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Pectin-like acidic polysaccharide from *Panax ginseng* with selective antiadhesive activity against pathogenic bacteria

Ji-Hye Lee,[a] Jin Sun Shim,[b] Jung Sun Lee,[b] Mi-Kyung Kim,[b] Mi-Sook Chung[b] and Kyung Hyun Kim[a,c,*]

[a]*Department of Food Technology, School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea*
[b]*Department of Food Science and Nutrition, Duksung Women's University, Seoul 132-714, Republic of Korea*
[c]*Department of Biotechnology, College of Science and Technology, Chungnam 339-700, Republic of Korea*

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Abstract—Previous studies have revealed the inhibitory effects of an acidic polysaccharide purified from the root of *Panax ginseng* against the adhesion of *Helicobacter pylori* to gastric epithelial cells and the ability of *Porphyromonas gingivalis* to agglutinate erythrocytes. In this study, this acidic polysaccharide from *P. ginseng*, PG-F2, was investigated further, in order to characterize its antiadhesive effects against *Actinobacillus actinomycetemcomitans*, *Propionibacterium acnes*, and *Staphylococcus aureus*. The minimum inhibitory concentrations (MIC) were found to be in a range of 0.25–0.5 mg/mL. However, results showed no inhibitory effects of PG-F2 against *Lactobacillus acidophilus*, *Escherichia coli*, or *Staphylococcus epidermidis*. PG-F2 is a pectin-type polysaccharide with a mean *MW* of 1.2×10^4 Da, and consists primarily of galacturonic and glucuronic acids along with rhamnose, arabinose, and galactose as minor components. The complete hydrolysis of PG-F2 via chemical or carbohydrolase enzyme treatment resulted in the abrogation of its antiadhesive activity, but limited hydrolysis via treatment with pectinase (EC. 3.2.1.15) yielded an oligosaccharide fraction, with activity comparable to the precursor PG-F2 (the MIC of ca. 0.01 mg/mL against *H. pylori* and *P. gingivalis*). Our results suggest that PG-F2 may exert a selective antiadhesive effect against pathogenic bacteria, while having no effects on beneficial and commensal bacteria.

Keywords: Bacterial adhesion; Hemagglutination; *Panax ginseng* (Araliaceae); Antiadhesive activity; *Helicobacter pylori*; *Porphyromonas gingivalis*; *Actinobacillus actinomycetemcomitans*; *Propionibacterium acnes*; *Staphylococcus aureus*

1. Introduction

The human body hosts a variety of microbial flora. These flora are comprised largely of bacteria with a total number of cells greater than the rest of the human body. The acquisition of normal microflora of the oral, respiratory, and gastrointestinal tracts, as well as the skin, provides crucial stimuli for the development of innate immunity. These commensal bacteria are antigenic and can elicit antibody responses.[1] They also generate metabolites that function as essential nutrients for human cells, and exert a protective effect against more pathogenic bacteria. An increasing body of evidence suggests that the cooperation between human and commensal flora, and the stability of the bacterial ecosystem, may play a more important role in human health than had previously been recognized.[2]

Helicobacter pylori is a highly motile, Gram-negative microaerophilic bacterium, and has been identified as a principal etiological agent of chronic active or type B gastritis, duodenal ulcer, gastric carcinoma, and mucosa-associated lymphoid tumors.[3–6] *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* are also Gram-negative bacteria, but are facultative anaerobes that can frequently be isolated from the periodontal pockets of patients suffering from advanced adult or

* Corresponding author. Tel.: +82 2 3290 3444; fax: +82 2 927 9028; e-mail: khkim@korea.ac.kr

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localized juvenile periodontitis, respectively.[7,8] *Propionibacterium acnes* is a Gram-positive bacteria and a major inhabitant of adult human skin. This bacterium has been primarily implicated in acne, the most common skin disease.[9] *Staphylococcus aureus* is also a Gram-positive bacteria, carried primarily on mucosal surfaces, and constitutes the predominant causative agent of life-threatening nosocomial infections.[10] All of these bacteria require iron to facilitate basic cellular processes, including respiration and DNA synthesis. They hemagglutinate erythrocytes and bind to receptors on different tissue culture cells, utilizing heme and hemoglobin for their growth.[11–13]

Bacterial adhesion to human cells is a key step initiating the infection that may lead to the development of diseases.[14] Cell-surface carbohydrates mediate host–bacterial recognition during this adhesion through single or multiple interactions. The specificities inherent to these host–bacterial interactions have been the subject of recent studies,[15,16] from which a number of putative host receptors for *H. pylori*, *P. gingivalis*, *P. acnes*, and *S. aureus* have been discovered.[17–22] Previously, acidic polysaccharides obtained from the roots of *Panax ginseng* C.A. Meyer (Araliaceae) and the leaves of *Artemisia capillaris* (Asteracae) were reported to exhibit a marked inhibitory effect against the adhesion of *H. pylori* to human gastric cells and erythrocytes.[23–25] In addition, a polysaccharide obtained from *P. ginseng* has been shown to exert a comparable inhibitory effect against *P. gingivalis*-mediated hemagglutination.[26] In the present study, we have evaluated the antiadhesive effects of this polysaccharide against *H. pylori*, *P. gingivalis*, *A. actinomycetemcomitans*, *P. acnes*, and *S. aureus*, as well as *Lactobacillus acidophilus*, *Escherichia coli*, and *S. epidermidis* by using hemagglutination assays. We then compared our results with those obtained with other carbohydrates, and a partially hydrolyzed oligosaccharide derived from the polysaccharide. Our results suggest that this active polysaccharide may act as a selective carbohydrate mimetic, thereby effecting the blockage of a variety of host–pathogen interactions.

2. Results

2.1. Purification and characterization of acidic polysaccharide PG-F2

The acidic polysaccharide, PG-F2, was purified from *P. ginseng* based on the procedure established previously,[23] with minor modifications including DNase and RNase treatment and a second ethanol precipitation. A typical elution profile from Q-Sepharose anion-exchange chromatography resulted in a broad major peak at 226 nm with a 0.2–0.5 M NaCl elution gradient. Polysaccharide fractions with high uronic acid content were detected at

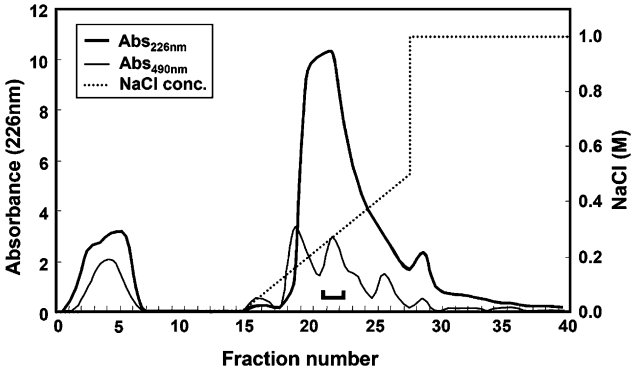

Figure 1. Elution profile of polysaccharides from *P. ginseng* by ion-exchange chromatography. A Q-Sepharose fast-flow column (2.5 × 10 cm) was used at a flow rate of 1 mL/min. The thin line represents absorbance at 490 nm from the phenol–sulfuric acid assay, and the dotted line represents the NaCl concentration gradient. The pooled fractions are indicated by the bar below the peak.

NaCl concentrations of 0.2–0.3 M (Fig. 1). These polysaccharide fractions were determined to exhibit profound antiadhesive activity against *H. pylori*, which were then further purified into a small peak of high molecular weight (PG-HMW) and a major peak of lower molecular weight (PG-F2), via Sephacryl S-200 gel-filtration FPLC (Fig. 2). Both of the peaks were of high uronic acid content, and no proteins were detected (Table 1). Notably, PG-F2 evidenced the highest degree of antiadhesive activity against *H. pylori* among the fractions. Its molecular mass, as estimated by gel-filtration chromatography using dextran molecular weight standards (5–150 kDa), was approximately 12 kDa (data not shown). This finding suggests that PG-F2 may be composed of approximately 60–70 sugar units, calculated from the average molecular weight of 180 Da per sugar unit.

Carbohydrate analyses of PG-F2 revealed that it contains significant amounts of galacturonic and glucuronic

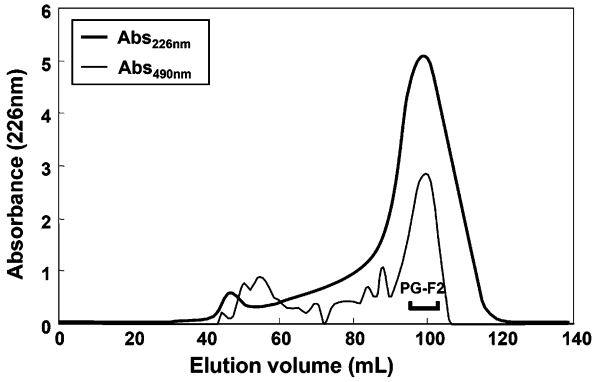

Figure 2. Elution profile of PG-F2 by gel-filtration chromatography. A Sephacryl S200 column (1.5 × 68 cm) was used at a flow rate of 0.5 mL/min. The thin line represents the absorbance at 490 nm from the phenol–sulfuric acid assay. PG-F2 is indicated by the bar below the peak.

Table 1. Characteristics of polysaccharides and an oligosaccharide purified from *P. ginseng*

	PG-HMW	PG-F2	GP-OF1
Carbohydrate (ug/mg)	10.8	16.0	0.7
Uronic acid (ug/mg)	255.3	578.9	455.1
Protein (ug/mg)	0.0	0.0	0.0
Yield (%)[a]	0.03	0.15	0.02
Molecular mass (kDa)	80	12	1
Sugar composition (mol %)[b]			
Rhamnose	6.4	2.3	0.3
Fucose	0	0.6	0
Arabinose	20.7	1.8	1.0
Xylose	2.6	0.1	0
Mannose	0	0	0
Galactose	6.3	1.6	0
Glucose	3.5	0	0
Galacturonic acid	41.2	76.6	72.7
Glucuronic acid	19.3	16.9	25.9

[a] Calculated as weight percent of applied material.
[b] Mole percent of total carbohydrate content.

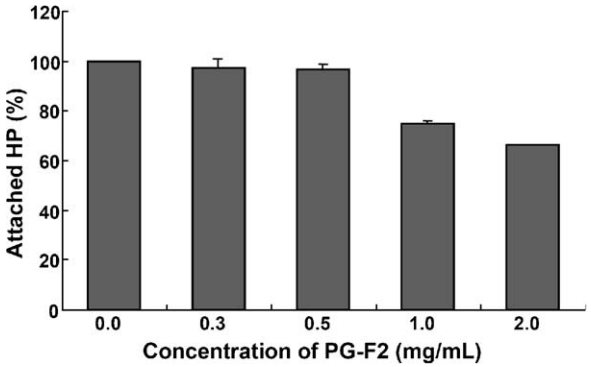

Figure 3. Inhibition of the binding of *H. pylori* to AGS cells. Attached *H. pylori* % = 100 − [(OD$_{experimental}$ − OD$_{negative}$)/(OD$_{positive}$ − OD$_{negative}$) × 100]. Negative control contained only epithelial monolayers without bacteria. Positive control contained bacteria and monolayer without added inhibitor, which was used to establish 100% attachment. Data are represented as the means of three separate experiments (mean ± s.d.).

acids, and small amounts of rhamnose, arabinose, and galactose as neutral sugars (Table 1). Interestingly, galacturonic acid represented the highest sugar compositional element (76.6%) and provided substantial amounts of uronic acids, implying that PG-F2 is composed primarily of acidic polysaccharides. Thus, PG-F2 may be an arabinogalactan-type polysaccharide, which is mainly composed of galacturonic and glucuronic acids, much like the pectin-type polysaccharides.

2.2. PG-F2 inhibits *H. pylori* adhesion to AGS gastric cells

In a previous study, the adherence of *H. pylori* to AGS gastric cells was determined via scanning electron microscopy (SEM) by counting microcolonies of *H. pylori* attached to the surfaces of gastric cells.[25] Administration of the acidic polysaccharide was found to attenuate the degree to which *H. pylori* attached to the gastric cells. This PG-F2-induced inhibition of the attachment of *H. pylori* to the AGS cells was re-examined using a urea phenol red assay. PG-F2 was not found to affect the viability of the AGS cell line at the concentrations used in this assay, but significantly inhibited the attachment of *H. pylori*, by ca. 40% at 2 mg/mL (Fig. 3). This finding was consistent with the results obtained in the previous SEM analysis,[25] which revealed a 36–67% inhibition at a concentration range from 0.2 to 2.8 mg/mL. Our results clearly indicate that PG-F2 treatment strongly inhibits the interaction between *H. pylori* and AGS cells.

2.3. Hydrolysis of PG-F2

The hydrolysis of PG-F2, accomplished by treatment with $NaNO_2$, α- and β-amylase, or cellulase, yielded oligosaccharide fractions of lower molecular weight, which were found to be non-inhibitory up to a concentration of 2 mg/mL (data not shown). By contrast, a limited enzymatic hydrolysis of PG-F2 by pectinase (EC. 3.2.1.15, poly-[1,4-α-D-galacturonide]glycanohydrolase) for 30 min at 37 °C resulted in the production of oligosaccharide fragments, which were then

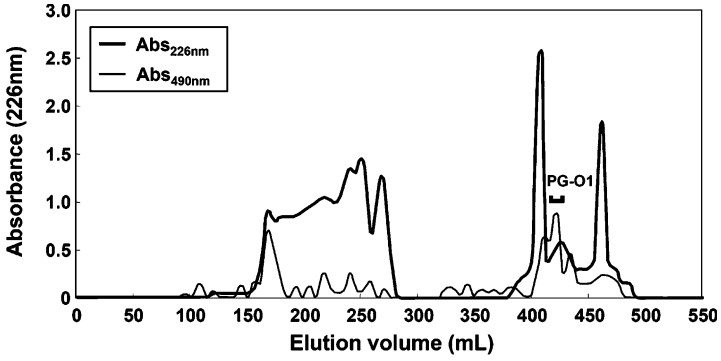

Figure 4. Elution profile of PG-OF1 by gel-filtration chromatography. A Bio-Gel P4 column (2.5 × 102 cm) was used at a flow rate of 0.3 mL/min. The thin line represents absorbance at 490 nm from the phenol–sulfuric acid assay. PG-OF1 is indicated by the bar above the peak.

purified by using Bio-Gel P-4 gel-filtration chromatography (Fig. 4). One of the oligosaccharide fractions, PG-OF1, exhibited activity comparable to that of the precursor PG-F2 against *H. pylori* attachment, with an MIC of 0.01 mg/mL.

Carbohydrate analyses of PG-OF1 revealed the highest uronic acid content among the fractions, and indicated a composition of galacturonic acid (72.7%) and glucuronic acid (25.9%), with minor amounts of arabinose and rhamnose (Table 1). The molecular mass of the predominant oligosaccharide in PG-OF1 was estimated by gel-filtration chromatography to be approximately 1 kDa and determined by MALDI-TOF mass spectrometry to be about 815 Da (see Fig. 1 in the Supplementary data). This suggests that PG-OF1 may be equivalent to 4–5 sugar units, calculated from the average molecular weight of 180 Da per sugar unit.

2.4. Antiadhesive activity of PG-F2 or PG-OF1 against oral, gastrointestinal, and skin bacteria

PG-F2 was assessed with regard to its antiadhesive effects against *A. actinomycetemcomitans*, *P. acnes* and *S. aureus*, as well as *H. pylori* and *P. gingivalis*, by using a series of hemagglutination assays. Surprisingly, PG-F2 was found to inhibit the attachment of these gastric, oral, and skin pathogenic bacteria, in a range of 0.1–0.5 mg/mL (Table 2 and Fig. 5). In contrast, when PG-F2 was examined regarding its possible effects against the beneficial or commensal bacteria, *L. acidophilus*, *E. coli*, and *S. epidermidis*, it showed little activity against them, even at concentrations of 2.0 mg/mL (Table 2).

It is worth noting that PG-OF1, a partially hydrolyzed oligosaccharide from PG-F2, also exhibited profound antiadhesive properties against *H. pylori* and *P. gingivalis*, with an MIC of ca. 0.01 mg/mL, which is 10-fold lower than that of its precursor PG-F2 (Table 2 and Fig. 6). The MICs of PG-OF1 against *A. actinomycetemcomitans* and *P. acnes* were as high as those of PG-F2, but it evidenced minimal inhibitory activity against *S. aureus*. Probably more importantly, PG-OF1 was also determined to exert no detectable activity against *L. acidophilus*, *E. coli*, or *S. epidermidis*, similarly to its precursor, PG-F2. The antiadhesive effects of both PG-F2 and PG-OF1 against *H. pylori* and *P. gingivalis* can likely be highly correlated with their inherent high uronic acid content. In this context, other carbohydrates harboring negatively charged groups were examined in order to elucidate the structure–activity relationships for the carbohydrates.

2.5. Antiadhesive activity of carbohydrates against oral, gastrointestinal, and skin bacteria

Among carbohydrates containing negatively charged groups, the antiadhesive effects evidenced by pectin were the most pronounced against *P. gingivalis* with an MIC of ca. 0.0001 mg/mL (Table 2). Pectin also exhibited significant antiadhesive properties against *P. acnes* and *S. aureus* with an MIC of 0.01 mg/mL. However, these effects disappeared at concentrations above 0.1 mg/mL. Alginic acid has lower but broad inhibition against bacteria at concentrations of 0.25–0.5 mg/mL. LMWH and SOS were highly active against *P. gingivalis*, both with MICs of 0.01 mg/mL (9 μM). Only xylitol was

Table 2. Inhibition of bacteria-induced hemagglutination by an acidic polysaccharide from *Panax ginseng* and by a range of carbohydrates

Inhibitors[b]	MW(Da)	Minimum inhibitory concentrations (MIC) in mg/mL[a]							
		H. p	*P. g*	*A. a*	*P. a*	*S. a*	*S. e*	*E. c*	*L. a*
PG-F2	12,000	0.1	0.1	0.25	0.5	0.5	—	—	—
PG-HMW	80,000	0.25	0.1	0.1	0.1	—	—	—	—
PG-OF1	1000	0.01	0.01	0.5	0.25	—	—	—	—
Pectin[c]	20,000	—	0.0001	—	0.01	0.01	—	—	—
Alginic acid	1,000,000	0.25	0.25	0.25	—	0.25	0.25		
Heparin	3000	—	0.02	—	0.1	1.0	—	—	0.1
SOS	1159	—	0.01	—	1.0	1.0	—	—	—
GlcA[d]	194	—	—	—	0.5	0.5	0.5	—	0.1
GalA[d]	212	—	—	—	—	—	—	—	0.01
GlcNAc[d]	212	—	0.01	—	—	—	—	—	—
Xylitol	152	—	—	0.01	—	—	—	—	—
Palatinose	342	—	0.1	—	—	—	—	0.01	—
Trehalose	342	—	0.01	—	—	—	—	0.1	0.01

[a] All the values are the MIC in mg/mL, corresponding to the average in triplicates. '—' represents no inhibition at high concentrations above 2.0 mg/mL. Aspartic and glutamic acids were used as non-carbohydrate acidic compounds and bacterial binding did not cause nonspecific reaction (data not shown). *H. p*: *Helicobacter pylori*, *P. g*: *Porphyromonas gingivalis*, *A. a*: *Actinobacillus actinomycetemcomitans*, *P. a*: *Propionicbacterium acnes*, *S. a*: *Staphylococcus aureus*, *S. e*: *Staphylococcus epidermidis*, *E. c*: *Escherichia coli*, *L. a*: *Lactobacillus acidophillus*.

[b] PG-F2 and PG-OF1 represent a polysaccharide and its hydrolyzed oligosaccharide from *P. ginseng*, respectively.

[c] Pectin showed the highest inhibition to *P. gingivalis* with an MIC of ca. 0.0001 mg/mL. However, pectin did not show any inhibitory activity above 0.1 mg/mL.

[d] GlcA, GalA, and GlcNAc are D-glucuronic acid, D-galacturonic acid, and *N*-acetylglucosamine, respectively.

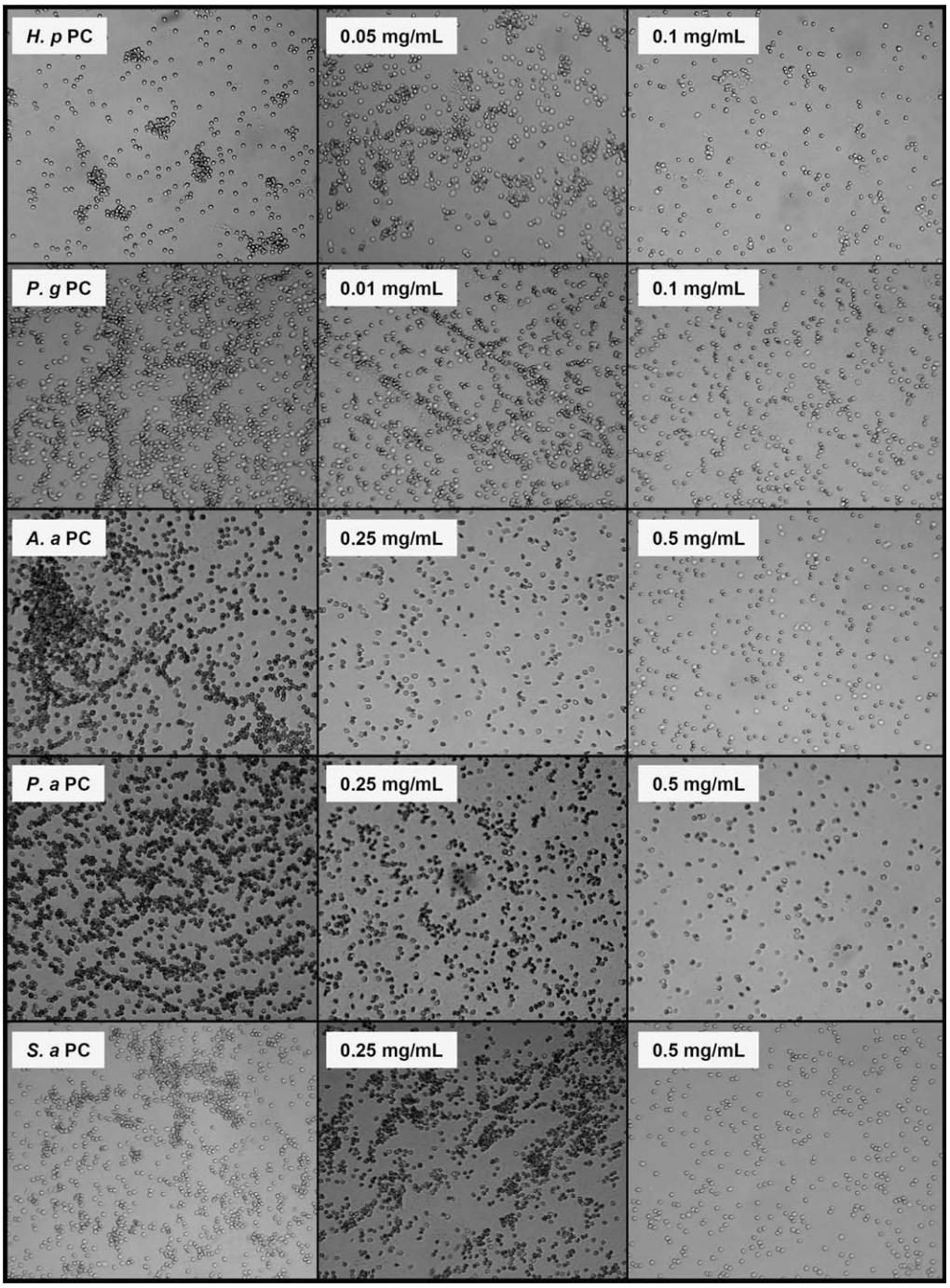

Figure 5. Micrograph images of a hemagglutination inhibition assay by pathogenic bacteria (magnification ×100). Hemagglutination was inhibited by PG-F2 from *P. ginseng*, which is active against *H. pylori* (*H. p*), *P. gingivalis* (*P. g*), *A. actinomycetemcomitans* (*A. a*), *P. acnes* (*P. a*), and *S. aureus* (*S. a*). The minimum inhibitory concentrations were in the range of 0.1–0.5 mg/mL. A positive control (without inhibitor) is shown along with each pathogenic bacterium.

found to be active against *A. actinomycetemcomitans* with an MIC of 0.01 mg/mL (66 μM). Palatinose and trehalulose exhibited significant *P. gingivalis*-mediated hemagglutination inhibitory effects, but they were also inhibitory against *E. coli*. However, glucuronic and galacturonic acids exhibited only minimal inhibitory activity against the pathogenic bacteria used in this study. Rather, *N*-acetylglucosamine exerted a significant effect against *P. gingivalis* and was comparable to LMWH and SOS in this regard. As is shown in Table 2, the asaccharolytic *P. gingivalis* was the most sensitive to the carbohydrates examined in this study, and *H. pylori* was the most resistant to the carbohydrates, with the notable exceptions of PG-F2 and PG-OF1.

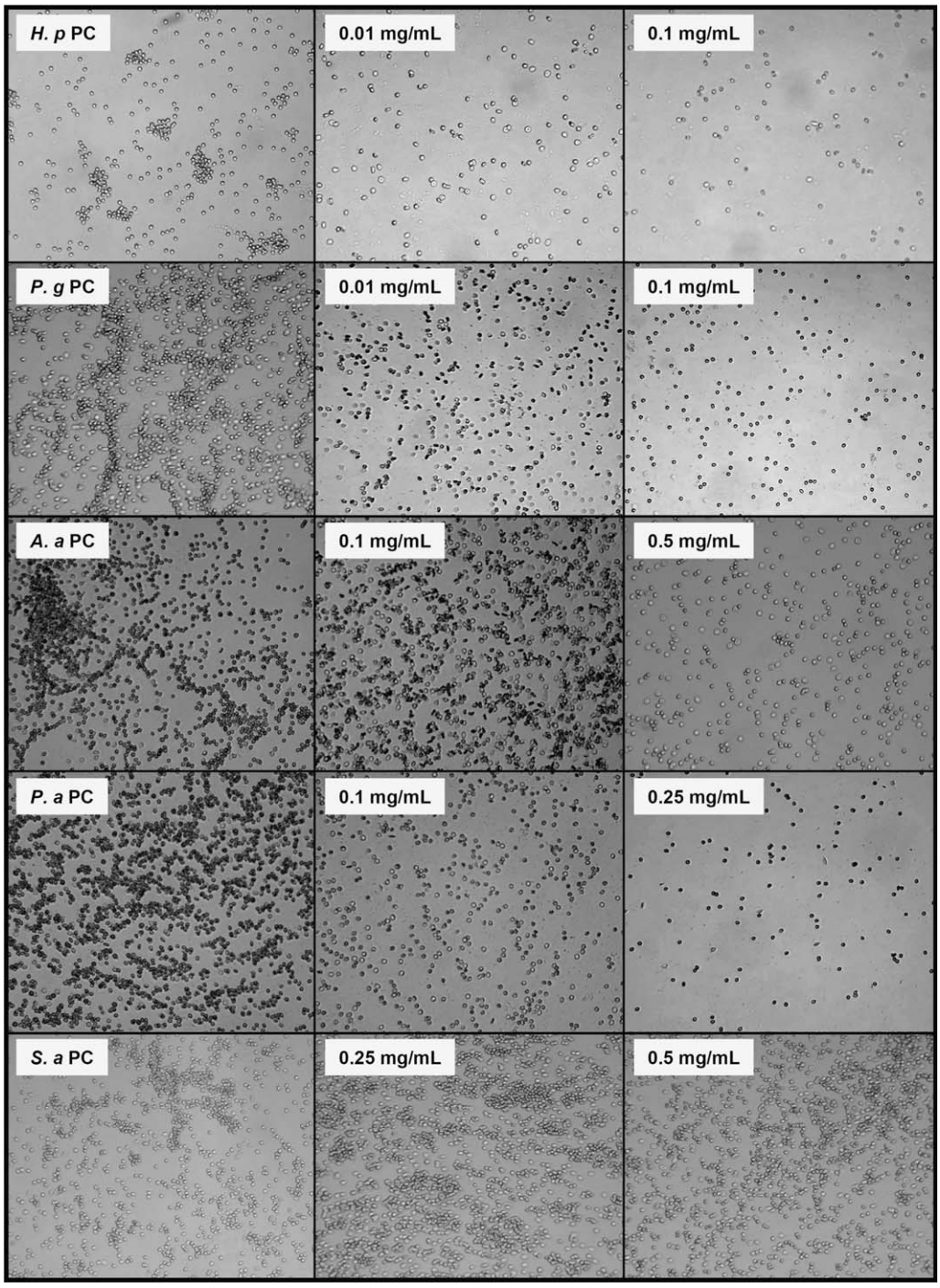

Figure 6. Micrograph images of a hemagglutination inhibition assay by pathogenic bacteria (magnification ×100). Hemagglutination was inhibited by the oligosaccharide PG-OF1, which is active against *H. pylori* (*H. p*), *P. gingivalis* (*P. g*), *A. actinomycetemcomitans* (*A. a*), *P. acnes* (*P. a*), and *S. aureus* (*S. a*), with minimum inhibitory concentrations between 0.01 and 0.5 mg/mL. A positive control (without inhibitor) is shown along with each pathogenic bacterium.

3. Discussion

The attachment or adhesion of pathogenic bacteria to human epithelial cells is a pivotal step in the initiation of the infection that leads to the development of various diseases.[14] Acidic polysaccharides from the roots of *P. ginseng* and the leaves of *A. capillaris* were previously determined to exhibit remarkable inhibitory effects against the adherence of *H. pylori* to human gastric cells and erythrocytes.[23,25] The polysaccharide from *P. ginseng* demonstrated a comparable inhibitory activity against *P. gingivalis*-mediated hemagglutination.[26] In

the present study, the PG-F2 polysaccharide further demonstrated its profound antiadhesive effects against *A. actinomycetemcomitans*, *P. acnes*, and *S. aureus*, in a concentration range of 0.25–0.5 mg/mL. Given its apparent molecular weight of 12 kDa, the effective concentrations of PG-F2 occurred in a range of 20–40 μM. These active concentrations are comparable to those of sialyl-3′-lactose, which has been considered to be the most active oligosaccharide.[27] In fact, sialyl-3′-lactose exhibited no significant inhibitory effects at a concentration of 0.1 mg/mL (ca. 150 μM), which is lower than that of PG-F2 with regard to the inhibition of *H. pylori*-mediated adhesion to AGS cells.[25] Whereas the inhibitory effect of sialyl-3′-lactose depended on *H. pylori* strains,[27] PG-F2 had similar activities against strains 43504 and 26695, in addition to 49503 previously reported.[23]

Notably, PG-F2 did not inhibit the adhesion capabilities of beneficial or commensal bacteria, including *L. acidophilus*, *E. coli*, and *S. epidermidis*. Although certain deleterious or pathogenic strains of *E. coli*, such as strain O157, are known to cause human disease,[28] many other strains live naturally and harmlessly within the human digestive system. *L. acidophilus* has been shown to provide health benefits.[29] Whereas *S. epidermidis* can be regarded as a pathogen primarily associated with nosocomial infections contracted from blood-contacting devices within hospitals,[30] it also exerts beneficial effects as a normal constituent of the flora of healthy human skin.[21] Collectively, our findings indicate that, whereas PG-F2 exhibits profound antiadhesive activity against pathogenic bacteria, it exerts no or minimal inhibitory effects against commensal bacteria. This suggests, then, that PG-F2 is selectively antiadhesive against human pathogenic bacteria.

PG-F2 may be a pectin-type polysaccharide composed primarily of galacturonic and glucuronic acids (93%). This uronic acid content appears to be significantly higher than previously reported,[25] and this can probably be attributed to improved techniques for the separation and calculation of neutral and acidic polysaccharides. Acidic polysaccharides obtained from plant sources have been shown to exhibit a variety of biological activities, including immunostimulatory, antioxidant, antitumor, and antiviral properties.[31–33] An acidic polysaccharide with immunomodulating activity, which was obtained from *P. ginseng* leaves, was found to consist of a highly branched glycan structure, composed of arabinose, galactose, rhamnose and galacturonic acid with a β-(1→3)-linked galactan backbone.[34] Glycosaminoglycan, *N*-acetylneuraminic acid and glucuronic acid were all previously implicated in *P. gingivalis*-mediated cell adhesion.[13,14] Negatively charged polysaccharides, such as dextran sulfate, also inhibit the adhesion of respiratory pathogens, including *P. aeruginosa*, *Burkholderia cenodepacia*, *Burkholderia pseudomallei*, *L. pneumophila*, *Bacillus anthracis*, and *Y. pestis*.[35] In addition, citrus pectin has proved effective with regard to the inhibition of adhesion to the specific cell-surface receptors.[33] Our findings also indicated that pectin exerts a profound antiadhesive effect against *P. gingivalis*, *P. acnes*, and *S. aureus*. These data are, therefore, suggestive that antiadhesive activity might be strongly associated with high uronic acid content, implying the potential role of carbohydrates harboring negatively charged groups in host–bacterial adhesion. In this context, it was not surprising to find that pectin, alginic acid, LMWH, and SOS were all effective in the prevention of *H. pylori* or *P. gingivalis*-mediated hemagglutination.

As is shown in Table 2, although pectin exhibited a high degree of antiadhesive activity against *P. gingivalis* with an MIC of 0.0001 mg/mL, it did not exhibit activity at concentrations above 0.1 mg/mL. Alginic acid, which is composed of guluronic acid and mannuronic acid, showed inhibition against bacteria at 0.25–0.5 mg/mL. Alginic acid was found to cause red cell hemolysis at concentrations above 1.0 mg/mL, and its inhibitory effect may be partly due to hemolysis. These results of pectin and alginic acid, although reproducible, have yet to be clearly elucidated. Glucuronic and galacturonic acids were not shown to have any inhibitory activity against the bacteria used in this study. Thus, we cannot rule out the possibility that specific carbohydrate components of PG-F2, other than uronic acids, may also play a role in the antiadhesion in host–bacterial interactions, thereby implying that host–bacterial adhesion may rely on multiple interactions.

Asaccharolytic *P. gingivalis* was shown to be the most sensitive to the carbohydrates used in this study, which involves binding to a variety of receptors containing collagen, fibronectin, laminin, vitronectin and cytokeratin.[7,36] The adhesion of *S. aureus* to the skin, as is seen in cases of atopic dermatitis, induces the formation of a biofilm, which binds to fibronectin, fibrinogen, laminin, and sphinganine.[15,16,21,22] Multiple agents specifically inhibiting each type of receptor adhesions, or a single agent exhibiting a broad spectrum antiadhesion activity, could be considered as candidates for the development of an antiadhesive therapeutic modality.[14,17] In this connection, PG-OF1, a partially hydrolyzed oligosaccharide from PG-F2, may mimic the effects of the core carbohydrate moiety in host–pathogen interactions, and should prove useful in the development of new carbohydrate-based antiadhesive agents with broad-spectrum activity.

4. Experimental

4.1. Materials

The ginseng root extract concentrates were kindly supplied by S&D Co. (Cheon-ahn, Korea). *H. pylori* (ATCC

43504 and 26695), *P. gingivalis* (ATCC 33277), *A. actinomycetemcomitans* (ATCC 29522), *P. acnes* (ATCC 6919), and *L. acidophilus* (ATCC 4356) cells were acquired from the Korean Culture Center of Microorganisms (KCCM) (Seoul, Korea). The *E. coli* strains, BL21(DE3) and BL21(DE3)pLysS, were purchased from Novagen (Madison, WI, USA). *S. aureus*, *S. epidermidis*, and human erythrocytes were obtained from Korea University Hospital (Ansan, Korea). Pectinase (EC. 3.2.1.15, poly-[(1→4)-α-D-galacturonide]glycanohydrolase), α- and β-amylases (EC 3.2.1.1. and EC 3.2.1.2, respectively), trypsin (EC 3.4.21.4), D-glucuronic acid, D-galacturonic acid, trehalulose (α-D-glucosylpyranosyl-(1→1)-D-fructofuranose), palatinose (α-D-glucopyranosyl-(1→6)-D-fructofuranose), $NaNO_2$, low-molecular-weight heparin (LMWH), citrus pectin, alginic acid sodium salt, ribonuclease A from bovine pancreas (RNase), deoxyribonuclease I from bovine pancreas (DNase), bovine serum albumin (BSA), methyl-β-cyclodextrin (CD), and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich (St. Louis, USA). Sucrose octasulfate (SOS) was purchased from Toronto Research Chemicals (North York, ON, Canada). Carbazole, phenol, and H_2SO_4 were purchased from Junsei Chemicals (Tokyo, Japan). The GC internal standards, *myo*-inositol and mannonic acid lactone, were also obtained from Sigma–Aldrich (St. Louis, USA).

4.2. Bacterial cell growth

The *H. pylori* cells were grown in Brucella broth (Difco) containing 10% (v/v) fetal bovine serum (FBS), 0.2% (w/v) 2,6-di-*O*-methyl-β-cyclodextrin (CD) and antibiotics (cefsulodin, vancomycin, trimethoprim, and amphotericin B). The cells were incubated in a 10% CO_2 atmosphere at 37 °C for 43 h. All stocks were maintained with 20% (v/v) glycerol in a liquid nitrogen tank until use. The *H. pylori* cells were quickly thawed and grown in Brucella broth containing 10% (v/v) FBS, CD, and antibiotics at 37 °C in a shaker for 43 h under a microaerobic environment afforded by BBL Campypak-Plus envelopes (Cockeyville, USA). The cultured bacteria were then identified via urease and catalase reactions, and the identifications were confirmed via polymerase chain reactions based on *VagA*, *Cag1*, *IceA1*, *IceA2*, *BabA*, and *UreA* primers (data not shown).[25] *P. gingivalis* and *A. actinomycetemcomitans* were grown for 2 days in trypticase soy broth (Difco) supplemented with 0.6% yeast extract, hemin (10 mL/L) and vitamin K3 (0.2 mL/L) (for *P. gingivalis*) at 37 °C under conditions of 80% N_2, 10% H_2 and 10% CO_2. *P. acnes* were grown for 2 days in brain–heart infusion (Difco) at 37 °C in an atmosphere of 80% N_2, 10% H_2 and 10% CO_2. *S. aureus* and *S. epidermidis* were grown aerobically in brain–heart infusion at 37 °C. *E. coli* was aerobically cultured on Luria–Bertani (LB) agar plates. The colony was inoculated and grown in LB broth without antibiotics for 6 h at 37 °C. All bacterial cell stocks were maintained in a liquid nitrogen tank until required. *L. acidophilus* was directly used from the stocks obtained from KCCM, without further growth.

4.3. Extraction and isolation of polysaccharide PG-F2

The extract (20 g) from the roots of *P. ginseng* was prepared as described previously,[23,37] which was dissolved in hot distilled water (200 mL). After centrifugation to remove insoluble materials, the supernatant was subsequently precipitated using abs EtOH (final, 70% concentration) at 4 °C. The precipitate was then dissolved in PBS containing 10 mM $MgCl_2$ and 1 mM $CaCl_2$, and incubated for 3 h with RNase and DNase at 37 °C. The enzyme reactions were stopped by heating at 100 °C for 5 min. After centrifugation, cold ethanol was added to the supernatant (final, 70% concentration). The precipitate was then dialyzed against 20 mM Tris–HCl (pH 8.0), and applied to a Q-Sepharose fast-flow column (2.5 × 10 cm) equilibrated with the same buffer. After extensive washing, the polysaccharide fractions were eluted with an increasing linear gradient of NaCl (0–1 M). The fractions were detected at 226 nm and analyzed by the phenol–sulfuric acid and carbazole assays at 490 nm and 525 nm, respectively. The polysaccharide fractions eluted at 0.2–0.3 M NaCl that exhibited antiadhesive activity were then pooled, dialyzed against distilled water, and lyophilized. It was dissolved in a minimum volume of 20 mM Tris–HCl buffer and applied to a Sephacryl S200 gel-filtration column (1.5 × 68 cm), which generated a major peak of polysaccharide (PG-F2) and a minor peak of high-molecular-weight polysaccharide (PG-HMW).

4.4. Partial hydrolysis of polysaccharide by enzyme treatment

In order to gain deeper insight into the structural and chemical characteristics of the functional moiety of the highly antiadhesive PG-F2, we attempted its partial hydrolysis using enzymatic and chemical methods. Partial HNO_2 cleavage was conducted as previously described[38] with some slight modifications. PG-F2 was dissolved in a 2% HOAc solution and $NaNO_2$ was added. After incubation at room temperature for 3 h, it was neutralized with 1 N NaOH and purified by gel-filtration chromatography over a Superdex peptide column (bed volume, 24 mL). The oligosaccharide fragments were also acquired via the partial hydrolysis of PG-F2 with enzyme treatment using either pectinase (final activity, 0.5 units/mg polysaccharide) in 20 mM NaOAc (pH 5.0) for 30 min,[23] or α- and β-amylase for 0.5–1 h. The hydrolyzed oligosaccharides with significant antiadhesive activities were pooled and subjected

to Bio-Gel P4 gel-filtration chromatography (2.5×102 cm) in distilled water. The fractions with high activity among oligosaccharide fractions were pooled. The molecular mass of the predominant oligosaccharide in PG-OF1 was estimated by using dextran standards (1–5 kDa). It was further analyzed with the Voyager DESTR MALDI-TOF mass spectrometry (PerSeptive Biosystems, USA) in the Korea Basic Science Institute.

4.5. General methods

Total carbohydrates, uronic acid, and protein contents were determined by the phenol–sulfuric acid, carbazole,[39] and Bradford methods,[40] respectively, using glucose, galacturonic acid and bovine serum albumin as respective standards. For gas chromatography (GC), the carbohydrate compositions of the polysaccharides were analyzed as alditol acetates.[41,42] A sample (2–4 mg) or a carbohydrate standard (0.5 mg) was hydrolyzed for 2 h with 3 M trifluoroacetic acid (1 mL) at 120 °C in a screw-capped vial. The hydrolysates were evaporated to dryness at reduced pressure with repeated additions of MeOH, and then reduced with 10 mg of $NaBH_4$ in 1 M NH_4OH solution. The reduced monosaccharides, either alditols or aldonic acids, were separated using Q-sepharose fast-flow ion exchange resin and then acetylated with 1:1 Ac_2O–pyridine, resulting in the formation of the alditol acetate derivatives. The resulting alditol acetates derived from the aldoses and those derived from uronic acids were then separately analyzed. After the complete removal of the reagents by vacuum evaporation, the dried residuals were extracted in 1:1 EtOAc–water, vortexed, and the EtOAc fractions were evaporated. This extraction procedure was repeated three times. After the final EtOAc was evaporated to a minimum volume (20 μL) and centrifuged, GC analyses were conducted on an HP5890 series II instrument, equipped with a flame-ionization detector and a DB-225 column (0.25 mm I.D. × 30 m). Chromatography was then carried out isothermally at 220 °C. The peak was identified via comparison with the monosaccharide standard peak, and the weights of each monosaccharide in the sample were quantified with internal standards, using *myo*-inositol and mannonic acid lactone as references from the integrated peak areas. The mole percent was calculated for each sample.[41]

4.6. Hemagglutination assay

Human erythrocytes suspended in 10 mL PBS(100 mM Na_3PO_4, pH 7.3, and 150 mM NaCl) were incubated for 3 h with trypsin (2 mg) at 37 °C, then washed three times with PBS. The trypsinized erythrocytes were suspended at 2% (w/v) in the same buffer and used for the hemagglutination assay,[43] the results of which were assessed by microscopic inspection. Erythrocytes in the bacterial suspension in the absence of antiadhesive agents were used as positive controls, and those without bacterial suspension were employed as negative controls.

PG-F2 and PG-OF1 were evaluated (final concentrations, 0.01–2.0 mg/mL) with regard to their hemagglutination inhibitory activities. The inhibitory effects of pectin, alginic acid, LMWH, SOS, glucuronic acid, galacturonic acid, and other carbohydrates were determined at final concentrations of 0.01–1 mg/mL. Aspartic and glutaric acids were tested for a nonspecific reaction and showed no inhibitory activity. Equal volumes of twofold dilutions of the bacterial suspensions and the purified inhibitor or carbohydrates were mixed and incubated at room temperature for 30 min. Each of the mixtures was then added to O-type blood samples in U-shaped 96 microwell plates. The hemagglutination inhibition assay was visualized by microscopic inspection.

4.7. *H. pylori* antiadhesion assay using urea phenol red method

AGS cells (ATCC CRL 1739, a human gastric adenocarcinoma epithelial cell line) were grown to confluence in tissue culture flasks (75 mm) in RPMI 1640 medium supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics–antimycotic in a 5% CO_2 incubator at 37 °C for 3 days. For the replating, the monolayers were detached using trypsin–EDTA treatment for 5 min, and then washed twice with RPMI medium supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics–antimycotics.

For the adhesion assay, the cells were seeded in flat-bottomed 96 microwell plates (Corning, USA) in 100 μL of complete culture medium at a concentration of approximately 5×10^4 cells/mL. After achieving confluence in 1.5–2 days, the cultures were evaluated for antiadhesive activity via urea phenol red (UPR) assays.[27] The confluent monolayers on the 96-well microtiter plates were washed three times with PBS. Nonspecific binding was blocked by incubation for 1 h at 37 °C with 0.5% BSA prior to two rinses in PBS. Equal volumes of twofold dilutions of *H. pylori* suspensions ($OD_{600nm} = 1.0$; 1×10^8 bacterial cells/mL) and purified inhibitor at various concentrations were incubated at room temperature for 30 min. The bacteria–carbohydrate mixture (100 μL) was added to the AGS cells in each well of the 96-well microtiter plates and incubated at 37 °C for 1 h. Control experiments were conducted in the absence of carbohydrate inhibitors. The 96-well microtiter plates were washed twice with PBS and then finally with 0.03% phenol red solution in PBS (pH 5.2). After removing the washing buffer, urea phenol red solution (0.03% phenol red and 2% urea in PBS, pH 5.2) was added to each well, and absorbance was measured at 560 nm using a microplate reader after

incubation for 15 min. The percentage of attached *H. pylori* cells was calculated as follows: attached % = 100 − [($OD_{experimental}$ − $OD_{negative}$)/($OD_{positive}$ − $OD_{negative}$) × 100]. The negative controls contained only epithelial monolayers without bacteria. The positive controls contained bacteria and monolayers without added inhibitor, and these were used to establish 100% attachment. Each experiment was conducted in triplicates on different days.

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Supplementary data

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