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Purification of Rotavirus Infection-Inhibitory Protein from *Bifidobacterium breve* K-110

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Abstract The inhibitory activity of fifty *Bifidobacteria* toward the infectivity of a rotavirus, which is the predominant cause of sporadic diarrhea in infants and young children, was investigated, and *Bifidobacterium breve* K-110 was found to have the most potent inhibitory activity. Accordingly, the rotavirus infection-inhibitory protein was purified, and its molecular weight was determined to be 76 kDa by SDS-PAGE. It was heat-labile and its 50% inhibitory concentration (IC_{50}) was 0.045 μg/ml.

Key words: Rotavirus, *Bifidobacterium breve* K-110, anti-rotaviral protein

Acute diarrhea, especially in children, is an extraordinarily common disease with worldwide distribution and a significant public health impact. Rotaviruses have been identified as the major cause of diarrhea, primarily in the neonatal period [5, 9], and are the most important etiological agent of severe diarrhea in infants and young children in both developed and developing countries, being responsible for approximately 35–50% of such illness [10, 11, 17].

Recent approaches in the treatment of rotavirus diarrhea include introduction of oral rehydration solutions within 4–6 h. However, an alternative practice for treating an enteric infection advocates rehydration through supplementation of milk products including viable lactic acid bacteria, which promote the fermentation process and are also beneficial for recovery, possibly acting to combat mucosal atrophy and mediating resistance to infectious challenge [6, 7]. However, the mechanism of this therapeutics is still unclear. *Bifidobacterium* spp. are considered to be one of the most beneficial probiotic organisms for improving human health, since they are one of the major bacterial flora in the human intestine and exhibit various biological activities [14]. In Japan and Korea, humans ingest enormous numbers of Bifidobacteria in fermented milk as dietary adjuncts.

During the screening program for discovering rotavirus infection-inhibitory bacteria, from lactic acid bacteria of healthy humans, various Bifidobacteria were screened. A rotavirus infection-inhibitory protein was purified from *Bifidobacterium breve* K-110, which exhibited the most potent rotavirus infection-inhibitory activity among the isolated Bifidobacteria.

MATERIALS AND METHODS

Materials

Macaccus Rhesus monkey kidney cells (MA104) were purchased from the Korean National Institute of Health, Korea. The Wa virus (a wild-type of human rotavirus) was kindly donated by the Toyama Institute of Health, Japan. Dulbecco's Modified Eagle's Medium (DMEM) and phosphate buffered saline (PBS) were purchased from Sigma Co. (St. Louis, MO, U.S.A.). The antibiotic-antimycotics, trypsin-EDTA (×10), trypsin (1:250), and fetal bovine serum (FBS) were all from Gibco Co. (Grand Island, U.S.A.).

Bacterial Strains and Culture

The *Bifidobacterium animalis* KCTC3126 and *B. longum* KCTC3215 were purchased from the Korean Collection of Type Cultures (Daejun, Korea). The *B. breve* JCM1192, *B. bifidum* JCM1254, and *B. infantis* JCM7007 were obtained from the Japanese Collection of Microorganisms (Tokyo, Japan). All other Bifidobacteria were isolated from healthy Korean males (in their twenties, 65 kg) according to the methods previously described [2, 15]. These isolated Bifidobacteria were separately inoculated into a general anaerobic medium (Nissui Pharm. Co. Ltd., Tokyo, Japan). Then, each cultured bacterium was inoculated into 500 ml of a tryptic soy broth (Difco Co., Detroit, MI, U.S.A.) containing 0.01% sodium thioglycolate and 0.1% ascorbic acid. Cultured cells

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were centrifuged at 5,000 rpm for 30 min, and the resulting precipitate was sonicated and centrifuged at 10,000 rpm for 30 min. The supernatant and precipitate were used as the cytosol and cell wall fractions, respectively.

Rotavirus Culture and Assay of Its Inhibitory Activity

The MA104 cells were cultured in DMEM containing 10% FBS, 1% antibiotic-antimycotics, and 3.5 g/l sodium bicarbonate (CO_2 incubator, 37°C). As a nonactivated virus, the Wa rotavirus (400 μl) was activated with 20 μl of 0.1 mg of trypsin per ml at 37°C for 0.5 h [1, 15]. Washed MA104 cell (1.5×10^6 cells/25 cm^2 flask) monolayers were then inoculated with the activated rotavirus at a multiplicity of infection of 1 to 5, incubated for 2 h, and then aspirated. Next, the cells were washed twice, refed with DMEM containing trypsin, and grown until a cytopathic effect was visible, usually within 3–5 days. Quantifying the rotavirus in the given sample was performed according to an end-point dilution assay. The assay of the inhibitory activity of the Bifidobacteria and their components was based on the inhibition of rotavirus-induced cytopathogenicity. Briefly, 50 μl of 10^{-3}-diluted Wa virus (1×10^3 pfu) was infected into 100 μl of MA104 cells (3×10^5 cells/ml) containing 50 μl of the sample. The cells were then grown until a cytopathic effect was visible and the inhibitory activity of the sample towards the rotavirus infectivity was measured.

Purification of Anti-Rotaviral Protein

To purify the anti-rotaviral protein from *B. breve* K-110, which exhibited the most potent rotavirus infection-inhibitory activity among the isolated Bifidobacteria, *B. breve* K-110 was subcultured in a synthetic medium of tryptic soy broth containing 0.1% ascorbic acid and 0.01% sodium thioglycolate, and pH was adjusted to 7.2. The subcultured medium (50 ml) was inoculated into 5 l of the same medium, and further cultured at 37°C for 20 h. The cultivated medium was centrifuged at 6,000 rpm for 20 min at 4°C. The collected bacteria were then washed with saline, and the resulting precipitate was suspended in 100 ml of 20 mM MOPS buffer, pH 7.0, followed by disruption by an ultrasonicator. The sonicated suspension was centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant (100 ml) was used as the crude enzyme solution, which was fractionated by 0% to 70% saturation with powdered ammonium sulfate and centrifuged at 12,000 rpm for 30 min. All purification procedures were performed at 4°C. The resulting precipitate was dissolved in 50 ml of 10 mM MOPS buffer (pH 7.0) and dialyzed three times against 10 mM MOPS. The dialysate was applied to a QAE-cellulose column (2.8×25 cm), equilibrated previously with 10 mM MOPS buffer. The column was washed with 200 ml of the same buffer, and a linear gradient elution was applied with 200 ml of the same buffer and 200 ml of 10 mM MOPS (pH 7.0) containing 1.0 M KCl. In addition, the column was washed with 100 ml of 10 mM MOPS (pH 7.0) containing 1.0 M KCl. Fractions (6 ml each) were collected and monitored by measuring absorbance at 280 nm and the rotavirus infection-inhibitory activity. The active fractions (Fr. Nos. 63–78) were pooled and the pooled sample was dialyzed against 10 mM MOPS. The dialysate was applied to a column of Q-Sepharose (2.8×20 cm) previously equilibrated with 10 mM MOPS buffer. The column was washed with 200 ml of the same buffer, and a linear gradient elution was applied with 200 ml of the same buffer and 200 ml of 10 mM MOPS (pH 7.0) containing 1.0 M KCl. Fractions (6 ml each) were collected and monitored by measuring absorbance at 280 nm and rotavirus infection-inhibitory activity. The active fractions were Fr. Nos. 59–63.

Protein Determination

The protein was measured using a Bradford protein assay kit with bovine serum albumin as the standard [4, 16].

SDS-Polyacrylamide Electrophoresis

Ten % SDS-PAGE was performed according to Laemmli's method [12] and the gel was stained with Coomassie blue.

Analysis of Amino Acid Composition

An amino acid composition analysis was performed by Applied Biosystem model 420/130 Derivatizer/Amino acid Analyzer using phenylisothiocyanate pre-column derivatization. The hydrolysis was carried out in 6 M hydrochloric acid and 0.1% phenol at 155°C for 1 h.

RESULTS AND DISCUSSION

Fifty Bifidobacteria were isolated from healthy Korean males, the cytosol and cell wall components were fractionated

Table 1. Inhibitory activity of representative Bifidobacteria to rotavirus infection.

Bifidobacterium spp.[a]	Inhibition (%)	
	Cytosol fraction	Cell wall fraction
B. animalis KCTC3126	14.3	14.3
B. longum KCTC3215	14.3	28.6
B. breve JCM1192	28.6	14.3
B. bifidum JCM1254	14.4	14.3
B. infantis JCM7007	28.6	0
B. cholerium K-103	42.9	28.6
B. bifidum K-105	49.1	14.3
B. breve K-110	85.7	28.6
B. catenulatum K-309	28.6	0
B. magnum K-311	0	0
B. minimum K-506	0	0
B. cuniculi K-513	0	0
B. infantis K-525	57.1	14.3

[a]The final concentration was 0.1 mg/ml.

Table 2. Summary of purification of the rotavirus infection-inhibitory protein from *B. breve* K-110.

	Total volume (ml)	Total protein (mg)	IC_{50} (μg/ml)
Crude solution	53	419.9	35
$(NH_4)_2SO_4$ precipitation	71	396.2	16
QAE-cellulose column chromatography	95	252.7	12
Q-Sepharose column chromatography	26	0.47	0.045

by sonication, and the inhibitory activities of the Bifidobacteria fractions on rotavirus infection investigated (Table 1). The cytosol components of most of the Bifidobacteria exhibited higher inhibitory activity than their respective cell wall components.

Among them, the cytosol component of *B. breve* K-110 showed the most potent inhibitory activity towards rotavirus infectivity, followed by *B. infantis* K-525 and *B. bifidum* K-105. When the active cytosol fractions of K-110 and K-525 were precipitated with cold acetone, the precipitates inhibited the infection of the rotavirus in the MA104 cell lines in a dose-dependent manner. However, when the same active cytosol fractions of K-110 and K-525 were heated at 100°C for 10 min or ashed at 500°C for 2 h, they did not inhibit the infection of the rotavirus in the MA104 cell lines, suggesting that the inhibitory component was a thermolabile protein. In addition, we investigated whether certain other organic acids produced by the *Bifidobacterium* spp. and human intestinal bacteria could inhibit the infection of rotavirus, however, the compounds were unable to inhibit the infection of the rotavirus (data not shown). Therefore, an attempt was made to purify the inhibitory protein from *B. breve* K-110, which exhibited the most potent activity against rotavirus infection. The inhibitory protein was purified to homogeneity by 70% ammonium sulfate precipitation, QAE-cellulose column chromatography, and Q-Sepharose column chromatography (Table 2).

The molecular weight of the purified rotavirus-inhibitory protein was determined to be 76 kDa by SDS-PAGE (Fig. 1). The inhibitory protein also exhibited a dose-dependent

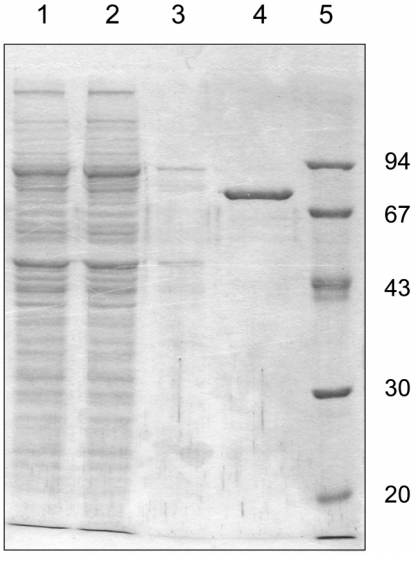

Fig. 1. SDS-PAGE of rotavirus infection-inhibitory protein at various steps in purification procedure.
Lane 1, crude solution; lane 2, preparation after ammonium sulfate fractionation; lane 3, preparation after QAE-cellulose column chromatography; lane 4, purified protein after Q-Sepharose column chromatography; lane 5, molecular marker.

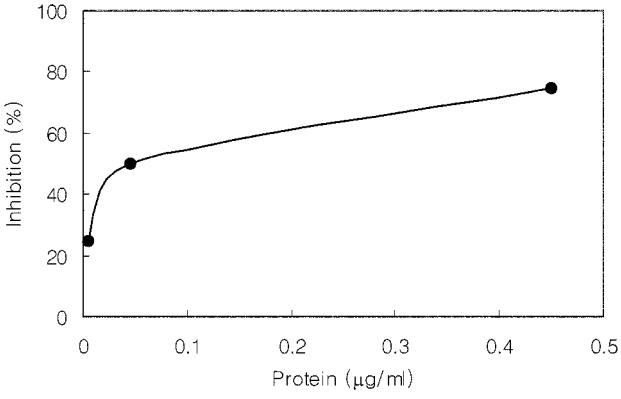

Fig. 2. Inhibition of rotavirus infection-inhibitory protein purified from *B. breve* K-110.

Table 3. Amino acid composition of rotavirus infection-inhibitory protein from *B. breve* K-110.

Amino acid	pmol	Mol %
Cys	N.D.[b]	0.00
Asx[a]	844.30	11.50
Glx[a]	720.75	9.82
Ser	343.19	4.67
His	87.65	1.19
Gly	1,675.00	22.82
Arg	176.62	2.41
Thr	274.47	3.74
Ala	610.03	8.31
Pro	437.41	5.96
Tyr	104.03	1.42
Val	850.54	11.59
Met	68.98	0.94
Ile	262.59	3.58
Leu	390.18	5.31
Phe	236.91	3.23
Trp	N.D.	0.00
Lys	258.87	3.53
Total	7,341.52	100

[a]Asx and Glx indicate the sum of asparagine and aspartic acid, and glutamine and glutamic acid, respectively.
[b]Not determined.

inhibitory activity towards rotavirus infection. The 50% inhibitory concentration of the protein was 0.045 μg/ml (Fig. 2). Based on the amino acid composition analysis, glycine and valine were the major predominant amino acids of the inhibitory protein (Table 3).

To prevent rotaviral diarrhea, many kinds of oral administration vaccines against each epidemiologically important serotype have been developed [3, 8]. However, such vaccines cannot completely prevent the occurrence of rotaviral diarrhea. On the other hand, rotavirus infection-inhibitory Bifibobacteria are not highly toxic and are considered to be one of the most beneficial probiotics. Therefore, such Bifidobacteria, which produce an inhibitory protein towards rotavirus infection, might contribute to the prevention of rotaviral illness.

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