J. Microbiol. Biotechnol. (2003), **13**(6), 853–858

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Inhibition of *Helicobacter pylori* Adhesion by Acidic Polysaccharide Isolated from *Artemisia capillaris*

WOO, JEUNG S.[1†], BYUNG H. HA[1], TAE G. KIM[1], YOONGHO LIM[2], AND KYUNG H. KIM[1,3*]

[1]*Graduate School of Biotechnology,* [3]*Department of Biotechnology, College of Science & Technology, Korea University, Seoul 136-701, Korea*
[2]*Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea*

Received: March 20, 2003
Accepted: August 10, 2003

Abstract *Helicobacter pylori* specifically adhere to host cells through a number of putative receptors and ligands, mainly based on carbohydrate-protein interactions. Polysaccharide fractions isolated from the leaves of *Artemisia capillaris* showed different inhibitory activities against *H. pylori* adhesion by using hemagglutination assay. Among these fractions, an acidic polysaccharide fraction F1A showed highly effective inhibitory activity, and its minimum inhibition concentration was 0.63 mg/ml. The inhibition results by the hemagglutination assay were consistent with those obtained by the enzyme-linked glycosorbent assay, which was developed by the conjugation of horseradish peroxidase with fetuin, a sialic acid-containing glycoprotein which was specific to *H. pylori* adhesion. F1A contained the highest carbohydrate content among polysaccharide fractions, and no protein was detectable when further purified by gel filtration FPLC. Sugar composition analysis using GC revealed the highest amount of galacturonic acid among sugars, which suggests that F1A contains essentially acidic polysaccharides. Our data suggest that acidic polysaccharides may play an important role in the inhibition of *H. pylori* adhesion to host cells.

Key words: Adhesion, *Artemisia capillaris*, *Helicobacter pylori*, polysaccharide, hemagglutination, enzyme-linked glycosorbent assay

Helicobacter pylori is a highly motile and Gram-negative bacterium which colonizes the epithelial surfaces of gastric mucosa in the stomach of patients with acute or chronic gastritis. It is now recognized as a major etiological agent of gastritis and peptic ulcer diseases [5, 11, 21, 29]. *H. pylori* colonization in gastric mucosa is also associated with the development of adenocarcinoma of the distal stomach [7]. The adherence of *H. pylori* to the gastric epithelium is a key step in this colonization, and has been suggested to be a means of initiating the infection [28]. Specificities of bacteria-host interactions have been the subject of many recent studies, and a number of putative receptors and ligands have been discovered, including glycoproteins such as fetuin and mucin, sialyllactose, and fucosylated Lewis b antigen [3, 10, 13, 17].

A recent discovery of an acidic polysaccharide fraction from the root of *Panax ginseng* C.A. Meyer (Araliaceae), which inhibited *H. pylori* adherence to host cells, was based on hemagglutinating activity [1]. The inhibition activity of *H. pylori*-mediated hemagglutination, which is evaluated visually or microscopically, was rather difficult to analyze quantitatively. Fetuin, which is able to express sialic acid-dependent binding specificity, has been known to be one of the most effective inhibitors of hemagglutination [17]. A spectroscopic method was thus devised, which quantitates the inhibition of *H. pylori* binding to carbohydrate epitopes, which are present on the glycoprotein, *via* conjugating with peroxidase [30]. This enzyme-linked glycosorbent assay (ELGA) system was further developed by attachment of bacteria to a microtiter plate format. ELGA was found to have high potential to be used as a complementary assay to subjective microscopic evaluation of the hemagglutination assay as well as a quantitiative assay for the relative inhibition potencies among many carbohydrates [30]. It was therefore used to survey inhibition activities of polysaccharide fractions, which were extracted from various plant sources. Results showed that an acidic polysaccharide fraction isolated from *Artemisia capillaris* was found to have relatively high inhibitory effect on *H. pylori* adhesion, comparable to that of the polysaccharide fraction isolated from *P. ginseng*.

Softly aromatic and native to northern China, Japan, and Korea, *A. capillaris* is a well-known natural herb medicine

*Corresponding author
Phone: 82-2-3290-3444; Fax: 82-2-3290-3945;
E-mail: khkim@korea.ac.kr

†Present address: Faculty of Biotechnology, Chungcheong University, Cheongwon, Chungbuk 363-792, Korea

used as a component of oriental traditional prescriptions. It is traditionally used as a sedative in health tea, decoction, and smoke, which has mainly been used as a choleretic, anti-inflammatory, and diuretic agent in the treatment of epidemic hepatitis [25]. Following the established isolation protocol by which the acidic polysaccharide fraction had been obtained from *P. ginseng*, a corresponding acidic polysaccharide fraction was isolated from the leaves of *A. capillaris*. Its acidic polysaccharide has high contents of arabinose, galactose, glucose, and galacturonic acids. Importantly, it showed high inhibitory effect on *H. pylori* adhesion, based on hemagglutination. The results were also shown to be consistent with those using the ELGA system. In the present study, we report the isolation, characterization, and inhibition analyses of an acidic polysaccharide fraction from *A. capillaris*.

Materials and Methods

Materials

Superdex 200 prepacked column and DEAE-Sepharose CL-6B were purchased from Amersham-Phamacia Biotech (Uppsala, Sweden). Asialofetuin, bovine serum albumin, fetuin, horseradish peroxidase (HRP; EC. 1.11.1.7), arabinose, fucose, galactose, glucose, D-galacturonic acid, D-glucuronic acid, rhamnose, xylose, and Dowex-1 were obtained from Sigma (St. Louis, U.S.A.). Fetal bovine serum was purchased from Difco (Detroit, U.S.A.). Mugwort leaf extract concentrates were kindly supplied by S&D Co. (Cheon-ahn, Korea). Erythrocytes obtained from local hospitals were suspended in an Alserver's solution to prevent clotting. The cells were washed three times with phosphate-buffered saline (PBS: 0.1 M sodium phosphate, pH 7.2, 0.15 M NaCl) and were suspended in the same buffer at a concentration of 2% (w/v), which was used for hemagglutination.

Helicobacter pylori Strains

Bacterial strains (ATCC 49503 and 43504) were grown in a Brucella broth containing 10% fetal bovine serum at 37°C under 10% CO_2, and harvested at 72 h into PBS (pH 6.0). The bacterial cells were washed in PBS and kept at -70°C until use. The cells were then dissolved in 0.1 M $NaHCO_3$ (pH 9.6) and 0.15 M NaCl and washed three times by centrifugation at 6,000 ×g. The bacterial suspension was diluted until its absorbance at 600 nm reached to 0.1.

General Methods

Total carbohydrate, uronic acid, and protein contents were determined by the phenol-sulfuric acid [8], carbazole [6], and Bradford [4] methods, respectively, using glucose, glucuronic acid, and bovine serum albumin as the respective standards. Carbohydrate compositions of polysaccharide fractions were analyzed by gas chromatography (GC) as alditol acetates [6]. The samples or standards were hydrolyzed with 2 M trifluoroacetic acid at 100°C for 2 h in glass ampules under N_2 and reduced with $NaBH_4$. The reduced monosaccharides, alditol or aldonic acid, which were separated by Dowex-1 ion-exchange resin, were acetylated with acetic anhydride to form alditol acetate derivatives [2, 14]. The resulting alditol acetates produced from aldoses and those from uronic acids were analyzed separately. After complete removal of the reagents by evaporation, the dried residuals were extracted by chloroform/water (1:1), vortexed, and the chloroform fraction was evaporated in vacuum. GC analyses were performed on a HP5890 series II instrument, equipped with an FID detector and a HP-5 column (0.25 mm i.d.×30 m). A temperature gradient from 150 to 220°C (Δ5°C/min) was used, and the molar ratios of monosaccharides were calculated from integrated peak areas.

Isolation of Polysaccharide Fractions

Forty grams of the extract concentrates of *A. capillaris* were dissolved in hot distilled water (200 ml). After centrifugation to remove insoluble materials, 5% cetylpyridinium chloride (CPC) (48 ml) was added to the supernatant, to precipitate polysaccharides [23]. The precipitation was maximized when 1% of the concentration was used, which was later found to be effective in isolating an active polysaccharide fraction. After discarding the supernatant, 10% sodium chloride (80 ml) was added to remove the remaining CPC. The supernatant was subsequently maximally precipitated with respect to carbohydrate by using 70% ethanol. After centrifugation, the precipitate was dissolved and dialyzed against Tris-HCl (pH 8.0). The solution was applied to DEAE-Sepharose column (2.5×16 cm) equilibrated with the same buffer. The eluants of polysaccharide fractions using linear gradient (0- 0.5 M) and step elution (0.5 M and 1.0 M) of NaCl were analyzed by the phenol-sulfuric acid and carbazole assays in addition to spectrophotometric assay at 226 nm. Three major polysaccharide fractions were obtained, and each fraction was dialyzed against distilled water, lyophilized, and dissolved in a minimum volume of 20 mM Tris-HCl (pH 8.0). The concentrated fraction was finally applied to gel filtration FPLC with prepacked Superdex 200 column equilibrated with 20 mM Tris-HCl (pH 8.0). The eluant was pooled and lyophilized.

Preparation of HRP-Fetuin Conjugate by Periodate

HRP-fetuin conjugation was performed by a modification of the method developed by Nakane and Kawaoi [19], as described previously [30]. HRP was first activated by sodium periodate, incubated with fetuin, reduced by sodium cyanoborohydride, and dialyzed against PBS (pH 7.4). Superdex 200 gel filtration FPLC was used to separate free fetuin and HRP from HRP-fetuin conjugate complexes.

Conjugate formation was detected using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by following the method of Laemmli [15] using 7% acrylamide. Protein bands were visualized by staining with Coomassie Brilliant Blue R250 and destaining with 10% acetic acid.

Binding Assay

Binding of a polysaccharide fraction to *H. pylori* was studied in a microtiter plate format using the ELGA system [30]. Briefly, *H. pylori* cells were attached to the wells of the microtiter plates using 0.5% glutaraldehyde solution. To minimize nonspecific binding, the microwells were blocked with 0.1 M Tris-HCl (pH 7.6) containing 1% (w/v) BSA and 0.1% (v/v) Tween 20, and kept overnight at 4°C. After washing each well, an inhibitor solution was diluted with a washing buffer (50 mM sodium acetate, pH 5.0, 150 mM NaCl, and 0.05% Tween 20) and incubated at 37°C for 1 h. HRP-fetuin conjugates were then added into each well and incubated at 37°C for 1 h. *o*-Phenylene diamine (0.05%) and H_2O_2 (0.015%) in 0.1 M citrate-phosphate buffer, pH 5.0, were used as substrates for HRP. After the reaction was stopped by 2 M sulfuric acid, absorbance at 490 nm was monitored. The inhibition plots were based on three replicates of each measurement. Negative control contained no bacterial cell suspension, whereas positive control contained bacterial cells without any added inhibitors. Fetuin and asialofetuin were also tested as inhibitor analogs.

Assays of Hemagglutination Inhibition

Hemagglutination was assayed, as described previously [1, 19]. The bacterial suspension was serially diluted with PBS (pH 6.0) in the microtiter U-plates. To the suspension of *H. pylori* (30 µl), 30 µl of the PBS-suspended erythrocytes were added. After being incubated at room temperature for 30 min, the hemagglutination was visually evaluated. For inhibition assays, the inhibition solutions (30 µl) of serially diluted purified polysaccharide fractions were used. The minimum inhibitory concentration was visually determined as an end-point inhibition and confirmed by microscopic inspection.

RESULTS

Isolation and Characterization of Polysaccharide Fractions

Among various plant sources, which were screened by ELGA, polysaccharide fractions from laver and mugwort were shown to have a prominently high inhibition activity on the bacterial adhesion [30]. Because of availability of one of the mugwort species, *A. capillaris* was first tested for isolation of polysaccharides. Thus, the crude polysaccharide fractions were extracted from the leaves of *A. capillaris* by hot distilled water, fractionated by CPC [23], and precipitated by 70% ethanol to obtain an ethanol-precipite of F1. It was further fractionated into neutral and acidic fractions by DEAE-Sepharose ion-exchange chromatography. Acidic polysaccharide fractions bound to the anion-exchange column were eluted, revealing three major peaks (Fig. 1). The first peak was eluted at approximately 0.3 M NaCl (fraction F1A) and found to have higher sugar contents than the other peaks (fractions F1B and F1C). Whereas unbound neutral polysaccharide fractions on the anion-exchange column were obtained from *P. ginseng* [1], little was observed from *A. capillaris*, possibly due to the CPC treatment. F1A was lyophilized and further purified by using gel filtration FPLC on the Superdex 200 prepacked column, where only a single peak (fraction F2) was detected with absorbance at 226 nm.

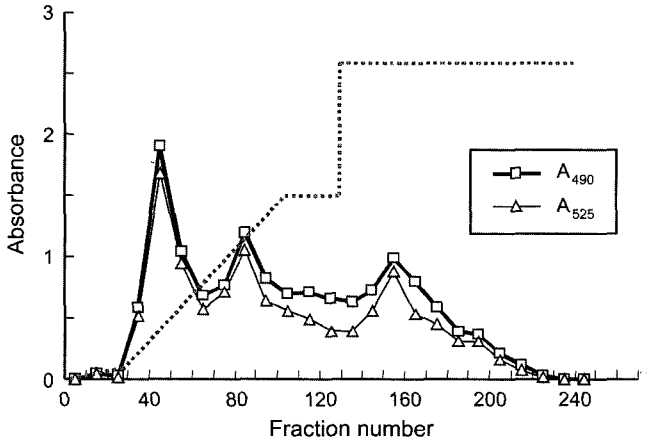

Fig. 1. Elution profile of polysaccharide fractions from *Artemisia capillaris* on DEAE Sepharose CL-6B ion-exchange chromatography.
Linear gradient (0–0.5 M NaCl) and step (0.5 and 1.0 M NaCl) elutions are represented as dotted lines. Total sugar (thick line) and uronic acid (thin line) contents were measured using the phenol-sulfuric acid ($Abs_{490\,nm}$) and carbazole ($Abs_{525\,nm}$) methods, respectively.

Biochemical analyses of these acidic fractions indicated that all the fractions, F1A, F1B, and F1C, contained 45–62% carbohydrates and 10–20% proteins. Among them, F1A had the highest carbohydrate content (Table 1) and F2 contained no detectable protein. Carbohydrate analyses of the fraction F2 indicated that it contained high amounts of glucose, arabinose, and galactose as neutral sugars (Table

Table 1. Characterization of polysaccharide fractions from *Artemisia capillaris*.

Fraction	Carbohydrate[a] (%)	Uronic acid[b] (%)	Protein[c] (%)
F1A	61.8	47.7	10.9
F1B	50.2	25.7	20.0
F1C	46.9	24.7	18.2
F2	72.4	22.3	-

[a]Phenol-sulfuric acid, [b]carbazole, and [c]Bradford methods were used.
[b]Uronic acid (%) in total carbohydrate.

Table 2. Carbohydrate composition of the polysaccharide fraction F2 purified from *Artemisia capillaris*. Values are indicated as relative mol %.

Arabinose	Xylose	Rhamnose	Mannose	Galactose	Glucose	Galacturonic acid	Glucuronic acid
19.5	11.9	3.6	7.9	15.6	19.7	21.7	0.0

2). Interestingly, galacturonic acid was the highest (21.7%) among sugars constituting the high amounts of uronic acids in F2 as well as in F1A. In contrast, glucuronic acid was found to be undetectable. High amounts of galacturonic acid implicate that the fraction F2 essentially contained only acidic polysaccharides. It was previously shown that the roots of *Panax ginseng* contain acidic polysaccharides whose structures were investigated [1, 26, 27]. An acidic polysaccharide was shown to inhibit *H. pylori* adherence to host cells that contain high amounts of galactose, arabinose, and uronic acids [1]. Other acidic polysaccharides showed immunomodulating activity that was mediated by nitric oxide synthesis [22]. The highly purified polysaccharides were found to have branched glycans, which composed of arabinose, galactose, rhamnose, and galacturonic acid with β-1,3-linked galactan as a backbone [27].

Inhibition of *H. pylori*-Induced Adhesion

Recent investigation on the specificities and mechanisms of bacterial binding has employed various techniques [1, 3, 9, 30]. In particular, it was shown that *H. pylori* bacteria possess hemagglutinating activity, because erythrocytes carry carbohydrate antigens which are responsible for adherence of *H. pylori* [11]. Hemagglutination test was first used to determine inhibitory activities of the polysaccharide fractions. Their inhibitory activities with various concentrations of polysaccharides were evaluated by microscopic inspection.

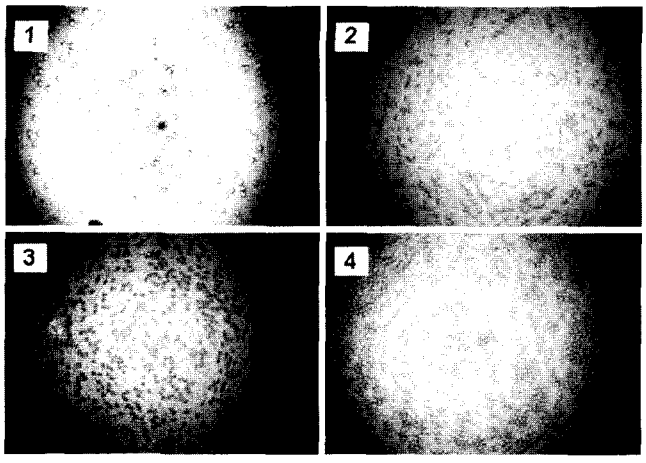

Fig. 2. Analysis of adhesion inhibitory activities of the acidic polysaccharide fraction F1A by using hemagglutination assay.
The hemagglutination was inhibited by F1A at the concentration of (1) 2.5 mg/ml, (2) 1.25 mg/ml, (3) 0.63 mg/ml, and (4) a positive control with erythrocytes and *H. pylori* (ATCC 43504).

Among three polysaccharide fractions (F1A, F1B, and F1C), F1A showed the most effective inhibition of hemagglutination, and its minimum inhibitory concentration was 0.63 mg/ml (Fig. 2). The minimum inhibitory concentration of the corresponding fraction (an acidic fraction on DEAE-Sepharose chromatography) from *P. ginseng* was 0.25 mg/ml [1]. ELGA was also employed to compare the inhibitory activities of the *A. capillaris* polysaccharide fractions, and confirmed that F2 and F1A were very effective in adhesion inhibition. In contrast, significantly low inhibition was detected in other fractions, F1B and F1C, and less than 20% or no inhibition was observed even at a concentration of 5 mg/ml (Fig. 3). F2 revealed the highest inhibitory activity, next to fetuin. At the concentration of 2 mg/ml, it showed about 50% inhibition. Based on ELGA, *A. capillaris* polysaccharide had at least 80% relative inhibition compared with that from *P. ginseng* (data not shown), indicating that *A. capillaris* polysaccharide may have slightly lower level of specificity for adhesion than that of *P. ginseng*.

Discussion

The bacterial pathogen *H. pylori* is a major causative agent of peptic ulcer diseases [5, 21, 29]. They show a wide

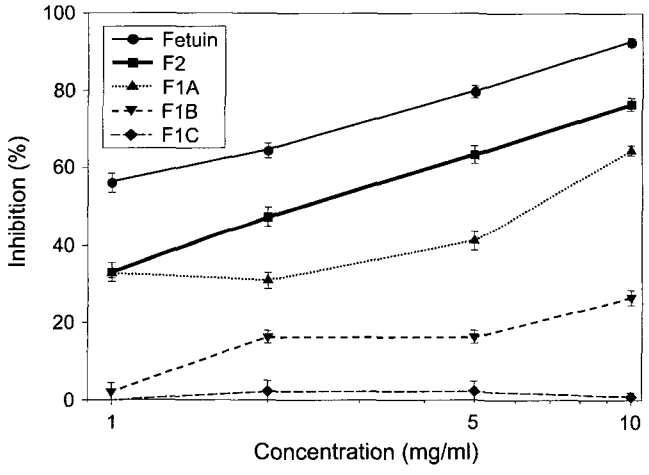

Fig. 3. A quantitative analysis of adhesion inhibition activities of various polysaccharide fractions from *A. capillaris*, by using enzyme-linked glycosorbent assay.
The inhibition % was calculated from [(R-S)/R]×100, where S and R represent the absorbance difference at 490 nm between the sample and negative control, and the positive and negative control, respectively. Each measurement is from three replicates.

spectrum of different specificities in adhesion to host cells, suggesting a multifactorial adherence. Interestingly, our results suggest that the inhibitory activity may correlate with the content of uronic acids, particularly galacturonic acid, reaching approximately 22% in total carbohydrate. F1A has higher uronic acid content than F2 (Table 1), probably due to interference caused by inherent pigments that originate from mugwort leaves. However, F2 was found to contain no trace amount of the pigment. Large amounts of uronic acids were also detected in the acidic polysaccharide fraction isolated from *P. ginseng*, which demonstrated remarkable inhibitory activity on *H. pylori* adhesion and immunomodulating activity [1, 22]. We could not rule out the possibility that other substances contaminated in F1A and F2, such as peptides or other undetected molecules, may be responsible for the activity, or that the inhibitory activity of F2 may have been caused by a combined or synergistic effect of these substances. Nevertheless, our data suggest that the acidic part of polysaccharides in F1A and F2 could be responsible for the inhibitory activity. *H. pylori* also binds sulfated glycosaminoglycans such as heparan sulfate [24]. These negatively charged groups may play a mechanistic role in the host-bacterial adhesion. Two strains of *H. pylori* in our study consistently revealed the presence of the acidic polysaccharides.

Microscopic evaluation of hemagglutination has disadvantages of less reproducibility and difficulties to distinguish relative inhibitory activities, when the number of samples increases [1, 9]. The results obtained by ELGA showed that the stable and selective HRP-fetuin conjugate exhibited binding capacity specific to *H. pylori* [30]. Both hemagglutination and ELGA assays used in this study proved that the acidic polysaccharides in F1A and F2 from *A. capillaris* had very effective inhibitory activity. Therefore, by using a stable and selective HRP-fetuin conjugate, ELGA made it possible to carry out quantitative analysis of inhibitor screening against *H. pylori* adhesion as an assay complementary to hemagglutination, and could be used to survey polysaccharide fractions from various plant sources.

Adherence of bacteria to sialylated oligosaccharides on glycoproteins and glycolipids, phosphatidylethanolamine and the fucosylated blood group Lewis b antigen has been reported [3, 10, 13, 16, 17]. In the case of sialic acid-mediated adherence, depending on growth conditions, bacteria express at least two types of sialic acid-dependent binding specificities [17]. Although ELGA has a limited specificity only in a sialic acid-dependent manner due to its fetuin, nonsialic acid samples such as galacturonic acid or heparin have also been found to display a high inhibitory activity [30], thus suggesting that HRP-fetuin conjugates may partly possess a nonsialic acid-mediated specificity. Specific carbohydrate structures of polysaccharides are likely to play an important role in this anti-adhesive activity. Preliminary results show that the elution volume of F2 on Superdex 200 FPLC corresponds to molecular weight of about 60 kDa, equivalent to a polysaccharide with 300-400 sugar units. Determination of the sequence structure of this active polysaccharide and its inhibitory activity on gastric cell lines are presently underway. The acidic polysaccharides may be a useful dietary substance for prevention of chronic gastric injury. Recent improvements in the preparation protocol of effective vaccine antigen and probiotics [11, 18, 20] may result in the development of a safe anti-*H. pylori* substance. The design of synthetic glycomimetics, based on the structure of carbohydrate epitopes, may be used as carbohydrate-based anti-adhesive drugs.

Acknowledgments

We thank Mr. K. M. Yeo at S&D Co. for supplying us with mugwort leaf concentrates. This work was supported by a grant from BioGreen 21 Program, Rural Development Administration. We are also grateful to the Korea Institute of Industrial Technology (ITIP program). J.S.W, B.H.H. and T.G.K. were supported by the BK21 program of the Ministry of Education, Korea.

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