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Characterization of conditions for bacteria-human norovirus capsid P protein complex (BPC) binding to and removal from Romaine lettuce extract

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ABSTRACT

Norovirus is a very contagious virus that causes acute gastroenteritis. Contaminated produce is a main vehicle for dissemination of human noroviruses (HuNoVs). As HuNoVs could bind to bacteria effectively, it is highly possible that produce could be contaminated by bacteria-HuNoVs complex. In this study, we used a bacterial-surface-display system to express genogroup I (GI) or genogroup II (GII) HuNoV capsid protein (P protein) on the surface of bacteria. The bacteria-P protein complex (BPC) was used to characterize the conditions for binding to Romaine lettuce extract and removal of the bound BPCs. We demonstrated both GI and GII BPCs could bind to extract from leaf (LE) and vein (VE) effectively. Carbohydrates in LE and VE were involved in GI BPCs binding, and both carbohydrates and proteins were involved in GII BPCs binding. Saliva from both type A and O secretors could completely block binding of both BPCs to LE and VE. Saliva from type B secretors only partially blocked binding of GII but not GI BPCs to LE and VE. However, LE- and VE-bound BPCs could not be reversely removed by washing solution containing free HBGAs from saliva. The binding of GI BPCs to LE and VE was enhanced when pH was below pI (6.1) of GI and reduced when pH was above pI of GI ($p < 0.05$). The optimal binding for GII BPCs to LE and VE occurred at pI (6.4) of GII. All LE- or VE-bound BPCs could be reversely removed by washing with low (3.0–5.0) or high (9.0–10.0) pH buffer. The effect of ionic strength (NaCl and $MgCl_2$, from zero to 100 g/L) on binding of BPCs to LE and VE was tested. The optimal ionic strength for binding of BPCs to LE and VE was 10.0 g/L (GI) and 5.0 g/L (GII) for NaCl, and 5.0 g/L for $MgCl_2$. LE- and VE-bound BPCs could be reversely removed by washing with high ionic solutions. All LE- or VE- bound BPCs could be released when washed with NaCl concentrations of above 75.0 g/L (GI) and 25.0 g/L (GII), or with $MgCl_2$ concentrations of above 75.0 g/L (GI) and 50.0 g/L (GII). Binding of BPCs to LE and VE was inhibited in the presence of Tween-80 (nonionic surfactant) as low as 0.05% (*v*/v). All LE- and VE-bound BPCs could be reversed by Tween-80 concentrations over 0.1% (v/v). The study provided important parameters for BPCs binding to and removal from lettuce extract.

1. Introduction

Human noroviruses (HuNoVs) are the major cause of outbreaks of acute gastroenteritis. Histo-blood group antigens (HBGAs) have been recognized as viral receptors. The fecal-oral route is recognized as the main mode of transmission. The outbreaks of HuNoVs are often associated consumption of food contaminated during production, harvesting or processing. An US CDC survey estimated that 58.3% of all food-borne disease outbreaks associated with leafy greens between 1973 and 2006 were associated with by HuNoVs (Herman et al., 2008). It has been demonstrated that GI.1 HuNoV viral like particles (VLPs) could bind to surface of Romaine lettuce by forming clusters along the veins (Gandhi et al., 2010). Esseili et al. (2012) reported that minor veins inside the Romaine lettuce leaf and leaf cut edges had the highest levels of GII.4 VLPs binding. The mechanism for the attachment of the HuNoVs or VLPs to Romaine lettuce was not well defined. Gao et al. (2016) demonstrated that GII.4 HuNoVs recognized HBGA-like carbohydrate in Romaine lettuce. They were able to detect type H-like

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molecules in Romaine lettuce by using corresponding monoclonal antibody (MAb). Esseili et al. (2012) reported that binding of HuNoV VLPs to cell wall carbohydrate of Romaine lettuce. They further demonstrated that protein and carbohydrate were primarily associated attachment of VLPs in young and old leaves, respectively (Esseili et al., 2012). However, none of MAbs against HBGAs inhibited binding of VLPs to Romaine lettuce. In this manuscript, we applied different approaches by using a competitive assay with saliva that contained free HBGAs to test if attachment of a bacteria-P protein complex (BPC) could be blocked. Although some knowledge on candidate receptors on Romaine lettuce have been obtained from these studies, the kinetic of viral associate (binding) and disassociate (binding reverse) in Romaine lettuce has not been studied. In addition, it remains unclear if the LE- or VE-bound virus or VLPs could be removed by washing in the presence of competitive binding ligands for the virus or VLPs.

Human stools from sick and recovered patients contain high titers of infectious viruses. Recent studies demonstrated that HuNoVs could bind to bacteria isolated from human gut (Almand et al., 2017). The naturally occurring strains of bacteria isolated from human stools could rapidly bind to both genogroup GI (GI) and genogroup GII (GII) HuNoVs. Currently, HuNoV-bacteria interaction is an emerging topic. Early studies demonstrated that direct interaction between poliovirus and bacteria could enhance viral infectivity and attachment to viral receptors (Kuss et al., 2011). Enteric bacteria also could promote HuNoVs and murine norovirus (MNV) infection of B cells (Jones et al., 2014). Binding of poliovirus to bacteria increased the stability of the viral capsid when exposed to heat (Robinson and Pfeiffer, 2014). Similar phenomenon was observed for HuNoVs (Li et al., 2015). HBGAs have been recognized as receptors/co-receptor for HuNoVs. Although certain bacteria could express HBGAs on their surface and bind to HuNoVs, non-HBGA expressing bacteria also bind to HuNoVs and surrogates such as Tulane virus (TV) and MNV (Jones et al., 2014; Li et al., 2017). It remains unclear if the virus alone or bacteria-virus complex play a major role in viral transmission and limited knowledge on effect of bacteria on viral attachment to receptors.

Noroviruses have a single-stranded, positive-sense RNA genome. The open reading frame 1, 2, and 3 in viral genome encodes nonstructural proteins, a capsid protein VP1, and a minor capsid protein VP2, respectively (Hardy, 2005; Jiang et al., 1993; Xi et al., 1990). The capsid protein (VP1) has two major domains, the shell (S) domain forming the interior shell and the protrusion (P) domain constituting the arch-like protruding structures of the virus. It has been demonstrated that P domain is responsible for major biological functions (Prasad et al., 1999). Expression of the S domain alone forms a smaller, thin-layer particle without binding function to viral receptors (Bertolotti-Ciarlet et al., 2002). Expression of P domain forms the P particle, which binds to viral receptors (Tan and Jiang, 2005). Previously, we demonstrated that expression of P domains (P proteins) from GII.4 HuNoVs on the surface of bacteria could form a bacteria-P protein complex (BPC). BPCs could bind to candidate viral receptors in Romaine lettuce extract (Wang et al., 2017) and human saliva (Xu et al., 2017). In this study, we used this system to determine conditions required for GI and GII BPCs binding to and removal from Romaine lettuce.

2. Materials and methods

2.1. Preparation of bacteria-P protein complex (BPC)

A novel system to present HuNoV P proteins on the surface of transformed bacteria was used (Niu et al., 2015). The new system uses N-terminal domain of ice nucleation protein (InaQN), which is a bacterial trans-membrane protein, to fuse with P domain of VP1 of HuNoV (P protein) to display the P proteins on the surface of transformed bacterial cells. *Escherichia coli* BL21 (Thermo Fisher, Shanghai, China) was used as competent cell for recombinant plasmid transformation and expression of P proteins as described previously (Niu et al., 2015, Wang et al., 2017). Briefly, pET28a-inaQn-P (GI.1) and pET28a-inaQn-P (GII.4) were constructed to display P proteins of GI.1 and GII.4 of HuNoVs on the surface of transformed bacteria, respectively (Niu et al., 2015; Wang et al., 2017). The localization of surface-displayed P proteins was determined by using immunofluorescence microscopy (Olympus, Japan) with genotype specific polyclonal antibodies as previously described (Li et al., 2009). Cells were cultured in 5.0 mL of LB medium containing 60 μg/mL kanamycin with shaking (220 rpm) at 37 °C, overnight to select transformed bacteria. The cells (50.0 μL) were subcultured in 5.0 mL fresh LB medium with 60 μg/mL kanamycin. When the OD_{600} reached 0.5, isopropyl-β-D-thiogalactopyrano-side (IPTG) was added to reach a final concentration of 0.4 mmol/L to allow P proteins expressing to form BPC cells. The BPC cells were incubated at 25 °C for 12 h, then washed and diluted to OD_{600} = 1.0 with sterile PBS (pH = 7.4), and then kept at 4 °C for further use up to 1 day.

2.2. Preparation of Romaine lettuce

Fresh Romaine lettuce was collected randomly from a local grocery store and stored at 4 °C. The leaves were washed three times with sterile distilled water. The Romaine lettuce was made into leaf extract (LE) and vein extract (VE), collectively referred to as Romaine extract (RE). LE was prepared from the top half of a Romaine leaf (15.0 g), while VE was prepared from the excised main veins. The same volume of PBS (wt/vol) was added to each vegetable matter sample, and blended for 5 min at 4 °C. The liquid phase was transferred to a sterile microcentrifuge tube and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was transferred to a fresh sterile tube and kept at 4 °C for further use up to 1 day.

2.3. Preparation of boiled saliva

Human saliva was collected from A, B, O blood type volunteers and treated according to the previous report, with minor modification (Wang et al., 2014). The secretor-status of individual saliva was indirectly determined by an ELISA assay for its ability to bind purified bacteria-expressed GI and GII VP1 and P domains (Niu et al., 2015) and directly determined with corresponding monoclonal antibodies. The study was approved by the Institutional Bio-safety Committees (IBC) of College of Agriculture and Biology, Shanghai Jiao Tong University, and written informed consent was obtained from the volunteers. Briefly, each type of saliva was collected from at least three volunteers and mixed. Then, each saliva sample was boiled for 5 min, and then followed with centrifugation at 10,000 × *g* for 5 min at 4 °C. The individual supernatant of saliva from each blood group was mixed, aliquoted and stored at −20 °C for further use.

2.4. Standard Romaine lettuce extract-binding based ELISA

The RE-binding enzyme-linked immunosorbent assay (ELISA) was performed as previously reported (Wang et al., 2017). Briefly, 1:5 PBS (pH 7.4)-diluted LE or VE was added into 96-well microtiter plates (100.0 μL per well) and incubated at 4 °C overnight. Plates were washed three times with PBS and blocked with 1% BSA at 37 °C for 2 h. Plates were washed three times with PBS. One hundred μL of BPCs (GI) or BPCs (GII) were added to each well, respectively, and incubated at 37 °C for 1 h. After washing three times with PBS, 100.0 μL of primary antibodies (1: 10,000 in PBS) for GI.1 (Niu et al., 2015) or GII.4 (Xu et al., 2017) HuNoV was added to each well for an incubation at 37 °C for 1 h. After washing three times with PBS, 100.0 μL of peroxidase-conjugated goat anti-mouse IgG (H + L) (Yeasen, Shanghai, China) at a dilution of 1: 5000 in PBS was added to each well for an incubation at 37 °C for 1 h. After washing five times with PBS, 100.0 μL 3,3′,5,5′-tetramethylbenzidine (Fedbio, Wuhan, China) was added to each well. Plates were kept in the dark for 5 min, and then 50.0 μL of 2.0 mol/L

H_2SO_4 was added to stop the reaction. OD_{450} value was measured using a Microplate Reader (TECAN, Switzerland). RE-coated wells with PBS were used as the blank control. The non-transformed *E.coli* BL21 which do not express P proteins was used as a negative control (N). Samples were considered as positive when the positive (P) to negative (N) ratio (P/N) was > 2.0.

2.5. Heat-denaturation or carbohydrate oxidation of LE and VE

LE or VE was boiled for 5 min, and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was diluted 5 times with PBS (pH = 7.4) and was used to coat the wells as described in Section 2.4. To determine whether carbohydrates were involved in BPC binding, 100.0 μL 4.0 mg/mL sodium periodate (Tansoole Co., Ltd., Shanghai, China) was added to wells coated with LE or VE (Esseili et al., 2012). The plates were incubated at 37 °C for 30 min prior to the addition of BPCs. LE or VE without heat-denaturation or oxidation was used as untreated controls. The percentage inhibition of binding was calculated based on difference between OD_{450} readings of untreated samples (0%) and OD_{450} at P/N ratio of 2.0 (100%). The percentage of binding was calculated as: Binding% = (P/N ratio of treated sample − 2.0) / (P/N ratio of untreated sample − 2.0). Inhibition% = 1 − Binding%.

2.6. Competitive inhibition and competitive binding removal ELISA assays

For binding inhibition assay, boiled mixed saliva was diluted five times with PBS. GII BPC or GI BPC cells (100.0 μL) were pre-incubated in the presence of 1:5 PBS-diluted boiled saliva (100.0 μL) for 30 min, and the ELISA was performed as described in Section 2.4. BPCs incubated with PBS were used as untreated control. To calculate percentage inhibition, the P/N ratio of untreated samples was equal to 0% inhibition and P/N ratio of 2.0 was equal to 100% inhibition. For competitive binding removal assay, after BPCs bound to LE or VE coated plate, the plate was washed three times with 1:5 PBS-diluted saliva (1×), 1:2.5 (2×) or undiluted (5×) followed by detection with genotype specific antibodies as described in Section 2.4. BPCs washed with PBS were used as an untreated control. Binding percentage was calculated as described in Section 2.5.

2.7. The pH effects on association and disassociation of BPCs to LE and VE

The isoelectric point (pI) of GI and GII VLP was 6.1 (Goodridge et al., 2004) and 6.4 (Wang et al., 2017), respectively. Buffers around the pIs were selected to test binding of BPCs to LE and VE. For binding removal assays, the BPCs were firstly bound to LE or VE coated plate as described in Section 2.4. Then, the bound BPCs were washed with solutions with pH ranging from 3.0 to 10.0 followed by detection with genotype specific antibodies as described in Section 2.4. For pH 3.0, 4.0, 5.0 and 6.0, citric acid/ sodium citrate buffer (0.1 mol/L) was used. For pH 7.0, 8.0 and 9.0, Tris-HCl buffer (0.05 mol/L) was used. For pH 10.0, glycine-sodium hydroxide buffer (0.05 mol/L) was used.

2.8. The effect of ionic strength on association and disassociation of BPCs to LE and VE

For binding assays, ELISA was performed as described in Section 2.4 except BPCs were resuspended in ddH_2O with NaCl or $MgCl_2$ at a concentration ranging from 0.0 to 100.0 g/L. For binding removal assays, BPCs were bound to LE or VE coated plates at the optimal salt concentrations, e.g. 10.0 g/L NaCl for GI BPCs, 5 .0g/L NaCl for GII BPCs; and 5.0 g/L $MgCl_2$ for both GI and GII BPCs. The bound BPCs were then washed with ddH_2O with NaCl or $MgCl_2$ at a concentration ranging from 0.0 g/L to 100.0 g/L followed by detection with genotype specific antibodies (mouse anti GI.1 and mouse anti GII.4) as described in Section 2.4. For removal experiments, the OD_{450} of GI BPCs washed at 10.0 g/L NaCl and OD_{450} of GII BPC washed at 5.0 g/L were used as 100% to calculate the binding/removal of the bound BPCs at the other conditions. The ODs of both GI and GII BPCs washed at 5.0 g/L $MgCl_2$ were used as 100% to calculate the binding/removal of the bound BPCs at the other conditions.

2.9. The effect of nonionic surfactant on association and disassociation of BPCs to LE and VE

For binding assays, Tween-80 concentrations ranging from 0.00% to 2.00% (*v*/v) in PBS (pH 7.4) were selected. For binding removal assays, the BPCs were firstly bound to LE or VE at described in Section 2.4. The bound BPCs were washed with PBS containing Tween-80 ranging from 0.05% to 2.00% (v/v) followed by detection with genotype specific antibodies as described in Section 2.4. BPCs washed without Tween-80 were used as untreated controls.

2.10. Statistics

Each experiment (N) was performed in triplicate ($n = 3$) and repeated at least three times ($N > 3$). The means and standard deviations from independent experiments were presented in all figures. One-way analysis of variance (ANOVA) was utilized for data comparison. Differences in means were considered significant when the $p < 0.05$.

3. Results

3.1. Effect of protein denaturation or carbohydrate oxidation of LE and VE on binding of BPCs

The OD_{450} for GI BPCs binding to LE and VE were 1.08 and 1.03. The OD_{450} for GII BPCs binding to LE and VE were 0.97 and 0.91. The background OD_{450} of bacteria BL21 without expression of P proteins was 0.31 and used as negative control. The corresponding P/N ratios for GI and GII BPCs binding to LE and VE were 3.49, 3.33 and 3.15, 2.94, respectively. After LE and VE were heat-denatured, the binding of BPCs to both LE and VE were unaffected ($p > 0.05$) for GI (Fig. 1A) but significantly reduced for GII ($p = 4.89 \times 10^{-6}$, $p = 9.92 \times 10^{-6}$) (Fig. 1B). The OD_{450} readings for binding of GI BPCs to heat-denatured LE (1.07) and VE (1.02) were not significantly different from the untreated LE (1.08) and VE (1.03). The heat-denaturation of LE and VE only resulted in 1.65% and 1.28% binding inhibition for GI BPCs, respectively (Fig. 1A). In contrast, the OD_{450} dropped from 0.97 to 0.85 and from 0.90 to 0.77 for GII BPCs binding to heat-denatured LE and VE, respectively. The binding of GII BPCs to heat-denatured-LE and -VE were significantly reduced 35.37 ± 1.27% and 46.81 ± 2.34% ($p < 0.01$) (Fig. 1B). Unlike the effect of heat-denaturation of Romaine lettuce extract, oxidation resulted in a complete inhibition of binding of GII BPCs to LE (97.37 ± 4.56%) and VE (100%) (Fig. 1B). Oxidation also caused a substantial inhibition of GI BPCs binding to LE (77.43 ± 2.39%) and VE (79.43 ± 1.58%) (Fig. 1A).

3.2. Competitive inhibition binding of BPCs to LE and VE and competitive binding removal of bound-BPC by free HBGAs

When pre-incubation BPCs with human saliva containing various HBGAs, type A and type O eliminated binding of GI BPCs (Fig. 2A) to LE and VE, and almost eliminated (> 90%) binding of GII BPCs (Fig. 2B) to LE and VE. In contrast, pre-incubation BPCs with type B human saliva had no significant effect on GI BPCs binding to LE (8.70% inhibition) and VE (6.13% inhibition). However, GII PBCs binding to LE (65.32% inhibition) and VE (70.96% inhibition) could be substantially inhibited by type B HBGA. Although, free HBGAs had significant effect on preventing BPCs binding to LE and VE, the effect of free HBGAs on removal of bound BPCs was very limited. The remained LE-bound GI BPCs was 96.94%, 97.20%, and 96.63% after washed with 1:5 PBS-diluted type A, B, and O saliva, respectively (Fig. 2C). Similarly, the remained LE-

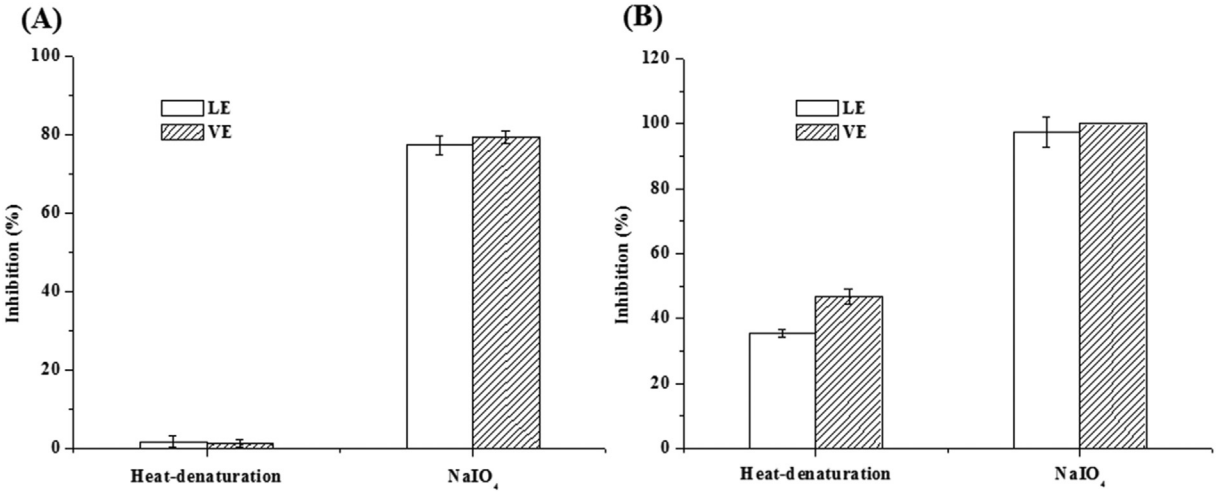

Fig. 1. Heat-denaturation and carbohydrate oxidation of LE or VE on the binding of GI BPC (A) or GII BPC (B). LE, leaf extract; VE, vein extract. Inhibition in % was shown in X-axis. Each experiment (N) was performed in triplicate ($n = 3$) and repeated at least three times ($N > 3$). Error bars represent standard deviation.

bound GII BPCs were 95.58%, 95.34%, and 96.07% after washed with 1:5 PBS-diluted type A, B, and O saliva, respectively (Fig. 2D). There was no significant difference between either PBS and PBS washing in the presence of HBGAs from human saliva or between different HBGAs from various human saliva. Similar phenomena for effect of saliva washed on VE-bound GI and GII BPCs was observed. Increasing saliva concentration in washing solution had no effect on removal of LE- or VE-bound GI and GII BPCs.

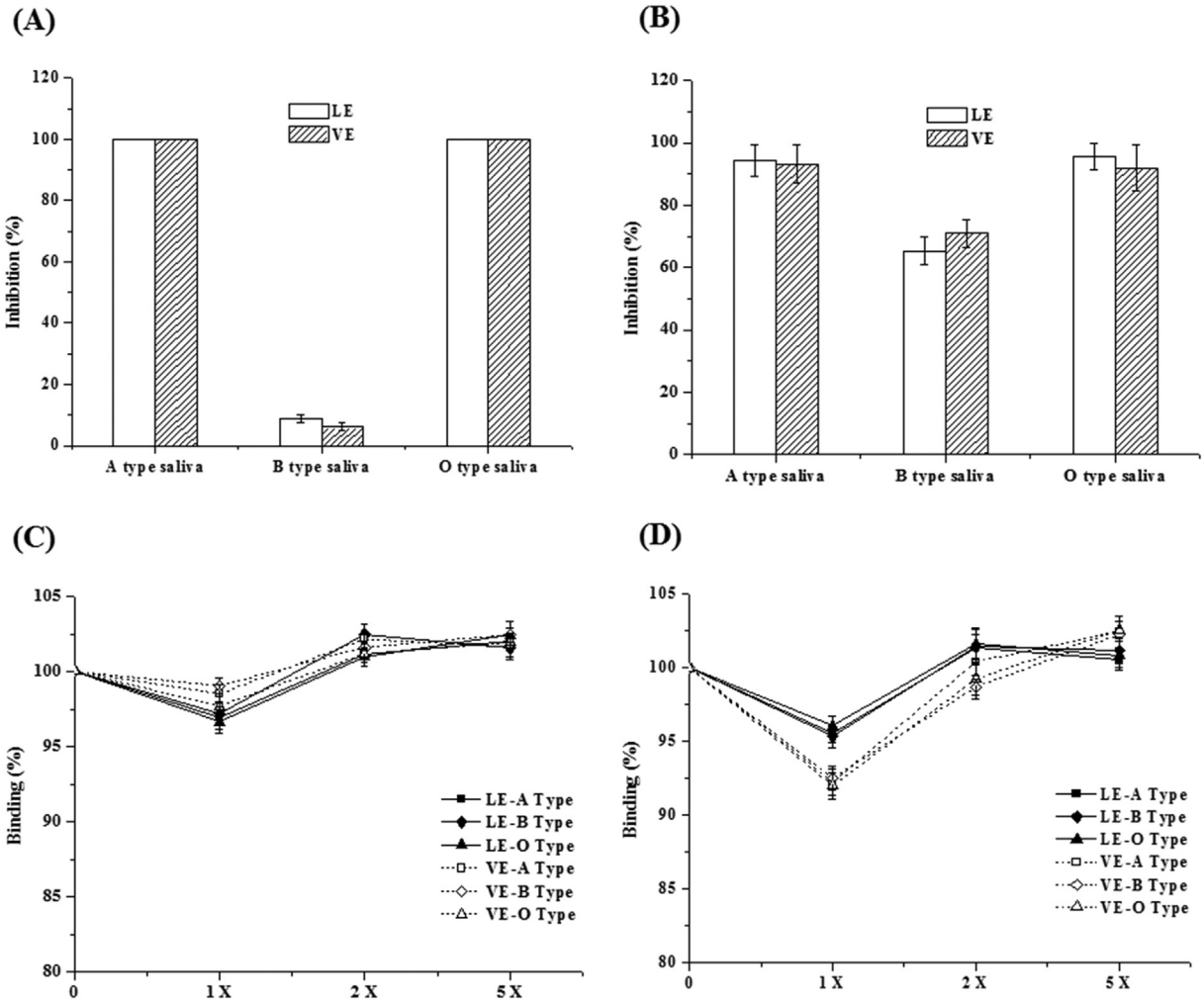

Fig. 2. Competitive inhibition binding of GI BPC (A) or GII BPC (B) to LE or VE and competitive binding removal of bound-GI BPC (C) or bound-GII BPC (D) by A, B, O-type saliva. LE: leaf extract; VE: vein extract; RE: romaine lettuce extract. Each experiment (N) was performed in triplicate (n = 3) and repeated at least three times (N > 3). Error bars represent standard deviation.

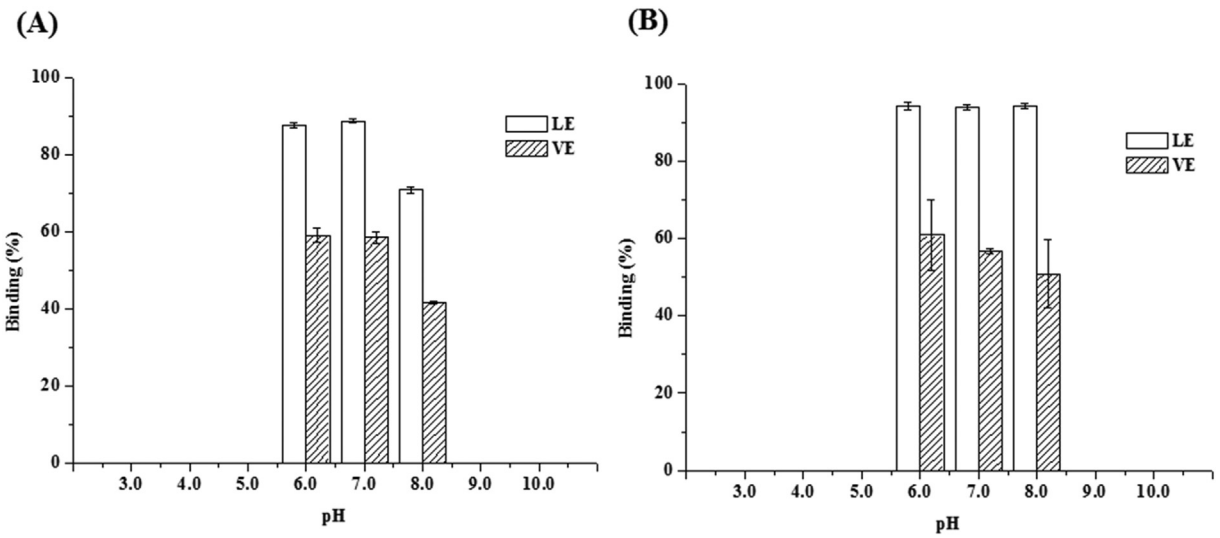

Fig. 3. The pH effects on the removal of GI BPC (A) GII BPC (B) from romaine lettuce. LE: leaf extract; VE: vein extract. Each experiment (N) was performed in triplicate (n = 3) and repeated at least three times (N > 3). Error bars represent standard deviation.

3.3. The pH effects on binding BPCs to LE and VE and removal of bound BPCs

As low or high pH resulted in a lysis of bacteria in BPCs, only pHs around pI were tested. Binding of GI and GII BPCs to LE or VE were affected slightly by variation of pH tested ranging from pH 5.1 to 7.6 and 5.4 to 7.9, respectively (Supplementary Table S1). The binding of GI BPCs to LE and VE was enhanced when pH was below pI of GI (pH 6.1) and reduced when pH was above pI of GI ($p < 0.05$). In contrast to GI BPCs, the best binding for GII BPCs to LE and VE occurred at its pI (pH 6.4). The binding of BPCs to LE and VE was reduced when pH was above or below its pI ($p < 0.05$). Washing the bound BPCs with buffer pH range of 6.0 to 8.0 could not effectively remove the bound BPCs (Fig. 3). The OD_{450} for LE-bound GI BPCs was 1.21, 1.22, and 1.10 when washed with buffer at pH 6.0, 7.0, and 8.0, respectively. The correspond P/N ratio was 3.96, 3.99, and 3.59. The OD_{450} for VE-bound GI BPCs was 1.00, 1.00, and 0.88 when washed with buffer at pH 6.0, 7.0, and 8.0, respectively. The corresponding P/N ratio was 3.36, 3.35, and 2.96. The OD_{450} for LE-bound GII BPCs was 1.38, 1.38, and 1.38, when washed with buffer at pH 6.0, 7.0, and 8.0, respectively. The correspond P/N ratio was 4.36, 4.35, and 4.36. The OD_{450} for VE-bound GI BPCs was 1.03, 1.00, and 0.96 when washed with buffer at pH 6.0, 7.0, and 8.0, respectively. The correspond P/N ratio was 3.39, 3.30 and 3.16. However, LE- and VE-bound GI and GII BPCs could be reversely removed (100%) by washing with low pH buffer (3.0–5.0) or high pH buffer (9.0–10.0).

3.4. The effect of ionic strength on binding BPCs to LE and VE and removal of bound BPCs

Binding of GI and GII BPCs to LE or VE were significantly affected by variation of NaCl concentrations ($p < 0.01$) (Supplementary Table S2). Proper concentration of ionic strength was required for optimal binding of BPCs to LE and VE (Fig. 4A). In the absence of NaCl, binding of GI BPCs to LE and VE was only 12.12% and 27.81% compared to their optimal binding abilities. The dose-dependent enhancement of BPCs binding was observed at NaCl concentration from 0.0 to 10.0 g/L for GI; and from 0.0 to 5.0 g/L for GII. The binding ability was 12.12%, 22.02%, 87.37%, 100%, and 27.81%, 42.38%, 84.34%, 100% for GI BPCs binding to LE and VE at NaCl concentrations of 0.0, 1.0, 5.0, and 10.0 g/L, respectively. For GII BPCs, the binding ability to LE and VE was 7.63%, 46.49%, 100%, and 5.30%, 20.38%, 100% at NaCl concentrations of 0.0, 1.0, 5.0, and 10.0 g/L, respectively. Binding of BPCs to LE and VE was dose-dependently reduced by increasing concentration of NaCl after their binding reached to the plateau. Washing the bound BPCs in the absence of NaCl resulted in a partial removal of the bound GI BPCs from LE (31.14%) and VE (25.46%), respectively (Fig. 4B). Washing the bound GII BPCs in the absence of NaCl resulted in a limited removal of the bound BPCs from LE (11.74%) and VE (2.23%), respectively. LE- and VE-bound BPCs could be effectively removed by washing with high NaCl concentrations. For LE-bound GI BPCs, 58.15%, 100% and 100% could be released when washed at NaCl concentrations of 50.0, 75.0 and 100.0 g/L, respectively. For VE-bound GI BPCs, 55.97%, 97.64%, 100% and 100% bound GI BPCs could be released when washed at NaCl concentrations of 25.0, 50.0, 75.0 and 100.0 g/L, respectively. Similarly, 100% bound GII BPCs in LE or VE could be reversely removed by washing with NaCl concentrations of 25.0, 50.0, 75.0 and 100.0 g/L, respectively.

The effects of $MgCl_2$ on binding of BPCs to LE and VE were like that of NaCl except the optimal concentration for binding was 5.0 g/L for both GI and GII BPCs (Fig. 5A). Binding of GI and GII BPCs to LE or VE were also significantly affected by the presence of $MgCl_2$ ($p < 0.01$) (Supplementary Table S3). There was a dose-dependent enhancement of BPCs binding from 0.0 to 5.0 g/L $MgCl_2$ for both GI and GII BPCs. The binding of BPCs to LE and VE was dose-dependently reduced by increasing concentration of $MgCl_2$ after their binding reached to the plateau at a concentration of 5.0 g/L (Fig. 5A). Washing with low concentration of $MgCl_2$ had limited effect on removal of bound BPCs except LE-bound GI BPCs (Fig. 5B). LE-bound GI BPCs could be partially removed by washing in the absence of $MgCl_2$ (22.48%) and 1.0 g/L $MgCl_2$ (23.85%). However, LE- and VE-bound BPCs could be effectively removed by washing with high $MgCl_2$ concentrations (over 75.0 g/L $MgCl_2$ for LE-bound GI BPCs and over 50.0 g/L $MgCl_2$ for VE-bound GI BPCs, LE- and VE-bound GII BPCs).

3.5. The nonionic surfactant effect on binding BPCs to LE and VE and removal of bound BPCs

Presence of Tween-80 had great impact on binding of BPCs to LE and VE (Fig. 6A and Supplementary Table S4) as well as removal of the bound BPCs (Fig. 6B). The binding of GII BPCs to LE and VE were eliminated in the presence of Tween-80 as low as 0.05% (*v*/v). For GI BPCs, the ability to bind to LE and VE was significantly reduced in the presence of 0.05% Tween-80 and was eliminated at concentrations of Tween-80 beyond 0.1%. GI LE- and VE-bound BPCs could be reversely removed by washing steps in the presence of Tween-80 as low as

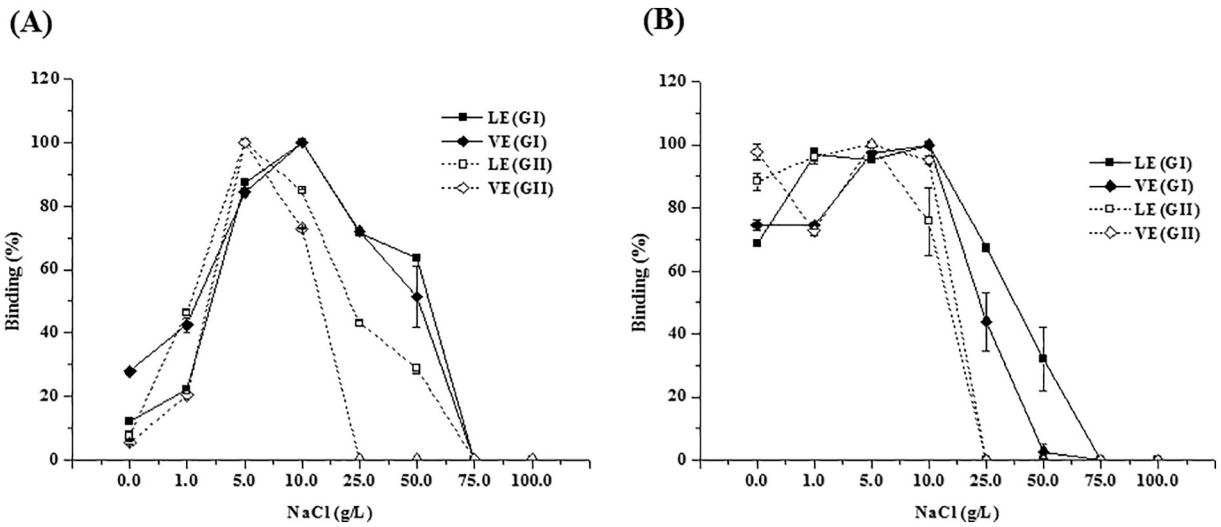

Fig. 4. The effects of NaCl on binding between GI BPC or GII BPC (A) to LE and VE and removal of bound-GI BPC or bound-GII BPC (B). LE: leaf extract; VE: vein extract. Each experiment (N) was performed in triplicate (n = 3) and repeated at least three times (N > 3). Error bars represent standard deviation.

0.05%, 100% of LE-bound GI BPCs and 90.91 ± 0.43% VE-bound G1.1 BPCs could be removed when washed in the presence of 0.05% (v/v) Tween-80, respectively. One hundred percent of LE- and VE-bound BPCs could be removed through wash steps in the presence of > 0.10% (v/v) Tween-80. Only bound GII BPCs could be completely removed through washes with 0.05% Tween-80.

4. Discussion

Over 58% of outbreaks of foodborne gastroenteritis are associated with HuNoVs (Scallan et al., 2011). Fresh fruits and leafy greens are increasingly being recognized as the source of many foodborne disease outbreaks (Berger et al., 2010). Due to lacking effective tissue culture system for HuNoVs, recombinant norovirus-like particles (rNVLPs), P particles and surrogate viruses such as feline calicivirus (FCV), MNV, and TV are often used as surrogates to study attachment of the virus to produce.

Attachment of surrogate viruses and rNVLPs to surfaces of various vegetables including Romaine lettuce, Iceberg lettuce, spinach, celery, and green onion have been reported. Vega et al. (2005) reported that bacteriophage, FCV, and echovirus could bind to lettuce through electrostatic force. Wei et al. (2010) reported that MNV could attach to Romaine lettuce surface and biosolids could enhance the binding. Gandhi et al. (2010) reported that rNVLPs could bind to Romaine lettuce through carbohydrate related molecules. Wang et al. (2012) reported that porcine saporovirus (SAV) attached to lettuce leaves significantly at its capsid pI and remained infectious for weeks. Esseilli et al. (2012) demonstrated that cell wall proteins and carbohydrates of Romaine lettuce leaves are primary source for young and old leaves binding to GII rNVLPs. Gao et al. (2016) reported that presence of HBGA-like carbohydrates in the cell wall of lettuce and the isolated cellular wall components could bind to GII rNVLPs. Almost all studies on interaction between lettuce and surrogate viruses and rNVLPs did not consider the impact of the presence of bacteria. Recent studies demonstrated that HuNoVs could bind different strains of bacteria effectively. Almand et al. (2017) recently reported that HuNoV and TV were able to bind to both Gram-positive and -negative bacteria selected from human gut microbiota, with transmission electron microscopy showing that the viruses could be found bound to the bacterial outer cell membrane, intestinal pili, or both. Previously, we demonstrated that TV could bind to bacteria regardless of HBGAs expression (Li et al., 2017). It remains unclear if binding of HuNoVs or VLPs to a bacteria

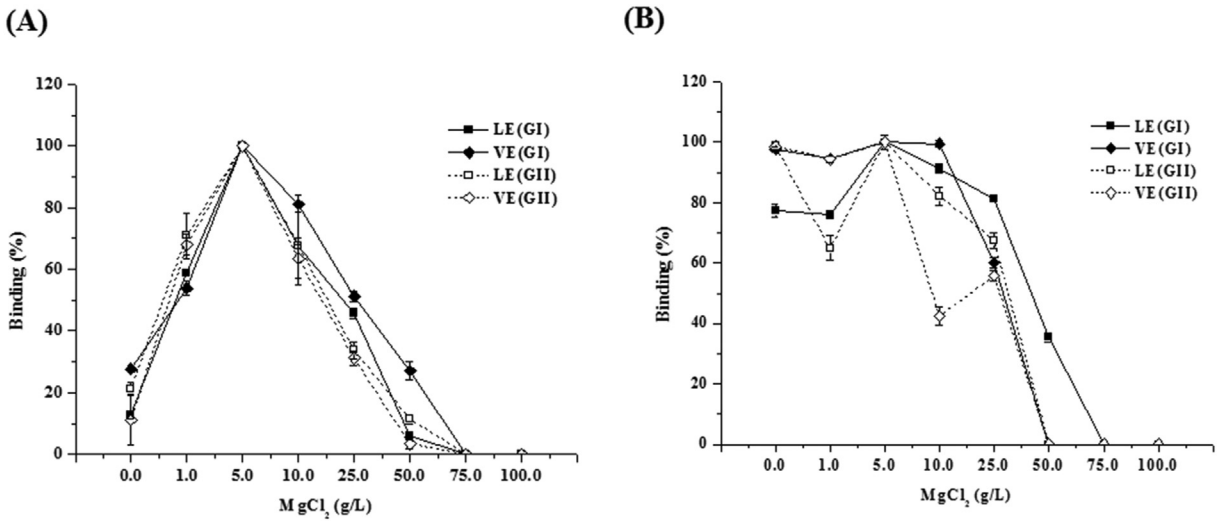

Fig. 5. The effects of $MgCl_2$ on binding between GI BPC or GII BPC (A) to LE and VE and removal of bound-GI BPC or bound-GII BPC (B). LE: leaf extract; VE: vein extract. Each experiment (N) was performed in triplicate (n = 3) and repeated at least three times (N > 3). Error bars represent standard deviation.

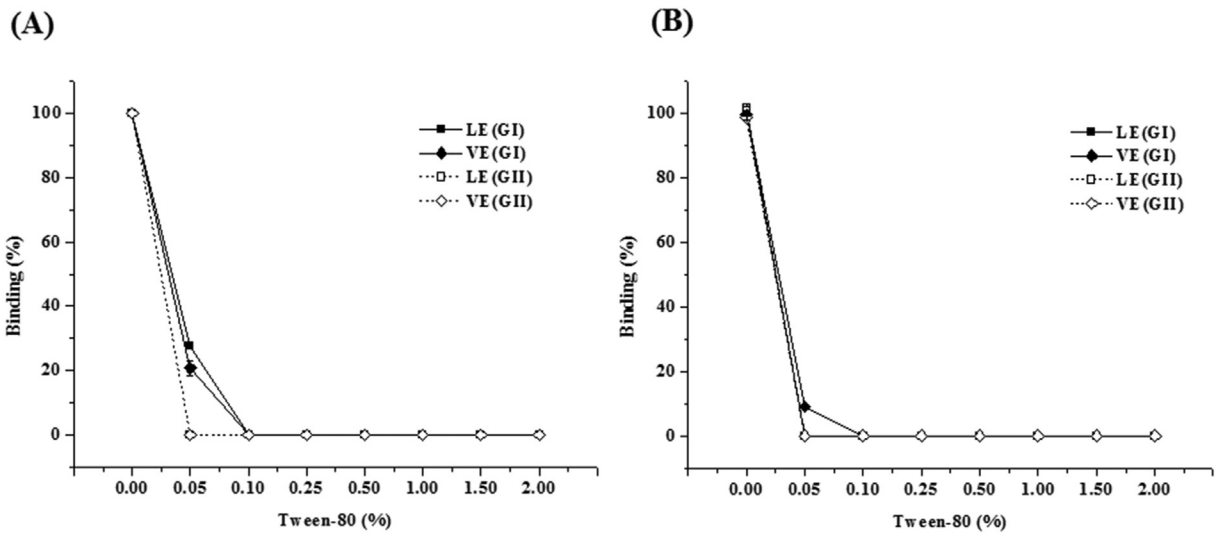

Fig. 6. The effects of Tween-80 on binding between GI BPC or GII BPC (A) to LE and VE and removal of bound-GI BPC or bound-GII BPC (B). LE: leaf extract; VE: vein extract. Each experiment (N) was performed in triplicate (n = 3) and repeated at least three times (N > 3). Error bars represent standard deviation.

could change the behavior of the attachment of the viruses or VLPs to Romaine lettuce. As it is not easy to obtain a stable bacteria-virus or bacteria-VLP complex, we used BPCs to test the binding of P proteins in the presence of bacteria in this study. We demonstrated that BPCs could bind to Romaine lettuce through a similar pattern as purified VLPs, HuNoVs and P proteins reported (Gandhi et al., 2010, Gao et al., 2016, Niu et al., 2015, Xu et al., 2017). The system we used was similar but not equivalent to bacteria-virus complex in native condition. The protein conformation might change in the new system used. Unlike the previous studies which either GI or GII VLPs were tested, we tested and compared the binding ability of both GI and GII BPCs to Romaine lettuce extract and factors affecting the binding to and removal from Romaine lettuce extracts.

Although it would have differences in basic biology, chemical components, and physiology between LE and VE, we did not notice significant difference in their ability to bind BPCs and in the factors influenced their binding and removal. Both GI and GII BPCs could bind to LE and VE effectively. The binding could be significantly inhibited by oxidation of carbohydrates in LE and VE for both GI and GII. Heat-denaturation of proteins in LE and VE could resulted a partial reduction of binding of GII BPCs but not GI BPCs to LE and VE. These data were consistent with previous reports that carbohydrates in Romaine lettuce were contributed factor for HuNoV VLPs binding in Romaine lettuce (Gandhi et al., 2010; Esseili et al., 2012; Gao et al., 2016). The competitive assays with human saliva further demonstrated that HBGA-like carbohydrates in LE and VE were related to BPCs binding. Esseili et al. (2012) demonstrated that cell wall proteins and carbohydrates of Romaine lettuce leaves are primary source for young and old leaves binding to GII rNVLPs. As we did not separate young and old lettuce leaves in our study, partial inhibition in heat-denatured LE and VE for GII BPCs was expected and consistent with previous report (Esseili et al., 2012). However, heat-denatured proteins in LE and VE did not contribute to GI BPCs binding. Overall, the features of ligands in LE and VE for BPCs and VLPs were similar which indicated that viral capsid proteins in rNVLPs and BPCs were driving force for binding to lettuce.

Other factors such as pH, ionic strength, and hydrophobicity in non-specific binding of virus to lettuce had been reported. Factors that affected the electrostatic forces included the pH and ionic strength of the solution. Studies had suggested that electrostatic forces contributed to the attachment of FCV and SAV to lettuce (Vega et al., 2005; Wang et al., 2012). Wang et al. (2012) reported that SAV attached to lettuce leaves significantly at its capsid isoelectric point (pI), Vega et al. (2005) reported that the attachment above the pI of FCV and echovirus 11 was reduced or eliminated in the presence of NaCl, indicating an electrostatic interaction between the animal viruses and lettuce. The pI of GI and GII VLP was 6.1 (Goodridge et al., 2004) and 6.4 (Wang et al., 2017), respectively. In our study, binding of GI and GII BPCs to LE or VE were affected slightly by variation of pH tested ranging from pH 5.1 to 7.9. Although the best binding for GII BPCs to LE and VE occurred at pI of GII, binding of GI BPCs to LE and VE was enhanced when pH was below pI of GI and reduced when pH was above pI of GI ($p < 0.05$). Overall, effect of pH on BPCs binding was very limited. Our results suggested that the specific interaction between the ligands in lettuce and viral capsid proteins played a dominant role in BPCs binding than electrostatic changed caused by pH changes.

The interaction between ligands in lettuce and BPCs were influenced by the change in ionic strengths. The optimal binding of BPCs to LE and VE required presence of ions. The binding of BPCs to LE and VE was poor in the absence of NaCl or $MgCl_2$. There was a dose-dependent enhancement of binding of BPCs to LE and VE when the salt concentrations increased until reached plateau. Our results were consistent with a previous report which showed that the attachment efficiencies of GI and GII VLPs to silica increased with increasing ionic strength in NaCl solutions (da Silva et al., 2011). The binding of BPCs to LE and VE were reduced when the salt concentrations were further increased. Our data suggested that the strength of an ionic bond supported initial transient interaction between ligands in lettuce and BPCs and promoted dissociation of the bioactive ligand from BPCs when the salt concentration was high.

Hydrophobic interactions significantly contributed to the non-specific adsorption of some viruses to selected sorbents (Chattopadhyay and Puls, 1999). In our study, hydrophobic interaction was also important to BPCs binding to LE and VE. The binding of BPCs was significantly reduced in the presence of Tween-80 as low as 0.05% (*v*/v) and eliminated at 0.1% (v/v). It is not surprised as changes in hydrophobic interaction resulted in movement of water in the binding-pocket of receptor or ligand and changes in the flexibility of a receptor/ligand reaction (Jasuja et al., 2009).

Few studies on removal of contaminated HuNoVs from contaminated produce has been reported. Bae et al. (2011) investigated the effect of various wash treatments on reducing HuNoVs on Iceberg Lettuce and Perilla Leaf. This study used artificially contaminated vegetables and washing was done with tap water. Wash treatments included immersion in water, rinsing with running water, a combination of immersion and rinsing, and in the presence of class I detergent. The most effective wash reduced 0.69- to 1.29-log reduction of HuNoVs.

DiCaprio et al. (2015a) reported that GII HuNoV, MNV, and TV attached efficiently to the Romaine lettuce leaves and roots and green onion shoots, and that washing with PBS or 200 ppm of chlorine removed < 0.4 log of viral RNA copies from the tissues (DiCaprio et al., 2015b). Predmore and Li (2011) reported that combination of a surfactant with a commonly used sanitizer enhanced the efficiency in removing viruses from fresh produce by approximately 100 times. However, the removal conditions for bacteria-norovirus complex have never been studied. In this study, we tried various conditions for removal of LE- or VE-bound BPCs.

As HBGA-like carbohydrate were involved in BPCs binding to Romaine lettuce, we firstly tried to wash the LE- or VE-bound BPCs in the presence of HBGAs from human saliva. However, the bound BPCs could not be washed off by free HBGAs. It is possible that the affinity of the ligands in Romaine lettuce is higher than HBGAs from human saliva. We demonstrated that the bound BPCs could be easily removed by washing with either low pH solutions (pH 3.0–5.0) or high pH solutions (pH 9.0–10.0). It has been reported that high pH resulted in rapid disintegrated of GI VLPs (da Silva et al., 2011) and destruction of Gram-negative food-borne pathogens (Mendonca et al., 1994). It has been reported that acidic electrolyzed water had a stronger bactericidal effect on most known pathogenic bacteria (Cao et al., 2009; Keskinen et al., 2009; Ongeng et al., 2006; Park et al., 2008; Venkitanarayanan et al., 1999). It might be a practical approach to use low pH solution to remove BPCs from contaminated Romaine lettuce and other produce. Although the presence of ions was required by BPCs for optimal binding to Romaine lettuce, limited effect of wash with solutions in the absence of salt or low salt. However, bound BPCs could be effectively removed by washing with solutions with high concentration of salt. The bound GI BPCs could be effectively removed at the 75.0 g/L NaCl or $MgCl_2$. Bound GII BPCs could be effectively removed by 25.0 g/L NaCl or 50.0 g/L $MgCl_2$. Although the bound BPCs could be effectively removed by high-salt wash, it might not be practical to use in produce industry. Therefore, we tested some nonionic surfactant which has been approved use in food industry. Tween-80 is a non-ionic surfactant that is widely used as an emulsifier in cosmetics, pharmaceuticals and food products. It is approved by the US Food and Drug Administration for use in up to 1% (v/v) in selected foods (Chassaing et al., 2015). We demonstrated the bound BPCs could be effectively removed by washing with Tween-80 as low as 0.1% (v/v). As the concentration of Tween-80 used is 10 times less than FDA approved dose, more studies are needed to further explore the safety of Tween-80 or similar product and their potential for removing the contaminated HuNoVs from produce.

In conclusion, we demonstrated that HBGA-like carbohydrate in Romaine lettuce might play a dominant role in BPCs binding although other factors also influenced the binding efficiency. The bound BPCs could be removed effectively by low or high pH, high salt concentration, and 0.1% (v/v) Tween-80. The study provided important information on BPCs binding to and removal from Romaine lettuce.

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Conflicts of interest

All authors report that they do not have a conflict of interest relating to conduct of the study or publication of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.03.008.

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