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Cleavage of the terminal *N*-acetylglucosamine of egg-white ovalbumin *N*-glycans significantly reduces IgE production and Th2 cytokine secretion

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ABSTRACT

Ovalbumin (OA) is one of the most abundant of the glycoprotein allergens, and induces a T-helper type 2 immune response that results in an IgE-mediated hypersensitivity. In this study, the terminal carbohydrates of *N*-glycans from intact OA were cleaved with the exoglycosidases galactosidase, mannosidase, and *N*-acetylglucosaminidase to generate degalactosylated-OA, demannosylated-OA, and de-*N*-acetylglucosaminylated-OA, respectively, in order to evaluate their role in allergenicity. The exoglycosidase digestion procedure did not result in either degradation or contamination of the three deglycosylated sample, and the digestion efficiency was confirmed by comparing the results of glycan analysis of the three exoglycosidase-treated OAs with that of glycans of intact OA. Mice were immunized with either intact or exoglycosidase-treated OAs, and their respective allergic reactions were compared. IgE production in the de-*N*-acetylglucosaminylated-OA group was reduced to 58.8% of that in the intact OA group. In addition, the production levels of the cytokines interleukin-4 and interleukin-5 were significantly reduced in the de-*N*-acetylglucosaminylated-OA group to 53.4% and 45.8% of the levels in the intact OA group, respectively. However, there were almost no changes (or only slight reductions) in the degalactosylated-OA and demannosylated-OA groups, respectively. These results indicate that cleavage of the terminal carbohydrate, and particularly *N*-acetylglucosamine, reduces the allergenicity of OA. This is the first report of the effect of cleavage of the terminal carbohydrate on glycoprotein allergenicity.

1. Introduction

Egg-white ovalbumin (OA) is the most abundant of the major allergens and can cause immunoglobulin E (IgE)-mediated food hypersensitivity [1]. OA is a 45 kDa glycoprotein with 385 amino acids and 3% carbohydrate content. It comprises approximately 54% of the protein in chicken egg white [2]. OA has a single *N*-glycosylation site at Asn-292. The glycosylation of OA has been studied by paper electrophoresis [3], high-performance liquid chromatography (HPLC) with nuclear magnetic resonance spectroscopy [4], and mass spectrometry (MS) [5,6]. OA glycans containing high-mannose-type, hybrid-type, and complex-type oligosaccharide have been identified. Moreover, *N*-glycosylation is important for OA biosynthesis; the nonglycosylated form of OA is misfolded and is not secreted from the cell [7].

OA-induced allergy is considered to be mainly an IgE-mediated, type I hypersensitivity that is characterized by increased production of IgE antibodies and T-helper type 2 (Th2) cytokines, which are common markers found in experimental models and human disease [8]. Th1 and Th2 cells can be distinguished according to the cytokines that each cell type produces: Th1 cells produce interferon-gamma (IFN-γ), interleukin (IL-2), and tumor necrosis factor-alpha, and Th2 cells produce IL-4, IL-5, and IL-13 [9]. B cells stimulated by IL-4 differentiate into Th2 cells, which secrete Th2 cytokines, which in turn induce class switching to IgE, leading to type I hypersensitivity [10,11]. When the IgE antibody is induced, it binds to the high-affinity IgE receptor FcεRI on mast cells. IgE-mediated hypersensitivity occurs when repeated stimulation of IgE with the same antigen triggers degranulation, which causes the release of various inflammatory factors, including histamine,

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prostaglandins, and leukotrienes from mast cells. Therefore, an increase in IgE production can be an important marker for such hypersensitivity [11].

Glycosylation of allergens may affect IgE-mediated hypersensitivity. For example, β1,2-xylose and core α1,3-fucose, which are carbohydrates found specifically in plant *N*-glycans [12], or galactose (Gal)α1-3Gal in mammalian meat products [13], cause allergic responses. Moreover, the glycans attached to proteins influence their physical properties, including their stability, solubility, hydrophobicity, and electrical charge, and these changes may affect the allergenicity and antigenicity of proteins by altering the B-cell and T-cell epitopes present on the protein surface [14]. Furthermore, glycosylation of proteins may affect their uptake and the availability of proteolytic sites, which may release T-cell epitopes [15].

Some studies have revealed reduced allergic responses in mice exposed to different OA preparations, such as heat-treated OA with secondary structural changes [16,17], gamma-irradiated OA [18], or an OA-IL-12 fusion protein [19]. In addition, the OA-induced allergic response in mice is reportedly suppressed after *Listeria monocytogenes* infection [20]. In contrast, the glycated form of OA is more immunogenic and allergenic than intact OA [21].

The nonreducing termini of *N*-glycans are potentially involved in antibody binding, and consist mainly of mannose (Man), fucose, xylose, and *N*-acetylglucosamine (GlcNAc) in different linkages [22]. However, the influence of the terminal carbohydrates at the nonreducing termini of OA glycans on allergenicity has not been reported previously.

This study used HPLC analysis with fluorescence derivatization and matrix-assisted laser desorption–ionization time-of-flight (MALDI-TOF) MS for the quantitative and sequential analysis of the glycans on OA. Furthermore, the role of terminal carbohydrates in OA allergenicity was investigated with three exoglycosidase-treated OA preparations: degalactosylated OA (de-G-OA), demannosylated OA (de-M-OA), and de-*N*-acetylglucosaminylated OA (de-N-OA). These preparations were obtained by cleavage of the terminal Gal, Man, and GlcNAc residues, respectively, from intact OA glycans using galactosidase, mannosidase, and *N*-acetylglucosaminidase digestion, respectively. The digestion efficiency was confirmed by comparing the results of glycan analysis of the three variants with that of glycans of intact OA. IgE production after immunization of mice with either intact OA or exoglycosidase-treated OAs was assessed. Cytokine levels in splenocytes obtained from the immunized mice were measured after restimulating the cells with the same antigen.

2. Materials and methods

2.1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the protocol reported by Laemmli [23] using a 15% acrylamide slab gel. The gel was stained with Coomassie blue R-250. Molecular mass markers (10–250 kDa) were purchased from Bio-Rad (Hercules, CA, USA).

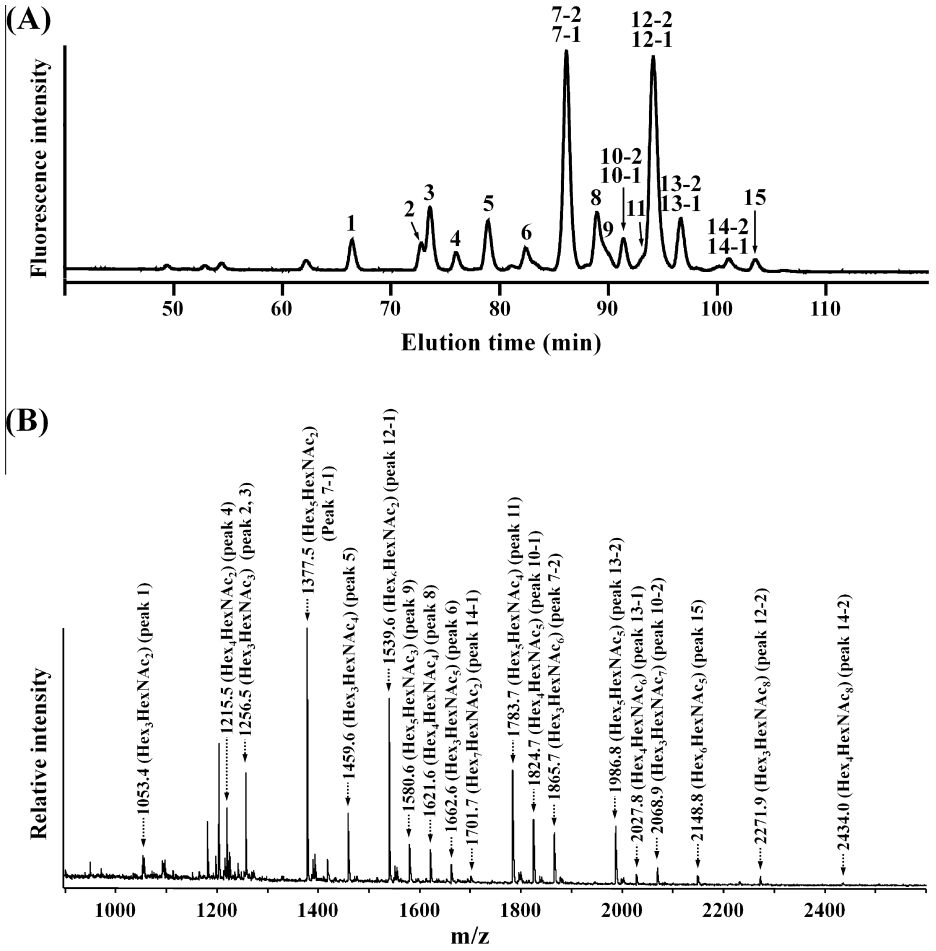
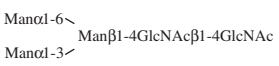
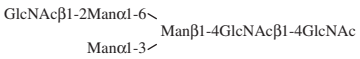
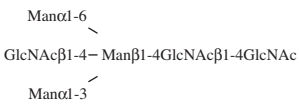
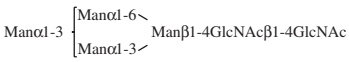
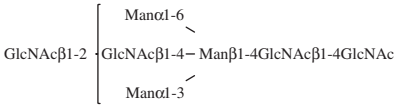
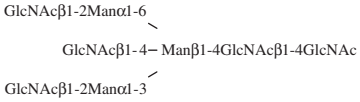
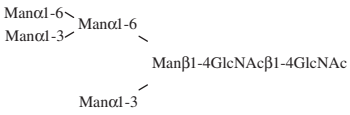
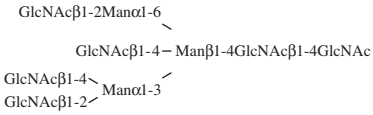
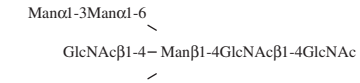
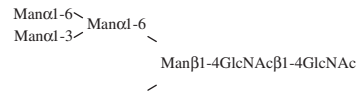
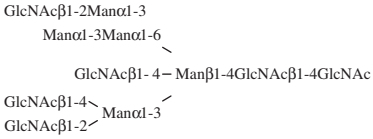
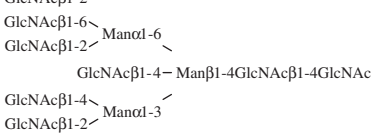
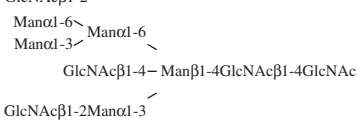
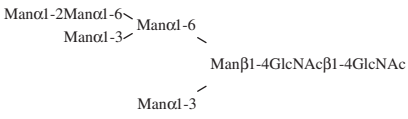
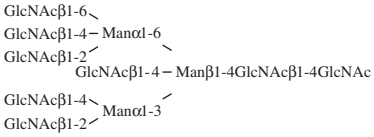


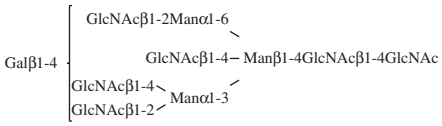
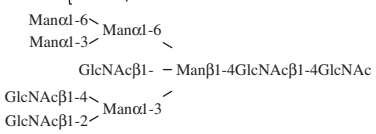
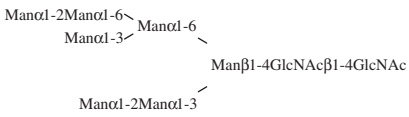
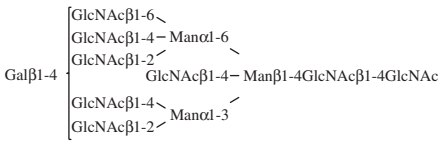
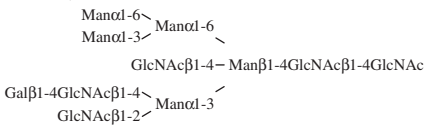
Fig. 1. (A) HPLC chromatogram including the peak numbers and (B) MALDI-TOF MS spectrum of 2-AB-labeled *N*-glycans from intact OA. Hex = hexose (Man, Gal), HexNAc = *N*-acetylhexose (GlcNAc). The peak numbers of the glycans in (A) and (B) are the same.

Table 1
Summary of MS, HPLC data, and relative quantity (%) of the 2-AB-labeled *N*-glycans from OA.

Peak no.	Proposed structure	MALDI-TOF MS *m/z* ([M+Na]$^+$)		GU		Relative quantity (%)
		Calculated	Detected	Reported	Observed	
1		1053.4	1053.4	4.40	4.42	3.0
2		1256.5	1256.5	4.93	4.93	2.5
3		1256.5	1256.5	4.97	4.99	7.2
4		1215.4	1215.5	5.31	5.21	1.7
5		1459.5	1459.6	5.31	5.47	5.5
6		1662.6	1662.6	5.77	5.81	1.7
7-1		1377.5	1377.5	6.19	6.19	27.0
7-2		1865.7	1865.7	–		
8		1621.6	1621.6	–	6.48	7.0
9		1580.6	1580.6	6.62	6.56	1.9
10-1		1824.7	1824.7	–	6.76	3.1
10-2		2068.8	2068.9	6.74		
11		1783.7	1783.7	–	6.96	0.9
12-1		1539.5	1539.6	7.06	7.08	30.6
12-2		2271.9	2271.9	–		

(*continued on next page*)

Table 1 (*continued*)

Peak no.	Proposed structure	MALDI-TOF MS *m/z* ([M+Na]$^+$)		GU		Relative quantity (%)
		Calculated	Detected	Reported	Observed	
13-1		2027.8	2027.8	–	7.38	5.9
13-2		1986.7	1986.8	–		
14-1		1701.6	1701.7	7.98	7.97	0.9
14-2		2433.9	2434.0	–		
15		2148.8	2148.8	–	8.30	1.1

2.2. N-glycan analysis

OA from chicken egg white that had been purchased from Sigma (St. Louis, MO, USA). The *N*-glycans were released from OA by glycoamidase A digestion, derivatized with 2-aminobenzamide (2-AB), and the *N*-glycan structures were confirmed by a combination of HPLC and MALDI-TOF MS analysis according to the methods described in our recent paper [24].

2.3. Cleavage of the terminal carbohydrates of glycan from intact OA

The terminal carbohydrates on intact OA were cleaved by β-galactosidase from *Escherichia coli* (Sigma), and α-mannosidase and *N*-acetylglucosaminidase from jack beans (Sigma). For galactosidase and *N*-acetylglucosaminidase digestion, OA was incubated in 50 mM sodium phosphate buffer (pH 6.0) for 18 h at 37 °C. For mannosidase digestion, OA was incubated in 50 mM sodium acetate buffer (pH 4.5) for 18 h at 37 °C. All exoglycosidases were used at a concentration of 0.25 U/ml. Exoglycosidases from each sample solution were separated with an Amicon Ultra-4 unit with a 100 kDa cutoff (Millipore, Billerica, MA, USA). The solution was then dialyzed against distilled water for 24 h to remove the buffer solution with the aid of a GeBAflex tube (Gene Bio-Application, Kfar Hanagid, Israel).

2.4. N-glycan analysis of exoglycosidase-treated OAs

The *N*-glycans were released from exoglycosidase-treated OAs, derivatized with 2-AB, and then analyzed using the methods described in Section 2.2.

2.5. Circular dichroism analysis

Circular dichroism (CD) measurements were performed with a Chirascan-plus CD spectrometer (Applied Photophysics, Leatherhead, UK) using a 0.02 cm quartz cuvette at 25 °C. The spectra were recorded in the 190–260 nm wavelength region. Secondary structure components were estimated using CDNN CD spectra deconvolution software [25].

2.6. Experimental animals

Six-week-old female BALB/c mice (Skh:HR-1) were purchased from Central Lab Animal (Seoul, South Korea). The experimental animals were provided with distilled water and pellet-type feed *ad libitum*. The animals were maintained under controlled temperature (22 ± 2 °C), humidity (50 ± 5%), and light-cycle (12 h/12 h light/dark) conditions. Experiments were conducted in accordance with the guidelines established by the Animal Care and Use Committee of the Korea Food Research Institute (No. KFRI-M-11007).

2.7. Immunization of mice and collection of antiserum

Groups of mice ($n = 5$) were immunized intraperitoneally on days 0 and 14 with 10 μg of OA or exoglycosidase-treated OAs adsorbed in 1 mg of aluminum hydroxide gel (Sigma). The immunized mice were bled on day 5 after the boosting immunization, and serum specimens were collected for the antibody test.

2.8. Measurement of IgE production

The amount of serum IgE produced in response to each antigen was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (BD Pharmingen, CA, USA) for mouse IgE according to the manufacturer's instructions. The IgE level was calculated based on the standard curve generated with a standard IgE antibody. ELISA antibody titers were expressed as the reciprocal of the highest serum dilution showing an absorbance value twice that of normal serum.

2.9. Measurement of cytokine production

Five days after the final immunization, splenocytes harvested from the immunized mice were plated onto 24-well plates and

coincubated with 100 μg/ml of intact or exoglycosidase-treated OAs at 37 °C in humidified air containing 5% CO_2. Three days later, the supernatants of the cultures were harvested, centrifuged at 800 × *g* for 5 min, and then frozen at −80 °C. The levels of various cytokines (including IL-4, IL-5, and IFN-γ) in the supernatant were determined using an ELISA kit (BD Pharmingen, San Jose, CA) according to the manufacturer's instructions.

2.10. Statistical analysis

Differences among experimental data were assessed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test and Duncan's multiple-range tests using SPSS statistical software version 10.0 (SPSS, Chicago, IL, USA). The results are expressed as mean ± standard deviation (SD) values from triplicate determinations.

3. Results

3.1. Analysis of N-glycans of OA

N-Glycans were released from tryptic- and chymotryptic-digested OA peptides by glycoamidase A. The glycans were isolated, derivatized with 2-AB, and analyzed by HPLC with a fluorescence detector [24]. The glycan structures were determined from their glucose unit (GU) values by comparison with the GU values of glycans reported in GlycoBase [26]. Their compositions were obtained from MALDI-TOF MS analysis. All analytical experiments were repeated three times, and the three sets yielded identical HPLC chromatograms and MS data.

Fifteen peaks obtained from HPLC chromatogram and their MS spectrum of 2-AB-labeled *N*-glycan from OA were analyzed (Fig. 1), and their structure and relative quantities (%) were identified (Table 1). The peak area comprising more than 0.9% of the total area was used to calculate the relative quantity of each OA glycan. Five peaks (peaks 7, 10, 12, 13, and 14) contained two different *N*-glycan structures that had a same GU value but a different mass:charge ratio (*m/z*). Eleven glycan structures were reported in GlycoBase, but not for nine glycans (7-2, 8, 10-1, 11, 12-2, 13-1, 13-2, 14-2, and 15 in Fig. 1A and Table 1). These glycan structures were expected based on the MALDI-TOF MS results (Fig. 1B) and previously reported *N*-glycans of OA [4–6]. The confirmed and proposed glycan structures are listed in Table 1.

3.2. SDS–PAGE and CD spectra of intact OA and exoglycosidase-treated OAs

The terminal carbohydrates of the *N*-glycans from intact OA were digested with the appropriate exoglycosidases: β-galactosidase [molecular weight (MW) = 465 kDa], mannosidase

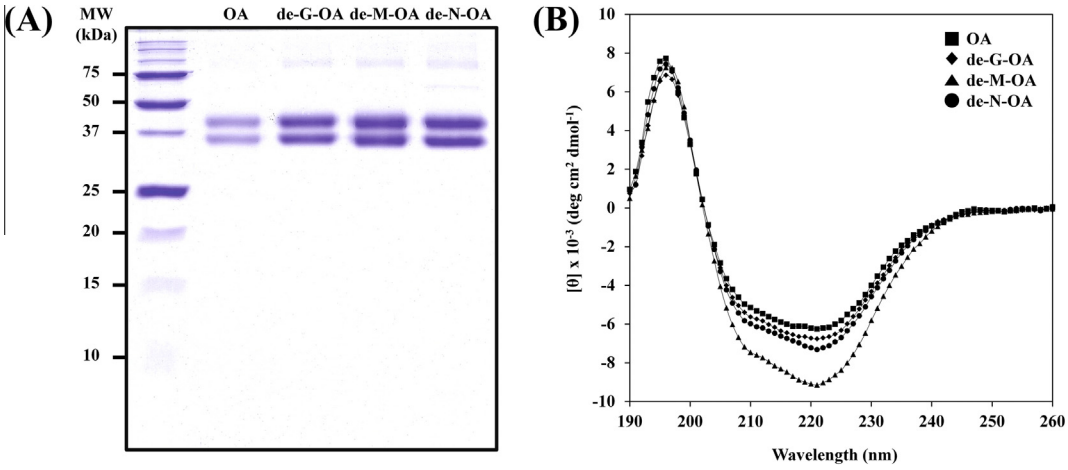


Fig. 2. (A) SDS–PAGE and (B) CD spectra of intact OA and the three exoglycosidase-treated OAs.

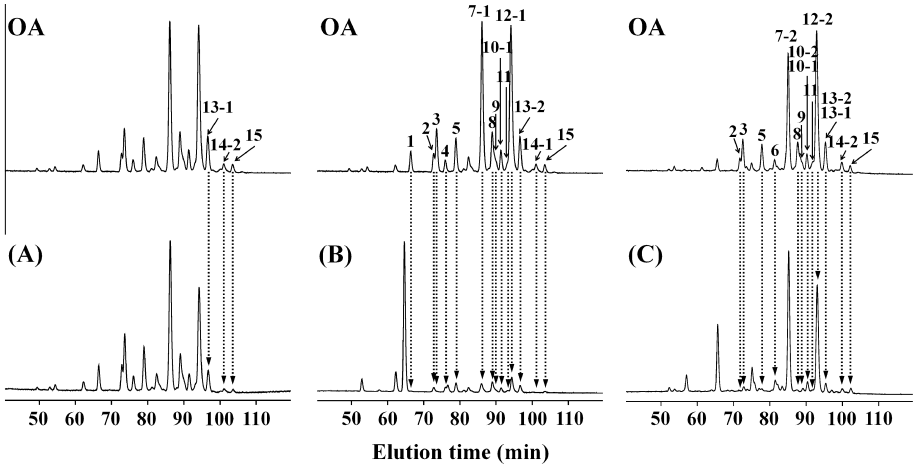


Fig. 3. HPLC chromatograms of *N*-glycans from intact OA with (A) de-G-OA, (B) de-M-OA, and (C) de-N-OA. The dashed line indicates the reduced peaks of exoglycosidase-treated OAs relative to those of intact OA.

(MW = 220 kDa), and *N*-acetylglucosaminidase (MW = 100 kDa). After digestion, the enzymes were removed from the sample solution using Amicon Ultra-4 units. Exoglycosidase-treated OAs were further purified as a single protein using HPLC with anion-exchange column chromatography (data not shown).

The molecular weight and purity of the exoglycosidase-treated OAs were confirmed by SDS–PAGE. The same major bands were evident for all of the samples; these were multiple bands due to the phosphorylation of OA [27] (Fig. 2A). In addition, far-UV CD spectra were measured for the secondary structural changes of intact OA and exoglycosidase-treated OAs (Fig. 2B). The contents of α-helix, β-sheets, β-turns, and random-coil structures in intact OA, estimated using CDNN CD spectra deconvolution software [25], were predicted to be 18.8%, 30.3%, 16.4%, and 34.5%, respectively. The far-UV CD spectra of exoglycosidase-treated OA observed in the same experimental conditions were recorded, and their secondary-structure contents were estimated to be similar to those of intact OA (de-G-OA, 21.4%, 27.2%, 16.6%, 34.8%; de-M-OA, 24.4%, 24.2%, 16.9%, 34.5%; de-N-OA, 22.3%, 26.1%, 16.7%, 34.9%, respectively).

3.3. Analysis of N-glycans of exoglycosidase-treated OAs

To confirm cleavage of the terminal carbohydrate of exoglycosidase-treated OA glycans, the glycans of de-G-OA, de-M-OA, and de-N-OA were analyzed with HPLC, following the same procedure used to analyze glycans from intact OA as described above. The relative quantities of all of the three galactosylated glycans (peaks 13-1, 14-2, and 15 in intact OA) were reduced in de-G-OA (Fig. 3A). In de-M-OA, all of the 14 mannosylated glycans (peaks 1, 2, 3, 4, 5, 7-1, 8, 9, 10-1, 11, 12-1, 13-2, 14-1, and 15) were reduced compared to OA (Fig. 3B).

All 15 GlcNAc-attached glycans (2, 3, 5, 6, 7-2, 8, 9, 10-1, 10-2, 11, 12-2, 13-1, 13-2, 14-2, and 15) were reduced in de-N-OA compared to OA with the exception of peaks 7 and 12 (Fig. 3C). Peak 7 coeluted with two glycans that had the same GU value but different *m/z* values, and 7-1 did not contain GlcNAc, so its elution time remained the same even after *N*-acetylglucosaminidase digestion. Moreover, three glycans (9, 11, and 13-2) containing GlcNAc shifted to peak 7 after *N*-acetylglucosaminidase digestion. Peak 12 also coeluted with two glycans that had the same GU value but different *m/z* values, and 12-1 did not contain GlcNAc, so its elution time remained the same even after *N*-acetylglucosaminidase digestion.

3.4. Effect of OA antigens on serum IgE production

The IgE production in mouse serum was compared after immunization with either intact OA or exoglycosidase-treated OAs (Fig. 4A). Immunization with de-N-OA significantly reduced IgE production (58.8%), compared to that obtained following immunization with intact OA. Compared to immunization with intact OA, immunization with de-M-OA yielded a reduced (71.9%) IgE level, while immunization with de-G-OA yield a similar (94.8%) level of IgE production.

3.5. Effect of OA antigens on Th1- and Th2-type cytokine production

Cytokine production in splenocytes from mice immunized with OA or exoglycosidase-treated OA is shown in Fig. 4B. Immunization with intact OA significantly increased cytokine production in splenocytes compared to immunization with media alone. Cytokine production was lower in splenocytes from mice immunized with exoglycosidase-treated OA than in those from mice immunized with intact OA. The IL-4 and IL-5 levels were significantly lower in splenocytes from the de-N-OA group (53.4% and 45.8%, respectively) than in those from the intact OA group. The IFN-γ level was also significantly reduced between the de-N-OA and

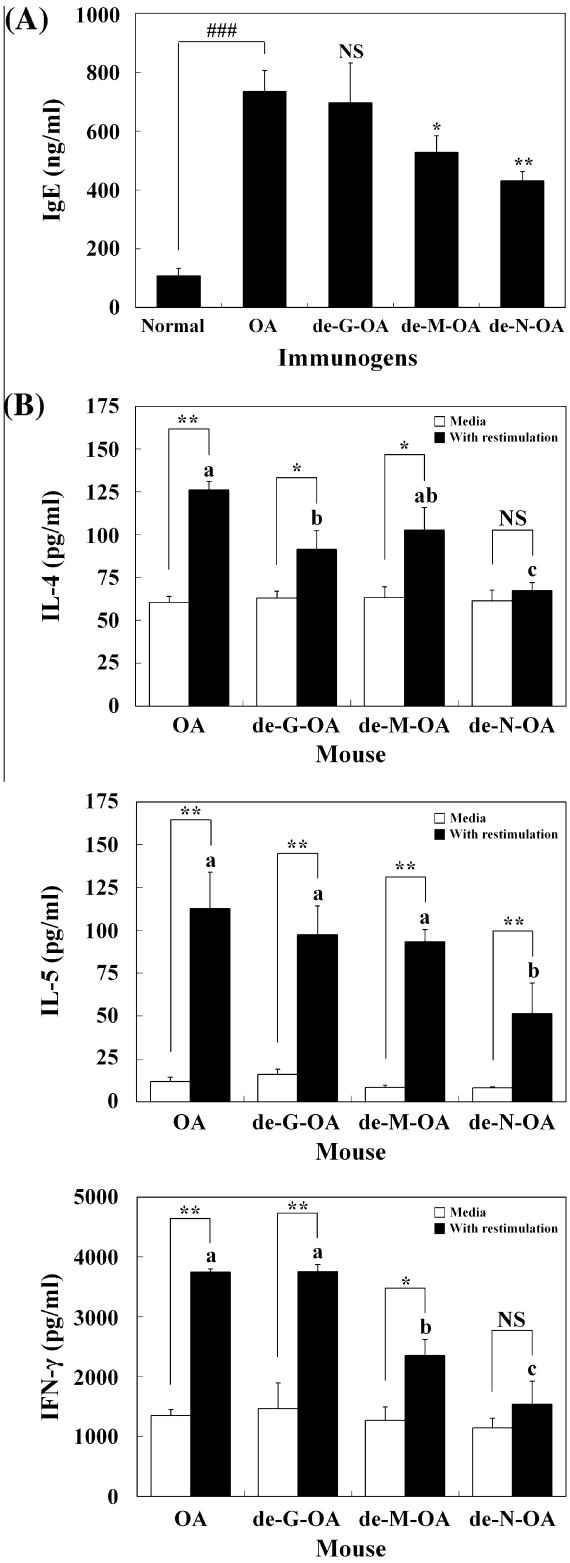


Fig. 4. (A) IgE production after immunization with either intact or exoglycosidase-treated OAs. The results are expressed as mean and SD values. $^{###}p < 0.001$, OA group vs. control; $^{*}p < 0.05$ and $^{**}p < 0.01$, exoglycosidase-treated OA groups vs. OA group. NS, not significant. (B) Cytokine production of splenocytes from mice immunized with either intact or exoglycosidase-treated OAs. The results are expressed as mean and SD values. $^{*}p < 0.05$ and $^{**}p < 0.001$, media group vs. restimulated group. Values not sharing a common superscript letter (a, b, and c) differ significantly at $p < 0.05$. NS, not significant.

intact OA groups (41.0%). The IL-4, IL-5, and IFN-γ levels in splenocytes from the de-G-OA (72.4%, 86.3%, and 100.2%, respectively) and de-M-OA (81.4%, 82.8%, and 62.8%, respectively) groups were similar or relatively slightly reduced compared to those from the de-N-OA group.

4. Discussion

The effects of removal of the terminal carbohydrates of *N*-glycans of OA on serum IgE and Th2-type cytokine production were assessed by immunizing mice with either intact or exoglycosidase-treated OAs. Exoglycosidase hydrolyzes the nonreducing terminal carbohydrates of a glycan, but does not cleave internal linkages, and is widely used for the sequential digestion of glycans to enable the determination of glycan structures. However, the present study used exoglycosidase to cleave the terminal carbohydrate from intact OA. Glycan analysis and SDS–PAGE confirmed that the three exoglycosidase-treated OAs were successfully cleaved at the corresponding terminal monosaccharides, with no accompanying degradation or contamination of the sample solution during the digestion. In addition, there was no difference between OA and exoglycosidase-treated OAs with respect to endotoxin content using the limulus amebocyte lysate (LAL) assay (data not shown).

The present results indicate that cleavage of GlcNAc from the nonreducing termini of intact OA (de-N-OA) can inhibit type I IgE-antibody-mediated hypersensitivity. In other words, de-N-OA was less allergenic than intact OA. The mechanism underlying this reduced allergenicity of de-N-OA is not clear; however, it is known that the nature of the terminal nonreducing carbohydrate residue of the oligosaccharide moiety influences the plasma survival of glycoproteins [28]. In addition, protease activity, surface features, and/or glycosylation patterns are important factors for glycoprotein allergens [29], and the allergenicity of a recombinant glycoprotein rich in Man was found to be significantly lower than that of the original glycoprotein [30]. Moreover, glycoproteins with Man or Gal as the terminal nonreducing sugar are cleared rapidly from rat or rabbit blood by Man receptors on liver endothelial cells or Gal receptors on liver parenchymal cells, respectively [31]. Glycated forms of OA with Man were shown to reduce the allergenicity of OA [32], and antibody-binding epitopes were altered or masked by different glycosylation patterns [33]. Together these previous findings and those of the present study suggest that the increase in glycans containing relatively homogeneously exposed inner Man after cleavage of nonreducing terminal GlcNAc is important for the control of allergenicity in intact OA.

Meanwhile, the CD spectra indicate that almost no changes were observed in exoglycosidase-treated OAs compared to OA; however, the double minima at 208 and 222 nm were increased in de-M-OA, and the α-helix content was increased from 18.8% to 24.4%. It has been reported that the carbohydrate-attached protein covers the hydrophobic surface patch and shields the α-helical conformation [34]. This means that the increase in α-helix content in de-M-OA was probably responsible for the decrease in OA allergenicity, although no significant decrease in allergenicity, like that observed for de-N-OA, was observed.

It has been reported that OA peptide 323–329 induces a Th2-like response in the mouse model, similar to intact OA, even though it has limited immunogenic potency [35]. In addition, OA peptide 347–385 in the C-terminal region is thought to be responsible for allergenic hypersensitivity [36]. However, little is known about the factors that determine the allergenicity of food proteins, and there are no structural motifs or conformational sequence patterns that are known to be common to all allergic food proteins [37]. The results of the present study suggest that the terminal GlcNAc on intact OA is closely related to its allergenicity. It is possible that these carbohydrates control antibody production by B cells and immune reactions mediated by $CD4^+$ T cells. Nevertheless, the reduced allergic effect caused by cleavage of GlcNAc from OA is unlikely to be a common feature of all glycoprotein allergens.

In conclusion, immunization of mice with the *N*-acetylglucosaminidase-treated OA induced significantly lower levels of serum IgE and Th2-type cytokine production in splenocyte cultures than those achieved following immunization with intact OA. Thus, cleavage of the terminal GlcNAc from intact OA dramatically affected the allergenicity of this glycoprotein. The results suggest that removal of the terminal GlcNAc is an efficient way of reducing the allergenic risk of OA. To the best of our knowledge, this is the first description of the effect of removing the nonreducing terminal carbohydrate from an intact glycoprotein on its allergenicity.

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References

[1] S. Elsayed, J. Apold, E. Holen, H. Vik, E. Florvaag, T. Dybendal, The structural requirements of epitopes with IgE binding capacity demonstrated by three major allergens from fish, egg and tree pollen, Scand. J. Clin. Lab. Invest. Suppl. 204 (1991) 17–31.
[2] A.D. Nisbet, R.H. Saundry, A.J. Moir, L.A. Fothergill, J.E. Fothergill, The complete amino-acid sequence of hen ovalbumin, Eur. J. Biochem. 115 (1981) 335–345.
[3] K. Yamashita, Y. Tachibana, A. Kobata, The structures of the galactose-containing sugar chains of ovalbumin, J. Biol. Chem. 253 (1978) 3862–3869.
[4] M.L. Corradi Da Silva, H.J. Stubbs, T. Tamura, K.G. Rice, ^{1}H NMR characterization of a hen ovalbumin tyrosinamide N-linked oligosaccharide library, Arch. Biochem. Biophys. 318 (1995) 465–475.
[5] S. North, H. Birrell, P. Camilleri, Positive and negative ion matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis of complex glycans released from hen ovalbumin and derivatized with 2-aminoacridone, Rapid Commun. Mass Spectrom. 12 (1998) 349–356.
[6] D.J. Harvey, D.R. Wing, B. Küster, I.B.H. Wilson, Composition of N-linked carbohydrates from ovalbumin and co-purified glycoproteins, J. Am. Soc. Mass Spectrom. 11 (2000) 564–571.
[7] K. Ito, T. Ishimaru, F. Kimura, N. Matsudomi, Importance of N-glycosylation positioning for secretion and folding of ovalbumin, Biochem. Biophys. Res. Commun. 361 (2007) 725–731.
[8] J.H. Savage, E.C. Matsui, J.M. Skripak, R.A. Wood, The natural history of egg allergy, J. Allergy Clin. Immunol. 120 (2007) 1413–1417.
[9] J. Zhu, H. Yamane, W.E. Paul, Differentiation of effector CD4 T cell populations (*), Annu. Rev. Immunol. 28 (2010) 445–489.
[10] D. Amsen, C.G. Spilianakis, R.A. Flavell, How are T_H1 and T_H2 effector cells made?, Curr Opin. Immunol. 21 (2009) 153–160.
[11] S. Kumar, A.K. Verma, M. Das, P.D. Dwivedi, Molecular mechanisms of IgE mediated food allergy, Int. Immunopharmacol. 13 (2012) 432–439.
[12] R. van Ree, M. Cabanes-Macheteau, J. Akkerdaas, J.P. Milazzo, C. Loutelier-Bourhis, C. Rayon, M. Villalba, S. Koppelman, R. Aalberse, R. Rodriguez, L. Faye, P. Lerouge, Beta(1,2)-xylose and alpha(1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens, J. Biol. Chem. 275 (2000) 11451–11458.
[13] S.P. Commins, T.A.E. Platts-Mills, Delayed anaphylaxis to red meat in patients with IgE specific for galactose alpha-1,3-galactose (alpha-gal), Curr. Allergy Asthma Rep. 13 (2013) 72–77.
[14] R.D.J. Huby, R.J. Dearman, I. Kimber, Why are some proteins allergens?, Toxicol Sci. 55 (2000) 235–246.
[15] S. Mouritsen, M. Meldal, I. Christiansen-Brams, H. Elsner, O. Werdelin, Attachment of oligosaccharides to peptide antigen profoundly affects binding to major histocompatibility complex class II molecules and peptide immunogenicity, Eur. J. Immunol. 24 (1994) 1066–1072.
[16] J. Golias, M. Schwarzer, M. Wallner, M. Kverka, H. Kozakova, D. Srutkova, K. Klimesova, P. Sotkovsky, L. Palova-Jelinkova, F. Ferreira, L. Tuckova, Heat-induced structural changes affect OVA-antigen processing and reduce allergic response in mouse model of food allergy, PLoS One 7 (2012) e37156.
[17] X.J. Ma, H.B. Chen, J.Y. Gao, C.Q. Hu, X. Li, Conformation affects the potential allergenicity of ovalbumin after heating and glycation, Food Addit. Contam. Part A Chem. Anal. Control. Expo. Risk Assess. 30 (2013) 1684–1692.
[18] J.H. Seo, J.W. Lee, J.H. Kim, E.B. Byun, S.Y. Lee, I.J. Kang, M.W. Byun, Reduction of allergenicity of irradiated ovalbumin in ovalbumin-allergic mice, Radiat. Phys. Chem. 76 (2007) 1855–1857.
[19] T.S. Kim, R.H. DeKruyff, R. Rupper, H.T. Maecker, S. Levy, D.T. Umetsu, An ovalbumin-IL-12 fusion protein is more effective than ovalbumin plus free

recombinant IL-12 in inducing a T helper cell type 1-dominated immune response and inhibiting antigen-specific IgE production, J. Immunol. 158 (1997) 4137–4144.
[20] D. Mizuki, T. Miura, S. Sasaki, M. Mizuki, H. Madarame, A. Nakane, Interference between host resistance to *Listeria monocytogenes* infection and ovalbumin-induced allergic responses in mice, Infect. Immun. 69 (2001) 1883–1888.
[21] T. Hilmenyuk, I. Bellinghausen, B. Heydenreich, A. Ilchmann, M. Toda, S. Grabbe, J. Saloga, Effects of glycation of the model food allergen ovalbumin on antigen uptake and presentation by human dendritic cells, Immunology 129 (2010) 437–445.
[22] K. Fötisch, S. Vieths, N- and O-linked oligosaccharides of allergenic glycoproteins, Glycoconj. J. 18 (2001) 373–390.
[23] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[24] H.S. Hwang, B.S. Kim, H. Park, H.Y. Park, H.D. Choi, H.H. Kim, Type and branched pattern of N-glycans and their structural effect on the chicken egg allergen ovotransferrin: a comparison with ovomucoid, Glycoconj. J. 31 (2014) 41–50.
[25] G. Böhm, R. Muhr, R. Jaenicke, Quantitative analysis of protein far UV circular dichroism spectra by neural networks, Protein Eng. 5 (1992) 191–195.
[26] L. Royle, C.M. Radcliffe, R.A. Dwek, P.M. Rudd, Detailed structural analysis of N-glycans released from glycoproteins in SDS–PAGE gel bands using HPLC combined with exoglycosidase array digestions, Methods Mol. Biol. 347 (2006) 125–143.
[27] E. Kinoshita-Kikuta, E. Kinoshita, T. Koike, Separation and identification of four distinct serine-phosphorylation states of ovalbumin by Phos-tag affinity electrophoresis, Electrophoresis 33 (2012) 849–855.
[28] G. Ashwell, J. Harford, Carbohydrate-specific receptors of the liver, Annu. Rev. Biochem. 51 (1982) 531–544.
[29] F. Shakib, A.M. Ghaemmaghami, H.F. Sewell, The molecular basis of allergenicity, Trends Immunol. 29 (2008) 633–642.
[30] R.J. Almond, B.F. Flanagan, A. Antonopoulos, S.M. Haslam, A. Dell, I. Kimber, R.J. Dearman, Differential immunogenicity and allergenicity of native and recombinant human lactoferrins: role of glycosylation, Eur. J. Immunol. 43 (2013) 170–181.
[31] G.M. Kindberg, S. Magnusson, T. Berg, B. Smedsrød, Receptor-mediated endocytosis of ovalbumin by two carbohydrate-specific receptors in rat liver cells. The intracellular transport of ovalbumin to lysosomes is faster in liver endothelial cells than in parenchymal cells, Biochem. J. 270 (1990) 197–203.
[32] P. Rupa, S. Nakamura, S. Katayama, Y. Mine, Effects of ovalbumin glycoconjugates on alleviation of orally induced egg allergy in mice via dendritic-cell maturation and T-cell activation, Mol. Nutr. Food Res. 58 (2014) 405–417.
[33] E. Lisowska, The role of glycosylation in protein antigenic properties, Cell. Mol. Life Sci. 59 (2002) 445–455.
[34] M.L. Blum, J.A. Down, A.M. Gurnett, M. Carrington, M.J. Turner, D.C. Wiley, A structural motif in the variant surface glycoproteins of *Trypanosoma brucei*, Nature 362 (1993) 603–609.
[35] L.Z. Sun, S. Elsayed, T.B. Aasen, T. Van Do, N.P. Aardal, E. Florvaag, K. Vaali, Comparison between ovalbumin and ovalbumin peptide 323–339 responses in allergic mice: humoral and cellular aspects, Scand. J. Immunol. 71 (2010) 329–335.
[36] K. Honma, Y. Kohno, K. Saito, N. Shimojo, T. Horiuchi, H. Hayashi, N. Suzuki, T. Hosoya, H. Tsunoo, H. Niimi, Allergenic epitopes of ovalbumin (OVA) in patients with hen's egg allergy: inhibition of basophil histamine release by haptenic ovalbumin peptide, Clin. Exp. Immunol. 103 (1996) 446–453.
[37] R. Bredehorst, K. David, What establishes a protein as an allergen?, J Chromatogr. B Biomed. Sci. Appl. 756 (2001) 33–40.