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Anti-*Helicobacter pylori* activity of crude N-acetylneuraminic acid isolated from glycomacropeptide of whey

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Helicobacter pylori colonizes the gastric mucosa of about half of the world's population, causing chronic gastritis and gastric cancer. An increasing emergence of antibiotic-resistant *H. pylori* arouses demand on alternative non-antibiotic-based therapies. In this study, we freshly prepared crude N-acetylneuraminic acid obtained from glycomacropeptide (G-NANA) of whey through a neuraminidase-mediated reaction and evaluated its antibacterial ability against *H. pylori and H. felis*. Overnight cultures of the *H. pylori* were diluted with fresh media and different concentrations (1-150 mg/mL) of crude G-NANA were added directly to the culture tube. Bacterial growth was evaluated by measuring the optical density of the culture medium and the number of viable bacteria was determined by a direct count of the colony forming units (CFU) on agar plates. For the *in vivo* study, mice were orally infected with 100 μL (5×10^8 cfu/mL) of *H. felis* four times at a day's interval, accompanied by a daily administration of crude G-NANA or vehicle. A day after the last infection, the mice were daily administered the crude G-NANA (0, 75, and 300 mg/mL) for 10 days and euthanized. Their stomachs were collected and bacterial colonization was determined by quantitative real-time PCR. Crude G-NANA inhibited *H. pylori*'s growth and reduced the number of viable bacteria in a dose-dependent manner. Furthermore, crude G-NANA inhibited bacterial colonization in the mice. These results showed that crude G-NANA has antibacterial activity against Helicobacter and demonstrated its therapeutic potential for the prevention of chronic gastritis and gastric carcinogenesis induced by Helicobacter infection in humans.

Keywords: N-neuraminic acid, glycomacropeptide, Helicobacter, antibacterial activity

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Helicobacter pylori is the causative agent of chronic gastritis, peptic ulcer, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [1,2]. *H. pylori* colonizes the gastric mucosa of 80% and 40% of adults in developing and industrialized countries, respectively [3]. After entering the stomach, *H. pylori* penetrates and adheres to mucin and gastric epithelial cells through various adhesion molecules [4]. Over 90% of *H. pylori* are thought to be present in the mucous layers while the remainder colonize the gastric surface [5]. There are two major *H. pylori* virulence factors that influence the pathogenesis of *H. pylori* infections: cytotoxin-associated genes pathogenecity island (CagPAI) and vacuolating cytotoxin A (VacA) [6,7]. The CagPAI encodes components of a type IV secretion system (T4SS) that can inject the CagA protein and other factors into the host cells, inducing more severe inflammatory disease [6]. VacA damages gastric epithelial cells by

forming pores in cell membranes [7]. Interferon (IFN)-γ-producing CD4⁺ T cells play a critical role in the control of *H. pylori* infections and in vaccine-induced protection [8,9]. However, these Th1 immune responses are known to be inhibited by transforming growth factors (TGF)-β produced by gastric epithelial cells and regulatory T (Treg) cells, which are induced by *H. pylori* on infecting the host [10]. Currently, the most effective therapeutic treatment is the combination of a proton pump inhibitor with antibiotics [11]. However, an increasing emergence of antibiotic-resistant *H. pylori* has led to eradication failures and a demand for alternative non-antibiotic therapies.

Sialic acids are a family of monosaccharides with a nine-carbon backbone and are present in the central nervous system and in body fluids including saliva, tears, and milk [12]. The predominant sialic acid found in mammals is N-acetylneuraminic acid (NANA or Neu5Ac) [12] and the highest concentration of sialic acid is found in the mammalian central nervous system, where it promotes brain development [13]. In addition, sialic acid has antimicrobial potential [14]. Pathogenic microorganisms including bacteria, viruses, and parasites attach to a host cell's surface carbohydrates during the initial stages of infection. As sialylated oligosaccharides in mucin and milk can be a decoy for binding of pathogens, sialylated oligosaccharides can inhibit infection by preventing the binding of pathogens [14]. In fact, sialylated oligo-saccharides in human milk prevent the binding of pathogenic virus, bacteria, and toxins to target cells [14-16].

Previous studies on the antimicrobial potential of sialic acid focused on its inhibition of the binding of pathogens. However, in this study, we prepared crude N-acetyl-neuraminic acid obtained from glycomacropeptide (G-NANA) and demonstrated its bactericidal activity against *H. pylori*. Furthermore, we examined the therapeutic potential of crude G-NANA against Helicobacter infection in a murine model.

Materials and Methods

Preparation of crude N-acetylneuraminic acid from glycomacropeptide (G-NANA)

Crude G-NANA was obtained from the glycomacro-peptide (GMP) of whey through an enzymatic reaction with a neuraminidase isolated from *Arthrobacter ureafaciens.* Briefly, *A. ureafaciens* was grown in an M9 medium containing 10% GMP as a carbon source for 16 h at 30ºC with shaking at 150 rpm. The cultures were filter-pressed, and crude enzymes with over 20 kDa molecular weight were collected by ultra-filtration. Ultra-filtrated crude enzymes were reacted with GMP of whey at 55ºC for 5 h and heat-inactivated at 95ºC for 30 min. Concentrations of G-NANA (65 mg/g) in the enzymatic reaction was determined by quantitative high-performance liquid chromatography (HPLC) analysis.

Bacteria and culture condition

H. pylori strains P1WT and SS1, and *H. felis* have been reported previously [17,18]. *H. pylori* was routinely grown on Campylobacter agar plates or Brucella broth (BD, Sparks, NV, USA) containing 10% of FBS, 10 μg/mL of vancomycin (Sigma-Aldrich, California, USA), 5 μg/mL of trimethoprim (Sigma), and 1 μg/mL of nystatin (Sigma) at 37ºC under microaerobic conditions using GasPak™ EZ Campy Container System (BD). *H. felis* was grown on Brucella broth containing 10% of FBS at 37ºC under microaerobic conditions using GasPak™ EZ Campy Container System (BD).

Growth inhibition and bacterial count

Overnight cultures of the bacteria were diluted with fresh broth media. Crude G-NANA was added at various concentrations (0-150 mg/mL) directly to the culture tubes and the turbidity was monitored at 12 and 24 h later by measuring the optical density at 600 nm. To determine the number of viable *H. pylori* P1WT and SS1 from the 24-h culture incubated with crude G-NANA, each culture was serially diluted and spread on agar plates. After 5-day incubation under microaerobic conditions, the colonies were counted.

Mouse infection

Wild-type (WT) C57BL/6 mice were purchased from Koatech (Pyeongtaek, Gyounggido, Korea). The mice were divided into two groups. Group 1 mice were fasted for 12 h and orally infected with 100 μL (5×10^8 cfu/mL) of *H. felis* for four times at 2-day interval; they were administered crude G-NANA (75 or 300 mg/mL) daily until the last infection. Group 2 mice were fasted for 12 h and orally infected with 100 μL (5×10^8 cfu/mL) of *H. felis* for four times at 2-day interval. One day after the last infection, mice from both group 1 and 2 were daily administered the crude G-NANA (75 or 300 mg/mL) for 10 days and euthanized by cervical dislocation. Their

stomachs were removed, opened longitudinally along the greater curvature, and washed with sterile PBS. The tissues were stored at –20ºC for DNA extraction to determine bacterial colonization. The animal studies were conducted under approved protocols by the Institutional Animal Care and Use Committee of Chonnam National University (Gwangju, Korea). [Approval No. CNU IACUC-YB-R-2015-58].

Quantitative analysis of *H. felis* colonization

An equal size of small sections of the stomachs, including the fundus was prepared for DNA extraction. DNA was extracted using the Tissue DNA kit (Omega Bio-tek, Norcross, GA, USA) according to the manu-facturer's instructions and real-time PCR was performed using the Qiagen SYBR green PCR kit (QIAGEN GmbH, Hilden, Germany). The following primer sequences were used. FlaB forward: 5'-TTCGATTGGTCCTACAG GCTCAGA-3', reverse: 5'-TTCTTGTTGATGACATTG ACCAACGCA-3'; Gapdh forward: 5'-TCAAGAAGGT GGTGAAGCAGG-3', reverse: 5'-TATTATGGGGGTC TGGGATGG-3'. PCR amplification was performed using a two-step cycle of 95ºC for 15 s followed by 58ºC for 1 min for 40 cycles in a Roter-GeenQ Real-time PCR system (QIAGEN, GmbH, Hilden, Germany). Relative gene expression of *H. felis* FlaB was calculated with the $2^{-\Delta\Delta Ct}$ method using Gapdh as the internal control [19]. Standards of relative gene expression were made by sequential 10-fold dilutions of known colony forming units (cfu) of *H. felis* in an uninfected stomach piece and used to estimate the cfu of *H. felis* in infected mice.

Statistical analysis

The differences in mean values among different groups were tested, and the values were expressed as mean ± SD. All of the statistical calculations were performed by one or two-way ANOVA with Bonferroni post-tests using GraphPad Prism version 5.00. Values of *$P<0.05$, **$P<0.01$, and ***$P<0.001$ were considered significant.

Results

Crude G-NANA inhibits the growth of *H. pylori*

We first evaluated the effect of crude G-NANA on the growth of *H. pylori*. 2×10^7 cfu/mL of the *H. pylori* strains P1WT and SS1 were incubated with various concentrations (0, 3, 15, 30, 75 and 150 mg/mL) of crude G-NANA for 12 and 24 h and bacteria growth was
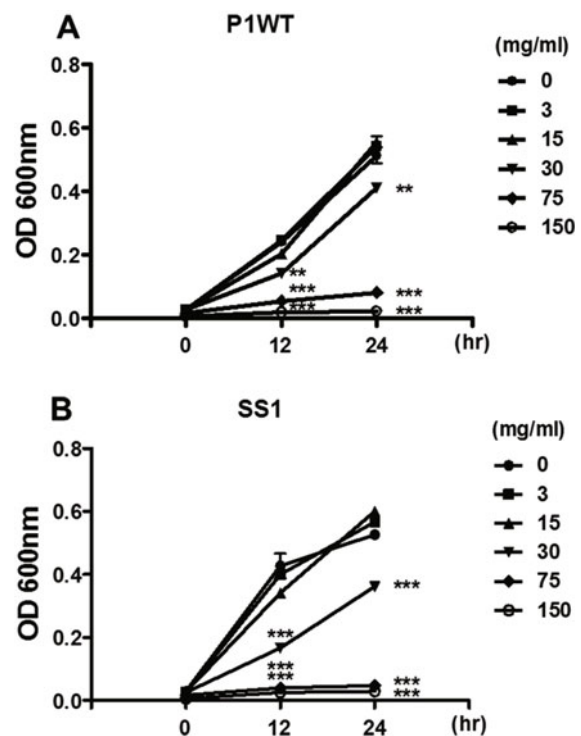

Figure 1. Crude N-acetylneuraminic acid obtained from glycomacropeptide (G-NANA) inhibits the growth curve of *H. pylori*. *H. pylori* P1WT (A) and SS1 (B) were grown overnight in the culture medium. The bacteria culture was diluted with fresh broth media. Crude G-NANA was added at various concentrations (0-150 mg/mL) directly to the culture media and turbidity was monitored 12 and 24 h later by measuring optical density of the medium at 600 nm. Data are shown as mean ± SD from one experiment representative of more than three independent experiments (**$P<0.01$ and ***$P<0.001$).

determined by measuring the optical density of the cultures at a wavelength of 600 nm [20]. The growth of both *H. pylori* P1WT and SS1 was inhibited by crude G-NANA at 30, 75, and 150 mg/mL (Figures 1A and B). This inhibitory effect increased in a crude G-NANA concentration-dependent manner from 30 to 150 mg/mL (Figures 1A and B). Next, we determined the number of viable bacteria from the 24 h culture of *H. pylori* incubated with crude G-NANA by counting colony forming units (CFU)/mL from serial tenfold dilutions of each culture. Crude G-NANA at the concentrations of 75 and 150 mg/mL drastically decreased CFU of both *H. pylori* strains P1WT and SS1 compared to that of the untreated bacterial cultures whereas 30 mg/mL of crude G-NANA slightly decreased the CFU of both *H. pylori* P1WT and SS1 cultures (Figure 2A and B). These results demonstrated that crude G-NANA has growth inhibition activity against the *H. pylori* strains P1WT and SS1.
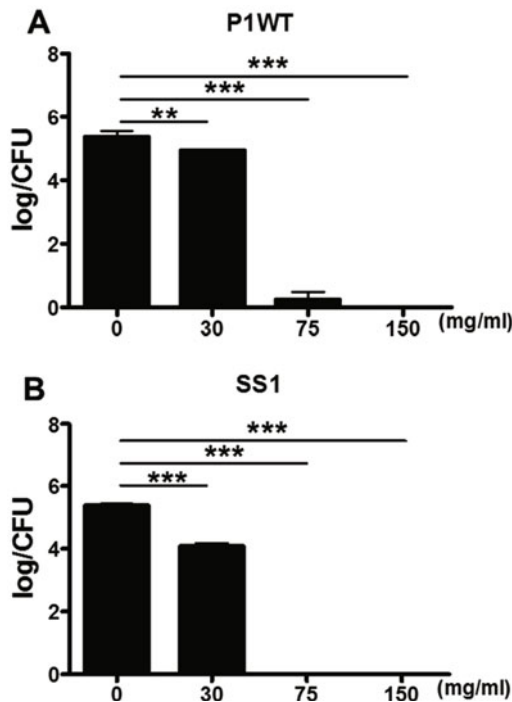
Figure 2. Crude N-acetylneuraminic acid obtained from glycomacropeptide (G-NANA) reduced the number of viable *H. pylori*. *H. pylori* P1WT (A) and SS1 (B) were prepared as described in Fig. 1. Twenty-four hours after bacterial culture with crude G-NANA at various concentrations (0-150 mg/mL), each culture was serially diluted and spread on agar plates. The colony was counted after 5 days incubation under microaerobic conditions. Data are shown as mean ± SD of triplicate samples from one experiment representative of three independent experiments (**$P<0.01$ and ***$P<0.001$).

Crude G-NANA inhibits Helicobacter colonization *in vivo*

To examine the *in vivo* effect of crude G-NANA in Helicobacter infections, we used an *H. felis* murine model [21]. Consistent with results on the growth of *H. pylori*, Crude G-NANA inhibited the *in vitro* growth of *H. felis* at concentrations of 15 mg/mL onwards (Figure 3). For *in vivo* experiment, as described in Materials and Methods, mice were divided into three groups; *H. felis* infected group, G-NANA treated group (from 1st day of infection) with *H. felis* infection (group 1), and G-NANA treated group (from the day after last day of infection) with *H. felis* infection (group 2). In group 1, both 75 and 300 mg/mL crude G-NANA significantly reduced bacterial colonization in the stomach (Figure 4). In group 2, 300 mg/mL of crude G-NANA, but not 75 mg/mL, administration for 10 days after the last bacterial infection significantly reduced bacterial colonization in the stomach (Figure 4). Although 75 mg/mL of crude G-NANA inoculation from a day after the last infection for 10 days
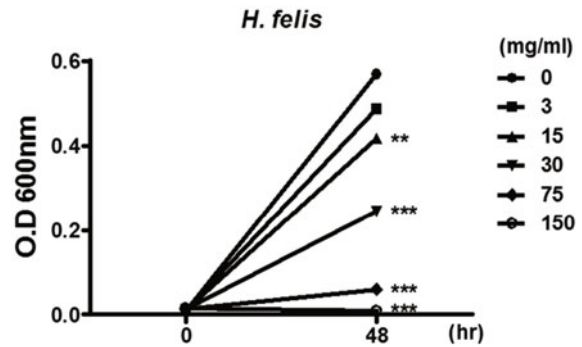

Figure 3. Crude N-acetylneuraminic acid obtained from glycomacropeptide (G-NANA) inhibits the growth curve of *H. felis*. *H. felis* was grown overnight in the culture medium. The bacteria culture was diluted with fresh broth media. Crude G-NANA was added at various concentrations (0-150 mg/mL) directly to the culture media and the turbidity was monitored 48 h later by measuring the optical density of the medium at 600 nm. Data are shown as mean ± SD from one experiment representative of more than three independent experiments (**$P<0.01$ and ***$P<0.001$).
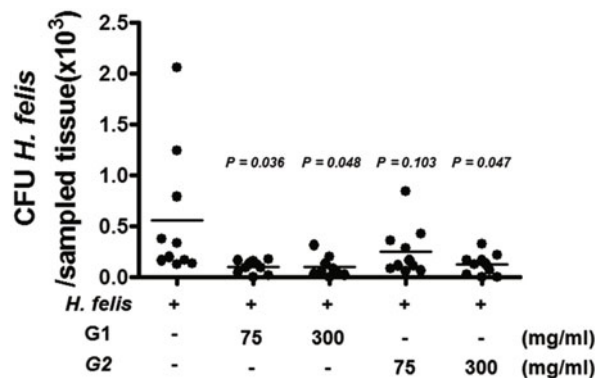

Figure 4. Crude N-acetylneuraminic acid obtained from glycomacropeptide (G-NANA) inhibits gastric colonization of *H. felis*. Mice were divided into two groups: Group 1 (G1); mice were fasted for 12 h and orally infected with 100 μL (5×10⁸ cfu/mL) of *H. felis* four times at 1-day interval with daily administration of crude G-NANA (75 or 300 mg/mL) until last infection, Group 2 (G2); mice were fasted for 12 h and orally infected with 100 μL (5×10⁸ cfu/mL) of *H. felis* four times at 1-day interval. One day after last infection, mice from both group 1 and 2 were daily administered the crude G-NANA (75 or 300 mg/mL) for 10 days and euthanized by CO_2 inhalation. The stomachs were collected and bacterial colonization was determined by quantitative real-time PCR as described in materials and methods. Data are shown as mean ± SD (n =6) from one experiment representative of two independent experiments.

showed little effect on bacterial colonization, the same concentration of crude G-NANA administered from the first infection until the last experiment reduced bacterial colonization (Figure 4). These results indicate that crude G-NANA has an inhibitory effect on the gastric colonization of *Helicobacter*.

Discussion

Although antibiotic-based therapies for *H. pylori* infections are available, the emergence of antibiotic-resistant bacteria coupled with the risk of re-infection and the high cost of antibiotic therapy make the bacterial eradication more difficult. Because of these challenges, many studies have focused on the development of effective vaccines and alternative treatments using non-antibiotic compounds. To develop a successful vaccine for inducing protective immunity against *H. pylori*, determining potent bacterial antigens, effective adjuvants and the best delivery route are critical. Due to its high expression in all *H. pylori* isolates, urease has been considered as a good candidate for vaccine antigens and has provided efficient protection against infections when used with other antigens [22]. Aluminum hydroxide is an approved adjuvant in clinical applications and has efficiently promoted antigen-specific humoral and cellular immune responses against intramuscularly delivered antigens in humans [23]. However, continued efforts to develop a safe and effective vaccination for humans are required.

There have been increasing studies on non-antibiotic-based therapies including plant extracts and probiotics as potential alternatives for the treatment of *H. pylori* infections. Green tea is one of the well-studied therapeutic candidates for *H. pylori* infection [24,25]. Green tea showed antibacterial activity against *H. pylori in vitro* and decreased bacterial colonization and inflammation in animal models [24,25]. In addition, catechins and polyphenols present in green tea appeared to be important for the inhibition of urease and the VacA toxin, respectively [25,26]. Although other plants such as broccoli, garlic and wine have been known to have antibacterial activity against *H. pylori in vitro*, very few of them show *in vivo* antibacterial activity [27-29]. Probiotics, which are live microorganism that provide health benefits when consumed, showed antibacterial effects against *H. pylori in vitro* and *in vivo* [30-32]. *Lactobacillus casei* strain Shirota, *L. acidophilus*, *L. rhamnosus,* and *Bacillus subtilis* inhibited *H. pylori* infections in mouse models [30-32]. The beneficial effects of probiotics on *H. pylori* infection included decreased gastric inflammation and reduced bacterial colonization in mice [33].

To our knowledge, this is the first report on the isolation of NANA from glycomacropeptides through a neuraminidase-mediated reaction. In addition, the discovery of its antibacterial activity against *H. pylori* is novel. Furthermore, crude G-NANA administration inhibited bacterial colonization in the stomach. Since sialic acid was shown to prevent the binding of *H. pylori t*o various human gastric epithelial cells *in vitro* [34] and inhibit bacterial colonization in rhesus monkeys [35], the reduced bacterial colonization could be attributed to the crude G-NANA-induced inhibition of bacterial binding to gastric epithelium. However, the observation that crude G-NANA administration reduced bacterial colonization significantly (Figure 2) while sialic acid effectively controlled *H. pylori* infection in mice when used with antioxidants, but not alone [36], indicates that the reduced bacterial colonization induced by crude G-NANA is most likely a result of its bactericidal activity against *H. pylori* rather than its inhibition of the bacteria's binding.

In this study, we freshly prepared crude G-NANA that showed antibacterial activity against *H. pylori in vitro* and *in vivo*, demonstrating its therapeutic potential in *H. pylori* infection.

Acknowledgment

Conflict of interests The authors declare that there is no financial conflict of interests to publish these results.

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