

Short Research Communication

# Inhibitory Effects of Anthocyanins on Secretion of *Helicobacter pylori* CagA and VacA Toxins

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## Abstract

Anthocyanins have been studied as potential antimicrobial agents against *Helicobacter pylori*. We investigated whether the biosynthesis and secretion of cytotoxin-associated protein A (CagA) and vacuolating cytotoxin A (VacA) could be suppressed by anthocyanin treatment *in vitro*. *H. pylori* reference strain 60190 (CagA<sup>+</sup>/VacA<sup>+</sup>) was used in this study to investigate the inhibitory effects of anthocyanins; cyanidin 3-O-glucoside (C3G), peonidin 3-O-glucoside (Peo3G), pelargonidin 3-O-glucoside (Pel3G), and malvidin 3-O-glucoside (M3G) on expression and secretion of *H. pylori* toxins. Anthocyanins were added to bacterial cultures and Western blotting was used to determine secretion of CagA and VacA. Among them, we found that C3G inhibited secretion of CagA and VacA resulting in intracellular accumulation of CagA and VacA. C3G had no effect on *cagA* and *vacA* expression but suppressed *secA* transcription. As SecA is involved in translocation of bacterial proteins, the down-regulation of *secA* expression by C3G offers a mechanistic explanation for the inhibition of toxin secretion. To our knowledge, this is the first report suggesting that C3G inhibits secretion of the *H. pylori* toxins CagA and VacA via suppression of *secA* transcription.

Key words: *Helicobacter pylori*, CagA, VacA, SecA, anthocyanin, cyanidin 3-O-glucoside.

## Introduction

*Helicobacter pylori* is a Gram-negative helix-shaped bacterium that is found in about half of the world's population<sup>1</sup>. *H. pylori* colonization of the human stomach is consistently associated with gastric mucosal inflammation and is a risk factor for the development of peptic ulcer disease and distal gastric adenocarcinoma<sup>2,3</sup>. The most intensively studied vir-

ulence factors of *H. pylori* are cytotoxin-associated protein A (CagA) and vacuolating cytotoxin A (VacA), which are both involved in the pathogenesis of *H. pylori*<sup>4</sup>. The East Asian CagA strains of *H. pylori* are known to have a significant correlation with gastric ulceration and cancer development<sup>5,6</sup>. VacA causes gastric epithelial cell damage by inducing vacuoles<sup>7</sup>.

Sustained damage caused by CagA and VacA over decades result in chronic superficial gastritis and chronic ulceration.

Numerous reports have demonstrated a correlation between consumption of anthocyanin-containing crops and fruits and disease prevention<sup>8-14</sup>. Data from these studies suggest that anthocyanins perturb peptic ulcer and gastric cancer development and at high doses anthocyanins exert antibacterial effects. Data from these studies suggest that anthocyanins may play a role in preventing ulceration and chronic inflammation due to *H. pylori* infection. In this study, we investigated the inhibitory role of four anthocyanins of cyanidin 3-O-glucoside (C3G), peonidin 3-O-glucoside (Peo3G), pelargonidin 3-O-glucoside (Pel3G), and malvidin 3-O-glucoside (M3G) on *H. pylori* toxin biogenesis and secretion.

## Materials and Methods

### Bacterial strains and culture

*Helicobacter pylori* reference strain 60190 (CagA<sup>+</sup>/VacA<sup>+</sup>) was purchased from ATCC (Manassas, VA, USA). Bacteria were grown under microaerophilic conditions at 37°C on Brucella agar plates (Becton-Dickinson, Brea, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Long Island, NY, USA). To examine the inhibitory effects of anthocyanins, *H. pylori* were cultured in Mueller-Hinton broth (Becton-Dickinson) containing 10% FBS.

### Anthocyanin preparation

C3G, Peo3G, Pel3G, and M3G were obtained from Extrasynthese (Lyon, France). Each compound was dissolved in sterile dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis, MO, USA) in 50 mM. The various anthocyanins (C3G, Peo3G, Pel3G, and M3G) were added to the Mueller-Hinton broth at a final concentration of 100 µM.

### Rabbit anti-*H. pylori* polyclonal antibodies production

Six-week old New Zealand White rabbits were purchased from Central Lab Animal Inc. (Seoul, Korea) and allowed to adapt to their new environment for two weeks before the first antigen inoculation. *H. pylori* 60190 bacteria (1 × 10<sup>8</sup>/ml) were fixed in 0.2% formaldehyde/saline for 24 hr, washed with saline and then injected intravenously every week for a total of six weeks. Blood was allowed to clot at 4°C overnight and the serum isolated after centrifugation. Pre-immune serum was harvested prior to immun-

ization. Collected anti-sera were purified and tested by ELISA

### Western blot analysis

*H. pylori* were lysed in ice-cold RIPA lysis buffer (Millipore, Billerica, MA, USA) for 30 minutes on ice and sonicated for 2 minutes with 10 second intervals (Sonicator XL-2020, Heat Systems Ultrasonics, Pittsburgh, PA, USA). Protein concentration was determined using NanoQuant spectrophotometer (Infinite M200, TECAN, Austria). Protein extracts were resolved on 7.5 or 10% SDS-PAGE and then transferred to a nitrocellulose membrane (Millipore). Membranes were blocked with 5% skim milk for 30 minutes and then incubated with mouse anti-CagA monoclonal antibody (Santa Cruz Biotechnology, CA, USA), rabbit anti-VacA polyclonal antibody (Santa Cruz Biotechnology) or rabbit anti-*Helicobacter pylori* polyclonal antibody (this study). Appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) were used and protein bands were visualized using enhanced chemiluminescence and X-ray film. The bacterial supernatant was concentrated 10-fold using a 3 kDa cut-off Centricon centrifugal filters (Millipore) at 3,000 rpm for 2 hours at 4°C.

### RT-PCR analysis

*H. pylori* 60190 bacteria (1 × 10<sup>8</sup> CFU/ml) were grown in Mueller-Hinton broth at 37°C under microaerophilic conditions with anthocyanins (100 µM) for three days. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and RNA concentration determined by spectrophotometry using Eppendorf BioPhotometer Plus (Eppendorf, Hamburg, Germany). Expression of *cagA*, *vacA*, *secA*, and *galE* (UDP-galactose 4 epimerase as an internal control) was determined by RT-PCR analysis. Total RNA (200 ng) was mixed with 0.25 ng of random hexamers (Invitrogen) and cDNA synthesized using 200 U of MMLV-RT (Invitrogen). PCR reactions were performed in a total volume of 20 µl consisting of 1/10 diluted cDNA, 2 µl of 10X PCR buffer (Tris-HCl [pH 9.0], 20 mM MgCl<sub>2</sub>, [NH<sub>4</sub>]<sub>2</sub>SO<sub>4</sub>), 2.5 mM dNTPs, 20 pmole of each primer, and 0.5 U *G-Taq* DNA polymerase (Cosmo Genetech, Seoul, Korea). PCR was performed using the GeneAmp PCR system 2700 (Perkin-Elmer Cetus, Boston, USA) and PCR products were analyzed by electrophoresis on a 2.0 % agarose gel containing 0.5 µg/ml of ethidium bromide. Gel images were captured and analyzed using the Quantity One System (Bio-Rad, Hercules, USA). Five independent cDNA samples were analyzed. The primer sequences and PCR conditions are listed in Table 1.

**Table 1.** Used primers in this study.

Primer	Relevant sequence (5'-3')	Size of amplicon	Amplification cycles	Annealing temperature	Reference
<i>cagA</i> -F	TGGCAGTGGGTTAGTCATA	278	27	45 °C	25
<i>cagA</i> -R	CCTGTGAGTTGGTCTTCTTTGT				
<i>vacA</i> -F	AAACGACAAGAAAGAGATCAGT	291	20	57 °C	25
<i>vacA</i> -R	CCAGCAAAAAGGCCCATCAA				
<i>secA</i> -F	AAAAATTTGACGCTGTGATCC	274	27	47 °C	this study
<i>secA</i> -R	CCCCAAGCTCCTTAATTC				
<i>galE</i> -F	ATGGCATTATTACACAGG	461	20	57 °C	26
<i>galE</i> -R	GCTCCATAAGGATTAATGGG				

## Results and Discussion

### Cyanidin 3-O-glucoside inhibits secretion of CagA and VacA

To test the inhibitory effects of anthocyanins, *H. pylori* 60190 bacteria were exposed to individual anthocyanins (100 µM) for three days and the levels of secreted CagA and VacA assessed by Western blot analysis. We found no change in the levels of secreted CagA and VacA when bacteria were cultured with peonidin 3-O-glucoside (Peo3G), pelargonidin 3-O-glucoside (Pel3G), and malvidin 3-O-glucoside (M3G). However, the levels of both secreted CagA and VacA decreased when bacteria were cultured with cyanidin 3-O-glucoside (C3G) (Fig. 1A). The bacterial numbers were unaffected by anthocyanins treatment (100 µM) suggesting that the decrease in CagA and VacA proteins was not due to decrease in bacterial numbers (data not shown). Furthermore, the levels of total secreted proteins were equivalent as determined by reactivity to rabbit anti-*H. pylori* polyclonal antibody showing that the decrease in CagA and VacA was specific to C3G treatment (Fig. 1B). The pre-immune serum showed negligible detection to *H. pylori* secreted proteins (data not shown).

### Cyanidin 3-O-glucoside induces intracellular accumulation of CagA and VacA

If the secretion of CagA and VacA was inhibited by C3G then these proteins may accumulate inside the bacteria. Therefore, we assessed intracellular accumulation of CagA and VacA by Western blot analysis. We found an increase in both intracellular CagA and VacA when bacteria were treated with C3G but not

with the other anthocyanins (Fig. 1C). The level of total intracellular proteins was equivalent as determined by reactivity to rabbit anti-*H. pylori* polyclonal antibody showing that the increase in CagA and VacA was specific to C3G treatment (Fig. 1D).

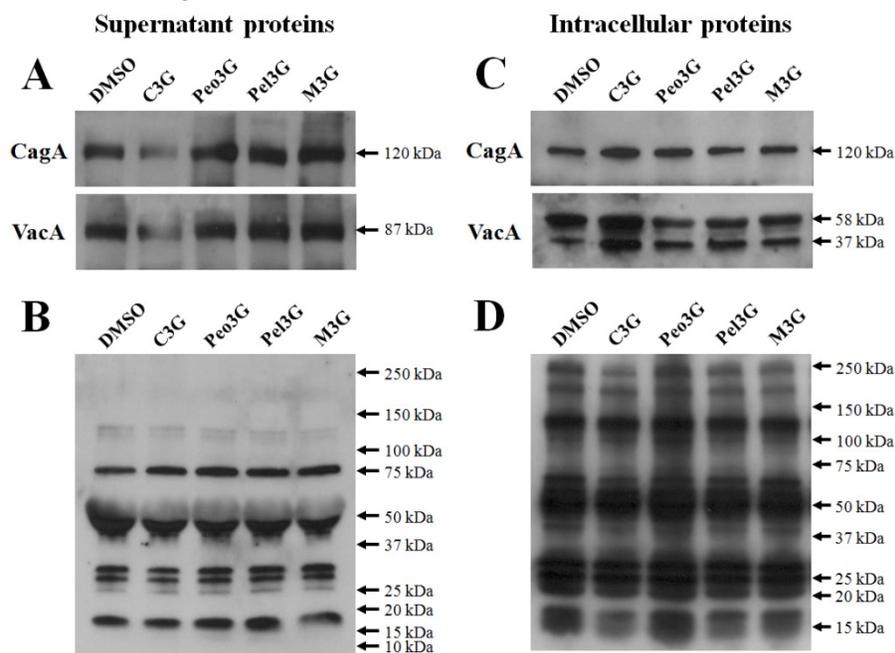
### Cyanidin 3-O-glucoside decreases *secA* expression

In *H. pylori*, VacA is secreted via the type Va secretion system (T5aSS) apparatus. The cytoplasmic protein SecA facilitates the ATP-driven translocation of bacteria proteins out of the bacterial plasma membrane<sup>7,15,16</sup>. Therefore, VacA secretion is considered to be *secA*-dependent. We tested whether the intracellular accumulation of VacA after C3G treatment was due to decrease in *secA* expression. Due to a lack of SecA-specific antibodies, we examined for *secA* expression by RT-PCR analysis. RT-PCR analysis of C3G treated *H. pylori* showed decreased expression of *secA* (Fig. 2) whereas the other three anthocyanins had no effect. In addition, we found no changes in *vacA* expression in C3G treated bacteria supporting the notion that C3G prevented *vacA* secretion by inhibiting the *secA*-dependent pathway. It has been reported that CagA secretion occurs via the type IV secretion system (T4SS) apparatus<sup>17,18,19</sup> and that CagA secretion is in part *secA*-independent<sup>20</sup>. In our studies we found that *cagA* expression was unaffected by C3G treatment (Fig. 2). Consistent with these observations, we found the possibility that C3G inhibits CagA secretion by inhibiting the *sec*-dependent pathway partially<sup>21-24</sup>.

There is a positive correlation between consumption of anthocyanin-containing foods and disease prevention<sup>8-13</sup>. The potential disease-preventive

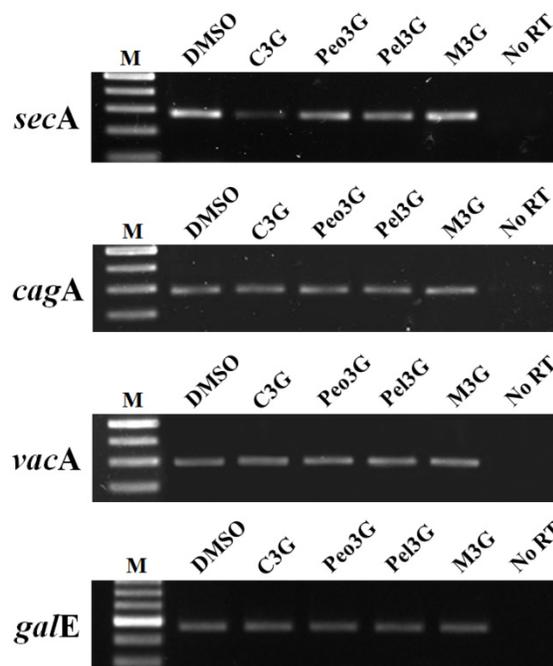
actions of anthocyanins include antibacterial effects<sup>10,11</sup>, inhibitory effects on the development of peptic ulcers and gastric cancer<sup>8,9,12</sup>, or reduction in interleukin-8 secretion<sup>13</sup>. However, these studies used anthocyanins contained in crude extracts and thus the beneficial effects attributed to anthocyanins are unclear<sup>8-13</sup>. In this study, we tested four different pure anthocyanins for their inhibitory effects on the bio-

genesis and secretion of *H. pylori* toxins. In conclusion, our data suggest that C3G inhibits secretion of CagA and VacA in *H. pylori* via down-regulation of *secA* expression. Given that black fruits and crops are an important source of C3G, consumption of these foods may be beneficial in reducing gastric inflammation or stomach cancer due to *H. pylori* infection.



**Figure 1. Effect of anthocyanins on secretion of *H. pylori* CagA and VacA.** *H. pylori* was cultured with 100  $\mu$ M of anthocyanin in Mueller-Hinton broth/10% FBS for 3 days and proteins assessed by Western blot. (A), secreted CagA and VacA in culture media. (B), secreted *H. pylori* proteins reacting with rabbit anti-*H. pylori* polyclonal antibody. (C), intracellular CagA and VacA. (D), intracellular *H. pylori* proteins reacting with rabbit anti-*H. pylori* polyclonal antibody. Representative image from five independent experiments.

**Figure 2. Effect of anthocyanins on *secA* transcription.** *H. pylori* was cultured with 100  $\mu$ M of anthocyanin in Mueller-Hinton broth/10% FBS for 3 days. After extensive washing, total RNA was extracted and cDNA synthesized. RT-PCR analysis was performed to assess expression of *secA*, *cagA*, and *vacA*. The *galE* (UDP-galactose 4-epimerase gene) was used as an internal control. No RT, no reverse transcriptase. A representative image from five independent experiments.



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### Competing Interests

The authors have declared that no competing interest exists.

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