlying the relationships between certain epitopes and allergy persistence. However, we believe that our results have provided for the first time a practical approach for generating peptide-based edible vaccines against major milk allergens using common food materials.

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Conflict of interest

H.M. Ueno and T. Nakano are employees of Bean Stalk Snow Co., Ltd. Other authors have no conflict of interests to declare. This study was funded in part by the Research and Development Program for New Bio-industry Initiatives (2005–2009) of the Bio-oriented Technology Research Advancement Institution (BRAIN), Japan. BRAIN had no substantial contribution except for the financial support.

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