

Mitigation of the allergenic activity of beta-lactoglobulin by electrolysis

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Beta-lactoglobulin (BLG) is the most prominent allergen causing milk allergy and contains disulfide (S-S) bonds that may be responsible for its allergic action. As S-S bonds may be reduced during electrolysis, this study was undertaken to evaluate modulation of the allergic action of BLG after electrolysis. A current of 85 mA/cm² was applied, and the allergic action was evaluated by means of competitive CAP-FEIA inhibition tests and skin prick tests (SPT). Modification of BLG was examined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS). Although the number of total free sulfhydryl (SH) groups of BLG did not differ between the cathode and anode sides, BLG on the cathode side showed 54% lower inhibition than untreated BLG in the competitive CAP-FEIA inhibition tests, and reduced wheal reactions, by 71%, in the SPT compared with those with untreated BLG. The SPT results with BLG on the anode side did not differ significantly from those with untreated BLG. The MALDI-ToF-MS results for the dimer of untreated BLG or BLG on the anode side showed two distinct peptide fragments (residues ⁴¹V-⁶⁰K and ¹⁴⁹L-¹⁶²I) but, on the contrary, the dimer of BLG on the cathode side did not give these fragments, this being similar to in the case of the monomer of BLG. The allergic action of BLG was markedly mitigated during electrolysis on the cathode side, a dimer of BLG with a different mass spectrometric pattern from that of the dimer of untreated BLG being simultaneously formed.

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Food allergies continue to be an important human health problem, and cow's milk ranks among the top foods causing childhood allergies. Hydrolysate formulas have been used as substitutes in infants with cow's milk allergy. However, together with their unpleasant taste, there are some worrying nutritional issues with those formulas, such as growth reduction (1, 2), decreases in plasma protein concentrations and iron-binding capacities, and unbalanced plasma and urinary concentrations of some amino acids (2, 3). Furthermore, animals given hydrolysed formulas exhibited enhanced proteolytic activities of trypsin and chymotrypsin in the intestine, higher levels of cytochromes in the liver and disordered metabolism of glucocorticoids (4). Therefore, the mitigation of the allergic action of

cow's milk may be required over procedures in being independent of proteolytic enzymes.

Beta-lactoglobulin (BLG) is the most prominent S-S protein allergen as well as the main whey protein. There is no counterpart in human milk. As B-cell epitopes are mostly conformational and dependent on the three-dimensional structure of antigens, heat denaturation of BLG has been studied as to modulation of its immunogenicity or allergenicity. Although a decrease in the IgE-binding ability of BLG has been demonstrated after heat treatment to some degree (5), heat-denatured BLG induces a more intensive local immunologic reaction in the gastrointestinal mucosa than native BLG (6). The antigenicity of BLG has been reported to depend on its intramolecular S-S bonds, and it

can be reduced by thioredoxin through its catalytically active S-S groups, which results in its lower allergenicity and enhanced digestibility (7). Although a reduction of S-S bonds was expected during electrolysis on the cathode side, only short-term modulation of the redox potential was observed for whey proteins with a carbon electrode and a low voltage difference (8, 9). Carbon electrodes are not approved for the modulation of food in Japan. Therefore, this study was undertaken to modulate the most prominent milk allergen, BLG, with a much higher voltage difference between platinum electrodes, and then to evaluate its allergic action after the electrolysis. Because an acute allergic skin response has been proposed to be a major read-out system for addressing the putative sensitizing capacity and the residual allergenicity of hydrolysate formulas (10), SPT were performed in patients with cow's milk allergy. Ovomucoid (OMC) has been identified as the most prominent allergen causing hen's egg allergy and contains S-S bonds that may also be responsible for its allergic action (11); therefore, OMC was chosen for the control study. Furthermore, a sensory test for whey after electrolysis was performed for children. This study may offer a new strategy as to formulas for patients with cow's milk allergy.

Materials and methods

Subjects

Fifteen patients with IgE-mediated cow's milk allergy were enrolled in this study. All of them exhibited a previous adverse reaction to cow's milk or dairy products, namely urticaria/angio-oedema ($n = 6$), wheezing ($n = 1$), gastrointestinal symptoms ($n = 1$), anaphylactic shock ($n = 2$), skin symptoms plus wheezing ($n = 3$), or skin symptoms plus wheezing and gastrointestinal symptoms ($n = 2$). Immediate adverse reactions were confirmed by oral provocation tests with cow's milk in all patients, except two collapsed cases. Five patients with IgE-mediated hen's egg allergy were also enrolled. All of them exhibited a previous adverse reaction to hen's egg or hen's egg-containing food, namely urticaria ($n = 3$) or skin symptoms plus wheezing ($n = 2$). The immediate adverse reactions were confirmed by oral provocation tests with boiled hen's eggs in all patients. In addition, a sensory test for whey after electrolysis was performed for 20 children, aged 5–8, whose parents were staff members of the laboratory. The panel consisted of children who were not allergic to milk, or

lactose intolerant, whether they liked milk or not.

The study was approved by the Human Research Advisory Board, and written informed consent for the investigation was obtained from all the study participants, the parents' consent being obtained for children.

Competitive CAP-FEIA inhibition tests

The IgE-binding activity of BLG was determined by calculation involving the inhibitory capacity of BLG by serum-specific IgE determination with a CAP system FEIA (Phadia, Uppsala, Sweden), as described mainly by Gleich et al. (12) Briefly, eight-microliter aliquots of diluted BLG were incubated at 37°C for 1 h with 0.4-ml aliquots of sera from five patients (E.R., O.K., T.Y., M.K. and N.Y.) with cow's milk allergy. The percent decrease in the IgE-binding activity of BLG after the electrolysis was calculated as follows; $(1 - \text{CAP-FEIA value with BLG before electrolysis} / \text{CAP-FEIA value without BLG}) \times 100 - (1 - \text{CAP-FEIA value with BLG after electrolysis} / \text{CAP-FEIA value without BLG}) \times 100$.

Furthermore, BLG adjusted with acetic acid to pH 3.50 or with sodium hydroxide to pH 11.20 was also examined. The mitigation of the IgE-binding activity of OMC after electrolysis was examined in the same way.

SPT

The SPT were performed with plastic bifurcate needles (DUOTIP-TEST; Lincoln Diagnostics, IL), and the diameters of the wheals (mm) were determined after 20 min. Normal saline with or without 1% histamine hydrochloride was applied as a control. The tests were performed for the untreated BLG, the BLG after the electrolysis, and 1:10 w/v each of the regular cow's milk and whey hydrolysate formulas. Each solution was placed at random on the forearms. The percent decrease in the wheal reaction with BLG after electrolysis as against with BLG before electrolysis and the percent decrease in the wheal reaction with the whey hydrolysate formula as against the regular cow's milk formula were calculated as follows:

$(1 - (\text{wheal size with BLG/whey after electrolysis or wheal size with the whey hydrolysate formula} - \text{wheal size with normal saline}) / (\text{wheal size with BLG/whey before electrolysis and wheal size with the regular cow's milk formula} - \text{wheal size with normal saline})) \times 100$.

In the case of the same wheal size with normal saline, the percent decrease in wheal size with

BLG/whey after electrolysis and wheal size with the whey hydrolysate formula were defined as 100%.

Reagents

Purified BLG, 80% purity, was obtained from Sigma Chemicals, St. Louis, MO, USA. A regular cow's milk formula (Meiji Hohoemi[®], Meiji Dairies Corp., Koto-ku, Tokyo, Japan) and a whey hydrolysate formula (Meiji Milfee HP[®], Meiji Dairies Corp., Koto-ku, Tokyo, Japan), i.e., an enzymatic hydrolysate (22–27% hydrolysis) of isolated whey proteins, were obtained from a local representative of Meiji Dairies Co., Tokyo, Japan. The whey was obtained from low-fat milk containing 0.4% fatty acid (Musibo-Gyu-nyu[®], Yamaguchi Kenraku Milk Industry Co., Ltd., Shimonoseki, Yamaguchi, Japan), Yamaguchi-Rakuno, Yamaguchi, Japan, after precipitation of casein at pH 4.6 and 50°C. Purified OMC, 10,000 BAEE u/mg trypsin inhibitor, was obtained from Nacalai Tesque Inc., Kyoto, Japan.

Electrolysis

Heat-tight, 30-mm bore glass tubes were used in an H-shaped electrolysis cell, connected with two tubes of 300 mm long and one tube of 40 mm long. To collect samples during electrolysis, a tap-equipped 5-mm bore glass tube was inserted through a rubber gasket into each platinum electrode of 15 mm wide and 30 mm long. In addition, 5-mm bore silicon tubes were coiled around the electrolysis cell, circulating –5°C water originating from a thermostated water bath (Model TBF210DA; Advantec MFS, Tokyo, Japan). Electrolysis was carried out for 30 min in a batch manner with an anode/cathode voltage difference of 120 V, 0.6 A (85 mA/cm²) for 1% BLG containing 1% sodium chloride and of 250 V, 1.0 A (142 mA/cm²) for whey using a power supply (Model PAS160-2; Kikusui-Denshi, Yokohama, Japan). Electrolysis was also carried out for 1% OMC containing 1% sodium chloride for 30 min with a voltage difference of 90 V, 0.23 A (30 mA/cm²). The electrolyte volume of 260 ml was used. The temperature in the electrolyte in both glass tubes was kept in the range of 12–18°C, and the temperature next to the electrodes ranged from 40 to 50°C during the electrolysis. After flushing out 1 ml, 5 ml samples were collected from around the electrodes at 5, 10 and 30 min, respectively. The BLG and OMC solutions were then filtrated through a 0.45-mm filter unit (Millex-HA; Millipore, Bedford, MA,

USA). To confirm their stability, samples were then kept at 4°C for more than 2 wk in sterilized polystyrene tubes (Falcon 2054; Beckton-Dickinson, Franklin Lakes, NJ, USA) prior to the tests.

Protein assay

The amounts of protein were determined by the method of Lowry et al. (13), using bovine serum albumin as the standard protein. The redox potential of the samples was measured with a redox electrode (model HM-21P; DKK-TOA Co., Tokyo, Japan) calibrated with a standard potassium chloride solution at 210 mV. Free SH groups were determined by quantifying total SH groups according to Ellman's method (14). The total amino acid composition was determined using a high-performance liquid chromatograph (HPLC) for amino acid analysis (model L-850; Hitachi, Ibaragi, Japan) after incubation of the samples with the same volume of 12 N HCl for 20 h at 100°C.

Gel electrophoresis and peptide mapping

SDS-polyacrylamide gel electrophoresis (PAGE) was carried out as described by Laemmli (15) using a separation gel containing 14% acrylamide under reducing conditions with 1% SDS, 10% glycerol, BPB, 1% 2-mercaptoethanol and 100 mM Tris-HCl (pH 6.8), and the proteins on analytical gels were visualized by rapid Coomassie staining.

Gel filtration was performed on a Superdex200 column (GE Healthcare, Tokyo, Japan) with 150 mM sodium chloride and 20 mM Tris-HCl (pH 8.0), the column having a fraction range of 10–600 kDa. The eluted fractions were then purified by anion-exchange chromatography on a Resource-Q column (GE Healthcare) with 50 mM sodium chloride and 20 mM Tris-HCl (pH 8.0), and a gradient of sodium chloride up to 500 mM in 20 min. The UV absorbance of the effluent was monitored at 280 nm. After digestion with lysylendopeptidase, the products were separated by reversed-phase HPLC with a UV detector (model L-6000; Hitachi, Ibaragi, Japan) on a C8 reversed-phase column (AP-202; YMC Co., Kyoto, Japan) equilibrated with 0.1% trifluoroacetic acid in water and a gradient of acetonitrile up to 60% in 1 h. The UV absorbance was monitored at 215 nm.

Mass spectrometry

After dissolving the BLG in a lysis buffer (7M urea, 2M thiourea, 2% CHAPS and 40 mM Tris),

two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed with 5 mM TATA-binding protein and 10 mM acrylamide applied per isoelectric focusing gel pI 3–10. The isoelectric focusing was performed until about 35,000 volt-hours, and the proteins separated on 2D-PAGE were visualized by rapid Coomassie staining. Some spots were cut out from the gel, and then tryptic digestion was performed simultaneously according to the procedure of Gevaert et al. (16) A peptide mass fingerprint database search was performed with the masses obtained by means of MALDI-ToF-MS, with a 337-nm nitrogen laser (Shimadzu Biotech, Kyoto, Japan, and Kratos Analytical, Manchester, UK) in the positive ion mode. The monoisotopic m/z values of tryptic peptide peaks were entered into Mascot (Matrix Science, http://www.matrixscience.com/search_form_select.html) for a database search for characterization of the proteins.

Sensory test

Children were tested individually and were presented with a tray bearing three 30-ml transparent plastic cups filled with 10 ml of whey at room temperature. The location of each of the three choices on the tray was random. Cups were labeled with two-digit codes on their bottoms for identification of the items. The cups contained one of two whey formation, i.e., whey before electrolysis (two of the three cups) or whey obtained from the cathode side after electrolysis (one of the three cups). Hedonic ratings for each milk formulation were obtained in the first phase of testing in a self-selected order. After testing each whey sample, the child was asked to place the cup in front of one of three cartoon faces. The faces had a smile (like), a frown (dislike) or a neutral expression.

Statistical analysis

Group means were compared by means of Student's paired *t*-test. The results of sensory tests were analyzed by means of the chi-square test. Probability values of <0.05 were considered significant.

Results

IgE assays

Electrolysis, even after 30 min, only slightly reduced the inhibition capacity of BLG present on the anode side, as shown in Table 1. The reduction was only slightly greater than that with

Table 1. Inhibition of IgE binding to BLG-specific CAP-FEIA

Additive	Direct binding in Ua/ml to BLG-CAP	% inhibition in binding to BLG-CAP	Reduced inhibition relative to untreated BLG
Normal saline	2.28		
Untreated BLG	0.46	79.8	
5 min			
Anode BLG	0.47	79.4	0.4
Cathode BLG	0.49	78.5	1.3
10 min			
Anode BLG	0.46	79.8	0
Cathode BLG	0.49	78.5	1.3
30 min			
Anode BLG	0.65	71.5	8.3
Cathode BLG	1.69	25.9	53.9
Acid BLG*	0.47	79.4	0.4
Alkaline BLG†	0.52	77.1	2.7

*Buffered to pH 3.50 with acetic acid.

†Buffered to pH 11.20 with sodium hydroxide.

acid or alkaline treatment. In contrast, the reduction of inhibition capacity at the cathode side was markedly reduced. The inhibition of IgE binding to OMC-specific CAP-FEIA with OMC after electrolysis was not different from that before electrolysis, there being 4.13 Ua/ml of OMC-specific CAP-FEIA in aliquots of sera from five patients with hen's egg allergy, 0.17 Ua/ml with the untreated OMC, 0.17 Ua/ml with the anode OMC and 0.18 Ua/ml with the cathode OMC.

Representative SPT results for a patient with cow's milk allergy are shown in Fig. 1, and the results for all 15 patients are summarized in Table 2. The wheal size with BLG on the cathode side was markedly reduced in the SPT compared with untreated BLG or BLG on the anode side, except in one patient (M.R.) with a seriously systemic anaphylactic reaction to milk. The percent decrease in the wheal reaction with BLG on the cathode side as against untreated BLG (average 71%) was almost the same as that with the hydrolysate as against the regular milk (average 79%), whereas the wheal size with BLG on the anode side was not affected significantly compared with untreated BLG.

Protein measurements

The pH and redox potential results for the BLG solution were 6.30 and -1 mV before electrolysis, which changed to 3.50 and $+182$ mV on the anode side and to 11.20 and -208 mV on the cathode side after 30-min electrolysis, respectively. The results for whey were 6.1 and ± 137 mV before electrolysis but were 4.5 and ± 350 mV on the anode side, and 8.9 and



Fig. 1. Representative results of the skin prick test: (BLG) Untreated BLG, (+) BLG on the anode side, (-) BLG on the cathode side.

-135 mV on the cathode side after electrolysis, respectively. The results for the OMC solution were 5.9 and ± 242 mV before electrolysis but were 2.8 and ± 302 mV on the anode side, and 11.2 and -15 mV on the cathode side after electrolysis, respectively. The protein concentration of BLG was 7.28 mg/ml before electrolysis, which decreased almost equally to 5.72 mg/ml on the anode side and to 6.21 mg/ml on the cathode side after 30-min electrolysis. The whey concentration was 33 mg/ml before electrolysis, which changed to 28 mg/ml on the anode side and to 33 mg/ml on the cathode side after electrolysis. The OMC concentration was 5.8 mg/ml before electrolysis, which changed to 6.1 mg/ml on the anode side and to 9.4 mg/ml on the cathode side after electrolysis. The quantity of SH groups of BLG was 159.2 nmol/ml before electrolysis, which decreased almost equally after 30-min electrolysis on both the anode side (24.1 nmol/ml) and the cathode side (24.6 nmol/ml). The quantity of SH groups of OMC was 1.2 nmol/ml before electrolysis, which markedly increased after electrolysis on the cathode side (14.8 nmol/ml) but did not change significantly

Table 2. Wheal size results for SPT in 15 patients with milk allergy (mm)

Patient (Age, yr), clinical features*	Saline	Untreated BLG	Anode BLG (% decrease)†	Cathode BLG (% decrease)	Regular milk	Hydrolysate (% decrease)
E.R.(1), SW	4	21	21 (0)	13 (47)	27	2 (100)
I.S.(1), U/A	2	23	16 (33)	3 (95)	6	3 (50)
M.R.(2), AS	4	17	22 (0)	20 (0)	19	9 (67)
K.K.(5), U/A	4	13	12 (11)	6 (78)	10	7 (50)
H.S.(2), SW	3	9	9 (0)	3 (100)	13	5 (80)
S.Y.(2), SW	2	5	5 (0)	3 (67)	10	4 (75)
O.K.(1), W	3	25	24 (5)	5 (91)	14	1 (100)
K.K.(13), SW	1	11	11 (0)	5 (60)	22	7 (71)
T.Y.(2), U/A	2	15	14 (8)	2 (100)	5	2 (100)
K.T.(4), W	2	15	9 (46)	8 (54)	19	10 (53)
M.K.(0), U/A	4	28	17 (46)	5 (96)	n.d.‡	n.d.
N.N.(4), U/A	2	4	4 (0)	2 (100)	n.d.	n.d.
S.N.(2), U/A	5	16	15 (0)	8 (73)	8	3 (100)
I.S.(5), GS	4	15	24 (0)	7 (73)	n.d.	n.d.
N.Y.(17), AS	3	6	16 (0)	5 (33)	n.d.	n.d.
			10 $\pm$ 15§	71 $\pm$ 28		79 $\pm$ 18

*SW, skin symptoms plus wheezing; U/A, urticaria/angioedema; AS, anaphylactic shock; w, wheezing; GS, gastrointestinal symptoms.

†Percent decrease in wheal diameter with the BLG after electrolysis as against the untreated BLG, or with the whey hydrolysate formula as against the regular milk formula.

‡Not done.

§Mean $\pm$ 1 s.d.

on the anode side (2.8 nmol/ml). The total amino acid composition with BLG changed equally after 30-min electrolysis on both the anode and cathode sides, resulting in decreases in cystine (C) residues, methionine (M) residues and tyrosine (Y) residues, as shown in Table 3. The quantity of lysine (K) residues was a little less on the cathode side than on the anode side. The total amino acid composition with OMC changed equally after electrolysis on both the anode and cathode sides, resulting in decreases in cystine residues from 4% to 3%, and tyrosine residues from 3% to 2%.

SDS-PAGE

SDS-PAGE with complete reduction gave a single 18-kDa band with untreated BLG and BLG on the anode side, whereas two bands corresponding to 35 and 16 kDa were seen for BLG on the cathode side. Because the untreated BLG was obtained from a single peak of effluent at around 36-kDa after gel filtration (Fig. 2), it seems likely that BLG forms a dimer under non-reducing conditions. Although the BLG on the cathode side was collected from effluent containing materials exhibiting a wide range of molecular weights after gel filtration, 35- and 16-kDa proteins were obtained on SDS-PAGE with

Table 3. Total amino acid levels in BLG before and after electrolysis (%)

	D	T	S	N	E	Q	P	G	A	V	C*	M*	I	L	Y*	F	H	W	K*	R
Untreated BLG	11	5	5	0	19	0	5	2	9	5	1	2	5	14	2	3	1	0	10	2
Anode BLG 10 min	11	5	5	0	19	0	5	2	9	5	1	2	5	14	2	3	1	0	10	2
Cathode BLG 10 min	11	5	5	0	19	0	5	2	9	5	1	2	5	14	2	3	1	0	9	2
Anode BLG 30 min	12	6	5	0	20	0	6	2	9	5	0	0	5	15	0	3	1	0	9	2

complete reduction. As the 35-kDa protein was obtained from the effluent at around 70-kDa, and the 16-kDa one from the effluent at around 34-kDa, as shown in Fig. 2, it is suggested that both the 35-kDa and 16-kDa proteins formed dimers in the electrolysis cell, respectively. Untreated BLG, and the 35- and 16-kDa proteins on the cathode side were purified by anion-exchange chromatography, and then digested with lysyl-endopeptidase. The reversed-phase HPLC results gave exactly the same UV absorbance curves for the first half of the effluent containing peptides derived from untreated

BLG, and the 35-kDa and 16-kDa proteins on the cathode side, respectively, as shown in Fig. 3.

The 2D-PAGE study demonstrated the aggregation of BLG during the electrolysis on both the anode and cathode sides, and it seemed likely that the aggregation was greater on the cathode side than on the anode side (Fig. 4). Spots #2, #4 and #7 were thought to represent the monomer of BLG, spots #1, #3 and #6 to represent the dimer of BLG and spot #5 to represent the tetramer of BLG, respectively, as judged from the molecular weights.

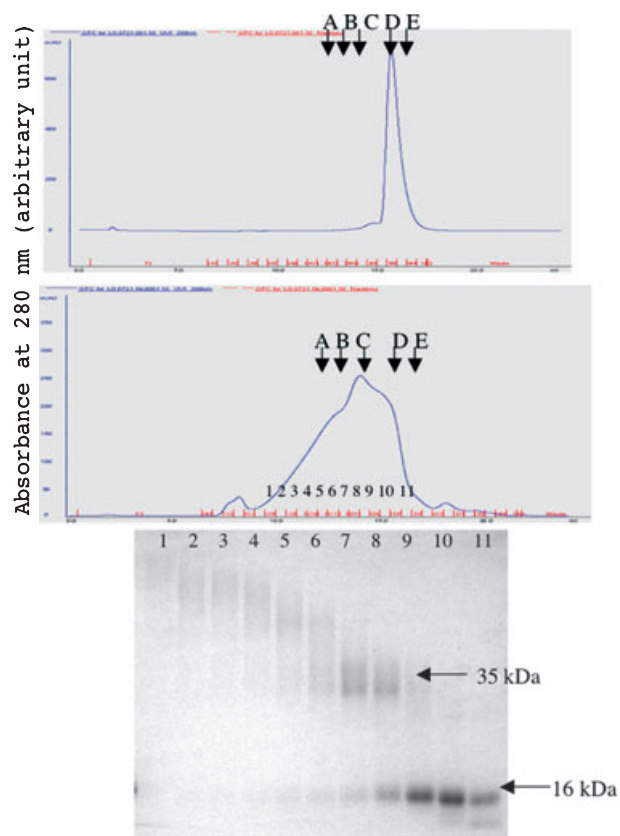


Fig. 2. Gel filtration chromatography with untreated BLG (upper) and BLG on the cathode side (lower): (A–E) molecular weight markers (A: 290 kDa, B: 140 kDa, C: 67 kDa, D: 34 kDa, E: 12.8 kDa), and SDS-PAGE results with BLG on the cathode side: 1–11, fractionated effluent numbers during the gel filtration chromatography.

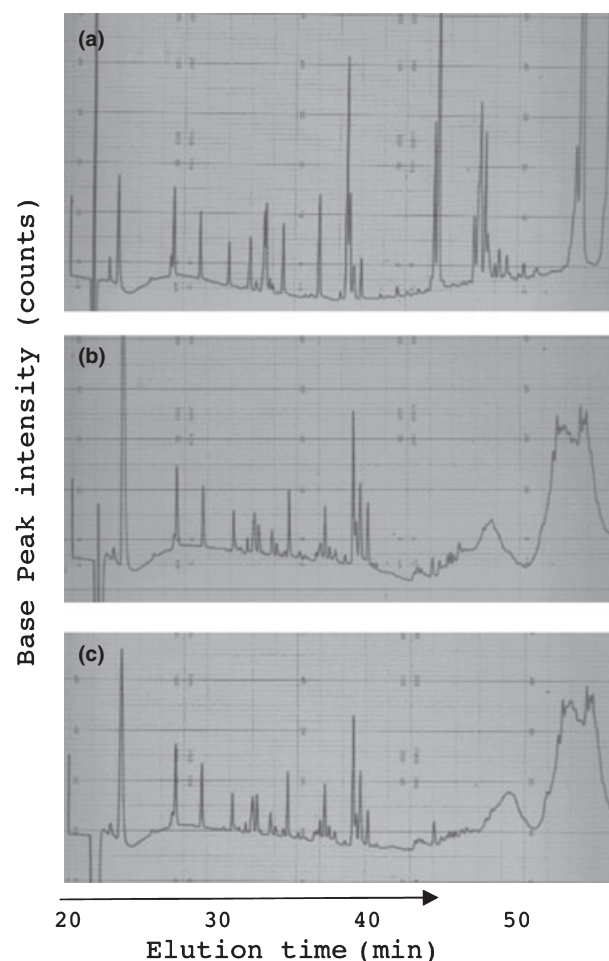


Fig. 3. HPLC chromatograms after lysylendopeptidase digestion: (a) Untreated BLG, (b) 16 kDa on the cathode side, (c) 35 kDa on the cathode side.

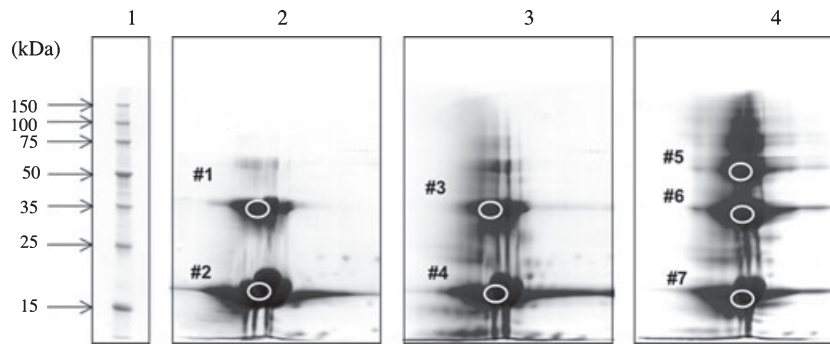


Fig. 4. 2D-PAGE before and after electrolysis: 1, molecular weight markers; 2, untreated BLG; 3, BLG on the anode side; 4, BLG on the cathode side. Spots 1–#7 were cut out from the gel.

Mass spectrometry

The materials in gel spots were analyzed by MALDI-ToF-MS after tryptic digestion, and the spectral results being summarized in Fig. 5. Spots #1 and #3 gave the same spectrum, but spots #5 and #6 showed the same spectrum as spots #2, #4 and #7. It seems likely that the dimer of BLG on the cathode side showed a different mass spectrometric pattern to that of untreated BLG or BLG on the anode side, indicating a similar mass spectrometric pattern to that of the monomer in each BLG solution category. Peptide mass fingerprinting showed that peak 17 contained a peptide fragment comprising residues 149–162 of the amino acid sequence of BLG with a c-terminal, i.e., LSFNPTQLEEQCHI. Peak 23 contained one comprising residues 41–60 of the BLG sequence, i.e., VYVEELKPTPEGDLEILLQK, and peak 27 one comprising residues 15–40 of the BLG sequence, i.e., VAGTWYSLAMAASDISLLDAQSAPLR, respectively, according to the database.

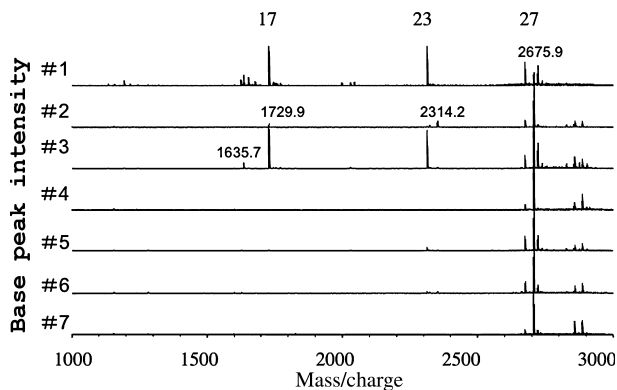


Fig. 5. MALDI-ToF-MS spectra of BLG from gels after tryptic digestion. The gels designated 1–#7 are shown in Fig. 4. Spots #1 and #3 exhibited the same mass/charge, but spots #5 and #6 showed the same spectrum as spots #2, #4 and #7.

Sensory test

Eighteen of the total 40 cups containing whey before electrolysis were placed in front of the smile face, 17 in front of the neutral expression one and five in front of the frown one, respectively. Ten of the total 20 cups containing whey obtained from the cathode side after electrolysis were placed in front of the smile face, seven in front of the neutral expression one and three in front of the frown one, respectively. The hedonic ratings for the two milk formulations were not significantly different.

Discussion

Because the quantity of SH groups of BLG was decreased almost equally on both the anode and cathode sides, the modulation of S-S bonds of BLG did not seem to be fundamentally associated with the mitigation of its allergic action. Although the quantity of SH groups of OMC markedly increased after electrolysis on the cathode side, mitigation of its allergic action was not observed in this study.

It has been postulated that the B-cell epitope of a protein with a rigid globular structure is localized on the surface of the molecule. Selo et al. (17) identified three peptide fragments as the allergenic sites on the BLG molecule after tryptic digestion, i.e., residues ⁴¹V-⁶⁰K, ¹⁰²Y-¹²⁴R and ¹⁴⁹L-¹⁶²I, all of which are exposed on the outside of its rigid three-dimensional structure (18). In the present MALDI-ToF-MS study, trypsin treatment of untreated BLG and anode BLG lead to clear identification of the two fragments ⁴¹V-⁶⁰K and ¹⁴⁹L-¹⁶²I, whereas no corresponding tryptic fragment was recovered for the cathode BLG, or the apparent monomeric forms of BLG derived from the untreated or anode spots (#2 and #4 from Fig. 4).

Untreated BLG was detected as a single peak of a dimeric form of BLG in the gel filtration study, suggesting that native BLG is present in milk as a dimeric form. An allergic action was observed with the untreated BLG and BLG on the anode side, and two antigenic fragments were distinctly separated from the gels containing the dimeric forms of untreated BLG and BLG on the anode side. Therefore, it seems likely that the dimeric form *per se* is highly involved in the allergic action of BLG, and the allergic action of the dimeric form of BLG is closely associated with two peptide fragments, ^{41}V - ^{60}K and ^{149}L - ^{162}I , on the surface.

In type I allergy, the cross-linking of cytophilic IgE on effector cells and that of membrane IgE on B lymphocytes by the respective allergens are key events. For the cross-linking of two IgE molecules, allergens require at least two epitopes. However, the availability of epitopes may be limited on small allergens such BLG (18-kDa). Scholl et al. (19) recently studied mice sensitized with the major birch pollen allergen Bet v 1 (17-kDa) and showed that skin tests in the mice were positive with the Bet v 1 dimer but were negative with the monomer. Therefore, dimerization seems to be a common prerequisite for the protein allergenicity of small allergens. Scholl et al. also indicated that, in contrast to dimeric Bet v 1, monomeric Bet v 1 was less capable of activating murine memory B cells as to IgE production *in vivo* (19). This is of special interest as the dimer and oligomers of BLG on the cathode side showed similar mass spectrometric patterns to that of the monomeric BLG. It should be crucial to mitigate the sensitization potential of the modified proteins in formulas for the nutrition of infants at high risk of developing food sensitization.

Beta-lactoglobulin is composed of nine beta-strands (beta-strand-A to beta-strand-I) and one alpha-helix, and it is assumed that the intermolecular hydrogen bonds between the beta-strand-I main chains and the intermolecular salt bridges between the side chains of the loop (beta-strand-A and beta-strand-B) play important roles in dimer formation (20). Allergenic peptide ^{41}V - ^{60}K contains beta-strand-B (^{41}V - ^{50}P), and the other allergenic peptide, ^{149}L - ^{162}I , includes a part of beta-strand-I (^{146}H - ^{151}F). The SDS-PAGE study with complete reduction showed 35- and 16-kDa bands for BLG on the cathode side, whereas a single 18-kDa band was seen with untreated BLG and BLG on the anode side, respectively. Alternatively, the structure of the dimeric form of BLG on the cathode side might be different from that of native BLG, resulting in conforma-

tional modulation with dislocation of the allergenic peptides from the surface of BLG.

Fifteen of the 162 amino acids in the sequence of BLG are lysine residues. Lysine is known as an essential amino acid for maintenance of the antigenic structure of BLG (21). The number of lysine residues was a little less on the cathode side than on the anode side. Various commercial BLG preparations have been reported to be lactosylated to some degree (22), and reducing sugars such as lactose favor the Maillard reaction via a Schiff base between the aldehyde of the glucose moiety of lactose and an amino group of a protein with heat and the optimum pH (23). As one of the first lactose-binding sites of BLG was identified as a lysine residue (22), the decrease in lysine residues might be because of the Maillard reaction during electrolysis.

As there are some worrying nutritional issues with hydrolysate formulas (1–4), mitigation of the allergic action of cow's milk may be required over procedures in being independent of hydrolysis for formulas. The taste of whey after electrolysis on the cathode side was not altered; therefore, this study may offer a new strategy as to formulas for patients with cow's milk allergy. Although further follow-up work with other allergenic milk proteins including casein and alpha-lactalbumin is necessary, my preliminary experiments showed that casein precipitated on the anode side, and alpha-lactalbumin aggregated on both the anode side and the cathode side during electrolysis.

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