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Glycosylated Compounds from Okra Inhibit Adhesion of *Helicobacter pylori* to Human Gastric Mucosa

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In Asian medicine the fruit of the okra plant, *Abelmoschus esculentus* (L.) Moench., is used as a mucilaginous food additive against gastric irritative and inflammative diseases. To find a rational basis for its use against these diseases, several crude and purified carbohydrate-containing fractions from immature okra fruits were isolated and analyzed, and their effects against *Helicobacter pylori* in an in situ adhesion model on sections of human gastric mucosa were determined. Pretreatment of the bacteria with a fresh juice preparation inhibited the bacterial adhesion almost completely. Lyophilization and reconstitution of an extract solution led to a reduction of this effect. A crude polysaccharide (RPS) isolated from the fresh juice by ethanolic precipitation showed strong inhibitory effects. Further fractionation of RPS revealed a purified, highly acidic subfraction (AF III) with high antiadhesive qualities. Carbohydrate analysis revealed the presence of rhamnogalacturonans with a considerable amount of glucuronic acid, whereas other inactive subfractions contained little glucuronic acid or were glucuronic acid-free. After heat denaturation of the fresh juice or protein precipitation with 5% TCA the antiadhesive activity of the fresh extract was reduced, indicating that besides polysaccharides, protein fractions also exhibited antiadhesive properties. SDS-PAGE analysis of the precipitate revealed several bands of glycosylated proteins between 25 and 37 kDa that were almost diminished in the nonactive supernatant. Preincubations of gastric tissue with any of the active fractions did not lead to reduced bacterial binding. The antiadhesive activity is therefore due to the blocking capacity of specific *Helicobacter* surface receptors that coordinate the interaction between host and bacterium. Neither of the active fractions showed inhibitory effects on bacterial growth in vitro. The antiadhesive qualities of okra were assumed to be due to a combination of glycoproteins and highly acidic sugar compounds making up a complex three-dimensional structure that is fully developed only in the fresh juice of the fruit.

KEYWORDS: ***Helicobacter pylori*; carbohydrates; polysaccharides; rhamnogalacturonan; okra; *Abelmoschus esculentus* (L.) Moench.; infection; antiadhesion; structure−activity; receptor**

INTRODUCTION

The okra plant, *Abelmoschus esculentus* (L.) Moench., Malvaceae, is an annual herb up to 2.5 m in height and bearing black-grounded yellow mallow-type blossoms. Its fruit is a greenish, slightly curved, six-chambered pod of fibrous texture that is rich in oily seeds. In Asia, okra is typically prepared as a vegetable and used in traditional medicine as a dietary meal in the treatment of gastric irritations, due to its high content of mucilages. So far, some general physiological studies in rat models with individual polysaccharide extractions have been accomplished indicating limited hypoglycemic and anticomplementary activity (*1*) as well as an ability to lower plasma cholesterol levels (*2*). Minimal attention has been focused on the question of whether there is a scientific justification for an application of the fruit in the field of gastric diseases. The use of herbal mucilages in the treatment of irritated mucous membranes has always been well appreciated and is understood as a bioadhesive interaction between exogenous polysaccharides and gastrointestinal mucins (*3*). Recently, the mechanism of this interaction was characterized for an acidic model glycan from sugar beet in ex vivo studies on several types of mucosal tissues (*4*). However, a raw polysaccharide from okra tested in analogous experiments exhibited only minor adhesive effects

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on any tested gastrointestinal tissue (data not published). Thus, it was not established that the assumed healing properties of okra are associated with extraordinary mucilaginous qualities of the drug.

For ~20 years *Helicobacter pylori* has been known to play a major role in the development of chronic gastritis (*5*), duodenal and gastric ulceration, adenocarcinoma, and gastric MALT-lymphoma (*6*). *H. pylori* is a spiral, Gram-negative, microaerophilic bacterium that lives exclusively in the human stomach, where it adheres to the gastric epithelium or its superficial mucus layers. One of its features is the enzyme urease, which enables the microbe to exist at pH levels down to 2.0 in the presence of urea. Associated with the outer cell wall, this protein binds several types of sialylated, fucosylated, or anionic glycans and glycoproteins such as mucins (*7*, *8*). Several different classes of adhesins have already been identified, ranging from sialic acid-specific lectins (*9*) to lipopolysaccharides with structural homology to human blood group antigens such as Lewis X (*10*). Such knowledge enables the development of new drugs that bind to these bacterial adhesins and inhibit further colonization on the mucous surfaces. Although the standard therapy of antibiotics in combination with a proton pump inhibitor is a safe way to eradicate individual acute infections, resistant strains are growing in number, prohibiting its use on whole populations (*11*). Here, antiadhesive carbohydrate-containing preparations could function as nontoxic food additives in therapeutical as well as preventive applications. To examine extracts from okra on their ability to interact adhesively with *H. pylori*, we have used an in situ adherence model on sections of human gastric mucosa previously reported (*12*). This system is easy to handle and gives clear and reproducible results that directly reflect the level of the microbe's adherence to material from its natural habitat.

MATERIALS AND METHODS

General Experimentation Procedure. *A. esculentus* fractions were extracted from imported fruits obtained at a local green grocery in Erlangen in November 2000. A lyophilized voucher specimen is retained in the documentation center at the Hochschule Wädenswil, University of Applied Science, Wädenswil, Switzerland. Sialyllactoses from human milk were kindly provided by the Milupa GmbH & Co. KG (Friedrichsdorf, Germany).

Fluorescence microscopy was performed using a Zeiss Standard 16 microscope with fluorescence condensor IV FI and camera system MC 63.

Preparation of a Fresh Juice Extract from Okra Fruits. Fresh okra fruits (*A. esculenti fructus*) were separated from stalks, cut into small slices, and homogenized in double-distilled water with a Polytron high-frequency homogenizer (Kinematika, Luzern, Switzerland). Insoluble material was removed by centrifugation at 15000*g* and 4 °C for 30 min. The supernatants were collected and dialyzed against double-distilled water or phosphate-buffered saline (PBS), pH 7.4. The total carbohydrate content of this fresh preparation was determined. One part of the material remained untreated, a second part was diluted to the value of a 0.1% dilution of the raw polysaccharide extract [45% ethanol (v/v) precipitate], adjusted to 0.2% BSA and 0.05% Tween 20 for use in in situ experiments, and prepared in samples of 1.0 mL volume. All samples were shock frosted in liquid nitrogen and stored at −80 °C. Half of the untreated frozen material was lyophilized.

Isolation and Fractionation of Okra Polysaccharides. Principally, polysaccharides were isolated according to the method of ref *13*. A fresh juice extract was prepared as described above. The soluble polysaccharide fraction was precipitated by the addition of ethanol 98% to final concentrations of 35, 45, and 60% (v/v). The precipitates were centrifuged at 15000*g* at 4 °C for 30 min, and residual ethanol was removed by vacuum filtration. The precipitated raw polysaccharides were resolved in double-distilled water. After dialysis, the solutions were frozen and lyophilized. The 45% ethanol precipitate (chosen because of best yields with high molecular polysaccharides and best solubility properties) was fractionated by anion exchange chromatography (AEX) on DEAE-Sephacel (Pharmacia, Freiburg, Germany). Conditions: gel in the phosphate form; column dimensions, 20 × 2 cm; flow rate, 60 mL/h; fraction size, 6 mL; mobile phases, bidistilled water, then step gradient with sodium phosphate buffers, pH 6.0; ion strengths, 0.1, 0.25, 0.5, and 1.0 mol/mL. Carbohydrate-containing fractions of each elution were pooled, dialyzed, and freeze-dried. Furthermore, the gel was treated with 1 N NaOH (batch eluation) for 15 min and was then neutralized with 1 N HCl. The carbohydrate-containing supernatant was dialyzed and freeze-dried. Generally, carbohydrate-containing solutions were concentrated under reduced pressure at temperatures below 50 °C. For dialysis, cellulose membranes with MWCO 3500 were used. Dialysis was repeated at least four times against 20 volumes of bidistilled water.

Polysaccharide Analysis. Total carbohydrates were assayed using the resorcinol sulfuric acid test (*14*) against a monosaccharide mixture representing the sugar composition of the sample (determination by TLC after hydrolysis of the polysaccharide sample) as a standard. Determination of total uronic acids was performed according to the method of ref *15* with *o*-hydroxydiphenyl in a modification for 96-well microtiter plates using galacturonic acid as a reference. Starch was detected using Lugol's solution. Separation and detection of galacturonic acid and glucuronic acid were accomplished on ion exchange PAD-HPLC (Dionex) with a tenary gradient (water, sodium hydroxide, sodium acetate on GP50 gradient pump system) on a Carbopac PA1 separation column with an ED 50 electrochemical detector. Polysaccharides were hydrolyzed with 2 N trifluoroacetic acid at 121 °C for 1 h. TLC was performed on glass plates sheeted with silica gel F_{254} using acetonitrile/water 80:20 as a mobile phase. Sugars were detected with a thymol sulfuric acid spray reagent and heating at 120 °C for 5 min. Neutral sugar content of polysaccharides was measured by GC-MS as their alditol acetates as described in ref *16*. Linkage analysis of neutral sugars was performed on their partially methylated alditol acetates by GC-MS according to the method of ref *17*, as modified in ref *18*. GLC was performed on a Hewlett-Packard HP 6890 system with mass selective detector at 250 °C (alditol acetates) and 200 °C (PMAA), respectively, with helium as a carrier gas (pressure = 1.5 bar) on an HP-1701 fused silica capillary column (30 m × 0.25 mm i.d., film thickness = 0.25 μm). Reduction of acidic polysaccharides to the carboxyl-reduced polymers was accomplished in the presence of carbodiimide and $NaBD_4$ following the method of ref *19*. Determination of the molecular weight distribution of polysaccharides was performed by FPLC (Pharmacia, Uppsala, Sweden) on a High Load 16/60 Superdex 200 prep grade column using standard dextrans (Sigma, Steinheim, Germany) as a reference. Quantification of residual protein was performed according to the method of ref *20* using standard BSA (PAA Laboratories GmbH) as a reference. SDS-PAGE was performed according to the method of ref *21* on 8% acrylamide gels with silver-staining.

Protein Separation. To precipitate soluble proteins, 10 mL of okra fresh extract was treated with 5% trichloroacetic acid (TCA) for 10 h at 4 °C. After centrifugation at 13000*g*, the protein-containing pellet was washed with ethanol 70% and diethyl ether and was redissolved in double-distilled water. Both the supernatant and the pellet were dialyzed against double-distilled water and freeze-dried. To obtain protein-free or protein-degraded samples of the raw polysaccharide (RPS) from okra, a protein digestion was performed using the self-digesting enzyme Pronase E (Sigma, Steinheim, Germany). Ten milligrams of okra RPS were dissolved in 10 mL of sterile PBS adjusted to pH 7.5. Pronase E was added to a final concentration of 2 units/mL. The incubation was carried out for 6 h at 37 °C and 300 rpm orbital shaking. As a control, a second RPS solution was treated identically but without enzyme added. After 6 h, both RPS solutions were dialyzed against double-distilled water and freeze-dried.

Viscosity Measurements. Kinematic viscosity was measured using a Ubbelohe-capilllary viscosimeter type 501 11/Ia (Schott Geräte) at 20 °C with a sample volume of 15 mL. Each sample was tested four times, and the average values were compared to the average value of bidistilled water. Rotation viscosimetry was performed at 20 °C using

a Haake VT 550 viscosimeter. Sample volume was 8 mL at a temperature of 20 °C. Shearing time was 50 s with a shear rate of 0−30 rotations per second (r/s) following a time program with 20 s at 0−30 r/s, 10 s at 30 r/s, and 20 s at 30−0 r/s. Evaluation was done using Rheowin Pro 2.52 software (Haake).

Bacterial Culture. *H. pylori* type I strains G27 and CCUG 17874 were kindly provided by Dagmar Beier, Lehrstuhl für Mikrobiologie, Universität Würzburg, Germany. *H. pylori* Sydney Strain SS1 as well as several clinical isolates were obtained from Christoph Schoerner, Institut für Medizinische Mikrobiologie, Universität Erlangen, Germany. Bacteria were grown on Columbia agar base (Oxoid) supplemented with 5% lysed horse blood (Oxoid) and *H. pylori* antibiotic selective supplement (Oxoid). Plates were incubated for 48 h at 37 °C under microaerophilic conditions in anaerobic jars with CampyPak (BBL Beckton-Dickinson).

In Situ Adherence Assay with *H. pylori*. The adhesion assay was performed according to the method of ref *12*. Briefly, 2 days after inoculation, bacteria were harvested from agar plates using a 2 μL sterile loop and resuspended in 1.0 mL of 0.15 M NaCl and 0.1 M Na_2CO_3, pH 9.0, in double-distilled water by gentle pipetting. Bacterial titers were adjusted to 3.0×10^7 CFU/mL by OD measurement at $\lambda = 550$ nm. Ten microliters of freshly prepared 1% fluorescein−isothiocyanate (FITC, Sigma) in dimethyl sulfoxide (DMSO) was added to the suspension, which was then incubated for 1 h at room temperature (RT) in the dark. Bacteria were recovered by centrifugation at 3000*g* for 5 min, resuspended by gentle pipetting in 1.0 mL of PBS−Tween 20 (0.05% v/v), and pelleted by centrifugation as above. The wash cycle was repeated three times. Aliquots (100 μL) were taken from the final suspension and used immediately. Multiple paraffin-embedded tissue sections from human gastric mucosa were kindly provided by Gerhard Faller, Pathologisches Institut Erlangen, Germany. The sections were taken from *Helicobacter*-negative individuals, and the antrum mucosa showed no major pathologic alteration. Tissue sections were deparaffinated in xylene and 2-propanol, rinsed in water followed by PBS, and then incubated for 15−30 min at room temperature in blocking buffer (0.2% BSA, 0.05% Tween 20 in PBS). The FITC-labeled bacteria were diluted 20-fold in blocking buffer of which aliquots (200 μL) were placed on the tissue slides. The slides were incubated for 1 h at room temperature in a humidified chamber in the dark. Slides were subsequently washed three or four times with PBS prior to microscopic inspection. To analyze the antiadhesive activity of test compounds, aliquots (100 μL) of FITC-labeled bacteria were preincubated with samples (0.01−0.1% in blocking buffer) for 2 h at room temperature in the dark. Bacteria were washed once in blocking buffer before aliquots (200 μL) were added to the gastric sections. To analyze the mucilaginous effect of the test polymers on the bacterial binding, tissue sections were preincubated for 2 h with the respective sample. After the sections have been carefully rinsed with PBS, FITC-labeled, untreated bacteria were added, and the assay was finished as described above.

Validation of Bacterial Adherence. Validation of bacterial adhesion was accomplished microscopically using a ranking list from (−) for "no bacterial binding" to (++++++) for "very strong bacterial binding". Each testing substance was examined at least three times in independent experiments and blind evaluation.

Toxicity Studies with *H. pylori*. To investigate possible toxic effects of the testing material on *H. pylori*, bacteria were freshly harvested from agar plates and adjusted to titers of 3.0×10^7 CFU/mL. From this suspension, aliquots (100 μL) were diluted 20-fold in 0.1% of test compound in blocking buffer. One aliquot was diluted in pure blocking buffer and served as an untreated control. After 2 h of preincubation at room temperatue, the samples were washed once in PBS and were diluted 100−1000-fold. Triplicate aliquots (100 μL) of the final dilutions were spread on agar plates and cultivated for 2 days at 37 °C under microaerophilic conditions. The resulting CFU were evaluated in relation to the CFU of the untreated control.

RESULTS

Fractionation and Characterization of Okra Extracts. A fresh fruit extract (FE) was prepared from the immature okra pod. Its lyophilized dry matter was collected with a yield of 1.9% based on the fresh weight of the starting material. From FE three raw polysaccharides were extracted by a fractionated precipitation with ethanol to final volumes of 35, 45, and 60% (v/v). The 45% precipitate displayed the best resolving qualities and the highest total sugar content as determined by the resorcinol−sulfuric acid test (*14*); thus, it was chosen for further investigations (referred to as RPS). It could be harvested with a yield of 1.24% based on the starting material. **Table 1** displays the chemical composition of FE and RPS. The total carbohydrate content of FE was significantly ($p = 0.0009$) lower than that of RPS, and the total uronic acid content was slightly lower ($p = 0.1331$). The total protein content of FE was 2 times higher than that of RPS ($p = 0.0207$). Protein analysis of FE by SDS-PAGE (8%) and silver-staining revealed several bands with molecular weights between 70 and 40 kDa and two major bands of brown color at 37 and 25 kDa, suggesting the presence of glycosylated proteins (data not shown). After precipitation of FE with 5% TCA, the total protein content of the extract was significantly ($p = 0.0001$) reduced by 72%, and total carbohydrate and uronic acid contents were reduced by 9.6% ($p = 0.9110$) and 33.5% ($p = 0.0601$), respectively (**Figure 1**). SDS-

Table 1. Composition of Okra Polysaccharide Fractions (Percent) and Sugar Composition (Mole Percent) after TFA Hydrolysis, Acetylation, and GC-MS Analysis[a]

	FE	RPS	NF	AF I	AF II	AF III
rhamnose	13.8	19.9	21.2	13.9	15.0	7.2
arabinose	1.5	2.1	14.4	1.2	2.3	0.3
xylose	0.8	1.3	2.9	0.5	0.4	0.5
mannose	0.9	0.5	3.6	0.9	1.5	0.3
galactose	24.7	18.2	43.6	30.1	20.6	15.3
glucose	6.0	3.7	14.3	3.2	6.7	1.0
total uronic acids	28.5	32.9		50.1	53.3	75.2
residual protein	23.7	11.9		<0.1	<0.1	<0.1
av mol wt (kDa)	1380	1380	30.2	692	977	1380
yield	1.9[b]	1.3[b]	<1.0[c]	34.4[c]	8.0[c]	16.1[c]

[a] All values were determined from the dry weight of the respective lyophilized fraction referred to [b] the fresh weight of the extracted okra fruits and [c] the amount of RPS used in AEX chromatography. FE, fresh extract; RPS, raw polysaccharide; NF, neutral AEX fraction; AF I–III, acidic AEX fractions I–III.

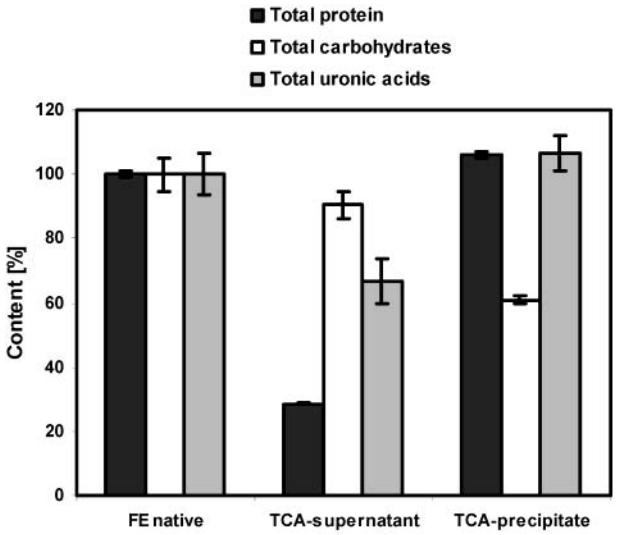

Figure 1. General composition of fractions obtained after precipitation with TCA, in comparison to the composition of the native fresh extract. Results (in percent) are given as MW ± SD with $n = 4$. All values were obtained from the dry weight of the respective lyophilized fraction.

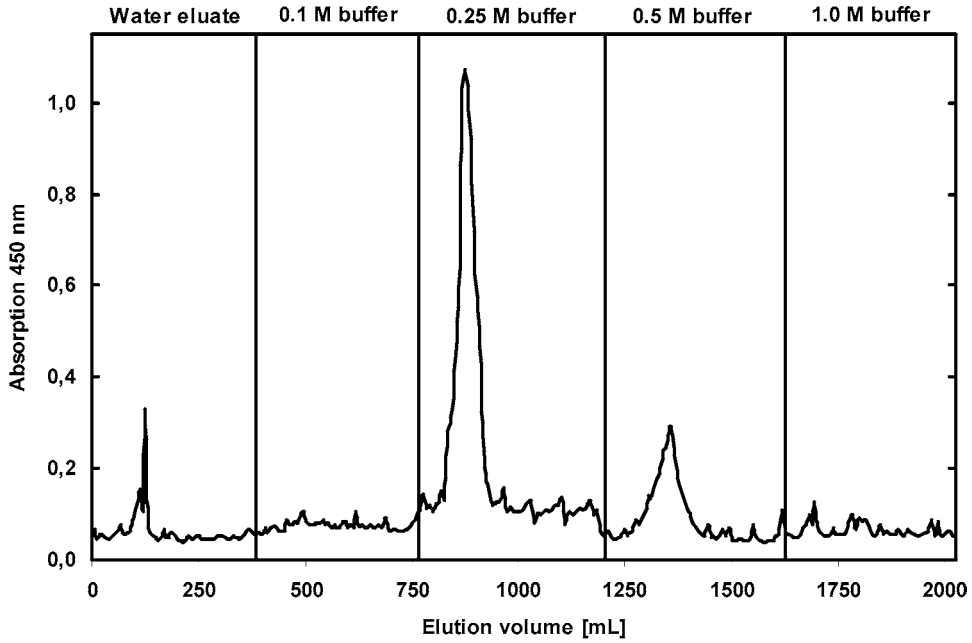

Figure 2. Elution profile of okra RPS in anion exchange chromatography on DEAE-Sephacel (buffer = sodium phosphate buffer).

PAGE showed that the bands at 37 and 25 kDa were almost completely diminished after precipitation, whereas the bands of higher molecular weight were still present in the supernatant (data not shown). SDS-PAGE of RPS showed one major band at 48 kDa. The protein content of RPS could not be significantly reduced after treatment with Pronase E, yet several new bands of lower molecular weight pointed to a partial degradation (data not shown), suggesting that the proteins of RPS were covalently linked to the carbohydrates, which inhibited a complete proteolytic digestion.

Fractionation of the RPS by anion exchange chromatography on DEAE-Sephacel (**Figure 2**) resulted in one neutral (NF) and two acidic polysaccharide fractions (AF I plus AF II). AF I eluted at 0.25 M sodium phosphate with a yield of 34.4% and AF II at 0.5 M sodium phosphate with a yield of 8.0%; NF was collected with a yield of <1.0% (**Table 1**). After a 15 min treatment with 1 N NaOH (batch elution, nonexhaustive), a fourth polysaccharide fraction, AF III, was collected with a yield of 16.1%. AF I−III were of a higher uronic acid content than RPS and were free of protein. As shown by gel permeation chromatography, all four fractions were homogeneous with average molecular weights of 30.2 kDa (NF), 692 kDa (AF I), 977 kDa (AF II), and 1380 kDa (AF III), whereas the respective RPS had a main peak at 1380 kDa and a slope ending at 347 kDa (**Table 1**). All fractions examined were free of starch. The monosaccharide composition of TFA-hydrolyzed FE, RPS, NF, and AF I−III was determined by GC-MS as their alditol acetates. **Table 1** reveals that all acidic fractions (FE, RPS, AF I−III) exhibited a dominant rhamnose−galactose moiety and that NF additionally contained considerable amounts of arabinose. Separation of uronic acids on PAD-HPLC revealed the presence of galacturonic acid as well as glucuronic acid in all acidic fractions.

Table 2 shows the results of linkage analysis of RPS, NF, and AF I−III as well as their carboxyl-reduced forms and GC-MS identification of the partially methylated alditol acetates (PMAA). The data indicated that NF consisted of carbohydrates with rhamnogalactan/-glucan backbone. The backbones of AF I−III might represent mainly galacturonans that increasingly consist of uronic acid clusters, from AF II to AF III with an increasing degree of glucuronic acid content. In contrast, the degree of branching and the degree of side-chain polymerization as well as their uronic acid contents probably decreased with increasing acidity of the fractions. Whereas the side chains of AF I were dominated by clusters of galacturonic acid, the side chains of AF II and AF III are thought to mainly consist of short galactose units. The side chains of NF probably contained galactose and arabinose residues in comparable molar relations.

Table 2. Methylation Analysis of Polysaccharide Fractions and Carboxyl-Reduced Acidic Fractions[a]

carbohydrate	linkage	RPS	NF	AF I	AF II	AF III
rhamnose	1,2-	2.68	1.65	2.21	8.02	4.25
	1,2,4-	19.80	19.42	0.27	6.96	3.01
arabinose	1-	0.44	1.81	0.87	0.51	
	1,3-	0.54	8.05	0.21	0.20	0.13
	1,4-	1.21		0.28	1.61	0.12
	1,2-	0.16	4.48			0.01
xylose	1,2-	0.56	1.94	0.09	0.27	0.48
	1,2,4-	0.96	1.54	0.46	0.16	
mannose	1,4,6-	0.53	3.53	1.06	1.52	0.32
galactose	1-	7.27	13.41	1.16	8.15	4.95
	1,2-	12.37	2.36	12.59	6.18	6.25
	1,4-	7.79	13.10	14.12	4.15	3.57
	1,6-	2.39	14.55	1.41	1.43	0.45
	1,4,6-	0.70		3.14	0.59	
	1,3,6-	1.29		0.71	0.14	0.14
galacturonic acid[b]	1-			11.97	-	
	1,2-	3.69		37.11	-	
	1,4-	18.72			31.92	31.78
	1,6-			5.66		
	1,4,6-	5.84			8.09	
	1,3,6-			2.08	1.54	
glucose	1-	0.26	1.28	0.30	0.86	0.07
	1,4	3.74	12.64	0.91	5.33	0.29
	1,6-					0.63
	1,3,6-	0.16	0.34	2.40	0.55	0.06
glucuronic acid[b]	1-	1.13			2.25	1.19
	1,4-					34.76
	1,3,6-	7.75			9.51	7.55

[a]Data indicate the respective molar composition. RPS, raw polysaccharide; NF, neutral AEX fraction; AF I–III, acidic AEX fractions I–III. [b] Determined as C-6-reduced galactose or glucose.

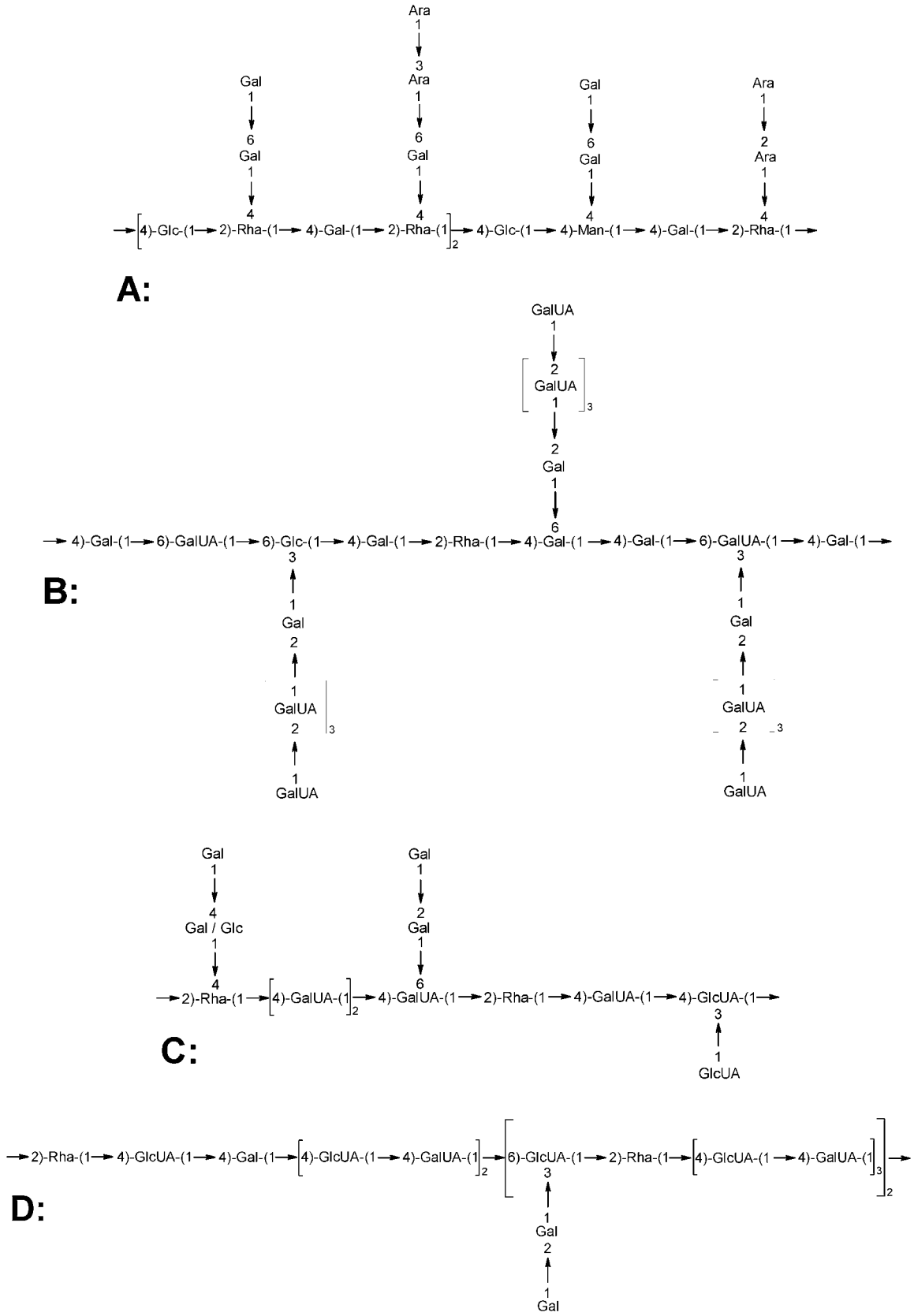

Figure 3. Proposed structures of polysaccharide fractions from okra RPS: general structures of NF (**A**), AF I (**B**), AF II (**C**), and AF III (**D**). The degree of substitution and the length of side chains may vary among the individual fractions. NF, neutral AEX fraction; AF I–III, acidic AEX fractions I–III; Ara, arabinose; Gal, galactose; GalUA, galacturonic acid; Glc, glucose; GlcUA, glucuronic acid; Man, mannose; Rha, rhamnose.

Models of the proposed structures are shown in **Figure 4**. Considering the molecular weights given in **Table 1**, NF should consist of ∼10, AF I of ∼170, AF II ∼490, and AF III ∼292 repeated units as shown in **Figure 3**.

As the immature okra fruits were peeled it became obvious that the juice had a quite high viscosity. Therefore, the kinematic viscosities of native FE, lyophilized FE, and RPS were determined at 20 °C. All solutions were diluted to the total

A:

Sample	Kinematic viscosity [mm²/s]
Water	1.000
RPS	1.240
FE lyoph.	2.093
FE native	3.186

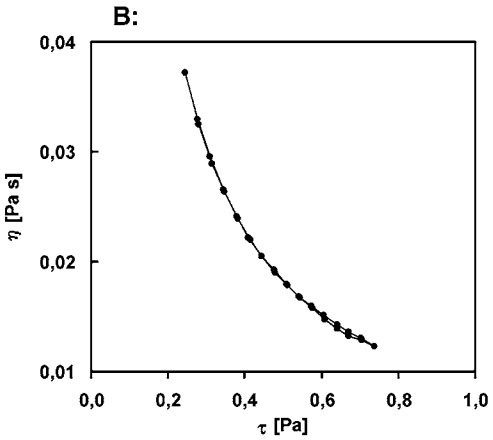

Figure 4. (**A**) Flow viscosimetry of 0.1% okra RPS in bidistilled water and okra FE (native and lyophilized) in bidistilled water diluted to equivalent total carbohydrate content. (**B**) Rotation viscosimetry of native FE reveals a decreased viscosity η (liquidification) with an increase in shear force τ: the solution shows a pseudoplastic rheology.

carbohydrate content equivalent to 0.1% RPS in double-distilled water. **Figure 4A** shows that the viscosity of native, nonlyophilized FE was 1.5 times higher than that of the lyophilized FE and 2.6 times higher than that of the RPS. The viscosity of RPS was only 1.2 times higher than that of double-distilled water. All differences were statistically significant ($p < 0.01$). For reasons of instrument sensitivity, rotation viscosimetry at 20 °C could be performed only with the native, nonlyophilized FE. **Figure 4B** shows the characteristic shearing curve for shear forces at 0−30 rotations per minute, which revealed that with an increased shear force τ the viscosity η of native FE decreases as the sample liquefied and vice versa: the mucilage displayed a pseudoplastic rheology without any thixotropic effects occurring.

Adherence of *H. pylori* to Material of Human Stomach. Prior to testing of antiadhesive effects, screenings had to be performed in order to find suitable combinations of bacterial strains and gastric material that allowed the development of a standard assay with reproducible binding rates. Initially, five different strains of *H. pylori* were examined for their ability to adhere to human gastric mucosal tissue, of which three, SS1, G27, and CCUG 17874, were established laboratory strains; the latter two were described previously (*22*) as type I strains of *H. pylori*. Two strains, 22880 and 23257 were clinical isolates recovered from gastric biopsy specimens. These strains were used in an adhesion screening on biopsy material from seven different human stomachs. With three of these gastric preparations strong and reproducible adhesive interactions could be observed. G27 displayed optimal binding properties on most of the respective stomach preparations, whereas SS1 and CCUG 17874 bound much more weakly and the two clinical isolates interacted only to a minor extent. Thus, G27 was chosen as a standard strain in all experiments. The adhesive situation of the test system was considered to be *Helicobacter*-specific, because in analogous experiments with FITC-labeled *Escherichia coli* strain DH5α no binding effects were observed.

Influence of Okra Polysaccharide Fractions on the Adhesion of *H. pylori* to Sections of Human Gastric Mucosa. For in situ studies on gastric human mucosa, aliquots of FITC-labeled *H. pylori* were pretreated for 2 h with lyophilized okra fractions diluted to 0.1, 0.5, and 1.0 mg/mL or with the native FE adjusted to the total carbohydrate content of RPS at 1.0 mg/mL in dilutions of 1:1, 1:2, and 1:10. For each test, one bacterial aliquot was incubated in pure blocking buffer and served as a negative control, indicating the normal level of bacterial adhesion. As a positive control, a 3′-/6′-sialyllactose mix (1.0 mg/mL) derived from human milk was used because 3′-sialyllactose is known as a strong antiadhesive agent against *H. pylori* (*23*−*25*). Pretreatment with 3′-/6′-sialyllactoses inhibited bacterial binding almost completely. After 2 h of pretreatment, bacteria were washed once in PBS and were spread on the deparaffinated tissue sections. For each test, a single stomach preparation was incubated with a given preincubation sample and two stomach preparations were incubated with the positive and negative controls, respectively. Each test polysaccharide of a given concentration was examined at least in two or three independent experiments. After preincubation with native FE diluted to the total carbohydrate content of 0.1% RPS and subsequent washing, the binding of FITC-labeled *H. pylori* to the gastric tissue was almost completely inhibited, compared with the binding of the nontreated bacteria (**Table 3**). The effect

Table 3. Effect of a 2 h Pretreatment with Okra Polysaccharide Fractions on the Adhesion of FITC-Labeled *H. pylori* to Sections of Human Gastric Mucosa[a]

pretreatment of bacteria	bacterial adhesion
untreated	++++++
0.10% 3′-/6′-sialyllactose	–
FE native	+
FE native (1:2)	++
FE native (1:10)	+++++
FE native, heat-denaturated	+++++
0.10% FE lyophilized	++
0.05% FE lyophilized	+++
0.15% TCA supernatant, lyophilized	+++++
0.10% RPS	+++
0.05% RPS	++++
0.01% RPS	+++++
0.10% AF I	++++++
0.10% AF II	+++++
0.10% AF III	+++

pretreatment of gastric epithelia	bacterial adhesion
untreated	++++++
FE native	+++++
FE lyophilized	+++++
0.10% RPS	+++++

[a] Treated were either the bacterial suspensions or the mucosal sections. 3′-/6′-sialyllactose served as a positive control for antiadhesive activity. FE native/lyophilized was diluted to the total carbohydrate content of 0.1% RPS. Each test polysaccharide of a given concentration was examined at least in two or three independent experiments. Quantification of bacterial adhesion: –, almost no binding; +, very weak binding; ++++++, very strong binding. FE, fresh extract; RPS, raw polysaccharide; AF I–III, acidic AEX fraction I–III; TCA, trichloroacetic acid.

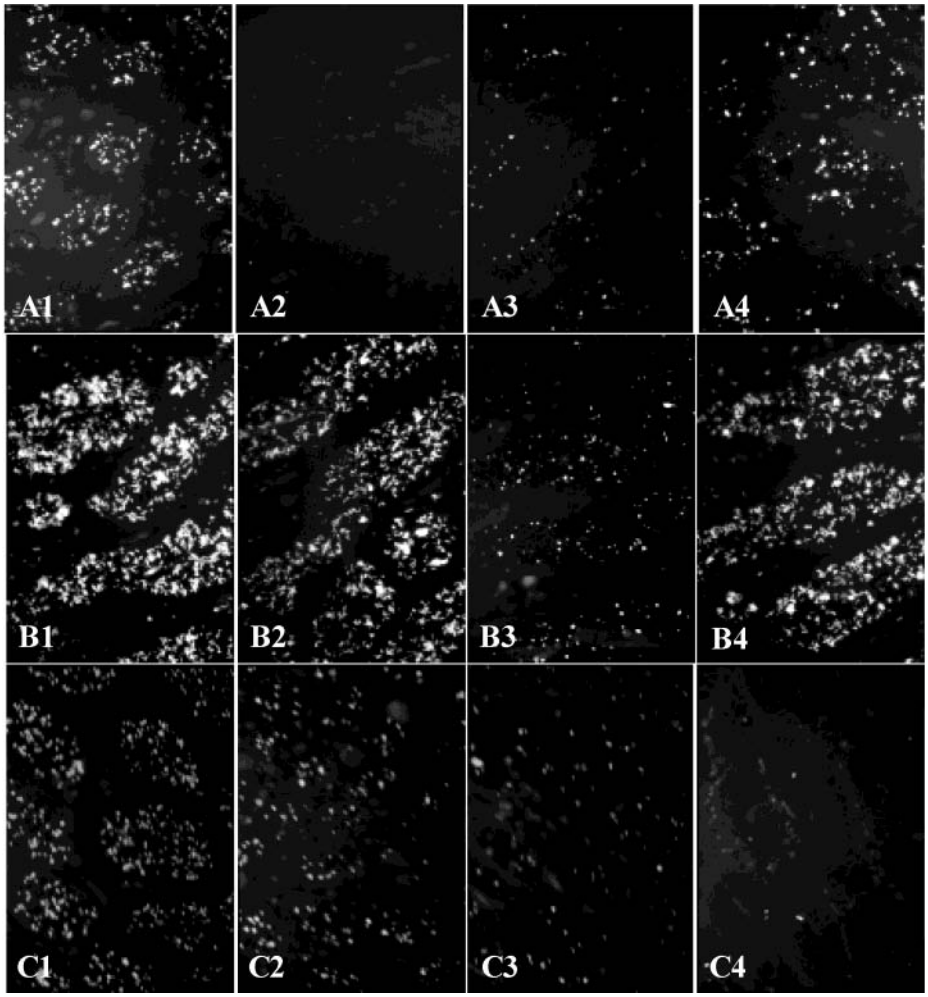

Figure 5. Fluorescent microscopy of three representative in situ experiments (**A**–**C**) with FITC-labeled *H. pylori* on human gastric mucosa: (**A**) adhesion of (**A1**) nontreated bacteria and (**A2**–**4**) bacteria pretreated with native FE (equivalent total carbohydrate content as 0.1% RPS) in dilutions 1:1, 1:2, and 1:10; (**B**) adhesion of (**B1**) nontreated bacteria and of bacteria (**B2**) pretreated with heat-denaturated, native FE, (**B3**) pretreated with 0.1% lyophilized FE, and (**B4**) pretreated with 0.15% TCA supernatant of FE; (**C**) adhesion of (**C1**) nontreated bacteria and of bacteria (**C2**) pretreated with 0.1% RPS, (**C3**) pretreated with 0.1% AF III, and (C4) pretreated with 0.1% 3′-/6′-sialyllactose. FE, fresh extract; RPS, raw polysaccharide; AF III, acidic AEX fraction III.

proved to be concentration-dependent. The lyophilized and resolved FE proved to be nearly as effective as the native FE, yet with visibly more bacteria bound to the epithelia. A pretreatment with RPS at 1.0 mg/mL reduced the adhesion of *H. pylori* strongly. **Figure 5** displays characteristic binding with pretreated and nontreated *H. pylori* on sections of human gastric mucosa.

To investigate the structural principle behind the antiadhesive activity of okra RPS, the two acidic AEX fractions, AF I and AF II, and the NaOH-eluated AF III with highest uronic acid content were tested. In experiments with AF III at 1.0 mg/mL, the adhesion of pretreated *H. pylori* was strongly reduced compared with the adhesion of the nontreated bacteria (**Table 3**; **Figure 5**). This effect was in the same activity range observed by pretreatment with RPS at the respective concentration, indicating that the antiadhesive effect of RPS is mainly caused by the AF III subfraction. AEX fraction AF II reduced the binding of *H. pylori* only to a minor extent, whereas fraction AF I with low uronic acid content displayed no antiadhesive activity at all.

To examine the effect of protein precipitation with TCA on the antiadhesive qualities of FE, the TCA supernatant was tested against the native, untreated FE. Because the precipitation had obviously reduced the amount of uronic acids, too (**Figure 1**), both fractions were diluted to equivalent uronic acid contents. It showed that the protein-degraded TCA supernatant had lost most of the antiadhesive potency of the initial extract, and the same effect occurred after heat denaturation of native FE (**Table 3**; **Figure 5**).

Preincubation of the gastric material instead of *H. pylori* with 0.1% RPS, lyophilized FE, or nonlyophilized FE did not result in reduced bacterial binding (**Table 3**), indicating that the inhibitory effects were due to interactions of compounds from okra with bacterial surface structures.

Results of Toxicity Studies. All tested polysaccharides were more or less acidic, with pH values ranging from 5.6 for RPS to 4.0 for AF III, each at 1.0 mg/mL. Although it is known that *H. pylori* is able to survive even at a pH of 4.0 in vitro in the absence of urea (*26*), it seemed necessary to ensure that the reduced binding of the bacteria to the gastric surfaces was not merely due to an increased lethality. Accordingly, in vitro toxicity studies were performed with all fractions that had shown to be effective. The tests revealed that preincubation with FE and RPS increased bacterial growth 3-fold ($p = 0.002$) and 9-fold ($p = 0{,}069$), respectively, and that AF II and AF III had no significant ($p = 0.826$ and 0.695) influence (**Figure 6**).

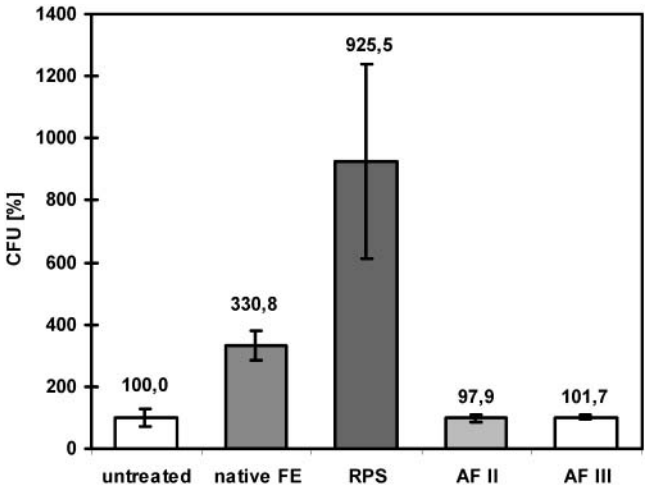

Figure 6. In vitro toxicity studies: evaluation of CFU on agar plates after 2 h of pretreatment of *H. pylori* with okra fractions (in percent) in relation to the untreated control. RPS, AF II, and AF III were at 1.0 mg/mL, and FE was diluted to a carbohydrate content equivalent to RPS 1.0 mg/mL. Each bar shows results as the MW ± SD of counted CFU on three plates. FE, fresh extract; RPS, raw polysaccharide; AF II/III, acidic AEX fraction II/III.

DISCUSSION

From immature okra pods an FE was prepared from which three crude polysaccharides were obtained by fractionated precipitation with ethanol to final volumes of 35, 45, and 60% (v/v). By choosing the raw polysaccharide of the 45% precipitate (RPS) for further experiments, a first selective step was taken focusing mainly on the high molecular weight carbohydrate fraction of the initial extract. Precipitations of higher ethanol concentrations generally lead to badly resolvable products and lower carbohydrate contents. Despite some differences in the extraction method, the yield, the general chemical composition, and the average molecular weight of RPS were comparable to results published in earlier studies on okra polysaccharides (*27*, *28*). Yet considering linkage analysis of its subfractions, our data suggest even more galacturonans than rhamnogalacturonans as the main structural elements. The second difference was that evidence (TLC, PAD-HPLC, GC-MS) was obtained for the presence of both glucuronic and galacturonic acid, whereas galacturonic acid was the only uronic acid mentioned previously in ref *27* or *28*. Linkage analysis based on GC-MS data did not suggest glucuronic acid as a structural element of fraction AF I, although PAD-HPLC data indicated the presence of minor amounts.

Concerning *H. pylori*, the central finding was that highest antiadhesive potential could be found in the native, nonlyophilized FE< which is a crude mixture of carbohydrates and protein forming a viscous mucilage with pseudoplastic rheology. This effect was caused by interactions of glycosylated compounds of FE with binding factors on the bacterial surface because a pretreatment of the gastric epithelial tissue instead of the bacteria was ineffective. Thus, the use of okra against the colonization of *H. pylori* cannot be justified by extraordinary mucilaginous properties of its extracts. For a practical use of FE as food additive it would not be likely to eliminate an already existing infection with *H. pylori*, but it might be possible to use it a prophylactic additive against further colonization.

The precipitated RPS obviously had less influence on the bacterial binding than FE. This could, on the one hand, be due to the reduction of low molecular weight sugars in RPS after precipitation in 45% ethanol (v/v), although a raw polysaccharide from precipitation in 60% ethanol (v/v) was not more effective (data not shown). On the other hand, the total protein content of FE was twice as high as that of RPS, and several different protein bands became visible after SDS-PAGE, with main bands of glycosylated proteins between 25 and 37 kDa. After TCA precipitation, these protein fractions were almost completely diminished, correlating with an almost complete reduction of antiadhesive activity. Therefore, besides the fraction of highly acidic carbohydrates, the main effectors against *H. pylori* could be seen in the glycoprotein fraction of the native, crude FE. Furthermore, there was a loss in antiadhesive activity with every lyophilization step from native FE to lyophilized FE to lyophilized RPS, along with a loss of viscosity. Here, denaturation effects during freeze-drying might have led to irreversible changes in the supramolecular structure that in the case of the raw polysaccharides also had an influence on their solubility.

From in situ experiments with AF I−III some information was gained concerning the relationship between structure and activity of the carbohydrates. Generally, a high uronic acid content seemed to be required for antiadhesive quality. This is supported by previous findings (*8*) indicating that sugar-associated negatively charged functionalities might be essential for antiadhesive activity against *H. pylori*. Along with effectiveness, a remarkable increase in glucuronic acid content could be noted, from 0% (AF I) to ∼12% (AF II) to >40% (AF III) of total sugars as determinable by GC-MS. Furthermore, in the carbohydrates of all active subfractions (AF II plus AF III) the negatively charged functions seemed to be increasingly located in the main sugar chain, probably with the tendency to build up boxes of repeated galacturonic and glucuronic acid units. On the other hand, AF II plus AF III probably displayed a low degree of branching with very short, mainly neutral side chains, whereas our data suggested that inactive AF I possessed more complex side chains carrying the total negative charge. All acidic fractions contained only minor amounts of rhamnose, suggesting that the carbohydrate chains possessed large areas of straight sugar array. The NF could not be tested on *H. pylori* for reasons of substance availability. However, previous studies (*8*) as well as our own testing with neutral sugar fractions from other plant-derived polysaccharides showed negative results (data not shown). Taken together, our data indicated that large straight sugar chains of a high uronic acid density, especially glucuronic acid, and a low degree of branching were necessary for the interaction of okra polysaccharides with surface adhesion molecules of *H. pylori*.

None of the tested okra fractions was toxic against *H. pylori*. On the contrary, after pretreatment with the crude fractions FE and RPS, bacterial growth was stimulated according to their total carbohydrate contents and acidities, which may simply have been due to bacterial sugar metabolism.

Some other plant-derived compounds with antiadhesive activity have already been identified. A high molecular mass constituent from cranberries has been shown to inhibit the adherence of *H. pylori* to immobilized human mucus in vitro (*29*). Although the testing substance was not further characterized, the data indicated that it interacted specifically with sialic acid-specific adhesins of *Helicobacter*. An acidic polysaccharide isolated from the root of *Panax ginseng* was shown to inhibit the in vitro hemagglutination of *H. pylori* (*29*). The fraction displayed a high uronic acid content and had lost activity after pectinase treatment. Different, nonacidic polysaccharide frac-

tions were ineffective; thus, the activity was correlated with uronic acid content of the pectine-type compound.

It is not yet clear which bacterial adhesins are responsible for the interactions reported here, although similar mechanisms reported previously (*29*) are possible. Possibly the surface-associated urease is involved, although it was described that the protein did not bind polygalacturonic acid in vitro (*8*). Experiments with cell wall preparations from *H. pylori* might be useful in answering this question. Further investigations will focus on the glycoprotein content of the fresh plant extract as the probably most important antiadhesive factor present in the fruit. Yet from a technical point of view and with regard to a possible use as a functional food additive, it is an enormous advantage to have maximum effects against *H. pylori* in the initial, nonpurified material with only minor losses in activity by freeze-drying.

ABBREVIATIONS USED

FE, fresh extract; RPS, raw polysaccharide precipitated in 45% ethanol (v/v); AEX, anion exchange chromatography; NF, neutral AEX fraction; AF I, low acidic AEX fraction; AF II, highly acidic AEX fraction; AF III, highly acidic NaOH eluate; FITC, fluoresceinisothiocyanate.

LITERATURE CITED

(1) Tomada M.; Shimizu, N.; Gonda, R.; Kanari, M.; Hikino, H. Anticomplementary and hypoglycemic activity of okra and hibiscus mucilages. *Carbohydr. Res.* **1989**, *190* (2), 323−328.

(2) Woolfe, J. A. The effect of okra mucilage (*Hibiscus esculentus* L.) on the plasma cholesterol level in rats. *Proc. Nutr. Soc*. **1977**, *36* (2), 59A

(3) Schmidgall, J.; Hensel, A. Evidence for bioadhesive effects of polysaccharides in an ex vivo bioadhesion assay on buccal membranes. *Planta Med.* **2000**, *66*, 48−53.

(4) Schmidgall, J.; Hensel, A. Bioadhesive properties of polygalacturonides against colonic epithelial membranes. *Int. J. Biol. Macromol.* **2002**, *30*, 217−225.

(5) Warren, J. R.; Marhall, B. J. Unidentified curved bacilli on gastric epithelium in chronic active gastritis. *Lancet* **1983**, *1*, 1273−1275.

(6) Covacci, A.; Telford, J. L.; Del Giudice, G.; Parsonet, J.; Rappuoli, R. *Helicobacter pylori* virulence and genetic geography. Review. *Science* **1999**, *284*, 1328−1333.

(7) Icatlo, Jr., F. C.; Kuroki, M.; Kobayashi, C.; Yokoyama, H.; Ikemori, Y.; Hashi, T.; Kodama, Y. Affinity purification of *Helicobacter pylori* urease. Relevance to gastric mucin adherence by urease protein. *J. Biol. Chem*. **1998**, *273*, 18130−18138.

(8) Icatlo, Jr., F. C.; Goshima, H.; Kimura, N.; Kodama, Y. Acid-dependent adherence of *Helicobacter pylori* urease to diverse polysaccharides. *Gastroenterology* **2000**, *119*, 358−367.

(9) Lelwala-Gurunge, J.; Ascencio, F.; Ljungh, A.; Wadstrom, T. Rapid detection and characterization of sialic acid-specific lectins of *Helicobacter pylori*. *Acta Pathol. Microbiol. Scand.* **1993**, *101* (9), 695−702.

(10) Edwards, N. J.; Monteiro, M. A.; Faller, G.; Walsh, E. J.; Moran, A. P.; Roberts, I. S.; High, N. J. Lewis X structures in the O antigen side-chain promote adhesion of *Helicobacter pylori* to the gastric epithelium. *Mol. Microbiol.* **2000**, *35* (6), 1530−1539.

(11) Karlsson, K.-A. Mini Review: The human gastric colonizer *Helicobacter pylori*: a challenge for host-parasite glycobiology. *Glycobiology* **2000**, *10* (8), 761−771.

(12) Falk, P.; Roth, K. A.; Borén, T.; Westblom, T. U.; Gordon, J. I.; Normark, S. An in vitro adherence assay reveals that *Helicobacter pylori* exhibits cell lineage specific tropism in the human gastric epithelium. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 2035−2039.

(13) Wu, A. M.; Jiang, Y. J.; Hwang, P. Y.; Shen, F. S. Characterization of the okra mucilage by interaction with Gal, GalNAc and GlcNAc specific lectins. *Biochim. Biophys. Acta* **1995**, *1243* (2), 157−160.

(14) Monsigny, M.; Petit, C.; Roche, A. C. Colorimetric determination of neutral sugars by a resorcinol sulphuric acid micromethod. *Anal. Biochem.* **1988**, *175*, 525−530.

(15) Blumenkrantz, N.; Asboe-Hansen, G. New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484−489.

(16) Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stone, A. B. A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res.* **1983**, *113*, 291−299.

(17) Hakamori, S. J. Rapid permethylation of glycolipids and polysaccharides, catalysed by sulfinyl carbanion in dimethyl sulfoxide. *J. Biochem.* **1964**, *55*, 205−210.

(18) Harris, P. J.; Henry, R. J.; Blakeney, A. B.; Stone, A. B. A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res.* **1984**, *127*, 59−73.

(19) Taylor, R. T.; Conrad, H. E. Stoichiometric depolymerization of polyuronides and glycosaminoglycanes to monosaccharides following reduction of their carbodiimid-activated carboxyl groups. *Biochemistry* **1972**, *11*, 1383−1388.

(20) Bradford, M. M. A rapid method for the quantification of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(21) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(22) Xiang, Z.; Censini, S.; Bayeli, P.; Telford, J. L.; Figura, N.; Rappuoli, R.; Covacci, A. Analysis of expression of CagA and VacA virulence factors in 43 strains of *Helicobacter pylori* reveals that clinical isolates can be divided in two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. *Infect. Immun.* **1995**, *63* (1), 94−98.

(23) Simon, P. M.; Goode, P. L.; Mobasseri, A.; Zopf, D. Inhibition of *Helicobacter pylori* binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides. *Infect. Immun.* **1997**, *65* (2), 750−757.

(24) Jagannatha, V. M.; Wiggington, T.; Simon, P. M.; Zopf, D.; Heman-Ackah, L. M.; Dubois, A. Treatment of *Helicobacter pylori* infection in rhesus monkeys using a novel antiadhesion compound. *Gastroenterology* **1999**, *117*, 1316−1325.

(25) Teneberg, S.; Jurstrand, M.; Karlsson, K. A.; Danielsson, D. Inhibition of nonopsonic *Helicobacter pylori*-induced activation of human neutrophils by sialylated oligosaccharides. *Glycobiology* **2000**, *10* (11), 1171−1181.

(26) Scott, D.; Weeks, D.; Melcher, K.; Sachs, G. The life and death of *Helicobacter pylori*. *Gut* **1998**, *43* (1), 56−60.

(27) Tomada, M.; Shimada, K.; Saito, Y.; Sugi, M. Plant Mucilages. XXVI. Isolation, and structural features of a mucilage, "okra-mucilage F", from the immature fruit of *Abelmoschus esculentus*. *Chem. Pharm. Bull.* **1980**, *28* (10), 2933−2940.

(28) Bhat, U. R.; Tharanathan, R. N. Fractionation of okra mucilage and structural investigation of an acidic polysaccharide. *Carbohydr. Res.* **1985**, *148*, 143−147.

(29) Burger, O.; Ofek, I.; Tabak, M.; Weiss, E. I.; Sharon, N.; Neeman, I. A high molecular mass constituent of cranberry juice inhibits *Helicobacter pylori* adhesion to human gastric mucus. *FEMS Immunol. Med. Micriobiol*. **2000**, *29*, 295−301.

(30) Belogortseva, N. I.; Yoon, J. Y.; Kim, K. H. Inhibition of *Helicobacter pylori* hemagglutination by polysaccharide fractions from roots of *Panax ginseng*. *Planta Med.* **2000**, *66* (3), 217−220.

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