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Analysis of anti-rotavirus activity of extract from *Stevia rebaudiana*

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Abstract

Anti-human rotavirus (HRV) activity of hot water extracts from *Stevia rebaudiana* (SE) was examined. SE inhibited the replication of all four serotypes of HRV in vitro. This inhibitory effect of SE was not reduced on the prior exposure of SE to HCl for 30 min at pH 2. Binding assay with radiolabeled purified viruses indicated that the inhibitory mechanism of SE is the blockade of virus binding. The SE inhibited the binding of anti-VP7 monoclonal antibody to HRV-infected MA104 cells. The inhibitory components of SE were found to be heterogeneous anionic polysaccharides with different ion charges. The component analyses suggested that the purified fraction named as Stevian with the highest inhibitory activity consists of the anionic polysaccharide with molecular weight of 9800, and contains Ser and Ala as amino acids. Analyses of sugar residues suggest uronic acid(s) as sugar components. It did not contain amino and neutral sugars and sulfate residues. These findings suggest that SE may bind to 37 kD VP7 and interfere with the binding of VP7 to the cellular receptors by steric hindrance, which results in the blockade of the virus attachment to cells.

Keywords: *Stevia rebaudiana*; Polysaccharide; Anti-rotavirus activity; Virus binding, VP7

1. Introduction

Rotavirus, a member of the Reoviridae, is a non-enveloped virus which has a segmented, double-stranded RNA genome surrounded by three concentric protein layers. Human rotaviruses (HRV) are the major etiologic agents of severe

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dehydrating gastroenteritis in children worldwide (Kapikian, 1996). They cause more than 850 000 deaths per year in the developing countries (Glass et al., 1994). To prevent the rotavirus infection, several vaccine candidates have been developed and subjected to extensive clinical trials. Among them, an oral tetravalent rhesus-human reassortant rotavirus vaccine was first licensed by the Food and Drug Administration. However, after administration of 1.5 million doses of the vaccine, it was withdrawn from the market because of rare but severe complications of intussusception (Abramson et al., 1999).

For the treatment of rotavirus gastroenteritis, intravenous fluid administration has been used successfully for dehydration from diarrhea. However, in the case of severe inpatients and immunocompromised hosts who are suffering from prolonged diarrhea and fever, virus-specific treatment will be necessary if possible. Several compounds, biomaterials, and plant extracts have been found to be inhibitory for rotaviruses of some species in vitro (Yolken et al., 1992; Kiefel et al., 1996; Koketsu et al., 1997; Clark et al., 1998). Some of them have prevented HRV-induced diarrhea in suckling mice (Ebina et al., 1990; Yolken et al., 1992), but none of them has yet been in clinical use.

Stevia rebaudiana is a family member of chrysanthemum, originating from Paraguay in South America, and has been used as a medicinal plant for a long time. Recently, Tomita et al. (1997) reported that the hot-water extract of *S. rebaudiana* (we refer this extract as SE) has a unique bactericidal effect against enterohemorrhagic *Escherichia coli* and other food-borne pathogenic bacteria, but not against non-pathogenic *Bifidobacteria* and *Lactobacilli*. We have found that the SE had inhibitory activity against the replication of HRV. In the present study we describe (1) in vitro anti-HRV activity of SE; (2) mechanism of anti-HRV activity of SE; (3) bioassay-directed fractionation and purification of the active component and (4) chemical nature of the purified anti-HRV component.

2. Materials and methods

2.1. Cells and viruses

MA104 cells (African rhesus monkey kidney cells) were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). Human rotavirus strains Wa (G1), S2, DS-1(G2), MO (G3), Hochi(G4), and a rhesus rotavirus, SA11 strain, were propagated in MA104 cells in the presence of trypsin (0.5 μg/ml), and harvested after two freeze–thaw cycles. Virus titer was determined by a plaque assay.

2.2. Preparation of extract from S. rebaudiana Bertoni

Dried stems and leaves (1 kg) of *S. rebaudiana* Bertoni was extracted with 11 l of hot water at 95°C for 1 h. The extract was then concentrated to 2 l by heating at 95°C, and naturally fermented at room temperature for 1 year. The *Stevia* extract (SE) was centrifuged at $10\,000 \times g$ for 10 min and sterilized by filtration (pore size, 0.45 μm) before use.

2.3. Antiviral assays

The inhibitory effect of *Stevia* extract on the replication of rotavirus was determined by the inhibition of virus-induced cytotoxicity in MA104 cells. A confluent monolayer culture of MA104 cells was infected with Wa strain of rotavirus (100 pfu per well) with various concentrations of SE in a 96-well microplate and cultured for 3 days. The number of viable cells was indirectly determined colorimetrically by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described earlier (Takahashi et al., 1997). The 50% effective concentration (EC_{50}) was defined as the reciprocal of dilution of SE required to prevent HRV-induced cytolysis by 50%. Cell cytotoxicity of SE or purified component of SE was defined as the 50% cytotoxic concentration (CC_{50}) expressed as the reciprocal of dilution of SE or the concentration of purified component of SE that reduced the absorbance of mock-infected cells by 50% of that of the control (without SE or purified com-

ponent). Selectivity index was expressed as the ratio of CC_{50}:EC_{50}.

2.4. Plaque assay

Rotavirus strains were treated with trypsin (10 μg/ml) at 37°C for 20 min and inoculated into confluent monolayers of MA104 cells in 6-well plates. Following adsorption of the virus at 37°C for 1 h, the inoculum was removed and cells were washed with MEM, overlaid with 2 ml of MEM containing 0.7% agarose (Seakem) and 0.5 μg/ml trypsin and incubated for 3 days at 37°C. The wells were overlaid with 1 ml of 0.7% agarose in phosphate-buffered saline (PBS) containing 0.33% neutral red and plaques were enumerated after 2-h incubation. For adsorption inhibition assay viruses were adsorbed to MA104 cells in serial dilutions of test materials for 1 h and thereafter the cells were treated as described above. The 50% effective concentration (EC_{50}) was defined as the reciprocal of dilution of SE or the concentration of purified component of SE required to reduce the number of plaques by 50%.

2.5. Virus binding assay

Virus binding assay was performed with radiolabeled purified Wa strain. Wa strain-infected MA104 cells were radiolabeled with 100 μCi ^{35}S-methionine for 12 h until massive CPE was observed. The radiolabeled virus was purified with CsCl gradient by ultracentrifugation as described earlier (Chen and Ramig, 1992). For a binding assay, a confluent monolayer of MA104 cells in a 96-well microplate was incubated with the radiolabeled virus (2×10^4 cpm) in the presence or absence of SE for 1 h on ice, after which cells were washed free of medium and excess labeled virus. The cells were lysed with lysing solution (1% Triton-X, 0.15 M NaCl, 10 mM Tris–HCl), and bound virus was counted in a liquid scintillation counter (Aloka). The percentage of bound viruses was calculated as (cpm of membrane-bound virus/cpm of total input Wa strain in medium) × 100%.

2.6. Immunofluorescence

MA104 cells in the 8-chamber slide were infected with Wa strain at m.o.i of 1 and cultured for 24 h at 37°C. The cells were washed with PBS, fixed in acetone for 5 min, and reacted with YO-2C2 anti-VP4 (Taniguchi et al., 1987) or KU-4 anti-VP7 (Taniguchi et al., 1988) monoclonal antibody diluted at 10^{-4} in PBS containing 0.1% bovine serum albumin (BSA) for 30 min at room temperature (RT). After washing three times with PBS, fluorescent isothiocyanate-labeled rabbit anti-mouse IgG was applied and incubated for 30 min at RT. After washing as described above, the slide was examined by fluorescent microscopy.

2.7. Preparation of polysaccharide of SE

Polysaccharides of SE were precipitated with two volumes of ethanol, dissolved in distilled water and further purified by precipitation in 1% cetyltrimethylammonium bromide. The precipitated polysaccharide was redissolved in 4 M NaCl, dialyzed against distilled water, and lyophilized.

2.8. Molecular sieve and ion exchange column chromatography of polysaccharides of SE

Polysaccharide of SE was fractionated by Sephacryl S-200 (Pharmacia-LKB) column chromatography. Each fraction was tested for anti-rotavirus activity by the MTT assay. Inhibitory fractions were collected, dialyzed against 0.01 M phosphate buffer (PB), pH 7.4, and applied on a DE52-cellulose column. After washing with 0.01 M PB, the polysaccharides were stepwise eluted with different concentrations of NaCl (0.2, 0.4, 0.6, 0.8, 1, and 2 M) in 0.01 M PB and finally eluted with 1 M NaOH. Each fraction was dialyzed against distilled water, lyophilized and used for testing antiviral activity and biochemical analysis.

2.9. Biochemical analysis

Biochemical analysis of polysaccharide of *Stevia* extract was performed as previously

described (Matsuda et al., 1999). In brief, electrophoresis was performed on cellulose acetate strips (Sartorius 11 200, 57 × 145 mm) in 0.2 M calcium acetate buffer (pH 7.5) or in 0.05 M sodium borate buffer (pH 9.4). Polysaccharide and monosaccharide bands were visualized with 0.1% alcian blue in 10% acetic acid or alkaline silver nitrate reagent, respectively.

Paper chromatography (PC) was carried out by the descending method on Whatman No. 1 paper with ethyl acetate/acetic acid/formic acid/water (18:3:1:4) as the solvent. Sugars were detected with alkaline silver nitrate, *p*-anisidine hydrochloride, or ninhydrin reagents.

Sugars were also analyzed by high-performance liquid chromatography (HPLC) with a Hitachi 655 HPLC equipped with a refractive index detector on a Wakopak WBT 130 E column (Wako Pure Chemicals, 7.8 × 300 mm) using water as a mobile phase at 60°C and at a flow rate of 0.5 ml/min.

The molecular weight of the purified polysaccharide was determined by a Hitachi 655 HPLC equipped with a refractive index detector on an Asahipak GFA 7 M column (Asahi Chemicals, 7.6 × 500 mm). To minimize the association effect of the polysaccharide solution, aqueous 0.1 M NaCl solution was used as a mobile phase at 40°C and at a flow rate of 0.6 ml/min. The molecular weight was calibrated with pullulans of various molecular sizes (Shodex Standard Kit P-82, Showa Denko, Japan).

For the component analyses of neutral sugars, the polysaccharide was hydrolyzed with 2 M trifluoroacetic acid (TFA) for 12 h at 100°C in a sealed glass tube. After the acid was removed by several evaporations under reduced pressure with water, the hydrolysates were analyzed by PC, electrophoresis, and HPLC.

For the analyses of amino acid and amino sugars, the polysaccharide was hydrolyzed with 4 N HCl for 12 h at 100°C, followed by a concentration to dryness over P_2O_5 and NaOH in vacuo. The residue was analyzed by PC, electrophoresis, and an amino acid analyzer.

Sulfate content was determined with a Shimadzu HIC-6A HPLC equipped with a Shimpack IC-A1 column (4.6 × 100 mm) and a conductivity detector using 2.5 mM phthalic acid containing 2.4 mM tris(hydroxymethyl) aminomethane (pH 4.0) as a mobile phase at 40°C after hydrolysis of the sample.

3. Results

3.1. Inhibitory effect of SE on the HRV replication and virus adsorption

Inhibitory effect of SE on the HRV replication was examined by MTT assay. SE showed the inhibitory activity against Wa strain with an EC_{50} of 220-fold dilution of the original SE solution. However, since the SE was relatively cytotoxic to MA104 cells under this condition (CC_{50}, 112-fold dilution; selectivity index, 2), the exact antiviral activity of SE was unclear. Therefore, a plaque assay was used to confirm the inhibitory activity of SE to HRV. First, we have examined whether or not SE might inhibit virus adsorption, because many plant extracts contain various kinds of polysaccharides, some of which have an inhibitory activity to virus adsorption. As shown in Table 1, the addition of SE during only the adsorption period inhibited the plaque formation of strain Wa (EC_{50}, 118-fold dilution). Cytotoxicity of SE was determined by an MTT assay in the MA104 cell culture which was exposed to SE for 1 h (adsorption period) and incubated for 3 days. The CC_{50} of SE was 32-fold dilution (selectivity index, 4) and cytotoxicity at 100-fold dilution was 5% of the control. These findings suggest that anti-HRV activity of SE is not due to cytotoxicity of SE to MA104 cells. For the strategy to be of value in a clinical application, it was necessary to determine whether exposure of SE to hydrochloride (pH 2) may lose activity or not. This inhibitory effect of SE was not reduced on the prior exposure of SE to HCl for 30 min at pH 2. To examine the virus specificity of SE we further tested whether SE may inhibit the four serotypes of HRV and a rhesus rotavirus (SA11 strain) or not. Interestingly, the SE inhibited the plaque formation of all four serotypes of HRV with similar EC_{50} among the serotypes but not that of SA11 strain (Table 1). Furthermore, SE did not inhibit the plaque

Table 1
Inhibitory effect of SE against rotaviruses on plaque formation

Serotype	Strain	EC_{50} (dilution)[a]	CC_{50} (dilution)[b]	Selectivity index
1	Wa	118	32	4
		121 (pH 2)	35	4
2	DS-1	137	32	4
	S2	153	32	5
3	MO	138	32	4
4	Hochi	114	32	4
Rhesus rotavirus	SA11	>32	32	nd[c]

[a] EC_{50} was expressed as the mean value from triplicate experiments of plaque assays.
[b] CC_{50}, cytotoxicity of SE was determined by an MTT assay in the MA104 cell culture which was exposed to SE for 1 h (adsorption period) and incubated for 3 days. CC_{50} was expressed as the reciprocal of dilution of SE that reduced the absorbance of control cells (without SE) by 50%.
[c] nd, Not determined.

formation of herpes simplex virus 1 (KOS strain) and influenza virus (Ishikawa strain) (data not shown). In addition, the pretreatment of MA104 cells with SE for 30 min at 10–200-fold dilution did not decrease the number of plaques in a plaque assay (data not shown).

3.2. Effect of SE on the binding of Wa strain to MA104 cells

To further examine whether SE inhibits virus binding or not, the binding of radiolabeled purified Wa strain to MA104 cells was tested. SE inhibited the binding of the virus to the cells in a dose-dependent manner (Fig. 1). In the presence of 50-fold dilution of SE, the bound virus reduced to approximately 20% of control bound virus in the absence of SE.

3.3. Effects of SE on the binding of anti-VP7 MoAb to HRV-infected MA104 cells

Since it has been shown that both viral proteins, VP4 and VP7, which constitute the outer layer of mature virions, play a role in the cellular entry of HRV (Ludert et al., 1996; Gilbert and Greenberg, 1997), we examined whether the SE might inhibit the binding of anti-VP4 MoAb (YO-2C2) and/or that of anti-VP7 MoAb(KU-4) to Wa strain-infected MA104 cells. As shown in Fig. 2, the SE considerably inhibited the binding of anti-VP7 MoAb to the infected cells (Fig. 2e). In contrast, it did not obviously inhibit the binding of anti-VP4 MoAb (Fig. 2b).

3.4. Isolation of the inhibitory component of SE against HRV

To identify the inhibitory component of SE against HRV, we thought polysaccharides of SE might be the component because some plant polysaccharides have been reported to be an adsorption inhibitor of a virus (Tabba et al., 1989).

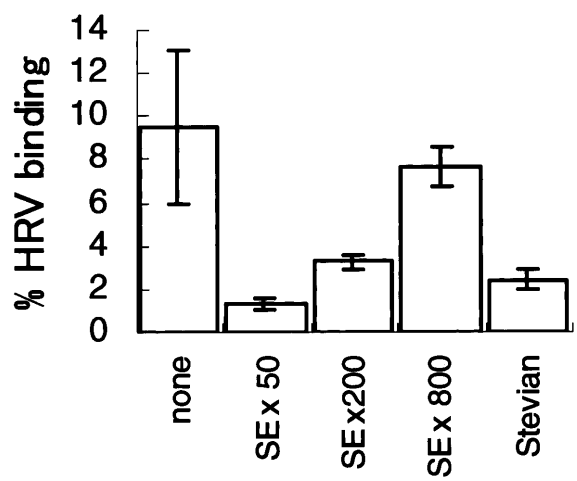

Fig. 1. Effect of SE on binding of the Wa strain to MA104 cells. Triplicate cultures of MA104 cells were incubated with ^{35}S-labeled Wa strain in various concentrations of SE or Stevian. Data represent mean percentages ($\pm$S.D.) of membrane-bound virus.

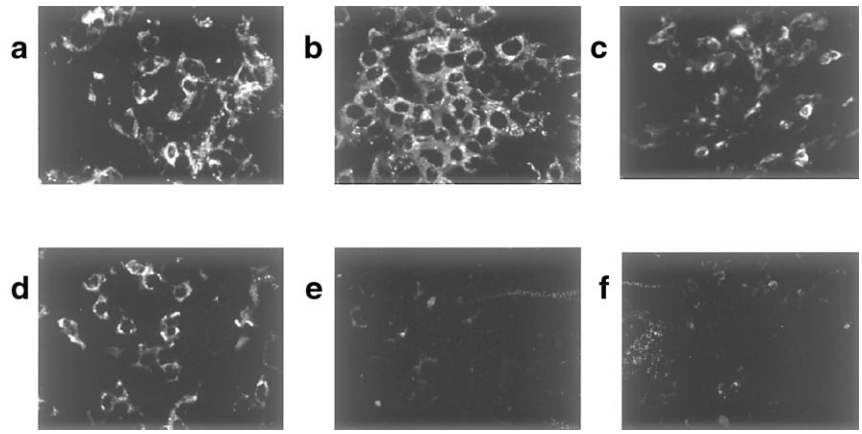

Fig. 2. Effect of SE or Stevian on binding of anti-VP7 MoAb to HRV-infected MA104 cells as visualized by immunofluorescence. Wa strain-infected MA104 cells were fixed and reacted with anti-VP4 (a, b, c) or anti-VP7 (d, e, f) MoAb in the absence (a, d) or presence (b, e) of SE (50-fold dilution) and in the presence of Stevian (c, f, 500 μg/ml). Binding of these MoAbs was visualized by FITC-conjugated rabbit anti-mouse IgG antibodies.

Therefore, according to the purification protocol shown in Fig. 3, polysaccharides of SE were first precipitated with ethanol and fractionated by Sephacryl S-200 column chromatography. The inhibitory activity to HRV of each fraction was examined by the MTT assay (Fig. 4). There were two major peaks of inhibitory activity. Higher inhibitory activity was found in fractions 48–61 (higher molecular weight (MW)) and secondary inhibitory activity was found in fractions 71–76 (lower MW). We further collected fraction 48–61 and purified it by DE52 ion-exchange column chromatography. Each fraction eluted with various concentrations of NaCl solution was dialyzed against water, lyophilized, and tested for anti-HRV activity by a MTT assay (Fig. 5). Anti-HRV activity was found at 500 μg/ml in all fractions eluted with 0.2–2 M NaCl and 1 M NaOH, with the highest activity in the 2 M NaCl fraction. Remarkable cytotoxicity was not observed in each fraction.

3.5. Component analyses of the fraction eluted with 2 M NaCl

To identify the component of inhibitory fraction, the 2 M NaCl fraction with the highest activity was subjected to biochemical analysis as follows. Purity of the fraction was evaluated by a cellulose-acetate membrane electrophoresis and gel filtration. The fraction showed one sharp homogeneous band in the electrophoresis and one peak in the gel filtration on an Asahipak GFA 7 M column on a Hitachi 655 HPLC (data not shown). The molecular weight of the fraction was determined to be 9800. Therefore, this fraction was found to be pure, and we referred to this purified fraction as 'Stevian'. Amino acid content of the fraction was determined by an amino acid analyzer, and it was revealed that two amino acids, Ser and Ala, were contained in the fraction at 5.4 and 3.7 μmol/mg fraction, respectively. Neutral and amino sugars were analyzed by a paper chromatography, cellulose-acetate membrane electrophoresis, and HPLC. No neutral and amino sugars were detected in the fraction by these methods (data not shown). Sulfate content was analyzed by HPLC (Shimpak IC-A1) and no sulfate residue was recognized (data not shown). Sugar component of this fraction was analyzed by cellulose-acetate membrane electrophoresis together with glucose and glucuronic acid. Although enough color development was not observed by the carbazole-sulfuric acid method (Tandavanitj et al., 1989), a uronic acid-like band was observed in electrophoretic analysis (data not shown).

3.6. Inhibitory activity of Stevian

To clarify whether Stevian represents the inhibitory activity of SE against HRV, we re-examined the inhibitory activity of Stevian on plaque formation, for binding of Wa strain to MA104 cells and for binding of anti-VP7 MoAb to Wa strain-infected MA104 cells. As shown in Table 2, the Stevian inhibited the plaque formation of all four serotypes of HRV with EC_{50} of 431–492 μg/ml. CC_{50} of Stevian was over 1000 μg/ml (selectivity index, >2). Since the cytotoxicity at 1000 μg/ml was 4% of the control, it is unlikely that the anti-HRV activity of Stevian is due to the cytotoxicity of Stevian to MA104 cells. Furthermore, the Stevian indeed inhibited binding of purified Wa strain to MA104 cells (Fig. 1) and also inhibited the binding of anti-VP7 MoAb, but not of anti-VP4 MoAb to Wa strain-infected MA104 cells (Fig. 2f and c). These experiments confirmed that Stevian represents the anti-HRV fraction of SE.

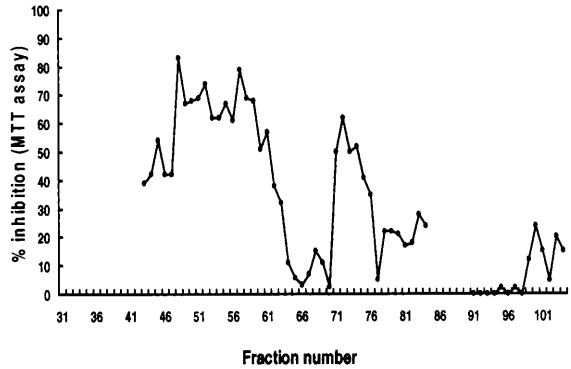

Fig. 4. Anti-HRV activity of fractions of SE from Sephacryl S-200 column. Polysaccharides of SE were gel filtered by Sephacryl S-200 column chromatography. Each fraction was examined for inhibitory activity to HRV by an MTT assay. Fractions which do not show the percent inhibition (i.e. no line) was cytotoxic to MA104 cells.

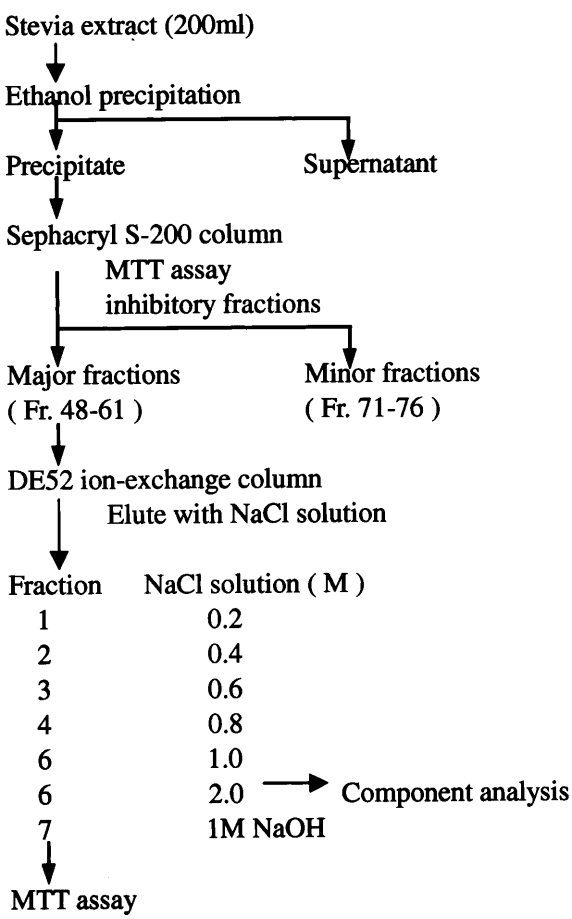

Fig. 3. Purification protocol for anti-HRV polysaccharide from SE.

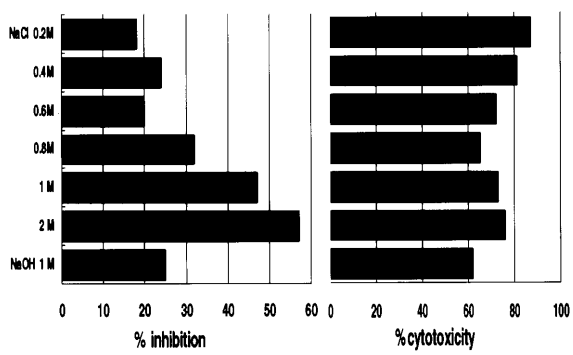

Fig. 5. Anti-HRV activity of fractions of SE from DE52 affinity column. Major inhibitory fractions of SE polysaccharides from Sephacryl S-200 were collected and fractionated by DE52 column chromatography. Each fraction was eluted by NaCl solution with stepwise increasing concentration and tested for anti-HRV activity at 500 μg/ml by an MTT assay. Inhibitory activities are expressed as percent inhibition.

Table 2
Inhibitory effect of Stevian against rotaviruses on plaque formation

Serotype	Strain	EC_{50} (μg/ml)[a]	CC_{50} (μg/ml)[b]	Selectivity index
1	Wa	487	>1000	>2
2	DS-1	452	>1000	>2
	S2	431	>1000	>2
3	MO	465	>1000	>2
4	Hochi	492	>1000	>2

[a] EC_{50} was expressed as mean value from triplicate experiments of plaque assays.
[b] CC_{50}, cytotoxicity of Stevian was determined by an MTT assay in the MA104 cell culture which was exposed to Stevian for 1 h (adsorption period) and incubated for 3 days. CC_{50} was expressed as the concentration of Stevian that reduced the absorbance of control cells (without Stevian) by 50%.

4. Discussion

In the present study, we have demonstrated that extract from *Stevia rebaudiana* has an anti-HRV activity in vitro, and one of the anti-HRV components (Stevian) is an anionic polysaccharide as revealed by the results of the DE52 anion-exchange column chromatography and component analysis. Although we have not identified other anti-HRV components eluted with 0.2–1 M NaCl and 1 M NaOH in DE52 column chromatography (Fig. 5), we presume that these components also represent anionic polysaccharides with different negative charges from the fraction eluted with 2 M NaCl solution, because these components were eluted with different concentrations of NaCl solution. We also have not identified the minor inhibitory peak in Sephacryl S-200 column chromatography (Fig. 4). These components may be polysaccharides with molecular weights of less than 10 000. Besides these fractions in the Sephacryl S-200 column chromatography, there were several fractions with different inhibitory activities. These findings indicate that the anti-HRV components of SE consist of heterogeneous anionic polysaccharides.

The finding that the addition of SE during HRV adsorption to MA104 cells resulted in inhibition of plaque formation indicates that the inhibitory mechanism of SE is the adsorption inhibition. In addition, binding inhibition experiments showed that SE and Stevian blocked the virus binding to MA104 cells in concentrations which are consistent with the result of a plaque assay (Table 1). Furthermore, the fact that SE and Stevian inhibited binding of anti-VP7 MoAb but not that of anti-VP4 MoAb to Wa-infected cells suggests that the SE and Stevian bind to at least the VP7 outer-layer glycoprotein, although this finding does not completely exclude the possibility that they may also bind to VP4 protein.

The outer-layer of rotavirus consists of trimers of the 37 kD VP7 surface and 60 spikes of the 88 kD VP4 (dimers), which interact with VP7 (Prasad et al., 1988; Yeager et al., 1990, 1994). VP4 is an important factor in receptor binding and cell penetration (Ludert et al., 1996) and VP7 also plays a role in cell attachment (Sabara et al., 1985) and may associate to receptor-binding specificity in interaction with VP4 (Mendez et al., 1996). In terms of cell receptors, Hewish et al. (2000) clearly demonstrated that integrins α2β1 and α4β1 are cell receptors for the simian rotavirus SA11. They have also indicated that VP5 of most human and animal rotaviruses has the amino acid sequence DGE at aa 308–310, which acts as a ligand of type I collagen for the α2β1 integrin (Staatz et al., 1991). VP7 of many rotaviruses has the sequence LDV at aa 237–239, which is the minimal essential sequence for adhesion of fibronectin to the α4β1 and α4β7 integrins (Komoriya et al., 1991). In addition, VP7 of all mammalian rotaviruses also has the sequence GPR at aa 253–255, which is a ligand of fibrinogen for the αxβ1 integrin. Furthermore, peptides LVDT and GPRP have been found to

block rotavirus infection in vitro, suggesting that the binding of VP7 to integrins on cell surface is the important first step of virus entry to cells (Coulson et al., 1997). Therefore, SE and Stevian with MW of 9800 bound to 37 kD VP7 are likely to interfere with the binding of VP7 to integrins by steric hindrance, which results in blocking of the virus attachment to cells. Although it is not clear whether SE may also bind to 88 kD VP4 or not, SE bound to VP7 may also inhibit the binding of VP4 to integrins by steric hindrance.

The binding mechanism of Stevian to VP7 is speculative. It is likely that anionic ions (probably carboxy residues) of Stevian may play an important role on the binding to VP7, because sulfations and carboxy groups of anionic polysaccharides have been shown to be key determinants for the inhibition of virus adsorption to host cells (Herold et al., 1996). Analysis of three-dimensional structures of VP7 may be required to understand the binding mechanism between Stevian and VP7.

Among anti-HRV agents previously investigated in vitro, agents originating from plants included PolySaccharide-Kureha (PSK), a protein-bound polysaccharide prepared from the extract of the mycelia of *Coriolus versicolor* of Basidiomycetes (Ebina et al., 1990), and tea extracts (Clark et al., 1998). The inhibitory component of PSK was found to be a protein (25%)-bound β-D-glucan, which is different from Stevian. In a mouse diarrhea model, the PSK, human milk mucin and a protease inhibitor are the agents which prevented the development of HRV-induced diarrhea besides animal anti-HRV IgA and egg yolk anti-HRV immunoglobulins (Ebina and Tsukada, 1991; Ebina et al., 1990; Yolken et al., 1992). However, none of them has yet been approved in clinical use. Since SE has been approved as a safe health food and has been commercially available for these 8 years in Japan, SE is expected as a candidate for clinical trials for treatment of rotavirus infection. Further studies are needed to evaluate whether SE may prevent the development of HRV-induced diarrhea or not, and whether SE may reduce the duration and severity of diarrhea in a mouse model.

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