Original Article

DOI: 10.5582/ddt.2018.01040

Bacterial polysaccharides inhibit sucrose-induced hyperglycemia in silkworms

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Summary **Diabetes and obesity result from sucrose-induced hyperglycemia. Prevention of hyperglycemia contributes to inhibit the onset of these life-related diseases. Here we show that polysaccharides obtained from soil bacteria inhibit sucrose-induced hyperglycemia in an *in vivo* silkworm evaluation system. Ethanol precipitates of extracellular polysaccharides were prepared from viscous bacterial colonies. Among 24 samples obtained from different bacterial species, oral administration of 6 samples from *Rhizobium altiplani*, *Cupriavidus* sp., *Paenibacillus polymyxa*, *Pantoea eucalypti*, *Variovorax boronicumulans*, and *Xanthomonas cynarae* suppressed sucrose-induced hyperglycemia in silkworm insect larvae. The *R. altiplani* fraction treated further with DNase I, RNase A, and proteinase K, followed by phenol extraction also exhibited suppressive activity. Our results suggest that silkworms provide an efficient screening system of bacterial polysaccharides that inhibit sucrose-induced hyperglycemia.**

Keywords: Bacteria, polysaccharide, silkworm, sucrose-induced hyperglycemia

1. Introduction

Transient and rapid postprandial increases in blood glucose levels, referred to as a blood glucose spike, are a potential risk factor for diabetes. Suppression of the blood glucose spike is expected to be useful for preventing the onset of diabetes. Generally, blood glucose levels and their suppression by drugs are evaluated in mammalian animal models. We previously reported that an increase in blood glucose levels after glucose intake could also be evaluated in silkworms, an alternative model animal (*1*-*3*). Not only glucose, but also sucrose intake increases silkworm blood glucose levels (*4*). Glucose level increases in the silkworm hemolymph after sucrose intake are suppressed by acarbose and voglibose, α-glucosidase inhibitors that are used clinically for human diabetes patients (*4*). We propose that the silkworm evaluation system is useful for screening substances that suppress sucrose-induced hyperglycemia.

Bacterial polysaccharides have various structures and biologic activities (*5*-*7*). We recently reported a bacterial polysaccharide with high innate immunity-stimulating activity in a silkworm evaluation system (*8*). Bacterial polysaccharides can be obtained in large quantities at low cost. We therefore propose the use of bacterial polysaccharide libraries for screening seeds of medicines and supplements for human health. In this paper, we describe the collection of polysaccharide-producing bacteria and preliminary screening of polysaccharides that suppress sucrose-induced hyperglycemia in a silkworm model system.

2. Materials and Methods

2.1. *Collection of polysaccharide-producing bacteria*

Bacteria isolated from soil and plants that formed viscous colonies on agar plates were collected. The bacteria grown on the plates (10 cm) were recovered with a spreader and 15 ml of saline, and the cells were removed by centrifugation (8,000 rpm, 5 min). Ethanol (final concentration: 67%) was added to the centrifuge

Released online in J-STAGE as advance publication August 24, 2018.

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supernatant, and fibrous precipitates were collected by centrifugation. Bacterial 16S rRNA was sequenced and homology searches were performed to determine the bacterial species using the EZBiocloud database.

The crude polysaccharide fractions were treated enzymatically as follows. DNase I (1,000 U/mL; Promega) and RNase A (10 μg/mL; NIPPON GENE CO., LTD.) were added to the ethanol precipitate, dissolved in water, and incubated overnight at 37°C, and then further incubated overnight at 37°C with protease K (100 μg/mL). Phenol: chloroform: isoamyl alcohol (50:49:1) was added to the fraction, and the samples were vigorously shaken, followed by the addition of two volumes of ethanol to the upper layer fraction. The precipitates were then collected by centrifugation.

Table 1. Homology search of 16S rRNA sequences of bacteria producing extracellular polysaccharides

Species	Strain	Identities (%)
Bacillus megaterium	129-19	99
Cupriavidus sp.	No.48	97
Curtobacterium plantarum	126-8	99
Enterobacter kobei	126-4b	97
Enterobacter tabaci	118-13A	97
Escherichia coli	118-18	98
Ewingella americana	221-5-2	99
Gluconobacter cerinus	221-2-2	99
Kosakonia sp.	118-14	98
Lelliottia amnigena	112-13-1	99
Novosphingobium panipatense	208-110	98
Paenarthrobacter nicotinovorans	208-57	99
Paenibacillus lupini	110-24	99
Paenibacillus polymyxa	126-6-1A	99
Pantoea eucalypti	118-5-1	98
Pantoea sp.	126-2	99
Pantoea vagans	126-1	99
Paraburkholderia insulsa	118-3-2	99
Paracoccus aestuariivivens	126-5b	98
Pseudomonas nitroreducens	No.24	98
Pseudomonas palleroniana	118-25A	99
Rhizobium altiplani	No.26	99
Variovorax boronicumulans	110-14	99
Xanthomonas cynarae	No.4	100

Bacterial species of 24 strains isolated in this study are listed. Bacterial species exhibiting the highest sequence homology are presented. When the species of the highest homologous bacterial strain could not be specified, only the names of the genus are shown.

2.2. *Sucrose tolerance test of silkworms*

Silkworms (Hu Yo x Tsukuba Ne, Ehime Sericulture Incorporated Company, Ehime, Japan) were reared as described previously (*9,10*). The silkworm sucrose tolerance test was conducted according to the previously reported method (*4*). Briefly, sucrose (10%) and test samples were mixed with silkworm artificial diet. Sucrose diet with or without polysaccharide samples was fed to 5th-instar larva of silkworms for 1 h, the silkworm hemolymph was collected, and glucose concentrations were measured with a glucometer (Accu-Chek, Roche).

3. Results

We collected bacteria that formed viscous colonies on YME agar plates. The isolated bacteria comprised 19 genera (Table 1). Ethanol precipitates of crude polysaccharides (see Materials and Methods) were mixed with silkworm diet containing 10% sucrose and fed to the silkworms. After 1 h, the blood glucose levels of silkworms were measured. Among the 24 polysaccharide fractions tested, 6 samples from *Rhizobium altiplani*, *Cupriavidus* sp., *Paenibacillus polymyxa*, *Pantoea eucalypti*, *Variovorax boronicumulans*, and *Xanthomonas cynarae* exhibited suppressive effects on the increase in the blood sugar level of silkworms (Table 2). The differences in the glucose level between controls without bacterial samples and those with bacterial polysaccharides were statistically significant.

The crude polysaccharide fraction from *R. altiplani* was treated with DNase I, RNase A, and proteinase K, and further extracted with phenol. The treatment had little effect on the sugar content, whereas the amounts of DNA and protein were greatly reduced to 1/20 and 1/28, respectively (Table 3). This phenol-extracted fraction also exhibited suppressive activity against sucrose-induced hyperglycemia (Figure 1).

4. Discussion

The findings of the present study demonstrated that bacterial polysaccharides from *R. altiplani*

Table 2. Bacterial polysaccharides that suppress sucrose-induced hyperglycemia by oral administration

Species	Strain	Sample weight (mg/g diet)	Blood glucose (mg/dL)		*p* value
			Exp. 1	Exp. 2	
No bacteria (control)		-	320 ± 64	366 ± 47	
Cupriavidus sp.	No.48	55	N.D.	207 ± 91	0.001
Paenibacillus polymyxa	126-6-1A	12	210 ± 27	N.D.	0.004
Pantoea eucalypti	118-5-1	9.4	226 ± 60	N.D.	0.02
Rhizobium altiplani	No.26	60	121 ± 22	N.D.	0.00003
Variovorax boronicumulans	110-14	21	253 ± 29	N.D.	0.05
Xanthomonas cynarae	No.4	25	N.D.	252 ± 37	0.0004

Ethanol precipitation fraction was mixed with 1 g of 10% sucrose-containing artificial diet. The mixture was fed to silkworms for 1 h, and the glucose levels in the silkworm hemolymph were determined. Statistically significant differences between control and testing groups were evaluated using Student's *t*-test. N.D., not determined. $n = 5$-10.

Table 3. Comparison of the amounts of DNA and protein in a crude polysaccharide fraction and an enzyme-treated fraction prepared from *Rhizobium altiplani*

Fraction	Sugar (mg/g)	DNA (mg/g)	Protein (mg/g)
Crude polysaccharide	490	10	56
Enzyme-treated sample	520	0.45	2.1

Crude polysaccharide was incubated with DNase I(1,000 U/mL) and RNaseA (10 µg/mL) 24 h at 37˚C. Then, proteinase K (100 µg/mL) was added to the samples and incubated 24 h at 37˚C. Phenol/chloroform/isoamyl alcohol was added and vigorously mixed, followed by centrifugation. The upper layer fraction was collected and mixed with ethanol (final concentration: 67%). Fibrous precipitates were collected by centrifugation. The amounts of sugars, DNA, and proteins in the crude polysaccharide fraction and the enzyme-treated fraction were measured by the phenol-sulfuric acid method, the fluorescent-based Qubit assay, and Bradford assay, respectively.

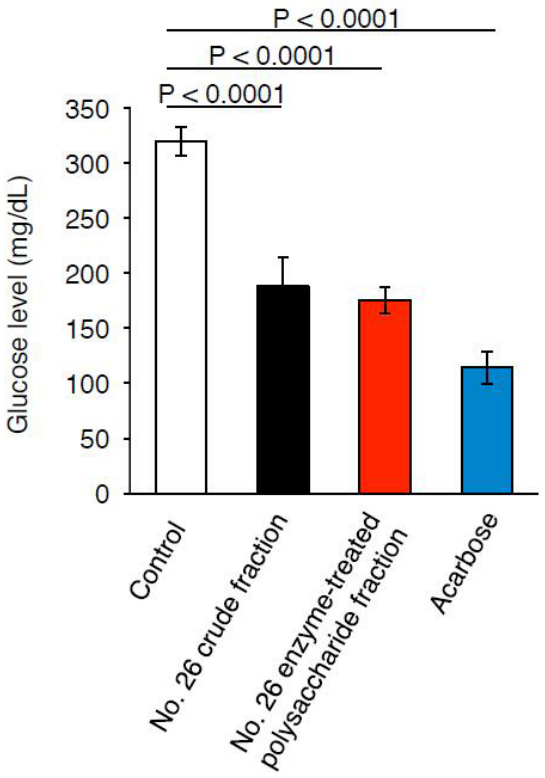

Figure 1. Effect of enzyme-treated polysaccharide fraction on sucrose-induced hyperglycemia. Crude fraction (50 mg/g diet) prepared from *R. altiplani* No. 26, enzyme-treated polysaccharide fraction (46 mg/g diet), and acarbose (40 mg/g diet) were mixed with 10% sucrose containing diet, respectively. Diets with the polysaccharide fraction were orally administered to silkworms for 1 h. The glucose levels in silkworm hemolymph were determined. Acarbose was used as a control according to a previous report (*4*). Statistically significant differences between control and testing groups were evaluated using Student's *t*-test.

markedly suppressed sucrose-induced hyperglycemia in silkworms. We propose that the polysaccharides screened using the silkworm model are promising candidates for healthy foods and medicines to prevent the onset and exacerbation of diabetes. The silkworm system is superior to mammalian systems in terms of cost and ethical issues (*11-13*). Therefore, the silkworm system is expected to be useful for screening bacterial polysaccharides that inhibit increases in blood glucose levels in humans. Bacteria secreting polysaccharides can be easily obtained as viscous colonies on agar plates. Polysaccharides secreted from bacteria have various structures depending on the bacterial species (*6,7*). Furthermore, industrial mass production of bacterial polysaccharides is possible. Based on these properties, it is expected that the library of bacterial polysaccharides will be useful for screening compounds with physiologic activities, such as agents with blood sugar lowering effects.

Acknowledgements

We thank Mari Maeda and Miki Takahashi (Genome Pharmaceuticals Institute Co., Ltd, Tokyo, Japan) for their technical assistance in rearing the silkworms. The project was supported by JSPS KAKENHI grant number JP15H05783 (Scientific Research (S) to KS), and JSPS KAKENHI grant number JP17K08288 (Scientific Research (C) to YM). The project was also supported by Genome Pharmaceuticals Institute Co., Ltd (Tokyo, Japan).

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(Received July 12, 2018; Accepted August 2, 2018)