Structural Features of Newcastle Disease Virus- and Anti-Ovomucin Antibody-Binding Glycopeptides from Pronase-Treated Ovomucin

Yoji Tsuge, Makoto Shimoyamada, and Kenji Watanabe*

The United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu 501-11, Japan

Pronase-treated ovomucin was applied on a Sephacryl S-400 column chromatography and separated into five fractions. The SDS−polyacrylamide gel electrophoretic pattern, and amino acid and carbohydrate compositions, of each of the obtained fractions were compared to those of ovomucin and its α- and β-subunits. Subsequently, bindings of each fraction to hen newcastle disease virus (NDV) and anti-ovomucin antibodies were estimated. It was found that the P1, P2, and P3 fractions from the β-subunit which were composed of O-glycoproteins, containing more or less clustered sialic acid moieties, had higher binding activity to NDV, while the peptide-rich P4 and P5 fractions mainly derived from the α-subunit had higher binding activity to the anti-ovomucin antibodies.

Keywords: *Ovomucin; newcastle disease virus; Pronase treatment*

INTRODUCTION

Hen egg white ovomucin, which accounts for about 3.5% of the protein in egg white, is a macromolecular and heavily-glycosylated glycoprotein, which consists of peptide-rich α-subunit (apparent molecular mass, AMM; 220 kDa) and carbohydrate-rich β-subunit (AMM; 400 kDa) (Itoh *et al*., 1987). The α-subunit released from ovomucin by disulfide reducing agents differs from β-subunit in that it maintains different amino acid and carbohydrate profiles (Donovan *et al*., 1970; Kato *et al*., 1973; Itoh *et al*., 1987).

Ovomucin serves physical functions such as maintaining the structure of egg white gel and vitelline membranes. On the other hand, it has been reported by Lanni and Beard (1948, 1949), Lanni *et al*. (1949), and Gottschalk and Lind (1949a,b) that ovomucin in hen egg white proteins has the greatest ability to inhibit hemagglutination by heated type B (Lee) influenza virus (IV). Our previous studies have also reported that ovomucin showed a high affinity for bovine rotavirus (RV), newcastle disease virus (NDV), and IV, which are the major pathogens for bovine, hen, and human, respectively. The appearance of hemagglutination inhibition (HI) activity of ovomucin against RV was accomplished by a macromolecule composed of α- and β-subunits, while that against NDV required β-subunit moiety only (Tsuge *et al*., 1996a,b).

A major goal in our ovomucin research is to relate the physical and biological properties of ovomucin to the chemical composition and three-dimensional structure of ovomucin and its subunits. The present paper describes the separation of fragments from Pronase-treated ovomucin and the characteristic chemical and structural properties of their fragments from the determinations of chemical composition and bindings to NDV and anti-ovomucin antibodies.

MATERIALS AND METHODS

Materials. Egg white was obtained from the White Leghorn hens within 24 h after laying. Pronase (from *Streptomyces griseus*), NDV (strain Ishii) and anti-NDV chicken serum, rabbit peroxidase-conjugated antibodies to chicken IgY (IgG), and chicken peroxidase-conjugated antibodies to mouse IgG were purchased from Calbiochem-Novobiochem Co., Kitasato Institute, and Chemicon International Inc., respectively.

Preparation of Ovomucin. Ovomucin was prepared by the method of Kato *et al*. (1970). Briefly, thick egg white was separated from total egg white by using a sieve, homogenized in a Waring blender for about 5 min, and diluted with 3 volumes of deionized water. The mixture was stirred for 1 h and then adjusted to pH 6.0 with 1 N HCl. After the mixture had been allowed to stand overnight at 4 °C, the crude ovomucin precipitates were washed with KCl solution (20 mg/mL) until the washing solution was free from proteins. The prepared ovomucin gel was dialyzed against 20 volumes of deionized water for 3 days and then lyophilized.

Preparation of Ovomucin Subunits. Ovomucin (40 mg) was dissolved in 4 mL of 10 mM potassium phosphate buffer (pH 7.0) containing 2-mercaptoethanol (2-ME, 20 μL/mL) and SDS (50 mg/mL). The reduced ovomucin was applied to gel filtration (Sephacryl S-400, 2.0 × 75.0 cm, Pharmacia LKB Products) to isolate the α- and β-subunits and eluted with 400 mL of 10 mM potassium phosphate buffer (pH 7.0) containing 2-ME (2 μL/mL) and SDS (5 mg/mL) with a flow rate of 0.5 mL/min. The eluent (5 mL/tube) was collected and assayed by the phenol−sulfuric acid method (absorbance at 490 nm) (Dubois *et al*., 1956) and by their absorbance at 280 nm. The obtained fractions corresponding to the α- and β-subunits were rechromatographed under the same respective conditions. Then each fraction was dialyzed against 50 volumes of 1 M urea, 1 M NaCl, and deionized water to remove SDS 12 h for 1 day, respectively (Rose *et al*., 1979), and then lyophilized.

Pronase Treatment of Ovomucin and Fractionation of Its Digests. Ovomucin (720 mg) was dispersed using a homogenizer in 200 mL of 10 mM potassium phosphate buffer (pH 8.5). Pronase (14.4 mg) was added to the suspension, and the mixture was incubated for 24 h at 37 °C. After incubation, the mixture was heated for 5 min at 100 °C. After cooling, the precipitate, which occurred during the incubation with Pronase, was excluded from the solution by centrifugation at 6000 rpm for 30 min. The supernatant was dialyzed against 15 volumes of deionized water for 2 days and then lyophilized. Its digests (50 mg) were dissolved in 4 mL of 10 mM potassium phosphate buffer (pH 8.5), applied to gel filtration (Sephacryl S-400, 2.0 × 73.0 cm), and eluted with the same buffer with a flow rate of 0.5 mL/min. The eluent (5 mL/tube) was collected and assayed by the phenol−sulfuric acid method (absorbance at 490 nm) and by the absorbance at 280 nm. The components in each tube were determined by SDS−polyacrylamide gel electrophoresis (SDS-PAGE). The fractions were dialyzed against 20 volumes of deionized water for 2 days and then lyophilized.

* To whom correspondence should be addressed (fax 058-293-2928).

S0021-8561(96)00753-4 CCC: $14.00

Preparation of Mouse Anti-Ovomucin Antibodies. Each of five mice (BALB/c, 6−8-week old, male; Shizuoka Agric. Assoc. for Lab. Animals) was immunized by intraperitoneal injection of 100 μg of ovomucin in 100 μL of phosphate-buffered saline (PBS) with Freund's complete adjuvant. Each mouse received two booster injections of 100 μg of antigen, 4 and 28 days after the first immunization. Blood was collected 38 days after the first injection. The serum was separated from the pooled blood and stored at −80 °C.

Enzyme-Linked Immunosorbent Assay (ELISA). Ovomucin and each Pronase-treated ovomucin fraction (about 100 μg) were dissolved in 1 mL of 50 mM carbonate buffer (pH 9.6). Flat-bottomed microtiter plates (Nunc) were coated overnight at 4 °C with 100 μL of these solutions diluted serially and washed with 10 mM potassium phosphate buffer (pH 7.2) containing 0.5 M NaCl and 50 mM Tween 20 (PBS-T). The plates were blocked with 150 μL of 1% bovine serum albumin (BSA) and incubated for 2 h at 37 °C. After the washing with PBS-T, 100 μL of NDV solution (0.2 mg/mL) was added to each well and the plates were incubated for 1 h at 37 °C. The plates were washed with PBS-T, 100 μL of anti-NDV serum (1:1000) was added to each well, and then plates were incubated for 1 h at 37 °C and washed with PBS-T. One hundred microliters of rabbit peroxidase-conjugated antibodies to chicken IgY (IgG) (1:5000) was added, and the plates were placed for 30 min at room temperature and washed. Then 100 μL of 0.1 M citric acid−0.2 M Na_2HPO_4 buffer containing O-phenylendiamine (0.4 mg/mL) and H_2O_2 (0.2 μL/mL) was added to each well, and the plates were placed for 30 min at room temperature in the dark. To stop the reaction, 100 μL of 3 N sulfuric acid was added to each well. The amount of peroxidase product in each well was quantitated spectrophotometrically at a wavelength of 490 nm. Each experiment was done in triplicate, and values were expressed in mean ±SD ($n = 3$).

In the experiment of the reactivity of each Pronase-treated ovomucin fractions to antibodies, mouse anti-ovomucin antibodies (1:500) and chicken peroxidase-conjugated antibodies to mouse IgG (1:1000) were used.

Analytical Methods. Protein was determined by the method of Lowry *et al*. (1951), in which BSA was used as the standard. Hexose was determined by the phenol−sulfuric acid method (Dubois *et al*., 1956), in which galactose was used as the standard. Hexosamine was determined by the modification of Elson-Morgen's method (Neuhaus and Letzring, 1957), in which galactosamine was used as the standard. Sialic acid was determined by the thiobarbituric acid method (Warren, 1959), in which *N*-acetylneuraminic acid (NeuAc) was used as the standard. Carbohydrate composition was determined by the method of Yamamoto *et al*. (1989). Briefly, dried samples (about 0.3 mg) were hydrolyzed for 6 h at 100 °C with 0.5 mL of 2 N (for hexose) or 4 N HCl (for hexosamine). After evaporation of HCl, the hydrolyzate was N-acetylated with 100 μL of acetic anhydride. Ten microliters of the coupling reagent (2-aminopyridine solution, 910 mg/mL) was added to the N-acetylated hydrolyzate, and the mixture was incubated for 20 min at 90 °C. After cooling, 10 μL of the reducing reagent (dimethylamine−borane solution, 60 mg/mL) was added to the mixture and incubated for 30 min at 90 °C. After cooling, the pyridylamino (PA) samples were washed with methanol, dissolved in distilled water, and then injected onto a Palpak Type A column (4.6 × 150 mm, Takara Biomedicals) on LC Module 1 (Waters). The column was kept at 65 °C and PA-monosaccharides were eluted with 0.6 M (for hexosamine) or 0.7 M (for hexose) potassium hydride−boric acid (pH 9.0)/acetonitrile (90/10, v/v). Amino acid analyses were carried out by high performance liquid chromatography (LC Module 1) of phenylisothiocyanate derivatives which had been previously hydrolyzed with 6 N HCl containing 1% phenol for 24 h at 110 °C. SDS-PAGE was done by the method of Laemmli (1970), using a separating gel of 7.5% acrylamide. The samples were dissolved in the buffer with or without 2-ME, heated for 3 min at 100 °C, and electrophoresed at a constant current of 17 mA. The gels were stained with Coomassie Brilliant Blue (CBB) for the detection of protein and Periodate−Schiff (PAS) reagent for the detection of carbohydrate.

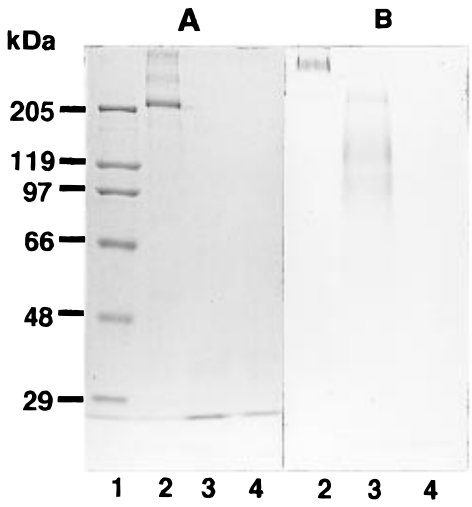

Figure 1. SDS-PAGE patterns of ovomucin and Pronase-treated ovomucin fractions. A: CBB stain; B: PAS stain. Lane 1 is a molecular weight standard. Lane 2 is prepared ovomucin. Lanes 3 and 4 are supernatant and precipitate fractions, respectively, obtained by centrifugation of Pronase-treated ovomucin.

RESULTS

As shown in Figure 1, ovomucin preparation was free of contaminating proteins as detected by CBB-staining as well as PAS-staining in SDS-PAGE (Figure 1A, lane 2, and Figure 1B, lane 2). The β-subunit displayed a low mobility on SDS-PAGE (AMM: 400 kDa) and was stained intensely with PAS-staining (Figure 1B, lane 2) and very weakly, if at all, with CBB-staining (Figure 1A, lane 2), whereas the α-subunit on SDS-PAGE (AMM: 220 kDa) was stained intensely with CBB-staining (Figure 1A, lane 2).

In the course of the Pronase treatments, the dispersed ovomucin suspension once turned to a clear solution, and then the precipitation partially occurred in the course of digestion. Therefore, the supernatant and precipitate were separated by centrifugation after the Pronase treatment, dialyzed, and lyophilized. The dry weight of precipitate corresponded to 10% for that of supernatant. When two fractions were applied to SDS-PAGE, the main three broad bands in the range from 90 to 220 kDa, which consisted of carbohydrate-rich fragments stained with PAS, were detected only in the supernatant fraction (Figure 1B, lane 3), and peptide-rich fragment of the relatively low molecular weight was observed in the samples of supernatant and precipitate (Figure 1A, lanes 3 and 4). These patterns in the presence of 2-ME showed virtually no change in the absence of 2-ME (data not shown), suggesting that carbohydrate-rich fragments in Figure 1 (B, lane 3) contained digests of β-subunit only, because the α-subunit linked to the β-subunit with disulfide bonds. It was also found that there were neither residual α- nor β-subunits in the Pronase-treated sample. From these results, it was estimated that digests from β-subunit were mainly contained in the supernatant fraction, and those from α-subunit were contained in both the supernatant and precipitate.

The elution profile of Pronase-treated ovomucin on gel filtration is shown in Figure 2. Three major peaks (one peak from measuring of absorbance at 280 nm and two peaks at 490 nm) were revealed. The first large peak from 490 nm was separated into three fractions on SDS-PAGE patterns as shown by the bars (P1−P3) in Figure 2, and the other peaks from 490 nm and 280

Table 1. Amino Acid Compositions of Ovomucin Subunits, Pronase-Treated Ovomucin, and Its Gel Filtration Fractions[a]

				Pronase-treated ovomucin						
	ovomucin	α-subunit	β-subunit	Sup[b]	Ppt[c]	P1	P2	P3	P4	P5
Asx	10.4	11.9	4.8	6.9	11.5	3.3	1.4	2.8	11.6	13.8
Glx	9.9	10.9	7.9	7.8	11.0	6.5	5.6	5.8	12.0	9.9
Ser	9.4	7.7	18.9	20.8	7.9	22.9	27.4	22.9	9.0	11.8
Gly	6.8	7.4	3.2	4.2	9.9	3.5	1.1	3.6	7.3	8.4
His	2.2	2.1	1.1	0.9	2.3	2.2	1.0	0.9	1.4	1.1
Arg	2.2	2.3	1.6	1.5	3.7	2.1	1.0	2.1	2.2	2.9
Thr	8.5	7.8	14.8	13.5	6.1	17.2	20.1	18.6	7.8	7.9
Ala	5.2	4.8	7.7	5.8	9.0	6.1	7.6	6.5	6.2	8.6
Pro	9.2	5.9	9.0	10.0	3.0	10.0	11.0	9.2	5.6	7.0
Tyr	3.0	3.5	1.7	1.0	2.8	1.2	0.4	1.2	3.2	2.2
Val	6.3	6.6	4.9	4.1	8.4	5.8	4.1	4.7	5.7	5.1
Met	2.2	2.2	4.4	4.8	2.4	2.0	2.2	1.5	1.6	1.9
1/2Cys	4.0	5.2	1.4	3.4	1.0	1.3	1.1	1.4	6.4	4.0
Ile	4.6	5.2	4.1	2.9	5.2	3.4	2.9	3.5	4.4	3.4
Leu	6.5	6.2	7.3	5.6	6.8	6.4	7.6	7.9	5.9	5.7
Phe	3.7	4.0	2.6	1.7	3.6	2.4	1.8	2.6	3.3	2.2
Lys	5.9	6.4	4.6	5.2	5.3	3.7	3.6	5.1	6.5	4.1
R[d]	1.3	0.9	3.4	3.0	0.8	5.1	8.3	5.9	0.9	1.1

[a] Values represented in mole percent. [b,c] Supernatant (Sup) and precipitate (Ppt) obtained by centrifugation of Pronase-treated ovomucin. [d] Ser + Thr + Pro/Asx + Glx. Tryptophan was not determined.

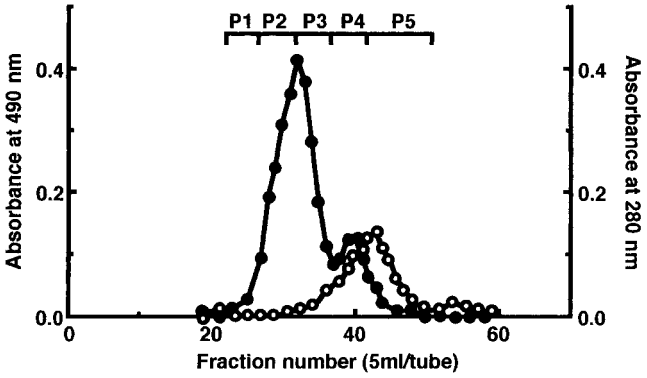

Figure 2. Sephacryl S-400 column chromatography of Pronase-treated ovomucin: ●, absorbance at 490 nm by the phenol−sulfuric acid method for the detection of carbohydrates; ○, absorbance at 280 nm for the detection of proteins.

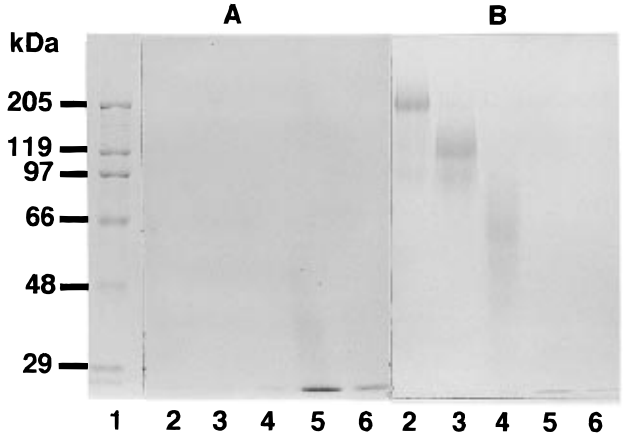

Figure 3. SDS-PAGE patterns of gel filtration fractions of Pronase-treated ovomucin. A: CBB stain; B: PAS stain. Lane 1 is a molecular weight standard. Lanes 2, 3, 4, 5, and 6 are P1, P2, P3, P4, and P5, respectively.

nm were separated into two fractions (P4 and P5), respectively. Their relative ratios in dry weight of the P1 to P5 fractions after dialysis and lyophilization were 7, 34, 26, 17, and 16%, respectively.

These fractions were applied to SDS-PAGE, as shown in Figure 3. The high molecular mass fragments seen in the patterns of P1, P2, and P3 fractions were found to be digests from β-subunit, because these fragments were only detected in PAS-staining (Figure 3B, lanes 2, 3, and 4) and the patterns evidenced almost no change in the absence of 2-ME (data not shown). The molecular masses of the main fragments in P1 and P2 fractions were about 220 and 120 kDa, with a minor fragment of about 100 kDa in SDS-PAGE, respectively. On the other hand, CBB-stained broad band (about 30−50 kDa) in P4 fraction and CBB- and PAS-stained bands of relatively low molecular weight (below 29 kDa) were detected in P4 and P5 fractions (Figure 3, parts A and B, lanes 5 and 6).

Subsequently, amino acid and carbohydrate compositions of the obtained fractions were compared to those of ovomucin and its subunits in order to clarify whether these fractions were derived from either ovomucin subunit. Amino acid compositions of supernatant and precipitate fractions from Pronase-treated ovomucin were nearly identical with those of β- and α-subunits, respectively, as shown in Table 1. This corroborated the aforementioned results that the precipitate was mainly derived from α-subunits. The characteristic of the amino acid composition of α- and β-subunits has been expressed as the difference of the ratio (R in Table 1) of Ser, Thr, and Pro contents to Asx and Glx contents (Itoh *et al.*, 1987). Each ratio in P1−P5 fractions was compared to those in ovomucin subunits. The ratios in P1, P2, and P3 fractions were much higher than those in β-subunit. These fractions were thought to contain a heavily O-glycosylated region because O-linked oligosaccharides bind to Ser and Thr. For most apomucins, a repeated amino acid sequence rich in Ser or Thr provides the sites for attachment of the majority of O-linked oligosaccharides (Gum, 1995). On the other hand, the ratios in P4 and P5 fractions were equal to and slightly higher than in α-subunit, respectively. Thus, P4 and P5 fractions mainly contain digests from α-subunit.

From the elucidation of the carbohydrate compositions shown in Table 2, it was found that P2 fraction consisted of only O-linked oligosaccharides, including sialic acid (NeuAc), because it did not contain mannose existing in N-linked oligosaccharides, whereas P1 and P3 fractions consisted of O-linked oligosaccharides with the minor part of N-linked oligosaccharides because they contained a small amount of mannose. Mucins have also been generally reported to contain a small propor-

Table 2. Chemical Compositions of Ovomucin Subunits, Pronase-Treated Ovomucin, and Its Gel Filtration Fractions[a]

	ovomucin	α-subunit	β-subunit	Pronase-treated ovomucin	P1	P2	P3	P4	P5
protein	65.3	90.8	33.3	40.7	11.8	6.4	11.1	71.2	64.6
carbohydrate	34.7	9.2	66.7	59.3	88.2	93.6	88.9	28.8	35.4
GalNAc	3.3	0	7.2	9.7	10.7	11.4	12.3	5.6	4.1
GlcNAc	11.0	3.1	11.4	17.9	26.7	18.5	26.9	10.2	12.8
Gal	10.0	1.1	29.3	27.5	40.2	35.3	34.0	8.5	5.8
Man	3.1	3.9	0.6	2.5	0.3	0	0.1	4.3	12.6
NeuAc	7.3	1.1	18.2	8.7	10.3	28.4	15.6	0.2	0.2

[a] Values represented in weight percent.

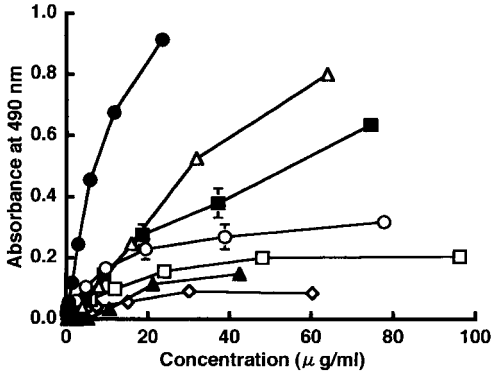

Figure 4. Bindings of Pronase-treated ovomucin and its gel filtration fractions to NDV: ●, prepared ovomucin; ○, Pronase-treated ovomucin; ▲, P1; △, P2; ■, P3; □, P4; ◇, P5.

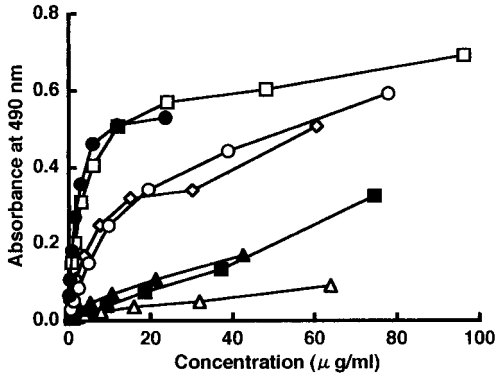

Figure 5. Bindings of Pronase-treated ovomucin and its gel filtration fractions to anti-ovomucin antibodies. The symbols are the same as those in Figure 4.

tion of N-linked oligosaccharides, as evidenced from the occurrence of mannose, usually at 1% or less of the total carbohydrate (Corfield *et al*., 1991). On the other hand, P4 and P5 fractions contained N- and O-linked oligosaccharides derived from the α- and β-subunits, because they contained N-acetylgalactosamine which only exists in the β-subunit. Eventually, P1, P2, and P3 fractions were found to be derived from the β-subunit, and P4 and P5 fractions mainly derived from the α-subunit. These results correspond to those for the amino acid compositions mentioned above, except for the fact that P4 and P5 fractions also contain fragments from the β-subunit at lower levels. When 1 mL of 50 mM carbonate buffer (pH 9.6) was added to the lyophilized P4 and P5 fractions, respectively, P4 fraction dissolved completely, but P5 fraction incompletely. We used the supernatant removed from the precipitate by centrifugation for each analysis. Thus, the carbohydrate content of P5 fraction may be higher than that of P4 fraction.

Bindings of the prepared fractions to NDV were evaluated by ELISA as shown in Figure 4. Binding of ovomucin to NDV was greatly decreased by the Pronase treatment, suggesting that the peptide moiety is also responsible for binding to NDV, and carbohydrate-poor peptides are involved in inhibition of the binding of carbohydrate-rich peptides to the plastic plates. Bindings of P2 and P3 fractions to NDV were higher than those of Pronase-treated ovomucin. It was interesting that the binding activity of P1 fraction was relatively lower, and the P4 and P5 fractions as well as P1 fraction also showed the low binding activity in the measured concentrations.

Bindings of the prepared fragments to anti-ovomucin antibodies were evaluated by ELISA, as shown in Figure 5. P4 fraction greatly bound to antibodies as well as ovomucin, and P5 fraction also bound to antibodies at slightly lower levels than P4 fraction. On the other hand, bindings of carbohydrate-rich fractions to antibodies were in the order of P1, P3, and P2 fractions, and less than half of that of P4 fraction, showing the recognition by anti-ovomucin antibodies of the β-subunit at lower levels than that of α-subunit. P1 and P3 fractions probably contained peptide-rich regions which could react more with antibodies than the P2 fraction.

In this study, we carried out heat treatment (100 °C, 5 min) for inactivation of Pronase. The effects of this heat treatment on the binding to NDV and to antibodies were examined using the Pronase-treated ovomucin (data not shown). There was lower binding to NDV and almost no change in bindings to antibodies with heated samples compared with unheated ones. The binding to NDV in the prepared fraction was presumably less than that of the corresponding unheated samples. However, the aforementioned obtained results are justified, because the binding of NDV among the prepared fractions was compared with the residual binding activities.

DISCUSSION

The ovomucin which was prepared by general methods and lyophilized could not be greatly solubilized with distilled water and neutral buffer solution. We previously reported that when ovomucin was digested with pepsin, trypsin, and Pronase, respectively, Pronase had the highest activity to solubilize the ovomucin on the production of some fragments through digestion (Tsuge *et al*., 1996b). Moreover, α- and β-subunits prepared after the reductions of the intra- and inter-disulfide bonds did not necessarily show the original biological functions corresponding to native ovomucin (Tsuge *et al*., 1996b). So, we used Pronase to prepare fragments having biological activities, because the procedure uses was thought to be effective in the investigation of the partial structure and functions of ovomucin.

Chemical compositions of ovomucin and its subunits have been studied by a number of researchers (Donovan *et al*., 1970; Robinson and Monsey, 1971; Yang and Gardner, 1972; Kato *et al*., 1973; Itoh *et al*., 1987). The values reported in these studies differed, especially in carbohydrate compositions, probably due to differences in the preparation method and the purity of ovomucin.

However, the chemical compositions in the present study were generally identical to those of Robinson and Monsey (1971) and Itoh *et al.* (1987). Fucose- (Donovan *et al.*, 1970) and sulfate-containing oligosaccharide (Donovan *et al.*, 1970; Robinson and Monsey, 1971; Kato *et al.*, 1973) have been reported to exist in ovomucin. However, we did not measure them because their amounts were negligible. Kato *et al.* (1973) reported that heavily glycosylated fractions obtained from ovomucin treated with Pronase and papain, respectively, contained sialic acid of 20% (w/w). We consider that these fractions correspond to the mixture of P1, P2, and P3 fractions in our study.

The results of SDS-PAGE patterns of ovomucin treated with Pronase indicated that incubation of ovomucin with Pronase resulted in the occurrence of three major fragments of 220 kDa (P1 fraction) and 120 and 100 kDa (P2 fraction) with some glycopeptides derived from β-subunit in ovomucin. Additionally, when the isolated P1 fraction was incubated with Pronase as described above, the main fragment of 60 kDa in the P3 fraction could be found in SDS-PAGE (data not shown). From these results, we supposed that the β-subunit consisted of a 220 kDa fragment (P1 fraction) and 120 and 100 kDa fragments (P2 fraction) showing with some carbohydrate-poor peptides.

It is generally accepted that mucin occupies a large region in the central part of the polypeptide, containing virtually all O-linked oligosaccharides linked to repeated amino acid sequences, and the central part of mucin is resistant to degradation by proteinases, while the protruding N- and C-terminal parts of the polypeptide are sensitive to proteolysis. The length of these repeated sequences as well as their number has been found to vary among the known mucin sequences (Strous and Dekker, 1992). So, it would appear that β-subunit contained O-glycopeptide chains having molecular masses of 220, 120, and 100 kDa in the central part, each linked with some peptides, and also some O- and N-linked glycopeptides in both the N- and C-terminal parts of the polypeptide. However, the arrangement of the fragments obtained in this study in ovomucin and its subunits remains an open question for further investigation. The experiment of time course digestion with Pronase is required.

Like most other mucins, the ovomucin contains aggregate content of Thr, Ser, and Pro of over 50%, which may serve as a region of abundant O-glycosylation. Also ovomucin is substantially similar to "typical" mucins, because it contains a high proportion of N-linked oligosaccharides, especially in α-subunits, for example, as seen in rat and human intestinal mucin which have link glycopeptide (118 kDa) (Roberton *et al.*, 1989).

Viral-ligands of NDV have been reported to recognize carbohydrate chains containing sialic acid as their receptor (Suzuki *et al.*, 1983, 1985). Our previous report indicated that binding of ovomucin to NDV was referable to the region of β-subunit and its binding decreased by the Pronase treatment, as estimated by HI test, and that the appearance of HI activity of ovomucin was not accomplished by the total amount of sialic acids alone but involved other factors (Tsuge *et al.*, 1996b). In the present investigation, the clustered sialic acid moieties in O-linked oligosaccharides bound to polypeptide were important for the binding between ovomucin and NDV, because the binding of P2 and P3 fractions, which contained the regions with the highest sialylate levels, was higher than that of P1 fraction and so on. Willoughby (1993) reported that higher avidity binding by rotaviruses could be explained by multivalent binding to clustered sialic acid moieities. Given the lower binding of P1 fraction than P2 and P3 fractions, we also suggest that there was a lower proportion of regions contributing to the binding to NDV in fragments of P1 fraction. If the concentration is expressed not by weight (in Figure 4) but by mole, the differences in the NDV-binding between P2 and P1 fractions would diminish because the molecular mass of the main fragment in P1 fraction was nearly twice as high as that of P2 fraction. It appears only natural that P4 and P5 fractions, containing less sialic acid, bound to NDV at lower levels than did P2 and P3 fractions. Thus, it might be concluded that the β-subunit consisted of heavily and slightly sialylated glycoprotein fragments. However, it is unclear why the binding activity of P1 fraction was lower than P4 fraction. The differences on the binding of each fraction to the plastic plates must be further investigated.

When the anti-ovomucin antibodies were prepared, we expected it would be generally easier to obtain antibodies against the non- or low-glycosylated part of the polypeptide, rather than carbohydrate-recognizing antibodies (Tytgat *et al.*, 1995). So, it would be natural to find lower binding to anti-ovomucin antibodies in Pronase-treated ovomucin which contained the carbohydrate-rich fractions higher than ovomucin, and similarly, the lower binding of P2 in which carbohydrate chains occupied a large part (Figure 5). The fact that bindings of P1 and P3 fractions to antibodies were slightly higher than of P2 fraction might imply higher levels of naked polypeptide chains in the former two fractions.

In this study, we used Pronase containing various proteases in order to obtain protease-insensitive fragments. However, we could assess the existence of polypeptide chains in P3 and P4 fractions which could react, although to a lesser degree, with the anti-ovomucin antibodies. It is also interesting that heat treatments after Pronase treatment did not significantly affect the binding to anti-ovomucin antibodies, suggesting that the conformation of the polypeptide in ovomucin is not so important for antigenic reactivity.

LITERATURE CITED

Corfield, A. P.; Corfield, C. D. A.; Veh, R. W.; Wagner, S. A.; Clamp, J. R.; Schauer, R. Characterization of the major and minor mucinous glycoproteins from bovine submandibular gland. *Glycoconjugate J.* **1991**, *8*, 330−339.

Donovan, J. W.; Davis, J. G.; White, L. M. Chemical and physical characterization of ovomucin, a sulfated glycoprotein complex from chicken eggs. *Biochim. Biophys. Acta* **1970**, *207*, 190−201.

Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Calorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350−356.

Gottschalk, A.; Lind, P. E. Ovomucin, a substrate for the enzyme of influenza virus. I. Ovomucin as an inhibitor of hemagglutination by heated Lee virus. *Br. J. Exp. Pathol.* **1949a**, *30*, 85−92.

Gottschalk, A.; Lind, P. E. Product of interaction between influenza virus enzyme and ovomucin. *Nature* **1949b**, *164*, 232−233.

Gum, J. R., Jr. Human mucin glycoproteins: varied structures predict diverse properties and specific functions. *Biochem. Soc. Trans.* **1995**, *23*, 795−799.

Itoh, T.; Miyazaki, J.; Sugawara, H.; Adachi, S. Studies on the characterization of ovomucin and chalaza of the hen's egg. *J. Food Sci.* **1987**, *52*, 1518−1521.

Kato, A.; Nakamura, R.; Sato, Y. Studies on changes in stored shell eggs. Part VI. Changes in the chemical composition of ovomucin during storage. *Agric. Biol. Chem.* **1970**, *34*, 1009−1013.

Kato, A.; Fujinaga, K.; Yagishita, K. Nature of the carbohydrate side chains and their linkage to the protein in chicken egg white ovomucin. *Agric. Biol. Chem.* **1973**, *37*, 2479−2485.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Lanni, F.; Beard, J. W. Inhibition by egg white of hemagglutination by swine influenza virus. *Proc. Soc. Exp. Biol. Med.* **1948**, *68*, 312−313.

Lanni, F.; Beard, J. W. Interaction of swine influenza virus and egg-white inhibitor of virus hemagglutination. *Proc. Soc. Exp. Biol. Med.* **1949**, *71*, 116−117.

Lanni, F.; Sharp, D. G.; Eckert, E.; Dillon, E.; Beard, O.; Beard, J. W. Egg white inhibitor of influenza virus hemagglutination. I. Preparation and properties of semipurified inhibitor. *J. Biol. Chem.* **1949**, *179*, 1275−1287.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

Neuhaus, O. W.; Letzring, M. Determination of hexosamines in conjunction with electrophoresis on starch. *Anal. Chem.* **1957**, *29*, 1230−1233.

Roberton, A. M.; Mantle, M.; Fahim, R. E. F.; Specian, R. D.; Bennick, A.; Kawagishi, S.; Sherman, P.; Forstner, J. F. The putative "link" glycopeptide associated with mucins glycoproteins. *Biochem. J.* **1989**, *261*, 637−647.

Robinson, D. S.; Monsey, J. B. Studies on the composition of egg-white ovomucin. *Biochem. J.* **1971**, *121*, 537−547.

Rose, M. C.; Lynn, W. S.; Kaufman, B. Resolution of the major components of human lung mucosal gel and their capabilities for reaggregation and gel formation. *Biochemistry* **1979**, *52*, 4030−4037.

Strous, G. J.; Dekker, J. Mucin-type glycoproteins. *Crit. Rev. Biochem. Mol. Biol.* **1992**, *27*, 57−92.

Suzuki, Y.; Suzuki, T.; Matsumoto, M. Isolation and characterization of receptor sialoglycoprotein for hemagglutinating virus of Japan (Sendai virus) from bovine erythrocyte membrane. *J. Biochem.* **1983**, *93*, 1621−1633.

Suzuki, Y.; Suzuki, T.; Matsunaga, M.; Matsumoto, M. Gangliosides as Paramyxovirus receptor. Structural requirement of sialo-oligosaccharides in hemagglutinating virus of Japan (Sendai virus) and newcastle disease virus. *J. Biochem.* **1985**, *97*, 1189−1199.

Tsuge, Y.; Shimoyamada, M.; Watanabe, K. Binding of egg white proteins to viruses. *Biosci., Biotechnol., Biochem.* **1996a**, *60*, 1503−1504.

Tsuge, Y.; Shimoyamada, M.; Watanabe, K. Differences in hemagglutination inhibition activity against bovine rotavirus and hen newcastle disease virus based on the subunit in hen egg white ovomucin. *Biosci., Biotechnol., Biochem.* **1996b**, *60*, 1505−1506.

Tytgat, K. M. A. J.; Klomp, L. W. J.; Bovelander, F. J.; Opdam, F. J. M.; Wurff, A. V.; Einerhand, A. W. C.; Buller, H. A.; Strous, G. J.; Dekker, J. Preparation of Anti-mucin polypeptide antisera to study mucin biosynthesis. *Anal. Biochem.* **1995**, *226*, 331−341.

Warren, L. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **1959**, *234*, 1971−1975.

Willoughby, R. E. Rotaviruses preferentially bind O-linked sialylglycoconjugates and sialomucins. *Glycobiology* **1993**, *3*, 437−445.

Yamamoto, S.; Hase, S.; Fukuda, S.; Sano, O.; Ikenaka, T. Structure of the sugar chains of interferon-γ produced by human myelomonocyte cell line HBL-38. *J. Biochem.* **1989**, *105*, 547−555.

Yang, L. L.; Gardner, F. A. Preparation of egg white ovomucin by gel filtration. *J. Food Sci.* **1972**, *37*, 8−11.

Received for review October 3, 1996. Revised manuscript received March 31, 1997. Accepted April 14, 1997.[⊗]

JF960753Y

[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1997.