Exfoliation of *Helicobacter pylori* from Gastric Mucin by Glycopolypeptides from Buttermilk

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ABSTRACT

In the human stomach, *Helicobacter pylori*, an ulcer pathogenic bacterium, colonizes the gastric mucosal layer primarily. The ability of glycopolypeptides (GPP) prepared from buttermilk to exfoliate *H. pylori* bound to gastric mucin was investigated. The GPP were prepared from buttermilk by digestion with trypsin, papain, pancreatin, bromelain, or pepsin. *Helicobacter pylori* ATCC 43504^T and 43579 adhered more strongly to all of the GPP tested than to whole buttermilk, the soluble fraction of buttermilk, gastric mucin prepared from mouse stomach, or commercial pig gastric mucin. The GPP digested with trypsin, papain, or pancreatin were significantly more adherent. When the GPP concentration was 10 mg/mL, bound *H. pylori* ATCC 43504^T, 43579, and 5 clinical isolates were exfoliated markedly from immobilized porcine gastric mucin following treatment with GPP digested with trypsin or pancreatin. This ability of GPP did not correlate with sialic acid content, indicating that sialic acid content is not important in the exfoliation of this microorganism. Such an ability may depend on the structure or number of sugar chains, or the position of sialic acid. We conclude that GPP promote the exfoliation of *H. pylori* bound to gastric mucin and prevent the de novo adherence of this microorganism. As such, GPP are a promising food material for preventing *H. pylori* infection. (**Key words:** *Helicobacter pylori*, exfoliation, glycopolypeptide, buttermilk)

Abbreviation key: GPP = glycopolypeptide, IC₅₀ = 50% inhibitory concentration.

INTRODUCTION

Since the successful culture of *Helicobacter pylori* in 1983 (Warren and Marshall, 1983), this gram-negative

bacterium has been recognized as one of the most common bacterial pathogens in humans (Taylor and Blaser, 1991). *Helicobacter pylori* is an etiologic agent in the development of gastritis (Lee et al., 1993), gastroduodenal ulcers (Marshall and Warren, 1984; Buck et al., 1986), gastric cancer (Goodwin, 1993; The Eurogust Study Group, 1993; Isaacson, 1994) and mucosa-associated lymphoid tumors (Parsonnet et al., 1994).

Many studies have examined the prevention of infection by and eradication of this microorganism. Antimicrobial agents are the main treatment of such infection in developed countries. In Japan, the Japanese Society for *Helicobacter pylori* Guidelines recommends the concurrent use of lansoprazole plus amoxicillin and clarithromycin for 1 wk. However, adverse reactions (14.8 to 45.1%) and the appearance of antibiotic-resistant bacteria are common problems (Kato et al., 2000). By contrast, some researchers have recently proposed a therapy that does not involve antimicrobial agents, including probiotics and anti-*Helicobacter* antibodies. Sakamoto et al. (2001) reported that the administration of probiotics suppresses *H. pylori* and reduces gastric mucosal inflammation, although the mechanisms of such suppression and reduction are as yet unknown. Although Shimamoto et al. (2002) demonstrated the inhibition of *H. pylori* infection by an orally administered yolk-derived anti-*H. pylori* antibody, this could not be put to practical use. It is desirable to investigate alternatives to antibiotic treatment to understand the nature of gastric *H. pylori*.

Two types of mucin secreted from surface- and glandular-type mucous cells in the mucous gel layer covering the gastric mucosa have reticular and band-like structures, respectively (Ota and Katsuyama, 1992). *Helicobacter pylori* is frequently found as small aggregates within the mucous gel layer of surface-type, mucous cell-type mucin (Hidaka et al., 2001). This mucin is a glycoprotein called MUC5AC, which has a basic structure that consists mainly of serine, threonine, proline, and cysteine (Van Klinken et al., 1997). In the stomach, *H. pylori* occurs in the surface-layer cell type

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mucin near epithelial cells. It travels toward the lumen due to the secretion of new mucin, and migrates back toward the epithelial cells via urea chemotaxis (Nakazawa, 2001). Many researchers have examined the binding of *H. pylori* to gastrointestinal epithelial cells and the gastric mucin/mucus. Evans et al. (1988, 1989) reported that this microorganism recognizes the sialic acid, 3'-sialyllactose (NeuAc α 2-3Gal β 1-4Glc). *Helicobacter pylori* adheres to sulfated oligosaccharides expressed on glycoproteins (Tsouveleakis et al., 1991) and glycolipids (Saitoh et al., 1991), as well as to basement membrane constituents, such as laminin and collagen (Trust et al., 1991). Various compounds inhibit *H. pylori* binding to gastrointestinal epithelial cells, gastric mucin/mucus, and glycolipid receptors in vitro, including sialic acid (Simon et al., 1997), cranberry juice (Burger et al., 2000), milk glycoprotein (Hirno et al., 1998), and lactic acid bacteria (Mukai et al., 2002).

We studied the effect of buttermilk, a by-product of butter production. Most of the milk fat globule membrane, which is composed of glycopeptides that predominantly contain sialic acid, serine, and threonine (McPherson and Kitchen, 1983), is found in buttermilk (Corredig et al., 2003). Previously, we noted that a polypeptide produced from buttermilk by bromelain digestion inhibits infection by bovine rotavirus, which uses cell surface sialic acid as a receptor (Matsumoto et al., 2002).

In this study, we examined the binding of *H. pylori* to the glycopolypeptides (GPP) prepared from buttermilk by digestion with 5 different proteases. Also, the exfoliation of *H. pylori* bound to gastric mucin by GPP was investigated.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Seven *H. pylori* strains were studied. *Helicobacter pylori* ATCC 43504^T (CagA and VacA-positive [Ohkusa et al., 2003]) and 43579 were purchased from the American Type Culture Collection (ATCC, Gaithersburg, MD). *Helicobacter pylori* LKM HP-2, LKM HP-3, LKM HP-4, LKM HP-5, and LKM HP-7 were isolated from patients with peptic ulcer. The bacteria were grown on tryptic soy agar (Merck, Darmstadt, Germany) containing 5% horse blood at 37°C for 96 h under humid microaerobic conditions using an AnaeroPack Helico (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). The purity of the cultures was established using gram staining, urease, oxidase, and catalase tests.

Glycopolypeptide Preparation from Buttermilk

Buttermilk powder was obtained from Matsumoto Factory of Kyodo Milk Industry Co., Ltd. (2 to 25 to

20 Minami-matsumoto, Matsumoto, Japan). All of the proteases (trypsin, papain, pancreatin, bromelain, and pepsin) used were obtained from Japan Bicon, Inc. (Nagoya, Japan). The optimal pH and temperature of each protease are shown in Table 1. Buttermilk was delipidized with chloroform:methanol (2:1). Nonfat buttermilk was thoroughly suspended in ion-exchanged water (60 g/L), which was adjusted to the optimal pH of each protease using HCl or NaOH, and then pasteurized at 95°C for 15 min. Each protease (0.3 g/L) was added to the buttermilk solution after it had cooled to the optimal temperature, and digestion proceeded at the optimal temperature for 24 h with gentle stirring. After 12 h, the pH of each buttermilk solution was readjusted, and more protease was added (0.3 g/L). After digestion, each buttermilk solution was adjusted to pH 7.0 and heated at 95°C for 15 min to inactivate the proteases. The precipitate was removed by centrifugation (10,000 $\times$ g for 30 min at 4°C), and the supernatant was filtered (0.45 μ m), lyophilized, and was used as GPP. The GPP were stored at 4°C in a refrigerator until use. The powder of the soluble fraction of buttermilk, which was treated similarly but without protease, was prepared as control.

The peptide content of each GPP was measured using the total nitrogen analyzer TN-05 (Mitsubishi Chemical Co., Tokyo, Japan). The carbohydrate content of each GPP was measured by phenol-sulfuric acid reaction.

Mouse Gastric Mucin Preparation

Ten 5-wk-old male BALB/c mice were obtained from Charles River Japan (Shizuoka, Japan). The mice were maintained on a 12-h light/12-h dark schedule with food and water available ad libitum. The temperature of the colony room was maintained at 24 to 25°C. The mice were killed by cervical dislocation after 1 wk, and the stomachs were collected, opened, and washed with PBS. The gastric surface mucosa was collected with a spatula and suspended in PBS containing sodium azide and a protease inhibitor. It was stirred slowly in an ice bath for 10 min, centrifuged (5000 $\times$ g for 20 min), and the supernatant was collected. Ethanol was added to this supernatant at a final concentration of 70%. This was then centrifuged (5000 $\times$ g for 20 min) and the precipitate was collected. This precipitate was lyophilized after desalination (molecular weight = 6000 to 8000), and was used as crude mouse gastric mucin. This experiment was performed in accordance with the protocols approved by the Kyodo Milk Animal Use Committee.

Test of *H. pylori* Adhesion to GPP

Treatments involving 5 types of GPP, untreated buttermilk, mouse gastric mucin, and porcine gastric mu-

Table 1. Proteases used in the current study, peptide and carbohydrate contents, and the recovery rate of each glycopolypeptide (GPP).

Protease ¹	Treatment		Peptide (mg/g of dry wt)	GPP	
	pH	Temperature (°C)		Carbohydrate (mg/g of dry wt)	Recovery rate (%) ²
Trypsin	8.0	60	721	75	66.7
Papain	7.0	60	904	39	69.1
Pancreatin	8.0	40	650	28	50.2
Bromelain	5.0	50	853	32	70.9
Pepsin	3.5	50	781	26	58.9
Untreated (buttermilk)	—	—	732	157	100

¹These proteases were obtained from Japan Bicon, Inc. (Nagoya, Japan).

²The recovery rate is shown as the percentage recovered from buttermilk.

cin (Sigma Chemical Co., St. Louis, MO) were prepared. The compounds were dissolved in 0.1 M carbonic acid buffer (pH 9.6) at 10 mg/mL, and 75 μ L was added to the wells of a 96-well microplate (Nunc A/S, Roskilde, Denmark) and incubated at 37°C for 2 h. The *H. pylori* cells suspended in PBS (pH 7.2) were added (50 μ L/well), after each well had been washed 3 times with sterilized PBS (250 μ L/well), and incubated with shaking (30 rpm) at 37°C for 120 min under microaerobic conditions using a shaking incubator. Nonadherent bacterial cells were removed by washing 5 times with sterilized PBS (250 μ L/well). To each well, 100 μ L PBS was added, and bound bacterial cells were exfoliated by pipetting, and counted on tryptic soy agar containing 5% horse blood. The count of adherent *H. pylori* was reported in colony-forming units. The bacterial colonies grown on tryptic soy agar containing 5% horse blood were suspended and adjusted to A660 = 0.4 $\pm$ 0.01 using a Spectronic 21 (Bausch & Lomb, Rochester, NY), and the suspension was diluted 100 times. The number of bacteria inoculated was 1.0 to 4.0 $\times$ 10⁴ cfu per well. All measurements were performed in triplicate.

Test of *H. pylori* Exfoliation from Gastric Mucin by GPP

Porcine gastric mucin was prepared at 10 mg/mL in 0.1 M carbonic acid buffer (pH 9.6), and 75 μ L of the solution was dispensed in each well of a 96-well microplate that was incubated at 37°C for 2 h. The *H. pylori* cell suspension in PBS was added (50 μ L/well) after each well had been washed three times with PBS (250 μ L/well), and incubated with shaking (30 rpm) at 37°C for 2 h using a shaking incubator under microaerobic conditions. Nonadherent bacterial cells were removed by washing twice with PBS (250 μ L/well). The GPP solution in PBS (10 mg/mL) was added to each well (50 μ L/well), and incubated with shaking (30 rpm) at 37°C for 60 min. After 2 washes in PBS, sterilized PBS was added to each well (100 μ L/well), and adherent

bacterial cells were liberated by pipetting. Viable cells were counted on tryptic soy agar containing 5% horse blood. The inhibitory rate was calculated from the cfu using the following formula:

$$\% \text{ of exfoliation} = \frac{[(\text{cfu}_{\text{control}} - \text{cfu}_{\text{experimental}})/\text{cfu}_{\text{control}}] \times 100}{1}$$

As a control, PBS was added instead of GPP suspension.

Sialic Acid Content of GPP

The sialic acid content of GPP was reported as *N*-acetylneuramic acid content. The concentration of *N*-acetylneuramic acid was measured using periodic acid–thiobarbituric acid reaction (Warren, 1959).

Statistical Analysis

The adherences of *H. pylori* to each GPP, and mouse and porcine gastric mucin were compared using Student's *t*-test. These calculations were performed using Statistica software (Design Technologies, Inc., Tokyo, Japan).

RESULTS AND DISCUSSION

The peptide and carbohydrate contents and the recovery rate of each GPP are shown in Table 1. Carbohydrate content decreased in GPP preparation. As the recovery rate is high, this is suitable for mass production in factories. The adhesions of *H. pylori* ATCC 43504^T and ATCC 43579 to each GPP, and mouse and porcine gastric mucins are shown in Figure 1. The numbers of *H. pylori* ATCC 43504^T cells bound to whole buttermilk, the soluble fraction of buttermilk, mouse gastric mucin, porcine gastric mucin, GPP treated by trypsin, papain, pancreatin, bromelain, and pepsin were 343, 350, 773, 850, 3691, 1987, 3309, 1158, and

Figure 1. Adhesion of *Helicobacter pylori* ATCC 43504^T (□) and 43579 (■) to GPP prepared by protease digestion of buttermilk, and mouse and porcine gastric mucin. The concentration of each sample was 10 mg/mL. Vertical bars represent standard errors. Statistically significant at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with untreated buttermilk. Statistically significant at † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ compared with mouse and porcine gastric mucins.

1668, respectively. The numbers of *H. pylori* ATCC 43579 cells bound to whole buttermilk, the soluble fraction of buttermilk, mouse gastric mucin, porcine gastric mucin, GPP treated by trypsin, papain, pancreatin, bromelain, and pepsin were 172, 200, 568, 720, 2690, 1856, 2786, 1309, and 1128, respectively. *Helicobacter pylori* ATCC 43504^T adhered more to GPP prepared using trypsin, papain, and pancreatin than to whole buttermilk or the soluble fraction of buttermilk significantly ($P < 0.05$). *Helicobacter pylori* ATCC 43504^T also tended to adhere more to GPP prepared using bromelain and pepsin than to whole buttermilk or soluble fraction of buttermilk. The adhesion of *H. pylori* ATCC 43579 to GPP prepared using trypsin ($P < 0.01$), papain ($P < 0.001$) and pancreatin ($P < 0.05$) was greater than to whole buttermilk or the soluble fraction of buttermilk. The adhesion of *H. pylori* ATCC 43579 to GPP prepared using bromelain and pepsin also tended to be greater

Table 2. The ability of glycopolypeptide (GPP) prepared using trypsin and pancreatin to exfoliate bound *Helicobacter pylori* from porcine gastric mucin (values are the means $\pm$ standard deviations of 3 determinations).

Strains	Exfoliation (%) of bound <i>H. pylori</i> by GPP digested by:	
	Trypsin ¹	Pancreatin ²
ATCC 43504 ^T	99.0 $\pm$ 1.1	99.8 $\pm$ 0.4
ATCC 43579	97.3 $\pm$ 4.3	99.0 $\pm$ 1.7
LKM HP-2 ²	100	100
LKM HP-3 ²	100	100
LKM HP-4 ²	86.5 $\pm$ 15.4	98.4 $\pm$ 2.4
LKM HP-5 ²	98.0 $\pm$ 2.0	100
LKM HP-7 ²	100	100

¹The concentration of GPP tested was 10 mg/mL.

²These strains isolated from gastric biopsy specimens of the patients with peptic ulcer.

than to whole buttermilk or the soluble fraction of buttermilk. These results show that *H. pylori* cells adhere to protease-treated buttermilk. The adhesion of *H. pylori* ATCC 43504^T to GPP prepared using trypsin was significantly more than to mouse and porcine gastric mucin ($P < 0.05$). The adherence of *H. pylori* ATCC 43579 to GPP prepared using trypsin ($P < 0.001$), papain ($P < 0.001$), and pancreatin ($P < 0.05$) was also significantly more than to mouse and porcine gastric mucin. The adhesion of both strains to GPP prepared using bromelain and pepsin tended to be more than to both types of gastric mucin. By contrast, the adhesion of *H. pylori* ATCC 43579 to whole buttermilk or the soluble fraction of buttermilk was significantly less than to both types of gastric mucin ($P < 0.05$). The adhesion of *H. pylori* ATCC 43504^T to whole buttermilk or the soluble fraction of buttermilk also tended to be less than to both types of gastric mucin. These results demonstrate that protease treatment increases the affinity of buttermilk to *H. pylori*, which is more than the affinity of gastric mucin to *H. pylori*. From these findings, GPP seem to prevent the de novo adhesion of this microorganism.

The ability of GPP prepared using trypsin and pancreatin to exfoliate bound *H. pylori* from gastric mucin is shown in Table 2. These GPP had very strong exfoliation effects against all the strains tested, including the clinical isolates. Many studies have examined the inhibition of *H. pylori* adhesion to gastric mucin in vitro using methods based on the inhibition of bacterial agglutination or the adhesion to a mucin-fixed plate by mixing *H. pylori* cells and the inhibitor simultaneously (Hirno et al., 1998, Burger et al., 2000). It is unclear whether these inhibitors can also exfoliate bound *H. pylori* from gastric mucin. We presume that *H. pylori* infection can be eradicated in vivo only by exfoliative

Figure 2. Dose-response curves of the exfoliative activity of bound *Helicobacter pylori* ATCC 43504^T from gastric mucin by glycopoly-peptide prepared using trypsin (●, filled) and pancreatin (○, open).

materials, and not by adhesive inhibitors. Few studies have examined the exfoliative effect of food ingredients on *H. pylori* adhesion to gastric mucin. In one report, Burger et al. (2000) found that a high-molecular-mass constituent derived from cranberry juice, which inhibited the sialic acid-specific adhesion of *H. pylori*, does not have an exfoliative effect on this microorganism in the gastric mucin. Therefore, our finding of the strong effect of GPP on all the strains investigated is very important. In many previous studies, the effect of inhibitors was dependent on the bacterial strains tested. For example, although 3'-sialyllactose inhibited the bindings of *H. pylori* strains 1832, CP22, 1351, 1512, and 1971 to Hep-2 monolayers, 8 other strains were not inhibited (Simon et al., 1997). We presume that GPP prepared from buttermilk are the first food material with the ability to exfoliate bound *H. pylori* from gastric mucin. The dose-response curves of the exfoliative activity of GPP prepared using trypsin and pancreatin against bound *H. pylori* ATCC 43504^T are shown in Figure 2. There was a dose-dependent removal of pre-bound bacteria from the gastric mucin. The 50% inhibitory concentrations (IC₅₀) of GPP prepared using trypsin and pancreatin were 0.52 and 0.22 mg/mL, respec-

Table 3. The 50% inhibitory concentration of the exfoliative activity of glycopoly-peptide (GPP) against *Helicobacter pylori* ATCC 43504^T and the sialic acid content of GPP.¹

GPP digested by	IC ₅₀ (mg/mL) ²	Sialic acid content (mg/g) ³
Trypsin	0.52	4.51 ± 0.98
Pancreatin	0.22	2.46 ± 0.34
No treatment	>10.0	20.76 ± 3.10

¹Values are the means ± standard deviations of three determinations.

²The IC₅₀ is the concentration required to reduce the number of bound microorganisms to 50% of the control value (i. e., in the absence of an inhibitor). Compounds are tested in serial dilutions beginning with a concentration of 10 mg/mL.

³The sialic acid concentration was adjusted using the *N*-acetylneuramic acid concentration, which was measured using the periodic acid-thiobarbituric acid reaction.

tively, whereas untreated buttermilk had no exfoliative activity (Table 3). These values are approximately 5 to 50 times higher than the IC₅₀ of 3'-sialyllactose reported by Simon et al. (1997) using epithelial cell monolayers. However, the sialic acid content of these GPP was lower than that of untreated buttermilk, so sialic acid content did not correlate with the exfoliative activity against *H. pylori* (Table 3). This indicates that sialic acid content is not an important factor in the exfoliation of this microorganism from gastric mucin, and suggests that exfoliative activity depends on the structure or number of GPP sugar chains, particularly the position of sialic acid. Hirno et al. (1998) reported that sialidase-treated mucin, in which sialic acid is present in the free form, inhibits the hemagglutination activity of a sialic acid-dependent *H. pylori* strain less than untreated mucin. They explained the residual inhibitory activity of the mucins after removing the sialic acids by the sulfate present in the mucin preparations acting as the binding site for the adhesion of *H. pylori*. When a HuTu-80 epithelial monolayer was pretreated with neuraminidase, an enzyme from *Clostridium perfringens* specific for the sialic acid-linked α2-3-galactose, the binding of isolated *H. pylori* was inhibited, whereas an enzyme from *Arthrobacter ureafaciens* that selectively cleaves the sialic acid-linked α2-6-galactose, had a weaker effect (Simon et al., 1997). They also reported that multivalent albumin conjugates of 3'-sialyllactose inhibit bacterial adhesion to epithelial monolayers more effectively than monovalent 3'-sialyllactose. We did not investigate the peptide/AA binding site of GPP. However, this appears to be an important factor that must be studied further in the future, because the main ingredients of both gastric mucins and GPP (MUC5AC and milk fat globule membrane, respectively) are serine and threonine. Although the GPP were heated during the preparation process, exfoliative activity against *H. py-*

lori bound to gastric mucin was sufficient in vitro. Also the recovery rate of GPP was high (Table 1). Therefore, GPP is a useful, heat-stable, functional food material, and mass production and long-term preservation are possible. We conclude that GPP promote the exfoliation of *H. pylori* bound to gastric mucin, making GPP a promising functional food material for countering *H. pylori* infection.

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