# Bindings of Ovomucin to Newcastle Disease Virus and Anti-Ovomucin Antibodies and Its Heat Stability Based on Binding Abilities

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The bindings of ovomucin, its chemically modified compounds, including its disulfide-reduced and alkylated α- and β-subunits, and desialylated ovomucin to NDV and anti-ovomucin antibodies were determined by ELISA. We found that the NeuAc residue in the β-subunit greatly contributed to the binding of ovomucin to NDV, and disulfide bonds in ovomucin contributed to the binding of ovomucin to antibodies. The conformational, biological, and chemical alterations of ovomucin heated at 60−100 °C for 10 min under the various pH conditions (pH 6−12) were examined on the changes in the ability to NDV and anti-ovomucin antibodies which were also determined by ELISA, along with determinations of SDS−PAGE patterns and CD spectra. Ovomucin degraded together with the increases in temperature and pH, depending on destruction of NeuAc in β-subunit, and cleavages of disulfide bonds in inter- and intrasubunits and peptide bonds in α- and β-subunits.

**Keywords:** *Ovomucin; newcastle disease virus; anti-ovomucin antibodies; heat treatment*

## INTRODUCTION

It has been reported that hen egg white ovomucin, which is a macromolecular and highly glycosylated glycoprotein, is a polymer of two subunits of protein-rich α-subunit (AMM of 220 kDa) and carbohydrate-rich β-subunit (AMM of 400 kDa) (Donovan *et al.*, 1970; Kato *et al.*, 1973; Itoh *et al.*, 1987). β-Subunit was also indicated to consist of heavily O-glycosylated and protein-rich regions by chemical analyses of fragments from ovomucin treated with pronase (Tsuge *et al.*, 1997). Such ovomucin is a unique gelatinous glycoprotein with excellent foaming and good emulsifying properties (Kato *et al.*, 1985). On the other hand, our previous studies on the biological function of ovomucin revealed its protein showed a high affinity to bovine rotavirus, hen NDV, and human influenza virus, using the hemagglutination inhibition test and, in part, ELISA (Tsuge *et al.*, 1996a,b, 1997).

Ovomucin was indicated to be physically stable under heat treatment under conditions in which egg white proteins such as ovalbumin and ovotransferrin coagulate or gel (Johnson and Zabik, 1981). The pronase-treated ovomucin was also found to be relatively stable against heat treatment such as heating at 100 °C for 5 min for the inactivation of its enzyme, from determinations of the bindings to NDV and anti-ovomucin antibodies (Tsuge *et al.*, 1997). However, how the conformational, biological, and chemical properties of ovomucin are altered by heat treatment has not yet been studied.

In this report, the bindings of ovomucin, its chemically modified compounds, including its disulfide-reduced and alkylated α- and β-subunits, and desialylated ovomucin to NDV and anti-ovomucin antibodies were at first determined by ELISAs, along with determinations of SDS−PAGE patterns and CD spectra. The conformational, biological, and chemical alterations of ovomucin heated under the various pH conditions were also examined by the same method described above. The bindings to NDV and antibodies were determined for analysis of the structural stability of each binding region in ovomucin, since the different regions in ovomucin independently bind to NDV or antibodies. The relationship between the heat treatment and structural alteration of ovomucin and its subunits was discussed.

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## MATERIALS AND METHODS

**Materials.** Mouse anti-ovomucin antibodies prepared in a previous paper (Tsuge *et al.*, 1997) were also used in this study. NDV (strain Ishii) and anti-NDV chicken serum were purchased from Kitasato Institute (Tokyo), and rabbit peroxidase-conjugated antibody to chicken IgY (IgG) and chicken peroxidase-conjugated antibody to mouse IgG were from Chemicon International Inc. (Tenecula, CA).

**Preparation of Samples.** Ovomucin from fresh egg white (White Leghorn hens) was prepared as the gel-like precipitate by the method of Kato *et al.* (1970) as described briefly in a previous paper (Tsuge *et al.*, 1997) and stored at −20 °C without freeze-drying. When necessary, the prepared and stored gel-like ovomucin (about 10 g) was dissolved in 100 mL of 10 mM carbonate buffer (pH 11.0) and used as samples for heat treatment after dialysis as later described in Heat Treatment. Part of the solubilized ovomucin solution was dialyzed against 0.5 M Tris-HCl buffer (pH 8.5) containing 7 M guanidine hydrochloride and 10 mM EDTA-2Na. Dithiothreitol (3 mg) was added to 1 mL of the solution (194 μg/mL), which was then stirred for 2 h room temperature. Then, iodoacetamide (7.5 mg) was added to the reduced ovomucin solution, incubated for 30 min, and dialyzed against 1 M NaCl and 50 mM carbonate buffer (pH 9.6), in that order, to prepare the RA-ovomucin (RA-α- and β-subunit mixtures). The A-ovomucin was prepared by the method described above without the addition of dithiothreitol. These procedures were carried out in the dark at 4 °C. The RA-ovomucin was applied to a gel filtration column (Sephacryl S-400, 2.0 × 75.0 cm, Pharmacia LKB Products, Uppsala, Sweden) previously equilibrated with 10 mM potassium phosphate buffer (pH 8.5) containing 0.2% 2-ME and 0.5% SDS and eluted with the same buffer. The obtained fractions corresponding to α- and β-subunits were rechromatographed under the same conditions. Each fraction was dialyzed against 1 M urea, 1 M NaCl, and 50 mM carbonate buffer (pH 9.6), in that order, to remove SDS (Rose *et al.*, 1979). The preparation of desialylated ovomucin from ovomucin solubilized with 10 mM carbonate buffer (pH

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11.0) and dialyzed against 10 mM acetate buffer (pH 5.0) was carried out as follows. Neuraminidase ($7.6 \times 10^{-3}$ units) from *Clostridium perfringens* (Sigma Chemical Co., St. Louis, MO) was added to 1 mL of the dialyzed ovomucin solution (184 μg/mL). The mixture was incubated at 37 °C for 24 h, heated at 100 °C for 3 min to stop the digestion, and then dialyzed against 50 mM carbonate buffer (pH 9.6). All sialic acid present in ovomucin was described as NeuAc (Robinson and Monsey, 1971), and no NeuAc residue when determined according to the methods described under Analytical Methods was found in the carbohydrate chain bound to the protein moiety in the desialylated ovomucin preparation.

**ELISA with NDV.** ELISA with NDV was carried out by the method described in a previous paper (Tsuge *et al*., 1997). Each sample of ovomucin (unheated and heated), A-ovomucin, RA-ovomucin, RA-α-subunit, RA-β-subunit, and desialylated ovomucin was dissolved to obtain a concentration of about 25 μg/mL in 50 mM carbonate buffer (pH 9.6). Flat-bottomed microtiter plates were coated with each solubilized solution and its serially diluted solutions. Each experiment was done in triplicate, and values were expressed as mean ± standard deviation (SD) ($n = 3$). In order to examine the effect of NeuAc on the abilities to bind to NDV, ELISA was performed as above using the NDV solutions preincubated with various concentrations of NeuAc (as the free type of NeuAc) and ovomucin (as the bound type of NeuAc) instead of NDV solutions to examine the inhibition. Its ratio, $I$(%), was calculated as $I(\%) = [1 - (A/B)] \times 100$, where $A$ is the amount of NDV bound to ovomucin when the NDV was preincubated with free NeuAc or bound NeuAc in carbohydrate chain of ovomucin and $B$ is the amount of NDV bound to ovomucin without preincubation. The minimum concentration required for a 50% inhibition was obtained from the relation between $I$(%) and NeuAc concentration (millimolar).

**ELISA with Anti-Ovomucin Antibodies.** ELISA with anti-ovomucin antibodies was also carried out by the method described in the previous paper (Tsuge *et al*., 1997). Each identical sample as described in ELISA with NDV was dissolved as described above, and each solubilized solution and its serially diluted solutions were coated on flat-bottomed microtiter plates. Each experiment was done in triplicate, and values were expressed as mean ± SD ($n = 3$).

**Heat Treatment.** The ovomucin solubilized with 10 mM carbonate buffer solution (pH 11.0) was dialyzed against each buffer solution of 0.1 M acetate buffer (pH 4.0), 0.1 M potassium phosphate buffer (pH 6.0 and 8.0), 0.1 M carbonate buffer (pH 10.0), and 0.1 M phosphate sodium hydroxide buffer (pH 12.0). Heat treatment of such solutions (about 200 μg/mL in each buffer) was conducted by placing 1.5 mL of the solution in test tubes (∅ 1.5 × 12.5 cm), which were positioned in a rack and immersed in a controlled-temperature water bath. The test tubes were then kept at fixed temperatures (60−100 °C) at 10 °C intervals for 10 min. Each sample was cooled immediately after heat treatment by placing the tubes in ice/water.

**Analytical Methods.** Protein was determined by the method of Lowry *et al*. (1951), in which bovine serum albumin was used as the standard. NeuAc was determined by the thiobarbituric acid method (Warren, 1959), in which NeuAc was used as the standard.

**Determination of NeuAc Content in Ovomucin Heated at pH 6.0.** NeuAc contents in ovomucin unheated and heated at pH 6.0 as described above were determined according to the method previously described.

**SDS−PAGE.** SDS−PAGE was performed using 7.5% gels according to the method of Laemmli (1970). The samples of unheated and heated ovomucin, RA-α-subunit, RA-β-subunit, and desialylated ovomucin were dissolved in buffers with and without 2-ME, heated at 100 °C for 3 min, and electrophoresed at a constant current of 17 mA. The gels were stained with CBB for the detection of protein and PAS reagent for the detection of carbohydrate.

**CD.** The CD spectra of the samples (about 200 μg/mL) unheated and heated at pH 6.0, 8.0, and 10.0 at 100 °C for 10 min between 200 and 250 nm were measured at 25 °C with a JASCO J-600 spectropolarimeter under continuous with dry nitrogen flashing with a 1 mm path length, strain-free UV-quartz cell, and mean residue ellipticities [θ] were calculated on the basis of average residue weight.

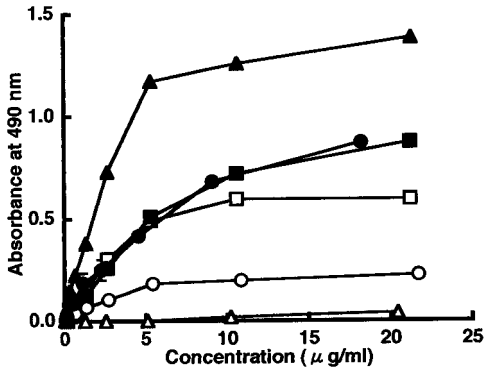


**Figure 1.** Bindings of chemically modified ovomucin and its subunits and desialylated ovomucin to NDV: (●) prepared ovomucin, (○) reduced and alkylated (RA)-α-subunit, (▲) RA-β-subunit, (△) desialylated ovomucin, (■) alkylated (A)-ovomucin, and (□) RA-ovomucin.

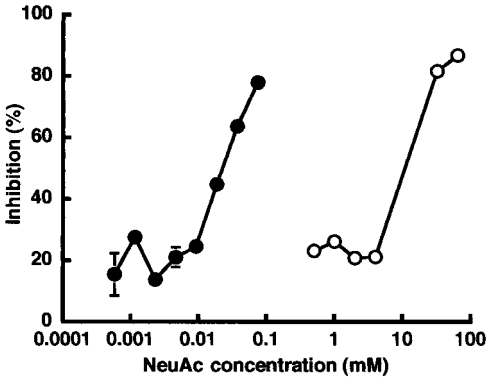


**Figure 2.** Effects of the preincubated free and bound type of NeuAc on binding of ovomucin to NDV: (●) bound NeuAc in ovomucin and (○) free NeuAc.

## RESULTS

Homogeneities of the ovomucin, RA-α- and RA-β-subunits, and desialylated ovomucin preparations were demonstrated by the absence of other proteins on SDS−PAGE before and after reduction of the disulfide bonds (data not shown). The mobility of β-subunit from desialylated ovomucin on SDS−PAGE was found to be slightly less than that from intact ovomucin, probably due to the loss of negative charge on the removal of NeuAc.

Figure 1 shows the bindings of ovomucin, A-ovomucin, RA-ovomucin, RA-α- and β-subunits, and desialylated ovomucin to NDV. The binding levels were found to be in the order of RA-β-subunit, ovomucin and A-ovomucin, RA-ovomucin, RA-α-subunit, and desialylated ovomucin. The bindings of ovomucin to NDV mainly depended on β-subunit. We found that NeuAc in ovomucin greatly contributed to the ability of ovomucin to bind to NDV, from the results which show that binding of desialylated ovomucin to NDV was at mere trace levels in the used concentration range.

Figure 2 shows the profiles of $I$(%) on the determinations by ELISA with NDV solutions which were preincubated with free and bound NeuAc. The amount of NeuAc required for 50% inhibition was about 25 mM in the free types and about 0.025 mM in the bound type. We demonstrated that the bound type of NeuAc, not the

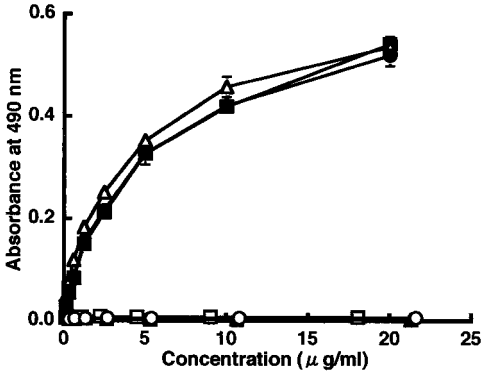


**Figure 3.** Bindings of chemically modified ovomucin and its subunits and desialylated ovomucin to anti-ovomucin antibodies. The symbols are the same as those in Figure 1.

free type, was mainly necessary for the activities of binding of ovomucin to NDV.

Figure 3 shows the binding of the same samples as indicated in Figure 1 to anti-ovomucin antibodies. Distinct from the abilities to bind to NDV in Figure 1, ovomucin, A-ovomucin, and desialylated ovomucin showed abilities to bind to anti-ovomucin antibodies. None of the RA-ovomucin and RA-α- and RA-β-subunits showed any binding abilities. The reduction and alkylation of disulfide bonds in ovomucin markedly altered its conformation and resulted in no ability to bind to anti-ovomucin antibodies. The binding of desialylated ovomucin to anti-ovomucin antibodies was also found to be slightly higher than that of ovomucin. This phenomenon might be due to the inhibitory effect of terminal NeuAc on the binding between ovomucin and anti-ovomucin antibodies.

When the ovomucin solution prepared by solubilization at pH 11.0 was dialyzed overnight against the buffers of pH 4.0, 6.0, 8.0, 10.0, and 12.0, in order to adjust the pH of its solution from 4.0 to 12.0, clear solutions could be obtained except for the sample treated at the pH 4.0 in which the precipitate occurred. Therefore, we used the solutions from pH 6.0 to 12.0, except for the sample at pH 4.0, to assess the symbiotic effects of the pH and temperature on their bindings to NDV and antibodies.

A given volume of each of the unheated and heated ovomucin solutions was applied to SDS−PAGE. In SDS−PAGE patterns of the samples solubilized with the alkaline buffer (pH 11.0) used in this study, both α- and β-subunits were detected in the presence of 2-ME, whereas the band, which was thought to be an aggregate of α-subunit, was slightly detected in its absence on the top of the running gel (data not shown). These results indicated that the disulfide bond in the solubilized ovomucin was slightly dissociated during the solubilizing procedure.

The representative electrophoretic patterns in the presence (Figure 4A,B) and absence (Figure 4C,D) of 2-ME on the samples unheated (25 °C) and heated (60, 80, and 100 °C) at pH 8.0 and 12.0, respectively, are shown in Figure 4. In the samples heated at pH 8.0 at 60 and 80 °C and at pH 12.0 at 60 °C, the stained bands of α- and β-subunits were clearly found in the same position as seen in the unheated sample stained with CBB (Figure 4A) and PAS (Figure 4B), but the intensities of the stained bands of α- and β-subunits, especially α-subunit, were decreased with the increase in heating temperatures. The results on the detection of β-subunit and no detection of α-subunit in the samples at pH 8.0

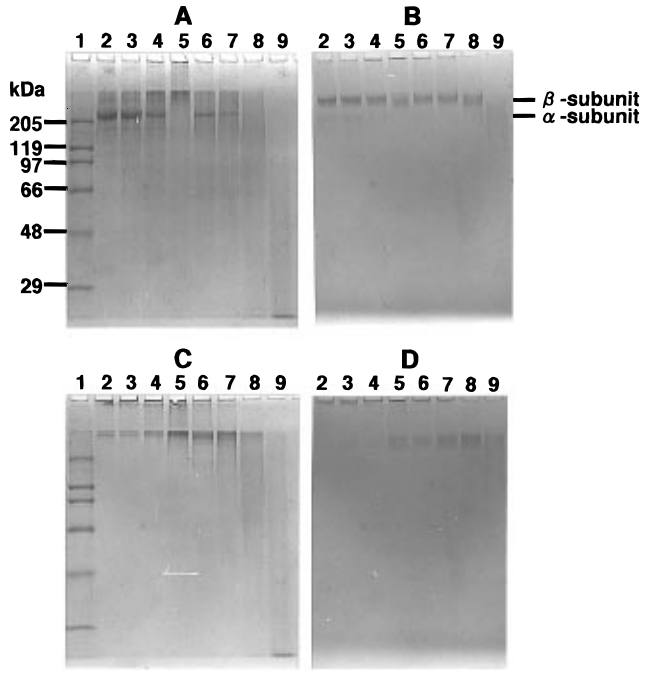


**Figure 4.** SDS−PAGE patterns of pH and heat treated-ovomucin. Electrophoresis was performed in the presence (A and B) and absence (C and D) of 2-ME, and gels were stained by CBB (A and C) and PAS (B and D): 1, molecular mass markers; 2, adjusted to pH 8.0, heat temperature of 25 °C; 3, adjusted to pH 8.0, heat temperature of 60 C°; 4, adjusted to pH 8.0, heat temperature of 80 °C; 5, adjusted to pH 8.0, heat temperature of 100 °C; 6, adjusted to pH 12.0, heat temperature of 25 °C; 7, adjusted to pH 12.0, heat temperature of 60 °C; 8, adjusted to pH 12.0, heat temperature of 80 °C; and 9, adjusted to pH 12.0, heat temperature of 100 °C.

at 100 °C and pH 12.0 at 25, 60, and 80 °C in SDS−PAGE in the absence of 2-ME also showed that disulfide bonds between α- and β-subunits and peptide bonds in intra-α-subunit were cleaved (Figure 4C,D). Further degradations of α- and β-subunits also occurred during heating at 100 °C at pH 12.0, as seen in the formation of peptide fragments. In the absence of 2-ME, the large particle complexes at the top of the gel, detected in the samples at pH 8.0, were almost never detected at pH 12.0 above 80 °C. This also indicated cleavages of disulfide and peptide bonds. The patterns of samples of pH 6.0 (60 and 80 °C) and pH 10.0 (25 and 60 °C) were similar to that of the unheated one of pH 6.0, whereas the cleavages of disulfide or peptide bonds were found in the other samples (data not shown). Thus, these cleavages and the degradations of both subunits themselves were considered to occur during heating, symbiotically with pH and temperature, greatly at pH 8.0 at 100 °C, at pH 10.0 at >80 °C, and at pH 12.0 at >25 °C.

The bindings of ovomucin treated under various heating conditions to NDV were determined by ELISA (Figure 5). The decreases in the binding to NDV in the heated samples occurred at temperatures higher than 70 °C at pH 6.0 and 8.0, and 60 °C at pH 10.0. The reactivity of the unheated sample (25 °C) at pH 12.0 was greatly decreased to less than 50% in comparison with that of the unheated samples at the other pH levels, and further decreases were found in binding to NDV with increasing temperatures.

The bindings of ovomucin treated under various heating conditions to anti-ovomucin antibodies were determined by ELISA (Figure 6). The binding activities

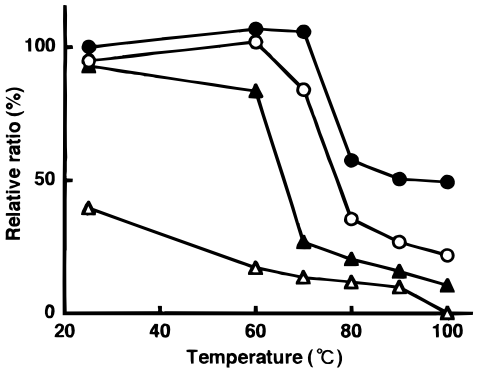


**Figure 5.** Effects of pH and heat treatment on binding of ovomucin to NDV. Values were expressed as the relative ratio of each unheated and heated ovomucin to the unheated one at pH 6.0: (●) pH 6.0, (○) pH 8.0, (▲) pH 10.0, and (△) pH 12.0.

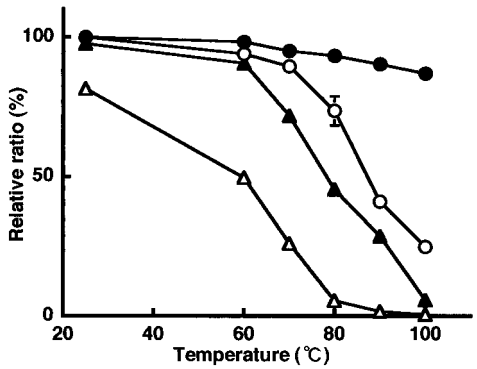


**Figure 6.** Effects of pH and heat treatment on binding of ovomucin to anti-ovomucin antibodies. Values were expressed as the relative ratio of each unheated and heated ovomucin to the unheated one at pH 6.0. The symbols are the same as those in Figure 5.

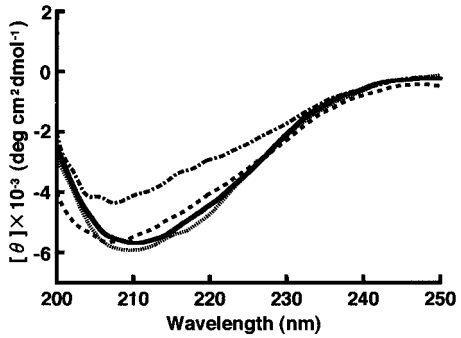


**Figure 7.** CD spectra of unheated and heated ovomucins: (heavy solid line) unheated ovomucin at pH 6.0; (hashmark line) heated ovomucin at pH 6.0 and 100 °C; (dashed line) heated ovomucin at pH 8.0 and 100 °C, and (dash-dot line) heated ovomucin at pH 10.0 and 100 °C.

at pH 6.0 decreased slightly with an increase in the heating temperature; above pH 8.0, they decreased greatly in the increasing order of pH, and were almost lost above 80 °C at pH 12.0.

CD spectra of the unheated and heated ovomucin solutions at pH 6.0 and those of heated ones at pH 8.0 and 10.0 are shown in Figure 7. The secondary structure of ovomucin was not greatly affected by heat treatment at pH 6.0 and 8.0, because the CD spectrum of the heated sample was almost similar to the spectrum of the unheated sample at pH 6.0. In the CD spectrum of the heated samples at pH 10.0, the partial destruction of secondary structure was found. The CD spectrum of ovomucin in this study was slightly different from that of the ovomucin solubilized with 1 M KCl (pH 6.5), as reported by Donovan *et al.* (1970). This was probably due to the differences in the ovomucin preparation method.

## DISCUSSION

In order to examine the heat stability of ovomucin, we should first clarify the conformational and biological properties of the intact ovomucin and its subunits. In this paper, the activities of binding of ovomucin and its subunits to NDV and anti-ovomucin antibodies were determined. However, preparation of intact α- and β-subunits could not be expected, because they might also contain the intrachain disulfide bonds in their individual subunit moieties. When each of the RA-α- and β-subunit preparations was used, higher and lower activities of binding to NDV were found in the β-subunit and α-subunit, respectively, and the binding activity of β-subunit was higher than that of intact and RA-ovomucin. On the other hand, almost no binding activities to anti-ovomucin antibodies were found in both its subunits (Figures 1 and 3). These results indicated that the binding to NDV was not greatly related to disulfide bonds in β-subunit, whereas the binding to anti-ovomucin antibodies was directly related to them. The three-dimensional structure of the protein moiety in ovomucin and its subunits which were formed by disulfide bonds might contribute to the binding activities between ovomucin or its subunits and anti-ovomucin antibodies. The carbohydrate chain moiety in mucin was reported to induce the formation of antibodies only with difficulty when the polyclonal antibody for such a protein was produced (Tytgat *et al.*, 1997). The prepared antibodies mainly depended on the tertiary structure of the protein moiety in ovomucin, but not on the carbohydrate chains containing NeuAc (Figure 3).

The NeuAc contents in ovomucin have been described to be 7.3% of the total weight (Tsuge *et al.*, 1997). The prepared desialylated ovomucin showed no ability to bind to NDV. The free NeuAc required about 1000 times as much bound-type NeuAc in ovomucin for 50% inhibition (Figure 2). This shows that the activity of binding of ovomucin to NDV is not expressed for the NeuAc alone, but by the carbohydrate chain containing terminal NeuAc bound to the protein moiety.

The contents and sites in the amino acid sequences of sulfhydryl and disulfide bond groups in ovomucin and its subunits have been not reported. In the previous paper, we indicated that the sulfhydryl and disulfide bond groups in ovomucin were not necessary for development of the hemagglutination inhibition activity of ovomucin to NDV (Tsuge *et al.*, 1996b). The activity of binding of A-ovomucin to NDV was almost the same as that of untreated ovomucin. These results suggest that the sulfhydryl groups in ovomucin were not involved in the activity of binding of ovomucin to NDV. On the other hand, the disulfide bonds contributing to the conformation of protein might have a small role in the development of its activity, because RA-ovomucin binding activity was slightly less than that of untreated ovomucin (Figure 1). It was found that the β-subunit moiety rather than the α-subunit moiety was necessary for binding to NDV, because the former contained about NeuAc levels 17 times higher than the latter (Tsuge *et al.*, 1997). Only carbohydrate chains containing NeuAc were reported to be receptors of NDV (Suzuki *et al.*, 1983, 1985).

When each pH-adjusted ovomucin solution was heated at the temperatures used in this study, no precipitation in any solutions was observed on the determination of protein concentration after centrifugation. Therefore, the criteria for the conformational and biological changes of heated ovomucin were obtained in the abilities of binding to NDV and anti-ovomucin antibodies.

It was unclear why the binding of ovomucin to NDV was greatly decreased by heat treatment at >80 °C at pH 6.0, because little change of its structure occurred. The changes in secondary structure of the unheated and heated ovomucins at pH 6.0 by CD analysis were observed at first to clarify this question. The secondary structure of ovomucin was not greatly affected by heat treatment (Figure 7). Therefore, we investigated whether NeuAc in ovomucin was degraded by heat treatment. The respective relative ratios of residual NeuAc contents in the heated samples at 60, 80, and 100 °C to the unheated one at pH 6.0 decreased to 94, 78, and 64%, respectively. The residual NeuAc contents in the samples heated under the pH conditions of 8.0−12.0 could not be correctly determined by the thiobarbituric acid method. When the ratios of the degradation of free-type NeuAc, heated at 60−100 °C for 10 min under the various pH conditions (pH 6−12) to the unheated one at pH 6.0 were also measured by the same method, NeuAc was degraded by heat treatments in the order of the combined increasings of pH and temperature. Although we do not think that this result precisely applies to the case of bound-type NeuAc, these results also supported the finding that the decreases in the binding to the NDV in the heated samples depended on the destruction of NeuAc residues in ovomucin. Kato *et al*. (1979) reported that the carbohydrate chains in ovomucin released from O-substituted serine and threonine by $\beta$-elimination with alkali were even further released by heat treatment. At alkaline pH, where heat treatments of ovomucin resulted in the loss of its ability to bind with NDV, the release and degradation of both NeuAc and the carbohydrate chain might occur.

In this study, the binding to NDV was determined to mainly analyze the structural alterations of heated ovomucin. Further studies are needed to elucidate whether the binding activity of ovomucin to NDV is related to its hemagglutination inhibition activity or neutralization activity to live virus.

It is well known that the cleavages of disulfide bonds in protein occur by heat treatment under the alkaline condition (Nashef *et al*., 1977). The SDS−PAGE patterns in the presence of the reductant of ovomucin heated at higher pH and temperature differed from those in its absence. The activities of binding of anti-ovomucin antibodies to heated ovomucin were much lower than that to unheated ones in the order of the combined increasings of pH and temperature (Figure 6), probably on the basis of the cleavages of intra- and intersubunit disulfide bonds. Thus, ovomucin was found to structurally and chemically degrade by heat treatments under pH >6.0, without any loss of solubility.

## CONCLUSION

The NeuAc residue in $\beta$-subunit greatly contributed to the binding of ovomucin to NDV, and inter- and intrasubunit disulfide bonds contribute to the binding of ovomucin to antibodies. From the changes in their binding abilities, SDS−PAGE patterns, and CD spectra of ovomucin heated at pH 6.0−12.0, the heated ovomucin was found to degrade biologically and chemically on the destruction of NeuAc, and on the cleavages of disulfide and peptide bonds in $\alpha$- and $\beta$-subunits, without any loss of solubility. Great destruction occurred by the heat treatments at pH 8.0 at 100 °C, at pH 10.0 at >80 °C, and at pH 12.0 at >25 °C.

## ABBREVIATIONS USED

NDV, newcastle disease virus; ELISA, enzyme-linked immunosorbent assay; NeuAc, *N*-acetylneuraminic acid; CD, circular dichroism; AMM, appearance molecular mass; A, alkylated; RA, reduced and alkylated; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CBB, Coomassie brillant blue; PAS, periodate-schiff; 2-ME, 2-mercaptoethanol.

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