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Purification and characterization of the Hemagglutinin-Neuraminidase of *Porcine rubulavirus* LPMV

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The Hemagglutinin-Neuraminidase (HN) from the LPMV strain of *Porcine rubulavirus* was purified from virions by ultracentrifugation in a continuous 20–60% sucrose gradient and by ion exchange chromatography. The HN is a glycoprotein of 66 kDa constituted by 50.5, 13.3 and 13.6% of non polar, uncharged polar, and charged polar amino acids, respectively. The HN contains 4% of carbohydrates, its glycannic portion is constituted by Man, Gal, GlcNAc, GalNAc, and Neu5Ac in 3:3:4:1:1 molar ratios. The HN possesses hemagglutinating activity in the presence of erythrocytes from several animal species, including human ABO, and treating the erythrocytes with neuraminidase or pronase abolishes this activity. The binding specificity of the purified HN was determined by hapten inhibition assays, indicating that the hemagglutinating activity of the HN is specific for sialic acid and Neu5Acα2,3Gal-containing structures.

Keywords: **Paramyxovirus, *Porcine rubulavirus,* Hemagglutinin-Neuraminidase, neuraminic acid, Neu5Ac(α2,3)Gal specific hemagglutinin.**

Introduction

The *Porcine rubulavirus* designated La Piedad Michoacán, México, virus (LPMV) has been identified as the causal agent of blue eye disease in swine [1], an endemic disease in México characterized by nervous, respiratory, and reproductive signs, with corneal opacity [2,3]. Analysis of nucleotide and amino acid sequences have shown that LPMV is closely related to mumps virus and simian virus 5 [4,5]. Its homology with the mumps virus led to classify LPMV as a species in the genus *Rubulavirus,* subfamily *Paramyxovirinae,* and family *Paramyxoviridae* [6]. Rubulaviruses possess two surface glycoproteins, the Fusion (F) and Hemagglutinin-Neuraminidase (HN) [7]. HN is involved in the recognizing and binding of cell receptors, which occur through interaction with sialic acid (N-acetyl-neuraminic acid, Neu5Ac) molecules; in addition, HN collaborates with F glycoprotein in the fusion of viral and cellular membranes [8]. In previous reports, we showed that Porcine rubulavirus recognizes Neu5Ac-containing structures on the surface of erythrocytes and tissue-culture cells [9]. We also showed that expression of Neu5Acα2,3Galactose moieties determines the cell and tissue susceptibility to *Porcine rubulavirus* infection [10]. In addition, we have found that the immune response of infected pigs is predominantly directed towards HN glycoprotein [11]. These findings indicate the relevance of HN as a potential target for the design of diagnosis and antiviral agents against *Porcine rubulavirus.* In this paper we describe the purification method and physicochemical properties of the *Porcine rubulavirus* LPMV HN glycoprotein and its sugar specificity aimed at elucidating the virus-cell receptor interactions.

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Material and methods

Cells and virus

The porcine kidney PK15 cell line was cultured in Eagle's minimal essential medium (MEM) supplemented with 2% fetal bovine serum. We inoculated 1.3×10^7 $TCID_{50}$ (Tissue culture infective dose 50%) of the *Porcine rubulavirus* LPMV (strain Michoacán/1985) into confluent cell cul-

tures, and incubated 1 h at 37°C, the supernatant was discarded and cells were washed with PBS. Fresh MEM was added to the cells and incubated for 72 h until cytopathic effects were apparent. Infected cells were freeze-thaw disrupted and supernatants were clarified by centrifugation at 3,200 rpm, 45 min. Virus in the supernatants were concentrated by precipitation with 10% (w/v) polyethylene glycol 8,000 during 3 h at 4°C under gentle stirring; then, centrifuged at 3,500 g, 20 min. The pellet was suspended and dialyzed in TEN (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, and pH 7.4).

Isolation of HN

Virions were disrupted by 3 cycles of ultrasound at 14 Hz for 30 sec, each. The samples were centrifuged in a discontinuous 30–60% sucrose gradient at 100,000 *g* for 4 h; then, 1ml fractions were obtained and dialyzed against TEN solution. Hemagglutinating activity and protein concentration of each collected fraction were determined. Further purification of HN glycoprotein was performed by ion exchange chromatography as follows: Fractions with the highest hemagglutinating activity were applied onto a prepackaged mono P 5/5 mm HR column (Pharmacia, Uppsala, Sweden). This column was previously equilibrated with 50 mM Bis-Tris buffer, pH 7.5, at a flow rate of 1 ml/min with a maximal pressure of 40 bars, in a 60-min program using the FPLC system (Pharmacia). The protein was then eluted with a 0–1 M NaCl stepwise gradient in Bis-Tris buffer; 1 ml fractions were collected and their optical density was monitored at 280 nm. Each eluted peak was exhaustively dialyzed against distilled water and was freeze-dried for further analysis.

Analytical methods

Protein concentration was determined by the method of Bradford [12] with Coomassie blue R-250, using bovine serum albumin as standard. Carbohydrate concentration was determined by the method of Dubois et al. [13], using lactose as standard. Carbohydrate composition analysis was performed by methanolysis in the presence of meso-inositol (Sigma Chem., St. Louis, MO, USA) as internal standard. The per-O-trimethyl silylated methyl glycosides (after N-re-acetylation) were analyzed by gas-chromatography using a 5% Silicone OV 210 capillary column (Applied Science Lab., Buffalo, NY), in a Varian 2100 gas chromatograph (Orsay, France), as described by Zanetta et al. [14]. Polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a slab gel apparatus according to the method of Laemmli [15].

The amino acid analysis of the purified HN from LPMV was determined as follows: a 100 µg sample was hydrolyzed under vacuum with 2 ml of 6 N HCl at 110°C in sealed tubes for 24, 48, and 72 h. The samples were analyzed in an automatic amino acid analyzer Durrum 500, according to Bidlingmeyer et al. [16], using Nor-leucine as internal standard.

Hemagglutinating activity

Erythrocytes from different animal species were obtained from the animal facilities at the Faculty of Medicine, UNAM. Human erythrocytes from healthy donors were obtained from the Central Blood Bank, IMSS, México. Hemagglutinating activity was assayed in 96-wells U-bottomed microassay plates (NUNC, Denmark) by the two-fold serial dilution procedure [9]. Hemagglutinating activity was also tested with 1% suspensions of untreated, neuraminidase- or pronase-treated erythrocytes dissolved in PBS (0.01 M sodium phosphate, 0.14 M sodium chloride, pH 7.2). For neuraminidase treatment, 0.5 ml of packed erythrocytes were incubated with 0.1 U of *Clostridium perfringens* neuraminidase at 37°C for 30 min. For pronase treatment, 0.5 ml of packed erythrocytes were incubated with 100 µg of *Streptomyces griseus* protease at 37°C for 30 min. The titer was reported as the inverse of the last dilution with hemagglutinating activity.

Sugar specificity

Carbohydrates, glycosides, and glycoproteins used in this study were from Sigma; Kdn (2-Keto-3-deoxynononic acid), sialylα2,3- and α2,6-lactosamine were donated by Prof. Gerard Strecker (Université des Sciences et Technologies de Lille, France) and sialic acid derivatives were donated by Prof. Ronald Schauer (University of Kiel, Germany). The sugar specificity of the purified HN from LPMV was determined by comparing the inhibitory activity of sugars, glycoproteins, their desialylated derivatives, or glycosides, on the hemagglutinating activity [17] of HN using human A_2 erythrocytes. Results are expressed as the minimal concentration of each compound required to inhibit four hemagglutinating units (HAU) of HN. Glycoproteins and glycans were desialylated by incubation at 100°C for 1 h in the presence of 0.02 N sulfuric acid, as described by Spiro and Bhoyroo [18], desalted on a Biogel P-2 column equilibrated with 0.5 M acetic acid and lyophilized until use. The molar concentrations of glycoproteins were determined according to their molecular weight. In desialylated glycoproteins, we subtracted the number of sialic acid molecules released from the native protein, and the molar concentration of glycans was calculated on the basis of their oligosaccharides content as determined by gas chromatography.

Physicochemical properties

The effect of ions on the hemagglutinating activity of the HN from LPMV was assessed after dialysis of the purified protein against 0.1 M EDTA/0.1 M acetic acid; then the protein was exhaustively dialyzed against distilled water and finally against PBS. Aliquots of treated HN were dialyzed against PBS supplemented with 5 mM $MnCl_2$, $CaCl_2$, or $MgCl_2$. The hemagglutinating activity of each fraction

was compared with non-treated HN dialyzed against PBS and tests were performed in the presence of a 2% suspension of human A_2 type erythrocytes. Temperature stability was determined by incubating aliquots of the HN at different temperatures and performing the hemagglutinating assay at several time intervals.

Results

Purification of the HN from LPMV

Virions from *Porcine rubulavirus* LPMV in infected-cell culture supernatants were concentrated 100X by PEG precipitation. The HN was separated from virions after sonication and sucrose gradient centrifugation. As indicated in Figure 1, most of the hemagglutinating activity was identified in the fractions corresponding to 40 to 45% of the sucrose gradient (fractions 16–20, bars), which represents 80% of the hemagglutinating activity deposed onto the gradient (Table 1). The most active fractions obtained by ultracentrifugation were further purified by ion exchange chromatography, using a mono P column in anionic form, the HN was eluted from the column by adding a stepwise NaCl gradient. Under these conditions, we obtained five fractions eluted at 0.12, 0.17, 0.22, 0.27 and 1 M NaCl; only the fraction eluted at 0.17 M NaCl (Fraction II in Figure 2) showed hemagglutinating activity. This fraction conserved 69.3% of the hemagglutinating activity originally contained in virions; moreover, the purification process increased 9.4 times the specific activity when compared with the virion activity (Table 1).

Chemical characterization

The purified HN from LPMV is a glycoprotein of 66 kDa, as determined in SDS-polyacrylamide gel electrophoresis (Figure 3). The amino acid analysis of HN indicated that it is rich in alanine, glycine, leucine, and proline. Methionine, cysteine, and tyrosine were identified at a lesser proportion (Table 2). Carbohydrate composition analysis showed that 4% of the total molecular weight of HN corresponded to its glycannic portion, which is formed by mannose, galactose, N-acetyl-glucosamine, N-acetyl-galactosamine, and N-acetyl-neuraminic acid in 3:3:4:1:1 molar ratios (Table 2).

Table 1. Purification of the Hemagglutinin-Neuraminidase from the *Porcine rubulavirus* LPMV

Fraction	*Protein (μg)*	*HAU**	*Specific activity (HAU/protein)*
Culture medium supernatant	21.4	26,100	1,219.0
Sonicated extract	15.2	25,600	1,684.2
Sucrose gradient	1.6	20,480	12,800.0
Ionic Exchange Chromatography (Fraction II)	0.9	14,200	15,777.7

*Hemagglutination units determined in the presence of human A_2 erythrocytes. Data obtained from 3×10^7 $TCID_{50}$. The Fraction II corresponds to the fraction with hemagglutinating activity obtained from the ionic exchange chromatography (see Fig. 2)

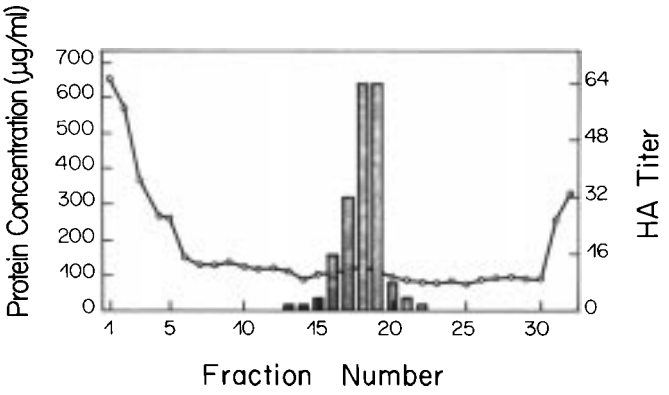

Figure 1. Isolation of the *Porcine rubulavirus* LPMV Hemagglutinin-Neuraminidase. Virions were disrupted by ultrasound and centrifuged in a discontinuous 30–60% sucrose gradient at 100,000 *g,* 4 h; 1 ml fractions were obtained, their hemagglutinating activity were tested with human A_2 erythrocytes (bars) and the protein concentration with the method of Bradford (continuous line).

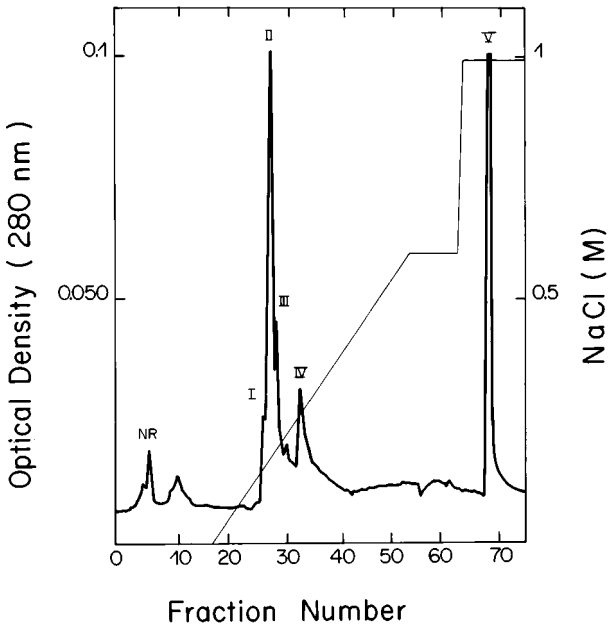

Figure 2. Purification of the *Porcine rubulavirus* LPMV Hemagglutinin-Neuraminidase by ion exchange chromatography. The HN isolated by sucrose gradient centrifugation was applied to a Mono P column in an FPLC system. The protein was eluted by a stepwise NaCl gradient (light line). Detection of protein was performed at 280 nm (dark line). Hemagglutinating activity in the presence of A_2 human erythrocytes was only identified in peak II, this activity corresponds to 69.3% of the hemagglutinating activity deposed onto the column.

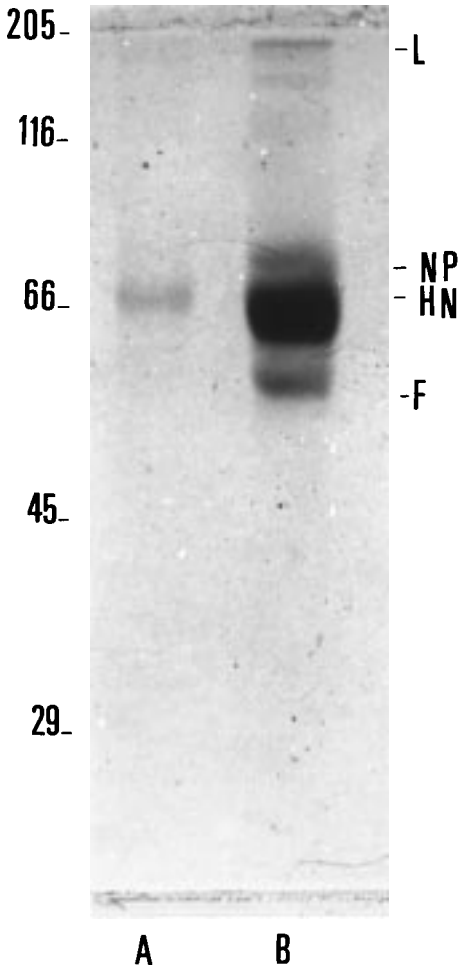

Figure 3. SDS-polyacrylamide gel electrophoresis of virions and purified *Porcine rubulavirus* LPMV Hemagglutinin-Neuraminidase. A) Purified HN, 5 μg. B) 25 μg of disrupted LPMV virions. Molecular weight markers are: Rabbit muscle myosin (205 kDa), *E. coli* β-galactosidase (116 kDa), bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), carbonic anhydrase (29 kDa). Viral proteins shown in the right-hand column are: L, large protein; NP, nucleoprotein; HN, hemagglutinin-neuraminidase; F, fusion protein.

Table 2. Chemical characterization of the *Porcine rubulavirus* LPMV Hemagglutinin-Neuraminidase

Amino acid residue	*Mol %*	*Res/Mol**
Asx	8.9	52.5
Glx	11.8	72.9
Ser	7.1	43.9
Gly	8.5	53.8
His	1.9	11.7
Arg	3.9	24.1
Ala	8.5	52.5
Pro	6.0	37.0
Tyr	1.3	8.0
Val	6.0	37.1
Met	1.4	8.7
Cys	0.5	3.1
Ile	4.7	29.1
Leu	10.0	61.8
Phe	4.2	26.0
Lys	7.8	48.2
	Total	570.4
Sugar 4.0% w/w.		
Mannose		3.1
Galactose		3.2
N-acetyl-D-galactosamine		1.0
N-acetyl-D-glucosamine		4.1
N-acetyl-neuraminic acid		0.9

*Considering a molecular weight of 66 kDa.

Hemagglutinating activity

The purified HN from LPMV agglutinates all the erythrocytes tested from different animal species, including human ABO blood groups, although the highest titers were observed with human A_2. The HN glycoprotein did not agglutinate neuraminidase- or pronase-treated red blood cells (Table 3).

Sugar specificity

The specificity of the HN from LPMV was analyzed in the presence of sugars, oligosaccharides, glycosides, and glycoproteins. As indicated in Table 4, Neu5Ac, Neu4,5Ac2, and Neu5,9Ac2 inhibited the hemagglutinating activity at 100 mM, and they were twofold more powerful inhibitors of the hemagglutinating activity of the purified HN that Neu5Gc. Kdn is not an inhibitor of the hemagglutinating activity even at 200 mM. A powerful inhibitory effect was observed with glycoproteins such as human orosomucoid (α_1-acid glycoprotein) and human IgA; whereas fetuin, IgG, IgM, ovalbumin, ovine, and bovine submaxillary gland mucin, as well as the asialo forms of orosomucoid, and IgA, were not inhibitors of the hemagglutinating activity of HN. The utilization of sialyl-glycosides clearly indicated that only α-sialyl glycosides are inhibitors of the HN hemagglutinating activity (Table 4). Neu5Ac(α2,3)lactosamine showed an inhibitory effect corresponding to 8-times that of Neu5Ac, but its isomer Neu5Ac(α2,6) lactosamine did not inhibit at all the activity of HN glycoprotein at a 100 mM concentration. Colominic acid, which is a large homopolymer constituted by Neu5Ac with α2,8 glycosidic linkages, was also an inhibitor.

Physicochemical properties

Incubation of HN in the presence of 0.1 M EDTA, or addition of 5 mM of divalent cations such as Ca^{++}, Mg^{++}, or Mn^{++}, did not modify its hemagglutinating activity; the hemagglutinating titer, in the presence of A_2 human erythrocytes, remained unaltered when compared with control

Table 3. Hemagglutinating activity of the HN from the *Porcine rubulavirus* LPMV

	*Hemagglutinating titer**		
Erythrocytes	*Native*	*Neuraminidase*	*Pronase*
Human A_1	32	NA	NA
Human A_2	64	NA	NA
Human B	32	NA	NA
Human O	16	NA	NA
Pig	32	NA	2
Horse	32	NA	NA
Rabbit	16	NA	NA
Rat	16	NA	NA
Mice CD-1	16	NA	NA
Chicken	32	NA	NA

The protein concentration of the purified HN is 60 μg/ml. *Represented as the inverse of the last dilution showing agglutinating activity in the presence of a 2% erythrocyte solution in PBS. NA, non agglutinating activity.

Table 4. Effect of sugars, glycosides and glycoproteins on the hemagglutinating activity of the Hemagglutinin-Neuraminidase from the *Porcine rubulavirus* LPMV*

Compound	*Concentration (μM)*	*Relative Inhibitory Potency***
Neu5Gc	200	1
Neu5Ac	100	2
Neu4,5Ac2	100	2
Neu5,9Ac	100	2
Kdn	200	NI
Neu5Ac α-methyl-glycoside	6.0	33.2
Neu5Ac β-methyl-glycoside	200	NI
(α2,3)sialyllactosamine	12.5	16
(α2,6)sialyllactosamine	100	NI
Colominic acid (α2,8)***	100	1
Fetuin	10	NI
α_1-acid-glycoprotein	0.1	2,000
Asialo-α_1-acid glycoprotein	10	NI
Human serum IgA	0.05	4000
Asialo-human serum IgA	10	NI

*Minimal concentration to inhibit 4 HAU (Titer = 4) of the HN from LPMV. **Inhibitory potency is the comparative value of the inhibitor concentration with N-glycolyl-neuraminic acid (Neu5Gc). Other sugars without inhibitory (NI) activity at 200 mM are L- and D-fucose, D-mannose, GalNAc, GlcNAc, D-glucose, D-galactose, D-galactosamine, D-glucosamine, D-mannosamine, melezitose, lactose, raffinose. Fetuin, asialo fetuin, hen ovalbumin, human IgG and IgM, ovine and bovine submaxillary gland mucin were not inhibitors at 10 μM. ***Values are represented in mg/ml since molecular weight is not known.

experiments with untreated HN (Titer 64 HAU), indicating that metals are not essential for the *Porcine rubulavirus* hemagglutinating activity. The activity of HN remained practically unaltered after incubation at 56°C or below, even for 1 h; however, the activity was drastically diminished after 15 min incubation at 65°C and was completely inhibited after 30 min incubation at 65°C or 3 min at 85°C.

Discussion

The Hemagglutinin-Neuraminidase is the main structure involved in the first stages of rubulavirus infection, i.e., host cell recognition, binding, fusion, and penetration [8]. Due to its participation in these processes its HN is an ideal target for neutralization of rubulavirus infection. In this work we purified a 66 kDa glycoprotein with hemagglutinating activity from virions of the *Porcine rubulavirus* LPMV. The purification process involves ultrasonic disruption, ultracentrifugation in a sucrose density gradient, and ionic exchange chromatography. These procedures increased 9.4 times the specific activity of pure HN with respect to virus extracts.

The HN is constituted mainly by hydrophobic and nonpolar amino acids and contains 4% of sugar by weight. The viral gene encoding this protein has been identified and sequenced, it has been predicted that it expresses a protein constituted by 576 amino acid, with a molecular mass of 63.3 kDa. Analysis of this sequence identified four potential sites of N-glycosylation [5]; our results agree with the predicted data, since we found that almost 4% of the HN molecular weight corresponded to sugars. The presence of N-glycosylation sites is confirmed in our study by the finding of mannose residues in the purified HN; nevertheless, the presence of N-acetyl-D-galactosamine also suggests strongly the existence of O-glycosylation sites. It could be possible that differences found between predicted glycosylation [5,19] and experimental data directly depend on the host cell, due to the fact that viruses make use of the host cell enzymatic machinery for the processing of oligosaccharidic structures, since until now no virus has been found to encode enzymes that can affect glycosylation of its proteins [20]. The high amount of hydrophobic amino acids identified in the purified HN also agrees with the secondary structure predicted for this protein, which implies the organization of its extracellular domain into a predominant β-loop-β conformation [19].

The *Porcine rubulavirus* LPMV HN agglutinates erythrocytes from several animal species, and this activity did not require divalent cations; however, when Neu5Ac or sialylated glycopeptides were removed from erythrocytes by enzymatic treatment, the hemagglutinating activity of HN was abolished, suggesting the relevance of sialic acid residues in the HN receptor. The specificity for sialic acid was confirmed by using sialic acid derivatives. Our results suggest that the presence of N-acetyl groups on C5 in the sialic acid is better recognized by the HN than by N-glycolyl in the

same position. Kdn, which lacks N-acetyl functional groups, does not inhibit the hemagglutinating activity of the HN protein. Our study also shows that the hemagglutinating motif of HN is mainly directed to Neu5Ac containing structures and that anomeric carbons play important roles in the binding process, since HN specificity is directed preferentially to α-anomers, which are linked to galactose through α2,3 linkages. The HN from the *Porcine rubulavirus* LPMV shows great affinity for glycoproteins with bi- and tri-antennary N-glycosidically linked glycans of the N-acetyl-lactosaminic type with Galβ1,3GlcNAc sequence substituted by Neu5Ac in α2,3 linkages, such as IgA and α1-acid glycoprotein [21,22]. It is also worthy to note that fetuin, which possesses N-acetyl-lactosaminic type glycans, does not show any effect on HN activity, probably because fetuin possesses Neu5Ac residues branched directly to GlcNAc in an α2,6 linkage or Galβ1,3GlcNAc sequence [23,24]. Relevance of Neu5Ac in the interaction with IgA and α_1-acid glycoprotein is suggested by the decreased inhibitory capacity of their asialo-forms. Hen ovalbumin, which possesses oligomannosidic- and hybrid-type structures and lacks Neu5Ac residues [22], does not inhibit the hemagglutinating activity of the HN nor do the sialylated O-glycosidically linked glycan glycoproteins such as bovine and ovine submaxillary gland mucin. These data agree with previous reports indicating that infectivity of LPMV depends on the presence of sialylated receptors in host cells [9–10]. The specific interaction of HN with sialylα2,3lactose residues seems to be responsible for the neurotropism showed by the porcine rubulavirus in infected suckling pigs [2,3].

Acknowledgments

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