

A new strategy for mitigation of the allergenic activity of ovomucoid in hen eggs and beta-lactoglobulin in cow milk

Jun Kido¹, Natsuko Nishi², Sachiko Misumi² and Tomoaki Matsumoto^{3*}

¹Department of Pediatrics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto City, Kumamoto, Japan

²Department of Pediatrics, Kumamoto Regional Medical Center, Kumamoto City, Kumamoto, Japan

³Pediatric Division, Aso Spa Hospital, Aso City, Kumamoto, Japan

Abstract

Recently, public interest in food allergies has been increasing because of increasing prevalence of these allergies among children. In Japan, hen egg and cow milk allergies account for nearly 50% of all cases of childhood food allergies. Patients with hen egg and cow milk allergies should avoid these foods and products that contain them until they have outgrown their allergies. However, regarding nutrition, quality of diet, and hyposensitization, it is significantly important for these patients to ingest hypoallergenic hen eggs, cow milk, and products that contain them. We previously demonstrated the mitigation of the allergenic activity of beta-lactoglobulin in cow milk and ovomucoid in hen egg by electrolysis. In this review, we discuss this new strategy for producing hypoallergenic hen eggs and cow milk.

Introduction

Food allergies continue to be an important human health problem. Recently, cases of food allergies have been increasing, with 5%-10% of infants and 1%-3% of school-age children in Japan affected by food allergies [1]. Hen eggs are most commonly responsible for food allergies, followed by cow milk. Patients with these food allergies develop adverse reactions, like urticaria, angioedema, wheezing, gastrointestinal symptoms, or anaphylactic shock, after ingestion of hen eggs, cow milk, or products that contain them.

Hen eggs are comprised of about 8%-11% shell, 56%-61% white, and 27%-32% yolk [2]. While the white is an aqueous protein solution (10% protein and 88% water), the yolk is composed of 50% water, 34% lipid, and 16% protein, giving it quite different properties. Egg white has been considered the most important source of allergens, with the ovomucoid (OMC; WHO/IUIS allergen name: Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3), and lysozyme C (Gal d 4) being the major allergens in it, but IgE-binding allergens have also been identified in the yolk. The characteristics of these four major egg white allergens are shown in Table 1. OMC and ovotransferrin are the most frequent allergens though it may depend on the reports.

Cow milk contains around 30-35 g of proteins per liter and includes more than 25 different proteins but only some of them are known to be allergenic. Table 2 provides the characteristics of cow milk allergens. Cow milk proteins consist of the coagulum containing the casein proteins and the lactoserum (whey proteins) representing 80% and 20% of the total milk proteins, respectively [3]. The casein fraction (Bos d 8) consists of four proteins that account for different percentages of the whole fraction: alphaS1-casein (Bos d 9), alphaS2-casein (Bos d 10), beta-casein (Bos d 11), and kappa-casein (Bos d 12), with alphaS1-casein being the most important allergen of the casein fraction [4]. Allergens in the whey fraction are alpha-lactalbumin (Bos d 4), beta-lactoglobulin (BLG) (Bos d 5), immunoglobulins (Bos d 7), bovine serum albumin (BSA)(Bos d 6), and traces of lactoferrin (Bos

d lactoferrin). Alpha-lactalbumin and beta-lactoglobulin are the most important allergens of the whey fraction, accounting for 5% and 10% of the total milk proteins, respectively [3,5]. There are only a few reports describing allergies to minor whey proteins such as immunoglobulin, BSA, or lactoferrin [6].

Diagnosis of Hen Egg and Cow Milk Allergy and Effectiveness of The Skin Prick Test

Conclusive diagnosis is crucial in patients with suspected food allergies. Double-blind, placebo-controlled food challenges are still the gold standard for diagnosing food allergies; however, these are time consuming, expensive, and troublesome for patients and involve risks of severe systemic reactions [7].

Table 1. Major egg white allergens.

Protein	Allergen name	Content in dried egg white (%)	Prevalence in patients (%)	Size (kDa)	pI	Amino Acids
Ovomucoid	Gal d 1	11	34-97	28.0	4.1	186
Ovalbumin	Gal d 2	54	9-100	45.0	4.5	385
Ovotransferrin	Gal d 3	13	22-94	77.7	6.0	686
Lysozyme	Gal d 4	3.5	6-69	14.3	10.7	129

pI: isoelectric point

Correspondence to: Tomoaki Matsumoto, Pediatric Division, Aso Spa Hospital, 1153-1 Uchinomaki, Aso city, Kumamoto 869-2301, Japan, Tel.: +81-967-32-0881; Fax: +81-967-32-4462; **E-mail:** matsumoto@a.asospahp.jp

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Table 2. Major cow milk allergens.

	Protein	Allergen name	Concentration (g/L)	Prevalence in patients (%)	Size (kDa)	pI	Amino Acids
Whey	Alpha-lactalbumin	Bos d 4	1-1.5	0-67	14.2	4.8	123
	Beta-lactoglobulin	Bos d 5	3-4	13-62	18.3	5.3	162
	Bovine serum albumin	Bos d 6	0.1-0.4	0-76	66.3	4.9-5.1	582
	Immunogloblins	Bos d 7	0.6-1.0	12-36	160		
	Lactoferrin		0.09	0-35	80	8.7	703
Whole casein	AlphaS1-casein	Bos d 9	12-15	65-100	23.6	4.9-5.0	199
	AlphaS2-casein	Bos d 10	3-4		25.2	5.2-5.4	207
	Beta-casein	Bos d 11	9-11	35-44	24	5.1-5.4	209
	Kappa-casein	Bos d 12	3-4	35-41	19	5.4-5.6	169

pI: isoelectric point

Several diagnostic tests have been developed to predict outcomes of oral food challenges. Analysis of food-specific serum IgE levels [8,9], the skin prick test (SPT) [10-12], and the atopy patch test [13] may be useful tools for diagnosing food allergies. However, the food challenge is still a crucial tool for definitively diagnosing food allergies, as analysis of food-specific serum IgE levels and the SPT do not currently render oral food challenges unnecessary in most cases [14]. The SPT is an important first-line procedure for evaluating food allergies, as it is quick and relatively inexpensive. Previously, we reported that measuring wheal sizes in the SPT and calculating the skin index (SI) could help diagnose many food allergies (e.g., to hen eggs, cow milk, wheat, and peanuts) [15]. Moreover, we performed the SPT in 126 children suspected to have CMA and 76 had positive oral provocation test results. We performed a logistic regression analysis to evaluate whether wheal diameters or SIs could predict CMA in these 126 patients. We found that wheal diameters and SIs could predict positive oral provocation test results. While the expected probabilities of having a positive oral provocation test result were ≥ 0.5 , the cut-off values for wheal diameters and SIs were ≥ 8 mm (79.5% sensitivity and 76.7% specificity) and ≥ 1.0 (77.3% sensitivity and 78.9% specificity), respectively.

Hill *et al.* [11] and Sporik *et al.* [16] showed that a 6-mm wheal diameter in children <2 years of age, or 8 mm in children >2 years of age, indicates 100% specificity of a clinical reaction. Saarinen *et al.* [17] reported 98% specificity and 92% positive predictive value (PPV) for >8-mm wheal diameter. Verstege *et al.* [12] reported 95% PPV for a 12.5-mm wheal diameter when pricked with fresh milk. Calvani *et al.* [18] reported 95% PPV for a 15-mm wheal diameter in infants; this evidence supports results of our study, which shows that a 15-mm wheal diameter had 90% PPV.

Treatment of Hen Egg and Cow Milk Allergy

Patients with hen egg allergy (HEA) or cow milk allergy (CMA) should avoid these foods or products containing them until they outgrow HEA or CMA. However, it has been recently reported that oral immunotherapy by ingestion of these foods was able to achieve effective desensitization to these food allergies [19]. The egg white has a higher allergenic content than the yolk of the hen egg. Moreover, because raw hen eggs have greater allergenic content compared to cooked eggs, patients with HEA are likely to develop adverse reactions after ingestion of raw egg whites in the oral provocation test (OPT).

Many patients with HEA can ingest the cooked yolk, and some can even ingest the cooked egg white in small amounts. We should therefore assess their upper dose limit in the oral hen-egg provocation test. It is certainly important for daily nutrition and quality of diet of patients to be able to ingest a small amount of cooked egg.

Extensively hydrolyzed formulas (ehCMF) have been recommended as milk substitutes for infants with CMA. However, together with their unpleasant taste, these formulas present nutritional issues, such as growth reduction [20], decreased plasma protein concentrations and iron-binding capacity, and unbalanced plasma and urinary concentrations of some amino acids [20,21]. Furthermore, animals given hydrolysate formulas exhibited enhanced trypsin and chymotrypsin proteolytic activities in the intestine, higher cytochrome levels in the liver, and disrupted glucocorticoid metabolism [22]. Therefore, mitigation of the allergic action of cow milk, independent of the use of proteolytic enzymes, may be required. Partially hydrolyzed cow milk formulas (phCMFs) present therapeutic effects in infants with mild to moderate atopic dermatitis [23]. Although phCMFs are not considered substitute formulas for infants with CMA, we recently reported that 40 of 53 (75%) children with CMA could ingest phCMF [24] without any adverse reactions. Additionally, most children presenting a >6-mm wheal diameter with phCMF could complete the OPT with phCMF [24]. Because phCMF includes larger molecular weight proteins compared to ehCMF [24], which do not pass through an ultra-filtration membrane, phCMF has higher allergenicity compared to ehCMF. Since phCMF is expected to retain more cow-milk antigens compared to ehCMF, early and long-term administration of phCMF may induce immunologic tolerance to cow milk antigens in children with CMA [25,26]. Extensive hydrolysis of cow milk generates considerable quantities of free amino acids, which causes bitterness. Since phCMF has been reported to taste better than ehCMF [27], phCMF may be more beneficial than ehCMF as a nutritional ingredient for drinks and food.

Beta-lactoglobulin

Beta-lactoglobulin (BLG), which is a prominent S-S protein allergen, is the main protein in whey, and has no counterpart in human breast milk. BLG is composed of nine beta-strands (beta-strand A-I) and one alpha helix; it is assumed that intermolecular hydrogen bonds between beta-strand-I main chains and intermolecular salt bridges between side chains of the loop (beta-strand-A and -B) play important

roles in dimer formation [28]. The allergenic peptide 41V-60K contains beta-strand-B (41V-50P); the other allergenic peptide, 149L-162I, includes part of beta-strand-I (146H-151F). As B-cell epitopes are mostly conformational and dependent on the three-dimensional structure of antigens, heat denaturation of BLG has been studied to modulate its immunogenicity and allergenicity. Although decrease in IgE-binding ability of BLG has been demonstrated after heat treatment to some degree [29], heat-denatured BLG induces a more intensive local immunologic reaction in the gastrointestinal mucosa compared to native BLG [30].

The antigenicity of BLG has been reported to depend on its intramolecular S-S bonds, and can be reduced by thioredoxin through its catalytically active S-S groups, which causes lower allergenicity and enhanced digestibility [31].

Ovomucoid

The allergen protein content in hen eggs is mainly included in the egg white. Ovomucoid (OMC) has been identified as the most prominent allergen causing HEA and contains S-S bonds that may cause its allergic action [32,33]. OMC, composed of 186 amino acids, is a 28-kDa protein with isoelectric point pH 4.1. OMC consists of three tandem domains, each with nearly 60 amino acids, called Gal.d1.1, Gal.d1.2, and Gal.d1.3 [34]. Gal.d1.1 contains an α -helix and three β -sheets. Each domain has three intramolecular S-S bonds, but no intermolecular S-S bond [35]. OMC is highly resistant to proteolytic enzymes and heat [36] and exhibits high allergic activity due to this structural feature.

Electrolyzed Beta-lactoglobulin

Although reduction of S-S bonds was expected during electrolysis on the cathode side, only short-term modulation of the redox potential and a low voltage difference was observed for whey proteins with a carbon electrode [37,38]. However, carbon electrodes are not approved for the modulation of food in Japan.

We reported that the allergic activity of BLG was markedly mitigated by a much higher voltage difference between platinum electrodes [39]. Briefly, we electrolyzed BLG as follows (Figure 1 in Kido *et al.* [40]). Heat-tight, 30-mm bore glass tubes were used in an H-shaped electrolysis cell, connected with two tubes of 300 mm in length and one tube of 40 mm in length. The electrolysis was carried out using 1% BLG containing 1% sodium chloride for 30 minutes with a voltage difference of 90 V, 0.23 A (30 mA/cm²). The temperature of 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°C to 50°C during the electrolysis. Each BLG solution was then filtered through a 0.45-mm filter unit.

In patients with CMA, the wheal size for BLG on the cathode side (cBLG) was markedly reduced in the SPT compared with untreated BLG (uBLG) or BLG on the anode side (aBLG), except in one patient with a serious systemic anaphylactic reaction to milk. The percent decrease in the wheal reaction with cBLG compared to uBLG (mean 71% \pm 28%) was similar to that observed on comparison of the ehCMF with regular milk (mean 79% \pm 18%), whereas the wheal size with aBLG was not affected significantly compared with uBLG [39]. Because the quantity of SH groups of BLG was decreased almost equally on both anode and cathode sides, the modulation of S-S bonds of BLG did not appear fundamentally associated with mitigation of its allergic action.

It has been postulated that the B-cell epitope of a protein with a

rigid globular structure is localized on the molecular surface. Selo *et al.* [41] identified three peptide fragments as allergenic sites on the BLG molecule after tryptic digestion, i.e., residues 41V-60K, 102Y-124R, and 149L-162I, all of which are exposed outside its rigid three-dimensional structure [42]. In our study, trypsin treatment of uBLG and aBLG clearly identified the two fragments, 41V-60K and 149L-162I, whereas no corresponding tryptic fragment was recovered for cBLG or apparent monomeric forms of BLG derived from the untreated or anode spots. The uBLG was detected as a single peak of a dimeric form of BLG in the gel filtration study [39], suggesting that native BLG is present in milk as a dimeric form. Allergic action was observed for uBLG and aBLG, and two antigenic fragments were distinctly separated in gels containing dimeric forms of uBLG and aBLG. Therefore, it seems likely that the dimeric form 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, 41V-60K and 149L-162I, on its surface [39]. This is of special interest, as the dimer and oligomers of cBLG showed similar mass-spectrometric patterns compared to monomeric BLG [39]. It is crucial to mitigate the sensitization potential of these modified proteins in nutritional formulas for infants at high risk of developing food sensitivity.

The allergenic peptide 41V-60K contains beta-strand-B (41V-50P), and the other allergenic peptide, 149L-162I, includes a part of beta-strand-I (146H-151F). An SDS-PAGE study with complete reduction showed 35- and 16-kDa bands for cBLG, whereas a single 18-kDa band was seen for both uBLG and aBLG [39]. Alternatively, the dimeric structure of cBLG might be different from that of native BLG, causing conformational modulation with dislocation of allergenic peptides from the surface of BLG. Fifteen of 162 amino acids in the sequence of BLG are lysine residues. Lysine is an essential amino acid for the maintenance of the antigenic structure of BLG [43]. There were slightly fewer lysine residues on the cathode side than on the anode side. Various commercial BLG products are reported to be lactosylated to some degree [44], and reducing sugars like 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, under conditions of heat and optimum pH [45]. Because a lactose-binding site of BLG was identified as being a lysine residue [44], the decrease in lysine residues may be due to the Maillard reaction during electrolysis.

Mitigation of the allergic action of cow milk, independent of hydrolyzation, may be beneficial, as there are some concerns about nutritional issues regarding hydrolysate formulas [20-22]. Additionally, the taste of whey, following electrolysis on the cathode side, was not altered. Therefore, our method may offer a new and more advantageous strategy of preparing hypoallergenic formulas for patients with CMA.

Electrolyzed Ovomucoid

There have been some reports of efforts to obtain hypoallergenic OMC. Cooke and Sampson [46] reported that the binding force of OMC to IgE antibodies decreased by approximately 28% when its intramolecular S-S bonds were cleaved by dithiothreitol (DDT), and this was particularly demonstrated in infants with hen egg allergy. Kato *et al.* [47] reported that the allergenic capacity of OMC was diminished when OMC was heated with wheat protein and was aggregated. This phenomenon was attributed to formation of intermolecular S-S bonds between the OMC and wheat protein, and the aggregation of OMC. When the solid component concentration of egg protein was 0.2%-0.3%, pH 10-11.5, and OMC was heated to >80°C, OMC was denatured and lost its high allergic capacity [48]. Moreover, when OMC was

treated at high pressure (100–400 MPa), the binding force between it and IgE antibodies decreased. Thus, tyrosine residues of OMC moved from the molecular entrail to the molecular surface.

We also showed that the allergenic capacity of OMC was attenuated after energization [40]. The electrolysis was carried out in the same way using 1% BLG containing 1% sodium chloride for 30 minutes with a voltage difference of 90 V, 0.23 A (30 mA/cm²). The wheal diameter for OMC on the polar opposites was reduced in the SPT compared with that of untreated OMC. In 21 patients with hen egg allergy, percentages of the wheal reaction with OMC on the cathode (cOMC) and anode (aOMC) sides, compared to untreated OMC (uOMC), were 77.6% ± 28.8% and 82.6% ± 33.8% ($P = 0.004$, $P = 0.030$), respectively [40].

This phenomenon of mitigated allergenicity for OMC was evidently demonstrated in the cOMC and the quantity of SH groups in cOMC significantly increased. Some of nine S-S bonds in an OMC molecule were expected to be cleaved, which is in accordance with Cooke and Sampson's report [8] that the binding ability between OMC and IgE decreased when S-S bonds were cleaved upon addition of DDT. Our study suggests that an intramolecular S-S bond of OMC was cleaved by electric energy without use of a reducing agent such as DDT, and this cleavage contributed to decreased allergenic capacity [40]. We performed 2D electrophoresis with an irreducible solvent for uOMC, cOMC, and aOMC, with some extremely diverse spots being obtained (Figure 4 in Kido *et al.* [40]). We cut these relatively large spots out of the three respective gels, treated these proteins with trypsin, and then performed mass spectrometry for the peptide fragments of each gel. Moreover, we calculated the theoretical molecular weight of OMC and compared the theoretical value to the actual one obtained using mass spectrometry. In cOMC, a new formation of S-S bonds in 15E-24K, 57E-82K and 122R-128R, or 15E-24K, and 165C-185K patterns were expected. In aOMC, a new formation of S-S bonds in 24M-14K, 130E-159K and 57E-63K, 165C-185K, and 18D-24K, or 90A-121K and 18D-24K patterns were expected. Therefore, the S-S bond regions in cOMC and aOMC were likely to have changed variously due to reactions other than reduction, and these changes were considered to contribute to the formation of hypoallergenic OMC.

Conclusion

In Japan, HEA and CMA account for nearly 50% of all cases of childhood food allergies. Although patients with HEA or CMA should avoid hen eggs, cow milk, or products that contain them, until they can outgrow HEA or CMA, it is significantly important for them to ingest hypoallergenic hen eggs, cow milk, or products containing them. An understanding of the mitigation of the allergenic activity of BLG and OMC by electrolysis may allow for its potential use in development of hypoallergenic hen egg or cow milk products or cooking methods.

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