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Diverse lectin-binding specificity of four ZP3 glycoprotein isoforms with a discrete isoelectric point in chicken egg coat

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ABSTRACT

The vertebrate egg coat corresponding to mammalian zona pellucida is a filamentous matrix composed of highly and heterogeneously glycosylated proteins designated ZP glycoproteins including ZP1 to 4, ZPD and ZPAX, and play important roles in species-specific egg–sperm interactions. Recent advance in structural biology of chicken ZP3 provided new insights into molecular mechanisms of the egg-coat function involving its carbohydrate moieties. In this study, chicken ZP3 was separated into four major and distinct isoforms with different pI in 2D-PAGE. To investigate the meanings of the ZP3 heterogeneity in egg–sperm interactions, we preliminary analyzed glycan diversity on the molecules by using lectin-staining assays. The four major ZP3 isoforms 4–7 (from acidic to basic) were recognized equally with PNA (Galβ1-3Gal*N*Ac), but the isoforms 5–7 were recognized dominantly with WGA ((β-Glc*N*Ac)n, clustered Sia), PHA-E (bi- and triantennary *N*-glycan containing Galβ1-4Glc*N*Acβ1-2Manα1-6) and RCA I (terminal Galβ1-4Glc*N*Ac), respectively. Despite such sugar chain diversity among the ZP3 isoforms, a partner in the egg coat, ZP1, showed specific binding to each isoform equally. Localization of ZP1 and ZP3 in the egg-coat matrix were also analyzed.

1. Introduction

The ovulated oocyte in vertebrates is surrounded by an egg coat corresponding to mammalian zona pellucida formed by a filamentous meshwork composed of zona pellucida (ZP) glycoproteins. The sperm–egg coat interaction is one of the most important steps in normal fertilization in nature, however, very little has been known about the molecular mechanisms of egg-coat formation, sperm–egg coat binding and sperm penetration through the egg coat [1]. The ZP glycoprotein is a secretory proteins characterized by an unique conserved sequence of ~260 amino acids so called ZP domain, or ZP module, containing conserved 8–12 Cys and divided in two novel immunoglobulin-like domains designated ZP-N and ZP-C [2], and classified into at least six subfamilies, ZP1, ZP2, ZP3, ZP4, amphibian/avian ZPD and non-mammalian ZPAX based on phylogenetic studies [3]. As a result of considerable efforts for more than three decades since the identification of them in 1980 [4], the crystal structure of full-length chicken ZP3, and the glycan-dependent molecular mechanisms of ZP glycoproteins in the binding of ZP3 to the sperm were elucidated [2]. A conserved Thr (designated site 1) of ZP3 located in the linker region connecting the ZP-N and ZP-C is occupied by a core 1 *O*-glycan (Galβ1-3Gal*N*Ac with and without a terminal Sia) that participates in the sperm binding in concert with the polypeptide moiety, probably corresponding to the so-called sperm combining site (SCS) previously identified in the C-terminal of mature ZP3 [5]. Whereas there have been two controversial models for the sperm–egg coat interaction mechanisms [5] that *O*-glycan(s) on the SCS mediate and regulate the interaction, and that the sperm recognize supramolecular structure of the egg coat regardless of the *O*-glycan and the binding is restricted by ZP2 cleavage, the structure-based studies described above strongly support the possible involvement of polypeptide and/or other moieties of ZP3, in addition to the third model that *O*-glycan(s) on the other site(s) mediate the sperm binding, and the ZP2 cleavage restrict it [5].

In our previous report, it was suggested that chicken ZP glycoproteins of mature egg coat have significant molecular heterogeneity caused by posttranslational modifications [6]. Therefore the *O*-glycosylation involved in the sperm binding is conserved among vertebrates [2] despite considerable species-selectivity in the egg–sperm interaction [1], we hypothesized that the species diversity in the sperm–egg coat interactions such that either mouse or chicken ZP3 binds to the sperm, while mouse ZP3, chicken ZP1 and ZPD but not chicken ZP3 have the ability to induce the sperm acrosome reaction [2,6], might be related to the diverse posttranslational modifications other than the *O*-glycosylation. To provide useful information to investigate whether the molecular heterogeneity of ZP glycoproteins affect the sperm–egg coat interactions, we

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initially analyzed the glycosylation profiles of chicken ZP3 by lectin blotting, and observed distributions of the sugar chains in egg-coat matrix by lectin immunofluorescent microscopy in this study. We also examined whether the heterogeneity of ZP3 affect the ZP1-ZP3 interaction involved in the diverse egg-coat formation [1,7,8]. Comparative studies of mammalian and avian egg coat in their biochemical and physiological features will provide new insights into either conserved or species-specific mechanisms of ZP glycoproteins to form the matrices and regulate the egg–sperm interactions, which is applicable to the reproductive medicine by developing novel non-hormonal contraceptives as well as therapies for zona-pellucida related infertilities [9].

2. Materials and methods

2.1. Antibodies and lectins

Mouse polyclonal antisera against recombinant chicken ZP1 repeat region and ZP3 (anti-ZP1 and anti-ZP3, respectively) were prepared previously [7]. Lectins (PNA, WGA, PHA-E, RCA I, ConA) and rabbit anti-lectin antibodies (anti-PNA, anti-WGA, anti-ConA) were obtained from Sigma-Aldrich® (St. Louis, MO) except for UEA-I and other rabbit anti-lectin antibodies (anti-PHA-E, anti-RCA I, anti-UEA-I) from EY Laboratories (San Meteo, CA).

2.2. Preparation of chicken serum and egg coat

Chicken serum was prepared as described previously [7]. Blood was collected from carotid artery of laying White Leghorn hens, coagulated overnight at 4 °C, and centrifuged at 1000*g* for 30 min to remove a clot. The supernatant was collected as the laying hen's serum and stored at −20 °C before use. Chicken egg coat was isolated mechanically with forceps from pre-ovulatory mature follicles of laying White Leghorn hens as described previously [6] and stored at −20 °C before use.

2.3. Gel electrophoresis, immunoblotting, lectin blotting and ligand blotting

SDS-PAGE was performed as described previously [6]. Protein samples were incubated at 100 °C for 5 min in SDS–PAGE sample buffer in the absence of DTT. 2D-PAGE was performed as described previously [6] using Invitrogen™ ZOOM® IPGRunner™ System (Life Technologies™, Carlsbad, CA). ZOOM® Strip pH3-10NL (Life Technologies™) was rehydrated in 140 μl of sample rehydration buffer (8 M Urea, 2% (w/v) CHAPS, 0.5% (v/v) ZOOM® Carrier Ampholyte pH3-10 (Life Technologies™), 0.002% bromophenol blue) containing 44.7 or 89.4 μg wet weight of egg coat for silver staining or electroblotting, respectively. Both the 1st dimensional isoelectric focusing (IEF) and the 2nd dimensional SDS-PAGE were performed in the absence of DTT. Proteins in the gel were electroblotted onto a nitrocellulose membrane. To remove the terminal Sia on the non-reducing ends of carbohydrate chains, proteins blotted on the membrane were hydrolyzed with 25 mM H_2SO_4 at 80 °C for 30 min followed by extensive washing with water. Silver staining, immunoblotting, lectin blotting and ligand blotting were performed as described previously [6,7]. Anti-ZP1 (1:2000), anti-ZP3 (1:2000), lectin (2.5 μg/ml) and rabbit anti-lectin antibody (1.0 μg/ml) described above were used as probes.

2.4. Lectin-immunofluorescent staining and confocal laser scanning microscopy

Small pieces of isolated egg coat were mounted onto aminosilane-coated glass slides, washed with PBS and blocked with 2% BSA in PBS (2% BSA/PBS) at 4 °C overnight. The egg coat was incubated with 2% BSA/PBS or 2% BSA/PBS containing PHA-E (2.5 μg/ml) at 4 °C overnight and washed with PBS followed by incubation with 2% BSA/PBS containing rabbit anti-PHA-E (1.0 μg/ml) and mouse anti-ZP1 (1:2000) or mouse anti-ZP3 (1:2000) at 4 °C overnight and washed with PBS. The egg coat was finally incubated with 2% BSA/PBS containing Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 568 goat anti-rabbit IgG (each 6.7 μg/ml; Molecular Probes, Eugene, OR) at room temperature for 30 min in darkness. After washing with PBS, the egg coat was observed under the confocal laser scanning microscopy. Imaging was performed on a Zeiss Axio Obserber.Z microscope equipped with LSM 700 laser scanning confocal optics (Carl Zeiss, Thornwood, NY). A 488-nm and 555-nm diode laser excitation in combination with a 490–555 nm band-pass emission filter were used for imaging the fluorescent probes. Differential interference contrast (DIC) microscopy images were taken on the same system.

3. Results

3.1. Molecular heterogeneity of chicken ZP glycoproteins

To separate the modified forms of ZP glycoproteins described above at higher resolution, the isolated chicken egg coat was subjected to 2D-PAGE (Fig. 1). Proteins in the 2-D gel were detected by silver staining (panel 1) and by immunoblotting with anti-ZP1 or anti-ZP3 (panel 2 or 4, respectively). Detected protein spots were assigned to ZP1 dimer and monomer (di- and mono-ZP1, respectively), ZPD and ZP3, according to their apparent MWs in non-reducing SDS–PAGE, the results of immunoblotting (panels 2 and 4) and our previous reports [6–8]. Either ZP1 dimer or monomer was separated into several spots in narrow range of pI (~7.5), although the abundance of the dimer migrated into the 2-D gel was significantly lower than that of the monomer, whereas ZPD and ZP3 were separated into distinct 4 and 9 spots, respectively, in wider range of pI (~5.0–6.5). Some unidentified proteins detected by non-reducing SDS–PAGE without IEF (right side lane in panel 1, and our previous reports [6–8]), were separated into multiple spots with (single asterisks) or without (double asterisks) affinity to the anti-ZP1. Furthermore, an unidentified ~35-kDa protein(s) non-immunoreactive with both antibodies were detected only by 2D-PAGE (triple asterisk).

3.2. Binding of serum ZP1 to the heterogeneous ZP3 and ZPD

To determine whether the heterogeneity of ZP3 affect the binding of serum ZP1, egg-coat proteins separated by 2D-PAGE (Fig. 1) followed by electroblotting onto nitrocellulose membrane were incubated with the serum containing ZP1, and probed with anti-ZP1 (panel 3). The bound anti-ZP1 was detected from the spots of ZP3 and ZPD in addition to ZP1 and other anti-ZP1 positive proteins on the membrane (single asterisks). Considering that the anti-ZP1 does not have any cross-reactivity with ZP3 and ZPD (panel 2), it is suggested that serum ZP1 bound to ZP3 and ZPD on the membrane and detected by the anti-ZP1.

3.3. Lectin-binding analyses to chicken ZP glycoproteins

To determine whether the heterogeneity of ZP3 correlate with heterogeneous glycosylation, ZP3 separated by the 2D-PAGE was detected by silver staining (inverted; panel 1 of Fig. 2) and lectin blotting (panels 2, 4, 6–8 of Fig. 2). Results of immunoblotting with anti-ZP3 (panel 4 of Fig. 1) and the ZP1-ZP3 binding assay (panel 3 of Fig. 1) were also shown for comparison (panels 3 and 5, respectively). To visualize the relative signal intensities of spots, the ver-

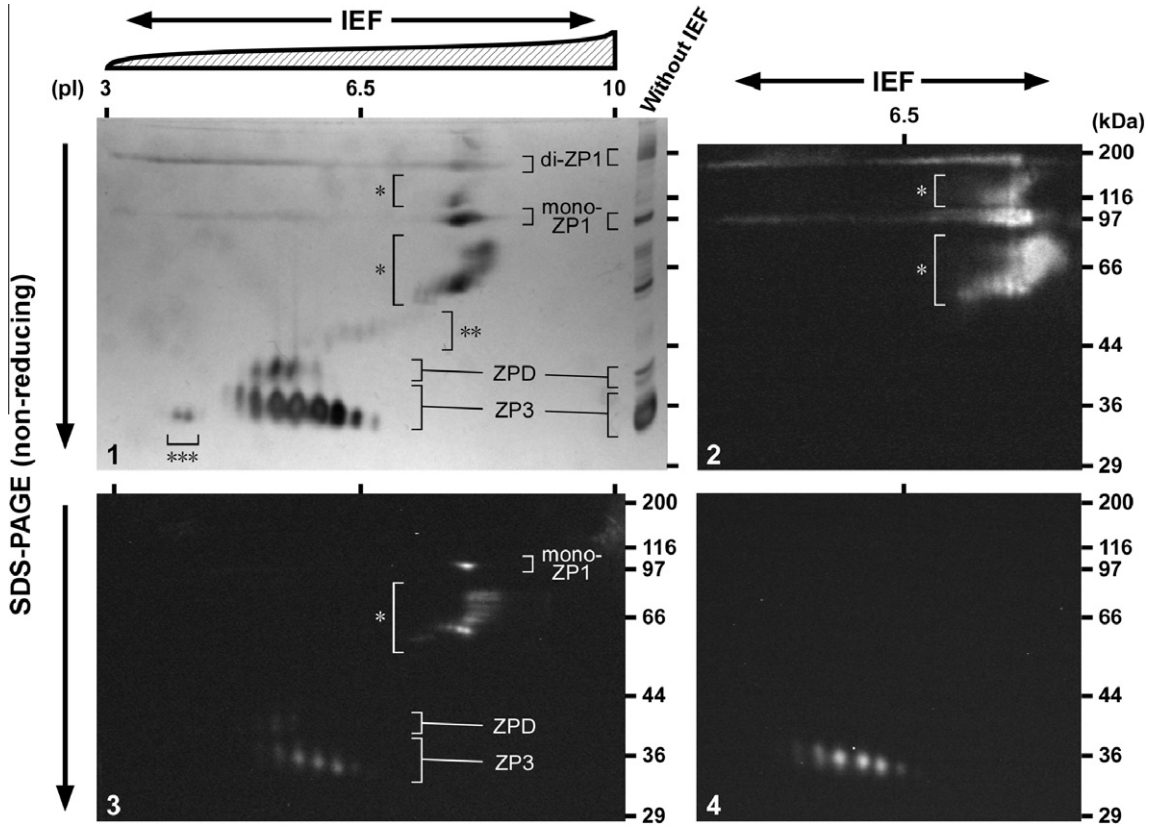

Fig. 1. Separation of egg-coat proteins by 2D-PAGE, and binding analysis of serum ZP1 to the separated proteins by ligand blotting. Chicken egg coat was subjected to 2D-PAGE. The egg-coat proteins were separated by IEF (pH 3-10, non-linear) in the first dimension followed by SDS–PAGE under non-reducing conditions in the second dimension, and detected by silver staining (panel 1) or immunoblotting with anti-ZP1 (panel 2) or anti-ZP3 (panel 4). For ligand blotting, the blotted membrane was incubated with laying hen's serum containing ZP1 before immunostaining with anti-ZP1 (panel 3). The egg-coat proteins were also separated only by non-reducing SDS-PAGE without IEF for comparison (right side lane of panel 1). Assignments of bands and/or spots are indicated in the images. Spots of unidentified proteins (∗: anti-ZP1 positive, ∗∗ and ∗∗∗: anti-ZP1 and ZP3 negative) are shown. Theoretical pIs and migration positions of the MW markers are shown on the top and right of each panel, respectively.

tically averaged pixel intensity of the ZP3 spot images (*Y*-axis of lower graph of the panels) was plotted against the horizontal distances of them (*X*-axis of lower graph of the panels). The anti-ZP3 staining confirmed that ZP3 was separated into 9 spots of distinct pIs, although the signal intensities of anti-ZP3 detected from spots 1–3, 8 and 9 were significantly lower than that from spots 4–7 (panel 3), and at least the spots 2, 3 and 4 were doublet with slightly different apparent MWs (panels 1 and 3). PNA and serum ZP1 bound to the ZP3 spots in the similar manner as anti-ZP3 (panels 3, 5, 7), whereas WGA, PHA-E and RCA I bound more specifically to the spots 5, 6 and 7, respectively (panels 2, 4, 6). ConA (panel 8) and UEA-I (data not shown) bound to ZP3 with quite low affinities.

To further characterize the carbohydrate moieties of chicken ZP glycoproteins, the egg coat was subjected to the lectin blotting with or without chemical desialylation by the H_2SO_4 treatment as described in Materials and Methods (Fig. 3). The amounts of proteins on the membrane were slightly decreased after the H_2SO_4 treatment (lanes 11 and 12). Regardless of the H_2SO_4 treatment, both ZP1 dimer and monomer were highly reactive with PHA-E (lanes 5 and 6) and RCA I (lanes 7 and 8), but less or not with PNA (lanes 1 and 2), WGA (lanes 3 and 4) and ConA (lanes 9 and 10), and almost all forms of ZP3 with various apparent MWs were reactive with PNA (lanes 1 and 2) but not with ConA (lanes 9 and 10). Interestingly, the ZP3 forms with higher, intermediate and lower apparent MWs were reactive with WGA (lane 4), PHA-E (lane 6) and RCA I (lane 8) without the H_2SO_4 treatment, respectively, whereas the reactivity of either ZP3 form with higher or intermediate apparent MW to RCA I was increased (lane 7) while that to WGA and PHA-E were remarkably decreased (lanes 3 and 5, respectively) by the H_2SO_4 treatment. UEA-I did not bind to the chicken ZP glycoproteins in detectable levels regardless of the H_2SO_4 treatment (data not shown).

3.4. Lectin-immunofluorescent staining of chicken egg-coat matrix

To determine how the heterogeneous ZP1, ZP3 and those distinctive carbohydrate moieties are distributed in the egg-coat matrix, the isolated egg coat was co-stained with PHA-E and anti-ZP1 or anti-ZP3 followed by immunofluorescent staining and confocal laser scanning microscopy (Fig. 4). The meshwork of filaments with a diameter of approximately 0.8–1.5 μm was observed in the DIC images (panels 1–4). Optical sections of the immunostained egg coat showed that anti-ZP1 tended to bind more specifically for the near surface of filaments (panels 5 and 6), whereas anti-ZP3 bound them more homogeneously (panels 7 and 8). PHA-E staining patterns of the same sections (panels 9 and 11) were approximately overlapped with the anti-ZP1 or anti-ZP3 ones (panels 13 and 15).

4. Discussion

ZP glycoproteins, the predominant components of vertebrate egg coat, are heavily and heterogeneously glycosylated [1], and the majority of studies based on *in vitro* binding assays demonstrated that the carbohydrate moieties of ZP glycoproteins play important role(s) in species-specific sperm–egg coat interaction(s)

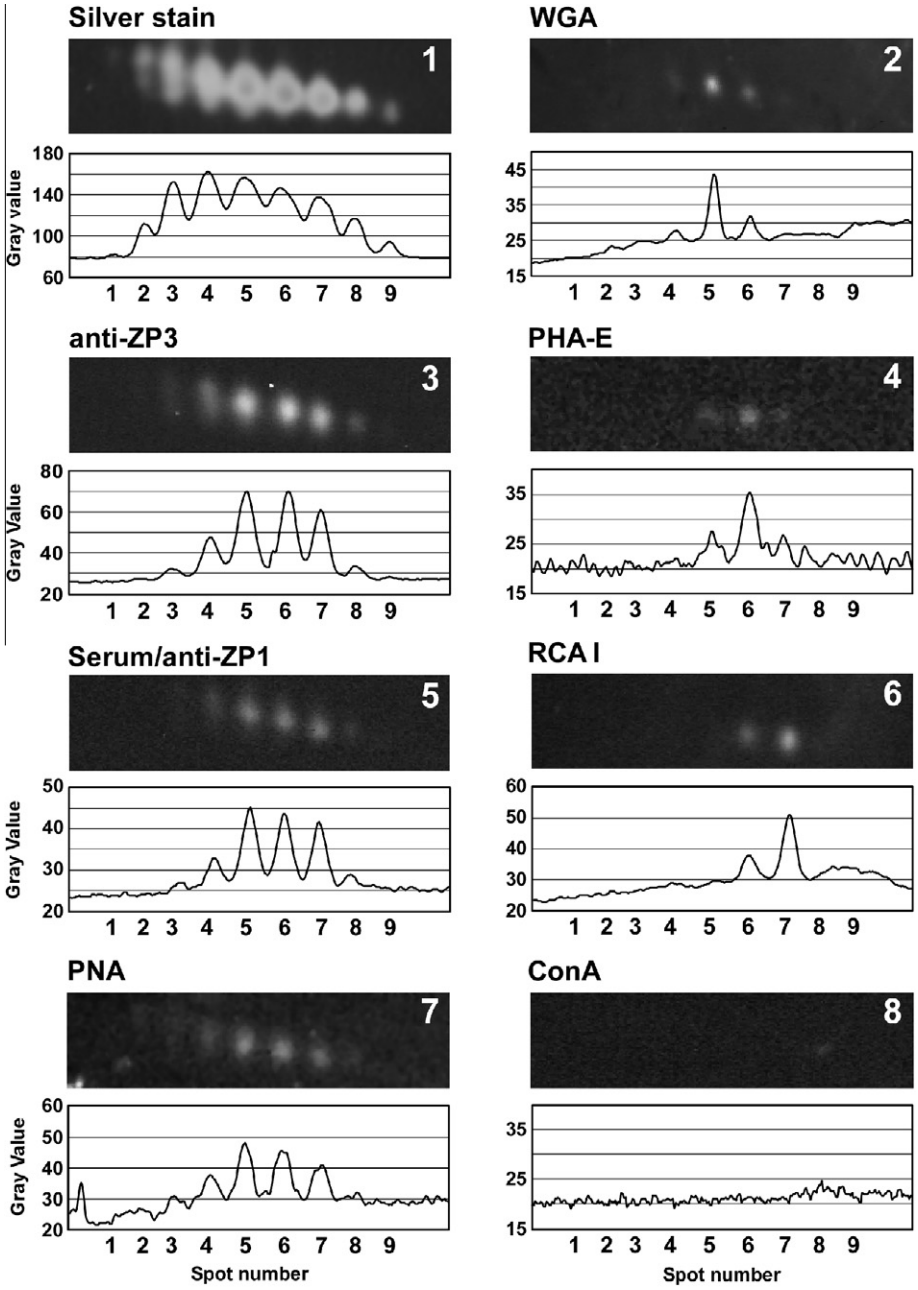

Fig. 2. Binding affinities of lectins and serum ZP1 to the ZP3 migrated at multiple pIs by 2D-PAGE. Chicken egg-coat proteins were separated by 2D-PAGE similarly to Fig. 1. Proteins were detected by silver staining (panel 1) or lectin blotting with PNA, WGA, PHA-E, RCA I and ConA (panels 7, 2, 4, 6, 8, respectively) and reprobed with anti-ZP3 (data not shown). The corresponding regions of 2-D gel and blotted membranes containing ZP3 spots (upper image of each panel) were arranged using the reprobed ZP3 signals as an internal control, and the vertically averaged pixel intensities (Gray Value) were plotted against the horizontal distance through the image (lower graph of each panel) using the ImageJ software (http://imagej.nih.gov/ij/). Corresponding positions of the Gray-Value peaks detected in the silver staining (panel 1) were shown on the *X*-axis of each graph as spot numbers (1–9). Results of immunoblotting with anti-ZP3 and ligand blotting with serum ZP1 in the Fig. 1 are also shown for comparison (panels 3 and 5, respectively).

[1,5]. Our previous study suggested that there are several forms of ZP1, ZP3 and ZPD with different posttranslational modifications including glycosylation in chicken mature egg coat [6]. In this study, we successfully separated the forms of ZP glycoproteins in higher resolution by 2D-PAGE (Fig. 1).

Chicken ZP3 possesses only one potential *N*-glycosylation site [10] and core 1 *O*-glycan(s) (Galβ1-3Gal*N*Ac) with or without terminal Sia occupying at least one Thr (site 1) [2]. In this study, it is suggested that ZP3 has at least 12 forms with individual apparent MWs and 9 distinct pIs (spots 1–9 in Fig. 2) including at least 3 pairs of the forms with same pIs (spots 2–4 in Fig. 2). The ZP3 forms with the most acidic and basic pIs are minor components (spots 1–3, 8 and 9 in Fig. 2).

Whereas it is suggested that chicken ZP1 might be predominantly modified by complex *N*-glycan(s) containing Galβ1-4Glc*N*Ac recognized by RCA I [11] and/or Galβ1-4Glc*N*Acβ1-2Manα1-6 structure with the bisecting Glc*N*Ac recognized by PHA-E [12] and lacking L-fucose recognized by UEA-I [11] (Fig. 3), ZP3 has more complicated glycosylation profile. A binding profile of PNA to the ZP3 forms separated by 2D-PAGE agreed well with that of anti-ZP3 (panels 3 and 7 of Fig. 2), and the binding affinity of ZP3 for PNA was not changed significantly after the

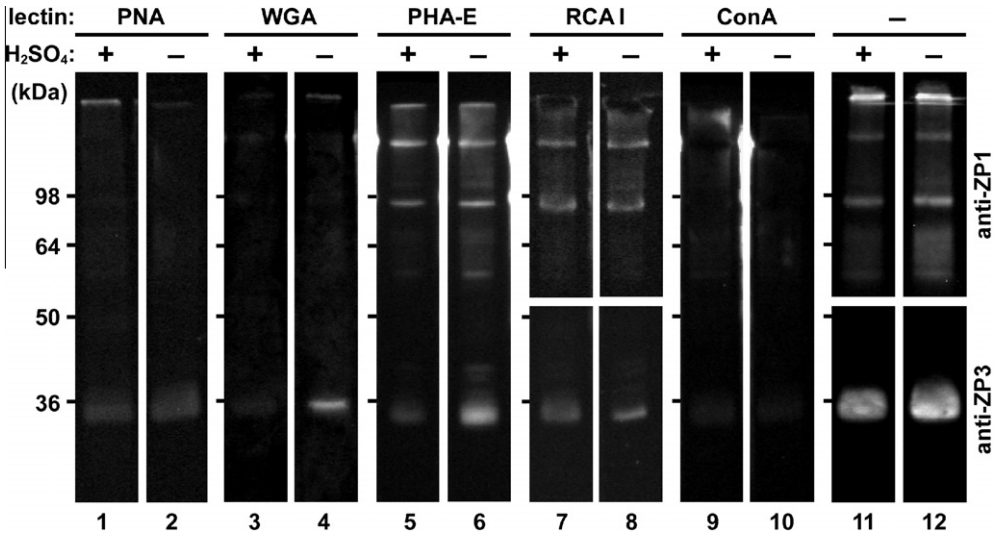

Fig. 3. Lectin blotting of egg-coat proteins separated by SDS–PAGE with or without H_2SO_4 treatment. Chicken egg-coat proteins were separated by SDS–PAGE under non-reducing conditions, electroblotted onto nitrocellulose membranes and treated with (+) or without (–) H_2SO_4 as described in Materials and Methods. The membranes were incubated with PNA, WGA, PHA-E, RCA I, ConA) or without (–) lectins followed by immunostaining with the respective anti-lectin antibodies, anti-ZP1 (upper panels of the lectin-minus lanes) or anti-ZP3 (lower panels of the lectin-minus lanes). Migration positions of the MW markers are shown on the left of each pair of lanes.

chemical desialylation (lanes 1 and 2 of Fig. 3), suggesting that almost all ZP3 forms possess a core 1 *O*-glycan without terminal Sia recognized by PNA [13]. The minor ZP3 forms modified by sialylated core 1 *O*-glycan being nonreactive with PNA [13] might be migrated to similar pI positions indistinguishable from another ZP3 forms in the 2-D gel. Interestingly, our data indicated that WGA, PHA-E and RCA I with individual carbohydrate binding properties [11,12] showed affinities for particular forms of ZP3, respectively (panels 2, 4 and 6 of Fig. 2). A core 1 *O*-glycan on major forms of ZP3 are not sialylated as described above, WGA bound to ZP3 form(s) separated into the spots 4–6 by 2D-PAGE (panel 2 of Fig. 2), and binding affinity of the higher apparent MW form(s) of ZP3 for WGA was significantly decreased after the chemical desialylation (lanes 3 and 4 of Fig. 3), suggesting that ZP3 forms with higher apparent MWs and lower pIs among the major ZP3 components possess *N*-glycan(s) with clustered Sia recognized by WGA [11]. In our data, PHA-E bound to ZP3 form(s) separated into the spots 5–7 by 2D-PAGE (panel 4 of Fig. 2), and the binding affinity of the intermediate apparent MW form(s) of ZP3 for PHA-E was significantly decreased after the chemical desialylation (lanes 5 and 6 of Fig. 3). Considering that PHA-E generally binds with the highest affinity to an *N*-linked glycans containing Galβ1-4Glc*N*Acβ1-2Manα1-6 structure and modified by the bisecting Glc*N*Ac as described above, still binds with high affinity to the similar glycans without the bisecting Glc*N*Ac but bearing terminal α2,3-linked Sia, and binds with lower affinity to the glycans possessing neither bisecting Glc*N*Ac nor α2,3-linked Sia, although the presence of α2,6-linked Sia has an inhibitory effect on the interactions [12], it is suggested that ZP3 forms with intermediate apparent MWs and pIs among the major ZP3 components possess *N*-glycan(s) containing Siaα2-3Galβ1-4Glc*N*Acβ1-2Manα1-6 structure modified by neither bisecting Glc*N*Ac nor α2,6-linked Sia. The α2,3-linked Sia on this glycan(s) might not be clustered because of the undetectable binding affinity of them for WGA (lanes 4 and 6 of Fig. 3). Furthermore, RCA I bound to ZP3 form(s) separated into the spots 6 and 7 by 2D-PAGE (panel 6 of Fig. 2), and binding affinity of the lower apparent MW form(s) of ZP3 for RCA I was not changed significantly, while that of the higher and intermediate apparent MW forms were increased obviously after the chemical desialylation (lanes 7 and 8 of Fig. 3), suggesting that ZP3 forms with lower apparent MWs and higher pIs among the major ZP3 components possess *N*-glycan(s) containing terminal Galβ1-4Glc*N*Ac structure interacting with RCA I and that these glycan(s) on ZP3 forms with higher and intermediate apparent MW forms are sialylated [11]. In addition, neither ConA (panel 8 of Fig. 2, and lanes 9 and 10 of Fig. 3) nor UEA-I (data not shown) bound to any ZP glycoproteins at detectable levels, suggesting that almost all *N*-linked carbohydrates on ZP3 are complex type, and there are not any fucose residues on glycans of ZP3.

Our previous studies showed that ZP1 expressed in liver cells of laying hen and secreted into the bloodstream interacts with ZP3 expressed by granulosa cells surrounding oocyte in the ovary to form ZP1-ZP3 heterodimer involved in the egg-coat matrix formation, and that the ZP1-ZP3 interaction can be analyzed by ligand blotting of ZP3 using the serum ZP1 as a probe [7]. In this study, we performed the ligand blot analysis for ZP3 separated by 2D-PAGE (Figs. 1 and 2). It is suggested that serum ZP1 also bound to ZPD in addition to ZP3 (panel 3 of Fig. 1), although the ZP1-ZPD interaction was not detected previously [7], probably because of much lower abundance of ZPD as compared to that of ZP3 in the egg coat. Furthermore, binding profile of serum ZP1 to the forms of ZP3 separated by 2D-PAGE (panel 5 of Fig. 2) agreed very well with that of anti-ZP3 (panel 3 of Fig. 2), suggesting that the heterogeneous glycosylation of ZP3 described above does not affect the interaction between serum ZP1 and ZP3 involved in the egg-coat formation mechanisms.

To examine how the forms of ZP1 and ZP3 possessing various glycans are distributed in the egg-coat matrix, chicken egg coat was co-stained with PHA-E interacting strongly either with ZP1 or ZP3 in combination with anti-ZP1 or anti-ZP3, and observed under a confocal laser scanning microscopy followed by immunofluorostaining (Fig. 4). Unexpectedly, anti-ZP1 bound more specifically to the near surface of egg-coat filaments (panels 5 and 6), whereas anti-ZP3 bound more homogeneously to whole region in the filaments (panels 7 and 8), suggesting that ZP1 is mainly distributed in the near surface of egg-coat filaments. Together with the significant differences between avian ZP1 and ZP3 in the expression site, regulation of biosynthesis and transportation [14,15], the localization of ZP1 in the egg-coat filaments might

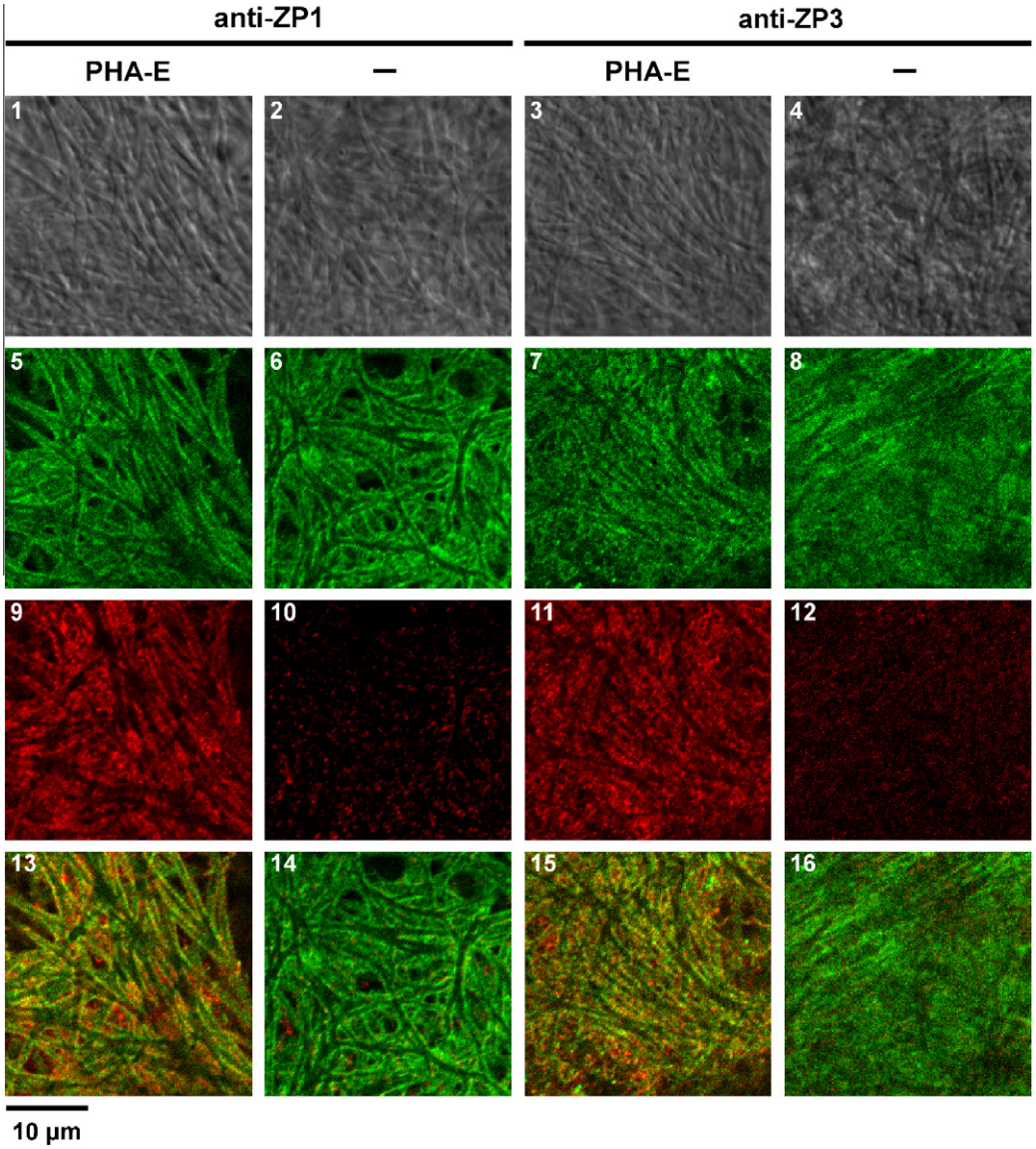

Fig. 4. Lectin-immunofluorescent staining of chicken egg-coat matrix. Small pieces of chicken egg coat were stained with (PHA-E; panels of odd number) or without (–; panels of even number) PHA-E followed by immunofluorescent staining with a combination of anti-PHA-E (red; Alexa Fluor 568) and anti-ZP1 (green; Alexa Fluor 488) (anti-ZP1; panels 1, 2, 5, 6, 9, 10, 13, 14), or anti-ZP3 (green; Alexa Fluor 488) (anti-ZP3; panels 3, 4, 7, 8, 11, 12, 15, 16), and then observed under a confocal laser scanning microscope equipped with a 488-nm (panels 5, 6, 7, 8) or 555-nm (panels 9, 10, 11, 12) excitation laser. Superimposed images constructed from the two color images (panels 13, 14, 15, 16) and light images of differential interference contrast microscopy (panels 1, 2, 3, 4) are shown. Bar = 10 μm.

indicate that the ratio of ZP1 to ZP3 incorporated in the egg-coat matrix is increased during the egg-coat formation or the oocyte maturation. In addition, the staining patterns with PHA-E (panels 9 and 11) and that with anti-ZP1 or anti-ZP3 were approximately overlapped (panels 13 and 15), suggesting that ZP1 and ZP3 are incorporated into the egg-coat matrix with homogeneous distributions of the modified forms.

In some vertebrates including chicken, *N*-linked carbohydrates are involved in egg–sperm interactions [1,16]. Considering that native chicken ZP1, ZPD and the *N*-glycans isolated from chicken egg coat, but not the native ZP3 have abilities to induce sperm acrosome reaction [6,16], and that the egg–sperm binding are mediated by the *O*-glycan of ZP3 [2], the *N*-glycan diversity of chicken ZP3 might play important role(s) in the modulation of species-specific sperm binding to the ZP3 together with the peptide containing SCS located in the most C-terminal part of the ZP module [2]. Our findings on the molecular heterogeneity of ZP glycoproteins will provide useful information to investigate molecular mechanisms of the complicated egg–sperm interactions.

Acknowledgments

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