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Sialic acid involves in the interaction between ovomucin and hemagglutinin and influences the antiviral activity of ovomucin

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ABSTRACT

Ovomucin (OVM) plays an important role in inhibiting infection of various pathogens. However, this bioactivity mechanism is not much known. Here, the role of sialic acid in OVM anti-virus activity has been studied by ELISA with lectin or ligand. Structural changes of OVM after removing sialic acid were analyzed by circular dichroism and fluorescence spectroscopy. OVM could be binding to the hemagglutinin (HA) of avian influenza viruses H_5N_1 and H_1N_1, this binding was specific and required the involvement of sialic acid. When sialic acid was removed, the binding was significantly reduced 71.5% and 64.35%, respectively. Therefore, sialic acid was proved as a recognition site which avian influenza virus bound to. Meanwhile, the endogenous fluorescence and surface hydrophobicity of OVM removing sialic acid were increased and the secondary structure tended to shift to random coil. This indicated that OVM molecules were in an unfolded state and spatial conformation disorder raising weakly. Remarkably, free sialic acid strongly promoted OVM binding to HA and thereby enhanced the interaction. It may contribute to the inhibition of host cell infection, agglutinate viruses. This study can be extended to the deepening of passive immunization field.

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1. Introduction

Ovomucin (OVM) has a unique antiviral activity, the mechanism of which is not entirely known. OVM is a highly glycosylated protein containing sialic acid (SA), which belongs to the mucin family [1]. Mucins are a major component of mucus, which are widely distributed in the body's internal surface and mucosal tissues, such as the respiratory tract and intestines. They provide an important innate immune barrier to potential toxins, particles, and pathogens [2]. It can prevent pathogens from being in contact with susceptible cells. It is thought that the adhesion of mucins to pathogens is an important mechanism with a potentially significant effect.

OVM has antiviral properties, that is similar in structure and composition to the influenza virus receptor and early findings suggest that OVM has an inhibitory effect on swine influenza virus-induced hemagglutination [3]. The interaction of HA on the surface of the virus with OVM leads to the release of glycopeptide complexes. Hemagglutination inhibition assays and enzyme-linked immunosorbent assays have revealed the high affinity of OVM for bovine rotavirus, chicken new castle disease virus and human influenza virus [4–6]. It was found that *N*-acetylneuraminic acid (NeuAc, a specific subtype of sialic acid in OVM) in the β-subunit could greatly facilitate the interaction between OVM and chicken new castle disease virus. The alteration of the conformation of OVM by the alkylation of disulfide bonds leads to loss of binding to OVM antibody [4]. However, evaluations of antiviral activity in these studies focused primarily on the inhibition of viral-induced hemagglutination and did not include other more accurate and intuitive antiviral methods.

Structurally, OVM is a highly glycosylated protein whose monosaccharides are predominantly in the forms of oligosaccharides and glycosides consisting of fewer than 10 monosaccharides [7,8], including N-glycosidic bonds and O-glycosidic bonds [9]. N-glycans are linked to the aspartic acid (Asp) residues of the polypeptide sequence Asn-X-Ser/Thr, where X represents any amino acid except proline, and O-glycans are predominantly linked to the serine (Ser) and threonine (Thr) residues [10,11]. These oligosaccharides mainly include mannose (Man), galactose (Gal), *N*-acetylgalactosamine (GalNac), *N*-acetylglucosamine (GlcNac), SA, fuctose (Fuc) [12] and sulfuric acid esters [13].

Sialic acid of OVM can promote interaction with chicken new castle disease virus. Meanwhile, various viruses such as influenza virus, coronavirus and rotavirus utilize glycoproteins containing sialic acid on the surface of host cells as recognition receptors [14–16]. Sialic acid can be recognized by the epitope on the globular head of the influenza virus HA, thereby inducing interaction with the corresponding HA receptor binding sites and interfering with or blocking the adsorption of the virus to the cells [17]. Sialic acid residues are generally located at the

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terminus of the N-linked oligosaccharide chain and the O-linked oligosaccharide chain with α2,3-, α2,6- and α2,8-linkages [18]. Different influenza viruses are capable of specifically recognizing different linked types of sialic acids. Human influenza viruses are more likely to bind to the α2,6-linkage sialic acid receptor, and avian influenza viruses preferentially recognize α2,3-linkage sialic acids [19,20].

OVM is a glycoprotein containing a large amount of sialic acid. Its antiviral activity has not been studied deeply, and its anti-infective mechanism is barely understood. The role that sialic acid plays and whether it is recognized as the same receptor of the influenza virus and binds to HA remain to be revealed. Therefore, this study aims to verify the interaction between OVM and HA and demonstrate the function of sialic acid in this interaction to explain the possible mechanism of OVM for satisfactory antiviral activity and increase the knowledge of the role of OVM in passive immunity.

2. Material and methods

2.1. Isolation and purification of OVM

OVM was crude extracted according to a previously reported method [21] with modifications. In brief, 200 mL of fresh whole egg white was stirred at 4 °C for 30 min and subsequently diluted with 600 mL of 100 mM NaCl. The pH was adjusted to 6.0 with 1 M HCl, and the solution was incubated overnight at 4 °C. The egg white solution was centrifuged at 10,000*g* for 10 min at 4 °C, and the precipitate was resuspended with 500 mM NaCl for 4 h and then centrifuged under the same conditions.

After the precipitate, which was crude OVM, was washed several times with distilled water, the above procedure was repeated. The extract was freeze-dried and stored at −20 °C. OVM crude extract was purified by gel filtration chromatography (Sephacryl S-300 HR, 26 mm × 60 cm) using the AKTA purification system (GE, USA). The target elution peak was dialyzed by distilled water, and the product was purified OVM.

2.2. Removal of terminal sialic acid of OVM

Purified OVM (2 mg) was mixed with 1980 μL of pH 5.6 sodium acetate buffer and 20 μL of NA enzyme and incubated in a 37 °C water bath for 18 h.

Desialylated OVM (dSA-OVM) was dialyzed against 14 kD dialysis bags for 24 h with pH 8.6 borate buffer. The solution was removed, stirred for 1 h and centrifuged for 10 min at 6000 r/min; the supernatant was saved for subsequent experiments.

The dSA-OVM supernatant was centrifuged for 10 min in a 10 kD ultrafiltration tube at 5000*g* and concentrated to 1.5 mL. A standard curve was used to quantify the concentration of OVM.

2.3. Determination of the OVM terminal sialic acid bond

The effect of enzymatic hydrolysis and the chemical bond of sialic acid in oligosaccharide chains was evaluated by ELISA using lectins *Sambucus nigra* (SNA) and *Maackia amurensis* (MAA). A 1:25 dilution of SNA was added to the ELISA plate at 100 μL/well, and the plate was incubated overnight at 4 °C. To the control group was added PBS buffer without SNA. The plates were washed 3 times for 5 min each with PBST and then again with PBST dissolved in 5% skim milk at 300 μL/well and incubated at 37 °C for 1 h for blocking. After the plates were washed, dSA-OVM and OVM diluted to 20 μg/mL were added to the experimental and control wells, respectively, at 100 μL/well, and the plates were incubated at 37 °C for 1 h. After the plates were washed, OVM antibody diluted 1:80000 was added at 100 μL/well, and the plates were incubated at 37 °C for 1.5 h. After the plates were washed, HRP-labeled goat anti-mouse IgG diluted 1:8000 was added at 100 μL/well, and the plates were incubated at 37 °C for 80 min, washed and stained.

2.4. OVM binding HA

The function of sialic acid was assessed by the change in the binding of HA to OVM with the removal of sialic acid. The HA proteins of the influenza viruses H_5N_1 (HA5) and H_1N_1 (HA1) were added to the ELISA plate at a dilution of 1:10 at 100 μL/well, and the plate was incubated overnight at 4 °C. To the control group was added PBS buffer without HA. Other operations were the same as in Section 2.3.

2.5. OVM oligosaccharide chain inhibition assays

The common components of oligosaccharide chains (Gal, Fuc, Man and sialic acid) were separately mixed with OVM for competitive binding analysis with HA. Subsequently, the effect of free sialic acid was analyzed by different additional sequences. The additional sequences were the addition of sialic acid followed by the addition of OVM, the addition of OVM followed by the addition of sialic acid, and the addition of preincubation mixture. The ELISA procedure was the same as previously described.

2.6. Circular dichroism (CD) changes in OVM

The protein solution was diluted 10-fold, the final protein concentration was 0.3 mg/mL, the optical path of the quartz cell was 0.1 cm, the sensitivity was 2 mdeg/m, the wavelength scanning range was 190–240 nm, the speed was 10 nm/s, and the resolution was 0.1 nm measured at room temperature.

2.7. The intrinsic fluorescence scanning of OVM

The optical path of the quartz cell was 1.0 cm. Using tyrosine (Tyr) as an intrinsic probe, the excitation wavelength was 274 nm, and the emission spectrum was scanned at 290–400 nm. Using tryptophan (Trp), the excitation wavelength was 295 nm, and the emission spectrum was scanned at 300–450 nm. The excitation and emission monochromators each had a bandwidth of 5 nm. OVM was incubated at room temperature for 1 h, and its concentration was diluted to 100 μg/mL. Blank samples were measured under the same conditions.

2.8. OVM surface hydrophobicity

Using l-anilinonaphthalene-8-sulphonate (ANS) as a fluorescence probe, the surface hydrophobicity of OVM was measured by the fluorescence method. OVM was diluted to 0.005–0.2 mg/mL with pH 8.6 borate buffer. Fluorescence spectra of ANS were scanned by adding 8 μL of diluted sample to 100 μL of 8 mmol/L ANS solution. The excitation wavelength was 365 nm, and the scan range was 300–600 nm. The fluorescence intensity at 365 nm excitation and 484 nm emission was used to making a standard curve for protein concentration. The control was blank ANS solution with addition of the corresponding sample buffer.

2.9. Statistical analyses

All values were expressed as mean ± s.e.m. ANOVA with Bonferroni's multiple-comparison test when more than two groups were compared. All the assays were run in triplicate and were representative of at least 3 independent experiments. A P value < 0.05 was considered statistically significant and the asterisks in all figures are defined, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

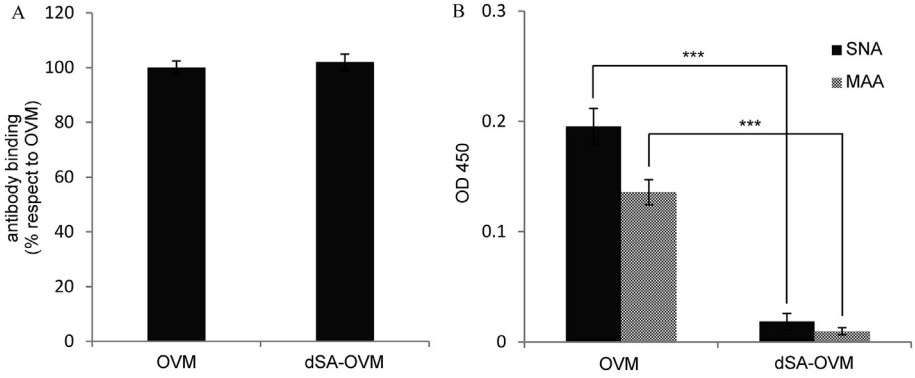

Fig. 1. Antigen activity and terminal sialic acid glycosidic bond of OVM. (A) Antigen activity of ovomucin before and after sialidase enzymolysis was consistent. dSA-OVM retained antigen binding ability. (B) Ovomucin interacted with lectin SNA or MAA, respectively. SNA recognize α2-6-SA whereas MAA prefer α2-3-. The terminal SA of OVM glycans include both two bonds. dSA-OVM's bonds content was significantly reduced both. OVM, natural ovomucin; dSA-OVM, ovomucin with the removal of the sialic acid residue; SNA, *Sambucus nigra*; MAA, *Maackia amurensis*; the average data are presented as mean ± s.e.m. Statistical significance is indicated by ***$p < 0.001$.

3. Results

3.1. OVM antigen activity and OVM terminal sialic acid glycosidic bond

In the ELISA reaction, a variety of factors together affected the final test results, and the antigenic epitope of the sample had a significant impact. To ensure the accuracy of the experiment, we needed to verify whether sialidase enzymolysis would affect the other functions of OVM. In this experiment, ELISA reaction of OVM was the key to analyzing physiological immune activity. The purified OVM was digested with sialidase to obtain dSA-OVM. The concentration of natural OVM was 1.394 mg/mL, and that of dSA-OVM was 1.677 mg/mL. For a concentration of 20 μg/mL, the binding changes in OVM and dSA-OVM to antibody were analyzed (Fig. 1A). Compared with that of natural ovomucin, the binding of dSA-OVM and antibody did not change significantly after enzymolysis, exhibiting a good consistency and no significant effect on the antibody binding activity.

Lectins SNA binds specifically to sialic acid linked by an α2-6-linkage, while MAA specifically recognizes an α2-3-linkage. As shown in Fig. 1B, the terminal sialic acid was effectively removed in dSA-OVM after enzymatic hydrolysis, and its binding activity was obviously lower than that in the natural OVM. The binding activity of natural OVM to lectin SNA was higher than that to lectin MAA, indicating that the terminal sialic acid glycosidic linkage in the OVM oligosaccharide chain was mainly α2-6 and that less α2-3 was present. After enzymolysis, all sialic acids in dSA-OVM significantly decreased ($p < 0.001$).

3.2. Binding of OVM to influenza virus HA

The surface antigen HA of the influenza virus could bind to related glycoproteins via protein-protein or protein-carbohydrate chain interactions. To verify whether sialic acid is an important site of the interaction between OVM and HA, we designed a deglycosylation experiment. As shown in Fig. 2, HA of both H_5N_1 and H_1N_1 bound to OVM and did not react with BSA (negative control). After sialic acid was removed, the binding of dSA-OVM to HA was significantly lower than that of natural ovomucin. At the same dose, the binding capacity of dSA-OVM to HA5 decreased 71.5%, and the binding capacity to HA1 also decreased by 64.25%. This indicated that HA recognizes sialic acid on OVM. Sialic acid in OVM is distributed on the oligosaccharide chain terminus. Therefore, it can be considered that this recognition involves the participation of sialic acid and oligosaccharide chains.

3.3. Influence of oligosaccharide chain components on the binding of OVM to HA

Based on the above results, it has been demonstrated that the carbohydrate chain is one of the recognition regions for the interaction between OVM and HA. Sialic acid is the critical target. To determine whether this interaction occurred with the participation of other sugar chain components, a sugar competition experiment was conducted. Different kinds of sugar were added to the plate coated with HA, followed by washing off and then adding OVM to measure the binding changes.

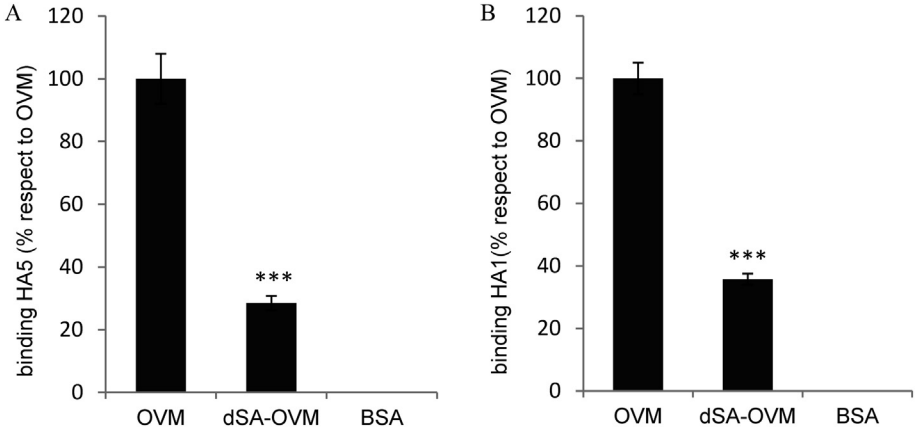

Fig. 2. Ovomucin binding with HA. (A) OVM bind with HA5 effectively. The binding of dSA-OVM to HA was significantly reduced. (B) Ovomucin bind with HA1. After sialic acid was removed, the trend of dSA-OVM interaction with HA1 weaken significantly. OVM, natural ovomucin; dSA-OVM, ovomucin with the removal of the sialic acid residue; HA5, hemagglutinin of avian influenza virus H_5N_1; HA1, hemagglutinin of avian influenza virus H_1N_1. The data are presented as mean ± s.e.m. Statistical significance is indicated by ***$p < 0.001$.

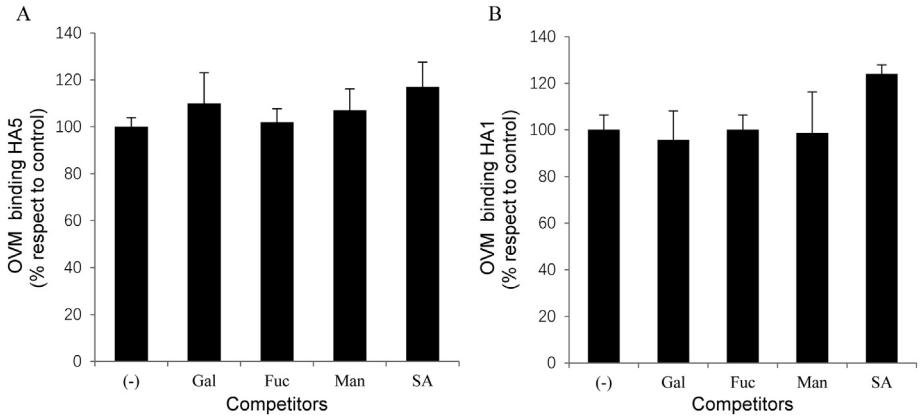

Fig. 3. OVM carbohydrate competitive binding. (A) OVM binding with HA5. Pre-mixing extra monosaccharide with HA5 did not reduce interaction with OVM. (B) OVM binding with HA1. Pre-incubation free monosaccharide with HA1 also did not interfere with the binding. Even the addition of free sialic acid induced increase slightly. (−), negative control, without adding monosaccharide; Gal, galactose; Fuc, fucose; Man, mannose; SA, sialic acid, here *N*-acetylneuraminic acid was used as specific subtype of sialic acid in ovomucin. The data are presented as mean ± s.e.m.

The results displayed in Fig. 3 indicate that no monosaccharide had an inhibitory effect on OVM binding HA. There was no significant difference in binding after pre-incubation of carbohydrates and HA compared to the negative control, and the addition of free sialic acid did not cause a decrease in binding. This binding trend was consistent with both HA5 and HA1.

According to the results in Fig. 3, when the reaction sequence in the experiment was the addition of sialic acid firstly and then the addition of OVM, it was unexpectedly found that free sialic acid promoted the interaction of OVM and HA. To analyze the role played by free sialic acid in the binding of OVM to HA, further experiments were carried out with different sequences of additions. Adding OVM at first and then adding SA or adding the two components together were analyzed (Fig. 4). When OVM was first added, followed by SA, neither OVM nor dSA-OVM showed any significant change compared with the negative control, and the binding intensity tended to be similar in HA5 and HA1. When OVM was mixed with SA and added at the same time, it is interesting found that the binding of OVM to HA was significantly enhanced far more than the negative control and other groups. This change in HA1 was as significant as in HA5. Based on the above results, it was found that free sialic acid enhances the binding of OVM to influenza virus HA.

3.4. Removing sialic acid influences the conformation of OVM

The changes in the secondary structure of dSA-OVM and OVM were analyzed by circular dichroism spectroscopy (CD), and the results were shown in Fig. 5A. The CD spectra of OVM showed weak negative peaks at 225 nm and 208 nm and a positive peak approximately 190 nm, characteristic of α-helical and β-sheet hybrids [22]. The negative peak of the dSA-OVM spectrum near 225 nm was weaker and closer to the short wavelength direction than OVM. Data on specific changes are shown in Table 1. After removing sialic acid, ovomucin α-helix decreased, while the random coil increased, indicating that the degree of disorder increased [23].

Fluorescence spectroscopy is a powerful tool for studying the changes in the protein microenvironment in solution. Aromatic amino acid (tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe)) residues in OVM molecules can fluoresce. Therefore, the changes in the OVM molecule before and after removing the terminal sialic acid can be reflected according to the change in endogenous fluorescence [24,25]. The endogenous fluorescence spectra of OVM before and after removing the terminal sialic acid at excitation wavelengths of 274 nm and 295 nm were shown in Fig. 5B and C, respectively. As shown in

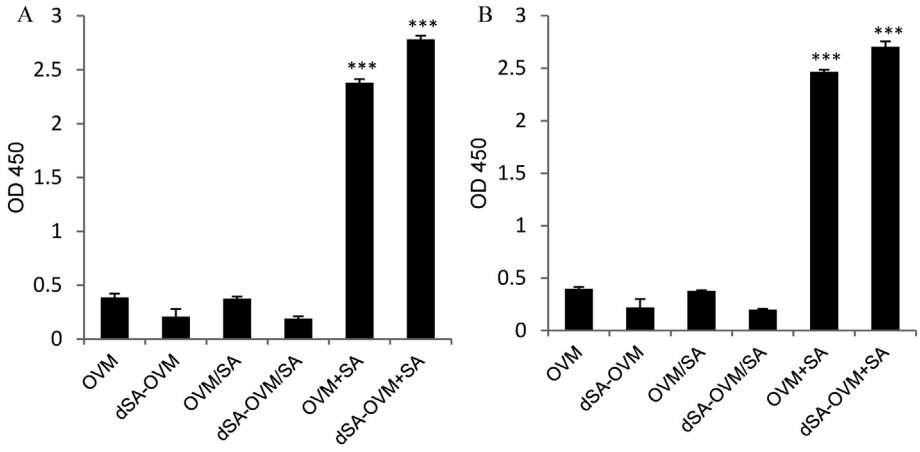

Fig. 4. Effects of SA different additional sequences on binding. (A) dSA-OVM binding with HA5 was reduced. Adding SA after adding dSA-OVM or OVM was not able to improve the binding. However, the addition of OVM premixed with SA greatly enhanced the interaction with HA5, and pre-incubation the dSA-OVM with SA had a significant improvement in its combination with HA5. (B) Adding SA after adding OVM or dSA-OVM did not influence its binding ability with HA1. The addition of dSA-OVM premixed with SA strengthened the interaction with HA1 significantly, and as much as pre-incubation the OVM with SA prior to addition to the HA1. OVM/SA represents the addition of natural ovomucin first then the addition of SA; dSA-OVM/SA represents the addition of dSA-OVM first and then the addition of SA; OVM + SA represents a pre-incubated mixture of natural ovomucin and SA being added at the same time; dSA-OVM + SA represents a pre-incubated mixture of dSA-OVM and SA being added at the same time; OVM, natural ovomucin; dSA-OVM, ovomucin with the removal of the sialic acid residue; SA, sialic acid, here *N*-acetylneuraminic acid was used as specific subtype of sialic acid in ovomucin.

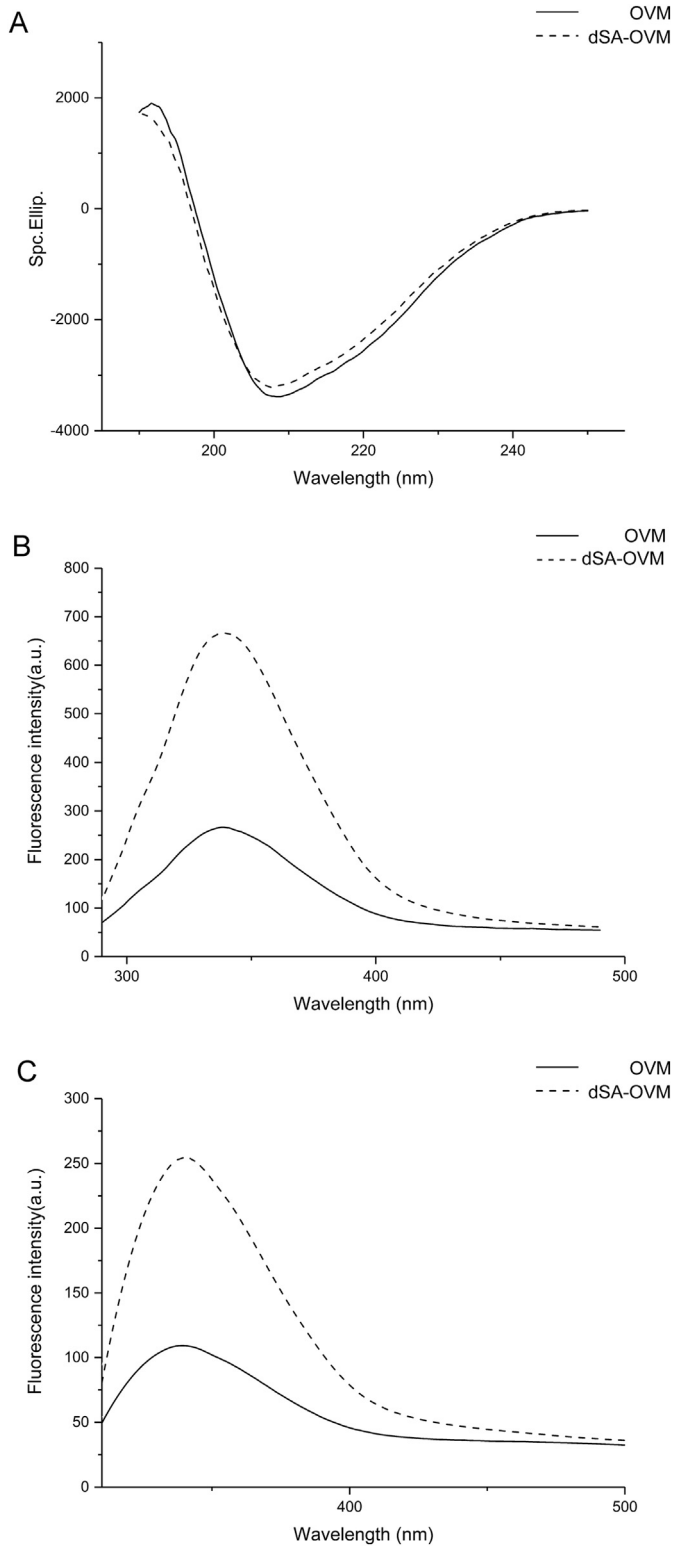

Fig. 5. The structure change of dSA-OVM. (A) The CD spectra of OVM and dSA-OVM. The curve of dSA-OVM occurred a mild blueshift and the negative peak was weaker. (B) Tyr endogenous fluorescence spectra of OVM and dSA-OVM. The fluorescence intensity of dSA-OVM was enhanced compared to OVM. (C) Trp excitation fluorescence of OVM and dSA-OVM. The fluorescence intensity of dSA-OVM was increased same to Tyr. The solid black line is OVM, and the black dotted line is dSA-OVM.

the Tyr fluorescence spectrum of Fig. 5B, removal of terminal sialic acid results in enhancement of the endogenous fluorescence intensity of OVM, but no obvious migration of the fluorescence peak occurs. The

Table 1
OVM and dSA-OVM secondary conformational change.

	OVM	dSA-OVM
α-Helix	11.2%	9.5%
β-Sheet	52.7%	55.5%
β-Turn	4.2%	2%
Random coil	31.8%	33%

fluorescence spectra of Trp was in Fig. 5C. The overall trend in fluorescence intensity agrees with the result of Tyr being a fluorescent probe. However, the λ_{max} of the Trp spectrum was blueshifted after removing the terminal sialic acid, suggesting that after removal of sialic acid, the OVM microenvironment hydrophobicity is enhanced. In order to verify this result, the changes in the ANS fluorescence spectra of OVM before and after the removal of terminal sialic acid were investigated by an ANS fluorescence probe.

According to the result, the fluorescence intensity of ANS was enhanced after OVM removal of the terminal sialic acid. The value of OVM surface hydrophobicity was 395.4 and the value of dSA-OVM was 713.9. This shows that OVM is located in a microenvironment with a non-polar increase and enhanced surface hydrophobicity. This is related to the unfolding of the OVM molecule and the increase in disorder, resulting in the exposure of the hydrophobic region, strengthening the binding of ANS to the OVM hydrophobic region, and enhancing the fluorescence intensity [26].

4. Discussion

The exact mechanism through which OVM exerts its antiviral function is not entirely clear at present. This research studied the interaction between OVM and the influenza virus HA and the role of sialic acid in this interaction. It was found that the OVM carbohydrate chain contains terminal sialic acid with both α-2-6- and α2-3-linkages (Fig. 1B). HAs cannot bind to other proteins such as BSA but can interact with OVM. This interaction is dependent on the presence of sialic acid (Fig. 2). Monosaccharides have no influence on the binding of OVM with HA. It is noteworthy that the binding of HA with OVM was strongly promoted by free sialic acid (Figs. 3 and 4). It is indicating that sialic acid is involved in the binding of OVM to influenza virus, and additional free sialic acid could enhance the OVM antiviral process.

Mucin, a natural barrier widely found in animals, always plays critical role in antiviral and antibacterial activities [27–30]. OVM is a mucin protein in egg. According to our previous research, OVM contains plenty of sialic acid, and its subtype is NeuAc. In this study, we demonstrated the glycosidic bond type of sialic acid linking the OVM carbohydrate chain terminus, which contains both α2-6- and α2-3-linkages. The content of sialic acid with the α2-6-linkage was higher than that with the α2-3-linkage (Fig. 1B). In the process through which the influenza virus infects the body, the first step is to recognize and bind to the cell surface receptor through the viral surface protein HA [20]. The main receptors are cell surface glycoconjugates, and sialic acid is required for this process. Human influenza viruses mainly recognize α2-6-linked sialic acids, whereas avian influenza viruses prefer α2-3-linked sialic acids [31,32]. Therefore, due to the type of sialic acid linkages in the OVM oligosaccharide chain, we believe that OVM has anti-infective activity against both human and avian influenza viruses. Furthermore, the anti-avian influenza virus function is closely related to α2-3-linked sialic acid.

To confirm this hypothesis, this study was carried out to enzymatically hydrolyze sialic acid. Using the HA of influenza viruses H_5N_1 and H_1N_1 as the binding protein, the changes in OVM as an acceptor with or without sialic acid binding HA were measured. Firstly, the interaction of OVM before and after removing sialic acid with its antibody was analyzed, and it was found that sialic acid was not the binding site. No

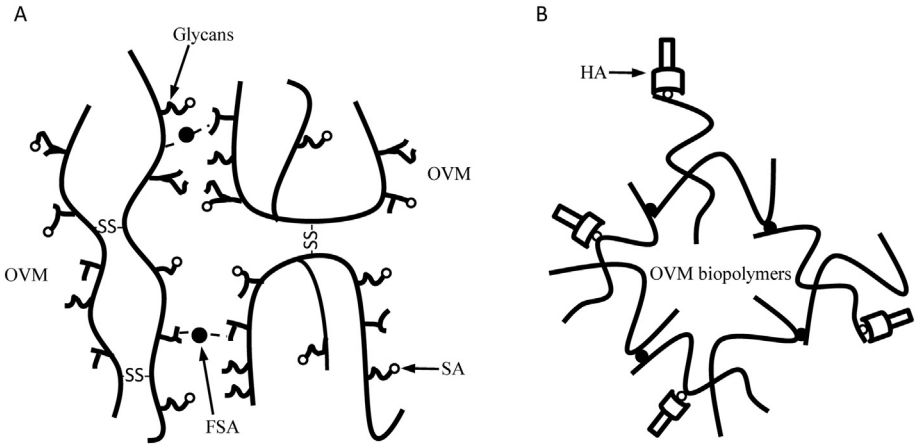

Fig. 6. Hypothetical model summarizing FSA-OVM interactions and ability of FSA to enhance virus agglutinating capacity of OVM. (A) Subunits constitute OVM via disulfide bonds in the non-glycosylated regions. FSA interacts with OVM to form OVM biopolymers through noncovalent bond. (B) FSA does not bind HA, but FSA enhance the agglutinating ability of OVM with HA by OVM biopolymers- and SA-dependent mechanism. Therefore, FSA enhance a biological function of OVM binding virus. ●, free sialic acid (FSA). ○, terminal sialic acid of OVM glycans (SA). -ss-, disulfide bonds. OVM, ovomucin. HA, hemagglutinin.

significant difference was found in the binding activity before and after the removal of sialic acid. This demonstrates that sialic acid does not participate in all the functions of OVM but also that follow-up experiments can use an ELISA reaction between OVM and an antibody. This ensures that the results are real and effective. Subsequently, the interaction of natural OVM and dSA-OVM with HA was investigated, and no binding reaction between HA and BSA was found, which indicated that the recognition of glycoprotein was specific (Fig. 2). However, the obvious interaction between HA and natural OVM indicates that HA can specifically recognize OVM and associate with it. On the other hand, the binding of OVM to HA was significantly reduced after sialic acid removal to only 30% to 40% of the original (Fig. 2). These results showed the same tendency in H_5N_1 and H_1N_1, which proves that sialic acid is involved in the recognition of HA and OVM. After sialic acid was removed, this binding could be inhibited. Thus, we demonstrate that one of the antiviral activity mechanisms of OVM is associated with sialic acid in the OVM oligosaccharide chain competing with the cellular receptor for binding HA. After removing sialic acid, OVM still retains some ability to bind HA, presumably through other recognition mechanisms. In fact, sialic acid was not completely removed after enzymatic hydrolysis (Fig. 1B). Therefore, this binding may be associated with sialic acid residues, and the role of sialic acid in this mechanism may be underestimated.

To further demonstrate the influence of the terminal monosaccharide of carbohydrate chain in the binding of OVM with HA, several monosaccharides that are commonly found near the end of the oligosaccharide chain were selected and tested. To determine whether Gal, Fuc, Man and free sialic acid could bind with HA, we conducted competition experiment. The results show that after adding of these free sugars first, the binding of OVM and HA was not significantly different and basically maintained at the same level. It suggests that these carbohydrates are not involved in binding and cannot prevent this interaction. Monomeric sialic acid is thought to be unable to compete effectively with the receptor on target cells for binding to influenza virus, while dendritic sialic acid structure is more effective against influenza virus [33]. The process by which influenza virus recognizes sialic acid is strongly related to its linked pattern [14,19,34,35]. This is similar to the finding that free sialic acid does not effectively bind with HA in this study (Fig. 3).

Sialic acid is a highly electronegative sugar molecule, and the role in OVM is worth in-depth exploration. For this reason, we designed the influence of free sialic acid on the interaction between OVM and HA under different sequences of addition. When OVM was added firstly and then free sialic acid, there was no significant change in the binding of OVM with HA, and the interaction remained at the same level as the control. However, it was exciting to note that when OVM was preincubated with free sialic acid, its binding to HA was significantly enhanced compared to the control. It had the same effect on H_5N_1 and H_1N_1 (Fig. 4). Sialic acid in OVM was involved in the recognition of HA. Free sialic acid does not directly bind to HA but mixed with OVM can effectively improve the interaction. Sialic acid is electronegative and carries multiple O atoms and N atoms that can have many unique physicochemical effects [27,36]. Taken together, this study proposes a novel mechanism hypothesis that sialic acid plays an active role in the antiviral activity of OVM (Fig. 6). First, sialic acid in the OVM carbohydrate chain is recognized by the influenza virus HA as a receptor [37]. On the other hand, free sialic acid is not directly involved in HA binding, but it can bind OVM to form an eupolymer by electrostatic interaction or hydrogen bond [38], promote OVM to bind and co-precipitate with HA. Therefore, OVM maintain effective defense against virus, and enhance its antiviral activity. In a future study, this hypothesis will be explored in vitro and in cell experiments.

In addition, we also analyzed the influence of OVM structure after removing sialic acid. The results exhibited that the secondary structure does not change too much but can cause OVM tertiary structural changes and that increasing the surface hydrophobicity causes molecular expansion [39,40]. This may increase the hydrogen bond binding sites of sialic acid to OVM. Therefore, the structural interpretation of OVM antiviral activity is worth further study.

5. Conclusions

Ovomucin contains sialic acids with both α2-6 and α2-3 linkages, and the α2-6 bond is higher than the α2-3 bond, which helps it against a wide variety of influenza viruses. The hemagglutinin of the influenza virus recognizes and binds to the ovomucin carbohydrate chain terminal sialic acid, and the interaction is greatly diminished after the sialic acid is removed. Therefore, sialic acid is an important recognition site for ovomucin to play a role in the binding of hemagglutinin competing with host cell surface receptors. At the same time, the removal of ovomucin sialic acid lead to a slight increase in the random coil of secondary structure and enhance surface hydrophobicity. We also found a unique role for free sialic acid. The addition of free sialic acid can promote the binding of ovomucin to hemagglutinin and enhance ovomucin anti-influenza virus activity. This in-depth study and exposition can complement the mechanism of ovomucin involved in innate immunity and provide new ideas and perspectives for the development of antivirus agents.

Abbreviations

OVM	ovomucin
dSA-OVM	ovomucin with the removal of the sialic acid residue
SA	sialic acid
AIV	avian influenza virus
HA	hemagglutinin
HA5	hemagglutinin of avian influenza virus H5N1
HA1	hemagglutinin of avian influenza virus H1N1

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