# Sialyloligosaccharides from Egg Yolk as an Inhibitor of Rotaviral Infection

**Keywords:** *Sialic acid; delipidated egg yolk; sialyloligosaccharide; rotavirus*

## INTRODUCTION

Rotavirus is known as a major pathogen of infectious gastroenteritis in infants. Rotaviral gastroenteritis kills more than 1 million infants a year in developing countries (Kaspikian *et al.*, 1991) and causes diarrhea and vomiting in about 150 million children around the world each year (Gouvea *et al.*, 1994; Tabassum *et al.*, 1994; Noel *et al.*, 1994; Kaga *et al.*, 1994).

Vaccines have been developed for the prevention of rotaviruses (Forrest, 1993; Greenberg, 1993), but the success of vaccination trials for rotavirus has remained questionable because of the difficulties in the introduction of the specific antibody into the intestinal tract of infants, whose immunity generally has not developed yet (DeMol *et al.*, 1986).

Bass *et al.* (1992) have reported that the early events of virus attachment and entry to the cells are critical for rotaviral replication. Keeping in view the above finding, prevention of rotaviral inhibition by certain food ingredients could be a good option to solve this global problem.

Egg yolk is not only a good nutritional balance food but also an excellent source of many essential nutrients. We reported the large scale preparation of *N*-acetylneuraminic acid from chalaza and egg yolk membrane (Juneja *et al.*, 1991) and delipidated egg yolk (DEY) of hen (Koketsu *et al.*, 1992). We also found that *N*-acetylneuraminic acid was the only sialic acid found in the egg yolk (Koketsu *et al.*, 1992). We isolated and characterized the major sialyloligosaccharide moieties of the water soluble fraction of the DEY as a step toward the elucidation of their biological and physiological functions (Koketsu *et al.*, 1993).

In the present investigation, the effect of sialyloligosaccharides from delipidated egg yolk on rotavirus inhibition was studied both *in vitro* and *in vivo*.

## MATERIALS AND METHODS

**Materials.** The delipidated egg yolk (DEY), as reported previously (Koketsu *et al.*, 1992), was used in the study. The SA-11 strain of rotavirus and MA-104 cells (an established cell line derived from rhesus monkey kidney) were obtained from the Department of Bacteriology, Tohoku University School of Medicine. Mice (ddY) were purchased from Japan SLC Inc., Hamamatsu, Japan.

**Release of Oligosaccharides from Glycoproteins of DEY.** The DEY was digested with protease (Orientase of *Aspergillus oryzae* origin, Hankyu Bioindustry Co., Ltd., Osaka, Japan) in 0.05 M phosphate buffer, pH 7.0, at 50 °C for 20 h. The reaction mixture was filtered and treated with a membrane of nominal molecular weight cutoff of 10 000 attached to an RUW-4 pump unit (Nitto Denko Co., Ltd., Tokyo, Japan). The digest containing oligosaccharides was collected and evaporated under reduced pressure at 45 °C.

**Separation of Sialyloligosaccharides by Ion Exchange Chromatography.** The oligosaccharide-enriched fraction was separated on a column of Dowex MSA-1 ($Cl^-$ type, 20–50 mesh). The stepwise elution performed with distilled water, and then 0.5 M NaCl, yielded the neutral oligosaccharide fraction and the sialyloligosaccharide fraction, respectively. The above sialyloligosaccharide fraction was loaded on a column of DEAE-TOYOPEARL 600M (2.5 × 100 cm, Tosoh Co., Ltd., Tokyo, Japan). The stepwise elution was performed with distilled water, 0.02 M pyridinium–0.01 M acetate, 0.1 M pyridinium–0.05 M acetate, and then 0.4 M NaCl at a flow rate of 1 mL/min. The sialic acids, hexoses, and amino acids were analyzed according to the periodate–resorcinol method (Jourdian *et al.*, 1971), the phenol–$H_2SO_4$ method (Hodge and Hofreiter, 1962), and the ninhydrin method (Horstmann, 1979), respectively. The weight of the sialyloligosaccharides was measured by collecting and drying fractions 2 and 3 (Figure 2).

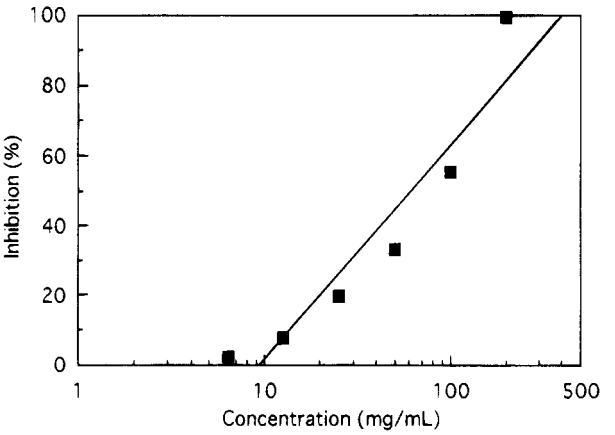


**Figure 1.** Effect of oligosaccharide-enriched fraction on rotavirus (SA-11) inhibition. Inhibition of rotavirus was measured as mentioned under Materials and Methods.

**Digestion by Neuraminidase.** The oligosaccharide-enriched fraction (200 mg) and sialyloligosaccharide fraction (100 mg) were treated with 500 mU of *Arthrobacter ureafaciens* neuraminidase in 50 mM sodium acetate buffer, pH 5.5, at 37 °C for 15 h to release their sialic acid.

Monolayers of MA-104 cells were treated with various concentrations of neuraminidase for the removal of sialic acid from their cell surfaces.

**Rotavirus Infectivity Assay *in Vitro*.** The ability of each fraction to inhibit rotavirus infection was measured according to the indirect immunofluorescence staining method (Fukudome *et al.*, 1989; Svensson, 1992). Briefly, MA-104 cells were grown to confluence on 96-well tissue culture microplates (Nunc Microwell Plate96F with lid) in the presence of Eagle's minimum essential medium (EMEM) and 10% fetal bovine serum at 37 °C in a $CO_2$ environment. The cells were washed with serum-free media before use. Aliquots of sample were diluted in EMEM, mixed with an equivalent volume of rotavirus (SA-11, $1 \times 10^5$–$10^6$ of FCFU/mL), and incubated at 37 °C for 1 h. This mixture was absorbed onto the cells. After incubation at 37 °C for 1 h, the inoculum was decanted, and the cells were incubated in EMEM at 37 °C for 17 h. After incubation, EMEM was decanted and cells were air-dried and fixed in cold MeOH (−80 °C) for 1 h. After MeOH was removed and cells were air-dried, guinea pig antiserum against rotavirus was added and incubated at 37 °C for 30 min. After cells were washed with PBS, FITC labeled goat IgG against guinea pig IgG was sailed and incubated at 37 °C for 30 min. Cells were washed and air-dried, the number of positively fluorescent cell focus forming units (FCFU) was counted under the fluorescence microscope.

**Rotavirus Infectivity Assay *in Vivo*.** The sialyloligosaccharide fraction (2.5 mg) was orally administered to 6-day-old mice (30 mice/group), and the mice were inoculated with 50 μL of $8.8 \times 10^6$ FCFU/mL of rotavirus. The control group was administered saline and rotavirus. All of the groups were inspected daily for diarrhea by gentle palpation of the abdo-

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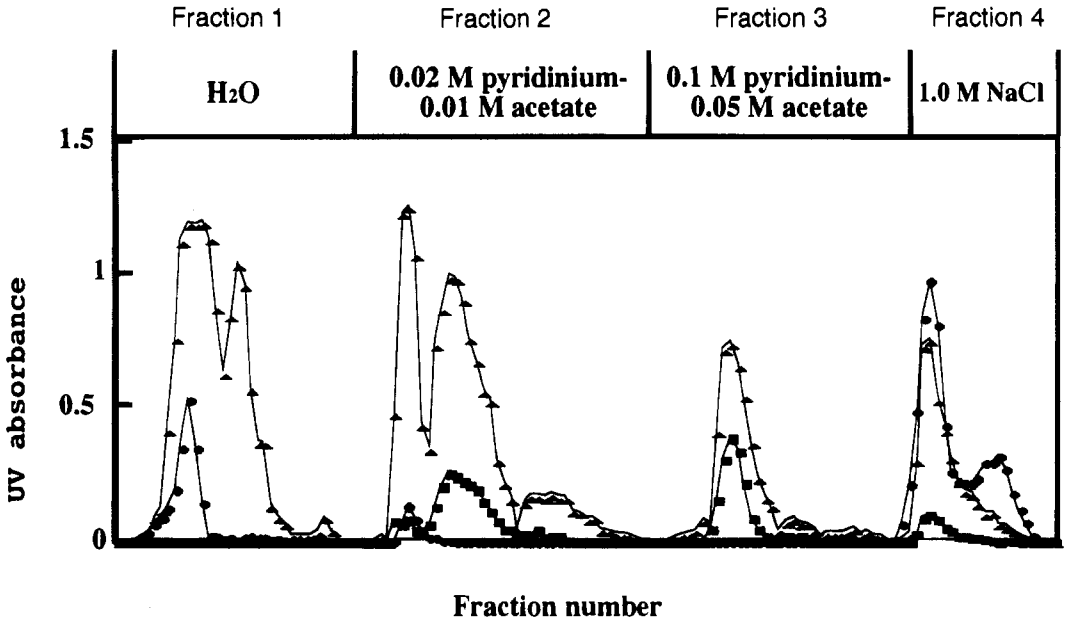


**Figure 2.** Elution profile of the sialyloligosaccharide fraction on DEAE-TOYOPEARL 650M: sialic acid (■); hexose (▲); amino acids (●). (3 mL/fraction).

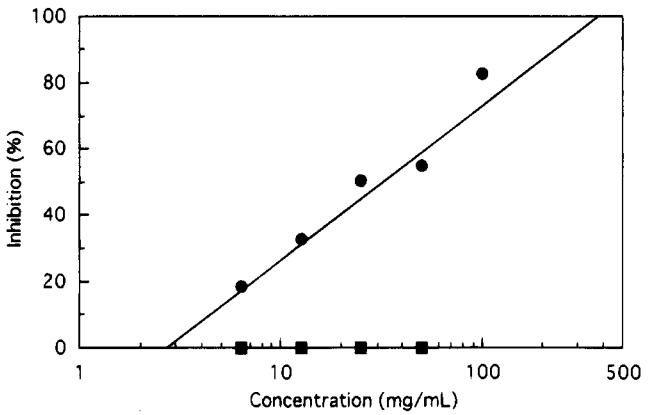


**Figure 3.** Effect of sialyloligosaccharide and neutral oligosaccharide fractions on rotavirus (SA-11) inhibition: sialyloligosaccharide fraction (●); neutral oligosaccharide fraction (■).

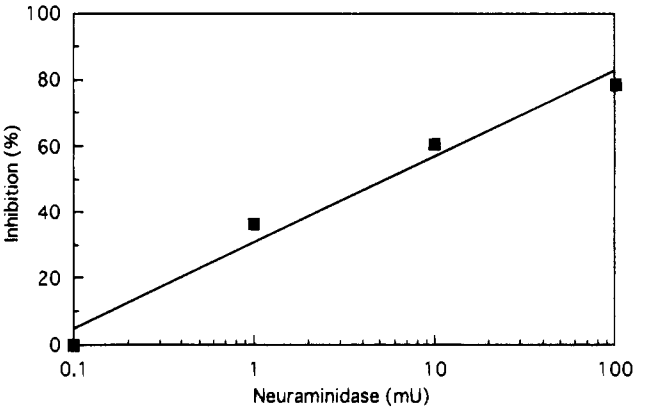


**Figure 4.** Effect of neuraminidase on rotavirus inhibition. MA-104 cell monolayers were treated with the indicated amount of neuraminidase for 2 h at 37 °C to release the sialic acid of the cell surface.

men, and the incidence of diarrhea was counted as reported previously by Hatta *et al.* (1993).

## RESULTS AND DISCUSSION

Various doses of the oligosaccharide-enriched fraction assayed by rotaviral neutralization assay *in vitro* showed the dose dependent inhibition against rotavirus. An amount of 200 mg/mL of the oligosaccharide-enriched fraction inhibited 99.4% of rotavirus infection (Figure 1). The $IC_{50}$ of this fraction was estimated as 60 mg/mL. The oligosaccharide-enriched fraction was separated into sialyloligosaccharide and neutral oligosaccharide fractions on an anion exchange resin (Dowex MSA-1) column. The sialyloligosaccharide fraction was loaded on an anion exchange column packed with DEAE-TOYOPEARL 650M, and the elution profile is shown in Figure 2. An amount of 100 mg of the sialyloligosaccharide fraction was loaded on the column and was found to contain 18.8 mg of asialyloligosaccharides when eluted with water (fraction 1, Figure 2). The fractions eluted with 0.02 M pyridinium−0.01 M acetate (fraction 2, Figure 2) and 0.1 M pyridinium−0.05 M acetate (fraction 3, Figure 2) contained 27.9 and 10.4 mg of sialyloligosaccharides, respectively. The rest of the sample was eluted with 1.0 M NaCl, and the fraction was found to contain peptides and other compounds (fraction 4, Figure 2). The ratio of the sialyloligosaccharide fraction and the neutral oligosaccharide fraction obtained was 1:1. The sialic acid content of the sialyloligosaccharide fraction was estimated as 6.9% according to the TBA assay (Warren, 1959). The neutral oligosaccharide fraction had no sialic acid, and the whole fraction was eluted with water. Therefore, the neutral oligosaccharide fraction did not contain any sialic acid derivatives. An amount of 100 mg/mL of the sialyloligosaccharide fraction inhibited 82.8% of rotavirus infection, but the neutral oligosaccharide fraction did not show any inhibition against rotavirus (Figure 3). The $IC_{50}$ of the sialyloligosaccharide fraction was 31.9 mg/mL. Furthermore, to confirm the role of sialic acid against rotavirus inhibition, the oligosaccharide-enriched fraction and the sialyloligosaccharide fraction were treated by *A. ureafaciens* neuraminidase. Both asialo fractions obtained showed no effect on rotavirus inhibition (data not shown). This suggests that sialic acid plays an important role in rotavirus inhibition.

In the above experiments, the samples were incubated with the SA-11 strain of rotavirus at first and added onto MA-104 cells. When MA-104 cells were incubated with samples first, washed with EMEM, and later

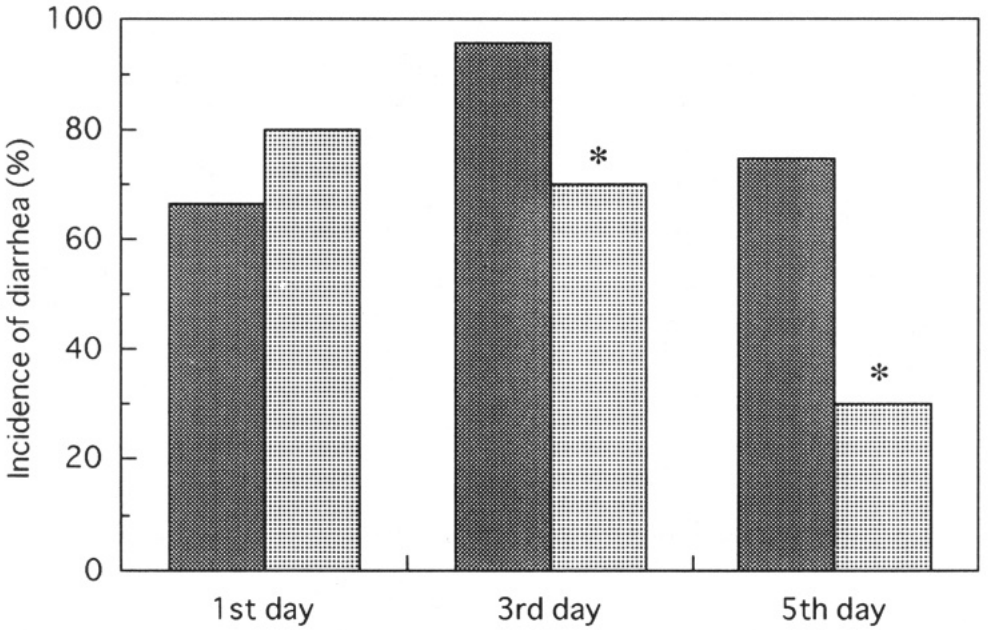


**Figure 5.** Effect of the sialyloligosaccharide fraction administered to suckling mice after inoculation of rotavirus (SA-11): saline (left bar of each pair); sialyloligosaccharide fraction (right bar of each pair); *, $p < 0.05$.

inoculated with rotavirus, neither the oligosaccharide-enriched fraction nor the sialyloligosaccharide fraction inhibited the rotavirus (data not shown). When sialic acids of MA-104 cells were removed by neuraminidase, the inhibition of rotavirus (the SA-11 strain) infection was found to be based on the degree of digestion by the concentration of neuraminidase: 78.7% of rotavirus infection was inhibited by the treatment of 100 mU of neuraminidase with MA-104 cells (Figure 4). These results confirmed the earlier results reported by Yolken *et al.* (1987). From these findings, it might be considered that the first step in rotavirus (SA-11 strain) infection is the binding of the virus to the sialic acids at the surface of the target (MA-104) cells. Therefore, it could be presumed that when sialic acid derivatives used in this study were preincubated with rotavirus, sialic acid derivatives masked the receptor of the virus and prevented attachment to the host cells.

The effect of the sialyloligosaccharide fraction on rotavirus inhibition was investigated *in vivo* on suckling mice which were inoculated with rotavirus SA-11 ($4.4 \times 10^5$ of FCFU/mouse). The incidence of diarrhea was observed 1, 3, and 5 days after oral administration of 2.5 mg of sialyloligosaccharide fraction/mouse. The group that was administered the sialyloligosaccharide fraction showed significant decreases in the incidence of rotavirus diarrhea by 24% and 43% in suckling mice on days 3 and 5, respectively, as compared to the control group (Figure 5).

## CONCLUSION

The oligosaccharide-enriched fraction and the sialyloligosaccharide fraction significantly inhibited rotavirus infection both *in vitro* and *in vivo*. The sialic acid moiety of the oligosaccharides was shown to play an important role in the inhibition of rotaviral infection.

In previous papers, ovomucoids and ovalbumins of egg white origin were found to inhibit rotavirus infection (Yolken *et al.*, 1987). This is the first study on the compounds of egg yolk origin having this effect on the SA-11 strain of rotavirus inhibition. In another paper, we have reported the large scale preparation and characterization of the sialyloligosaccharide fraction (Koketsu *et al.*, 1995).

The oral administration of the egg yolk sialyloligosaccharide prepared from hen egg, which is a common food, could be a good solution for the prevention of rotaviral infection. The egg sialyloligosaccharides could be easily prepared and it could prevent deaths by rotaviral infection in countries where it is difficult to get the vaccinations.

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