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RESEARCH PAPER

Assembly of the Capsid Protein of Red-spotted Grouper Nervous Necrosis Virus during Purification, and Role of Calcium Ions in Chromatography

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Abstract Currently virus-like particles (VLPs) are receiving much attention as platforms for next generation vaccines. However, chromatography-based methods for purifying VLPs remain challenging. Unlike traditional methods using density gradient for purifying VLPs, there have been few advances in explaining how assembled particles can be obtained by chromatography. Nervous necrosis virus (NNV) infects over 30 species of fish and leads to large economic losses in the farmed fish industry. Previously we developed a heparin chromatography-based method for purifying red-spotted grouper NNV (RGNNV) VLPs. However it is unclear how the assembled RGNNV VLPs are obtained by this method. It is known that assembly of NNV capsid proteins depends on calcium ions. In the present study, we found that the yield of purified RGNNV capsid protein in heparin chromatography was enhanced when calcium ions were present during binding. Also, it appears that the capsid protein of RGNNV undergoes partial disassembly and reassembly during sample preparation prior to heparin chromatography and the protein finally undergoes assembly during the chromatography. Therefore, our results indicated that heparin-binding affinity of RGNNV capsid protein is linked to its ability for VLP formation. The assembly of RGNNV capsid proteins recombinantly produced is a good model for explaining VLP formation during chromatography-based purification processes.

Keywords: virus-like particles, assembly, nervous necrosis virus, heparin chromatography, calcium ion

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1. Introduction

Remarkable developments in recombinant DNA technologies and genetic engineering strategies have facilitated the developments of subunit vaccines [1]. Also strategies for synthesizing peptides have allowed assessment of the potential of pathogen epitopes as vaccine antigens [2]. Subunits of pathogen proteins provide opportunities for neutralizing pathogens [3], and monomeric proteins or peptide fragments of pathogens have long been considered vaccine candidates [4]. However, the small size of such protein subunits tends to limit the strength of immune responses to vaccines [3,5].

Virus-like particles (VLPs) are multimeric protein complexes that mimic the structural properties of native viruses [6,7]. The advantages of VLPs are their conformation-specific neutralizing epitopes [8] and the fact that their size is similar to that of the native virions [9]; they are therefore

efficiently recognized by immune cells, since the latter have been programed in evolution to recognize antigens of the size of pathogens. The high density of repetitive epitopes on the surface of VLPs can facilitate the induction of effective immune responses and neutralizing antibodies [10,11]. Also, VLPs share the advantage of subunit vaccines in not containing unnecessary components such as viral nucleotides and non-structural proteins [12]. Two types of VLP-based vaccines, hepatitis B virus (HBV) and human papillomavirus (HPV) vaccines, are commercially available, and numerous fish and animal vaccines based on VLPs are under development [13,14].

Despite the great advantages of VLP-based vaccines, the downstream processing of VLPs remains enormously challenging [15,16]. The exquisitely detailed structures of VLPs make it difficult to develop scalable downstream processes. Numerous types of VLPs have been purified by traditional methods such as ultracentrifugation using sucrose cushions or cesium chloride density gradients to investigate their potential as vaccines, and these types of purification methods are still the main tools in the VLP field of study. There have been only a limited number of successful cases in which chromatography-based strategies were used to purify VLPs. The main requirement for chromatography-based strategies is to obtain fully-assembled forms of a capsid protein produced by the recombinant approach.

A recent study suggested that recombinantly-produced capsid proteins of HBV assemble during the purification process rather than intracellularly [17]. It was suggested that maturation process for HPV pseudovirus render its structure superior [18]. We also found that the capsid proteins of HPV formed intermolecular disulfide bonds during purification [19] and that this was critical for the assembly and stability of HPV capsid [18]. However, little effort have been paid into understanding what changes of the capsid proteins occur during purification and how assembled particles can be obtained by chromatography.

Betanodavirus, also known as nervous necrosis virus (NNV), infects more than 30 species of fish and causes massive mortality in hatchery-bred larvae and juveniles, leading to large economic losses for aquaculturists [20,21]. The development of high efficacy and cost-effective vaccines is ongoing. Previously, we developed heparin chromatography-based methods to obtain fully assembled VLPs of red-spotted grouper NNV (RGNNV) from *Saccharomyces cerevisiae* (*S. cerevisiae*) [22] and found that the RGNNV VLPs can provide protective immunity against RGNNV infection to the grouper *Epinephelus septemfasciatus* [23]. Fully assembled particles of RGNNV capsid proteins could be obtained with high purity when heparin chromatography was performed in 0.5 M NaCl pH 7.6 [22]. The capsid proteins had a greater affinity for the heparin-bound resin than contaminating proteins. Previous reports have suggested that the assembly of grouper NNV capsid proteins is calcium ion-mediated, and chelating agents can induce dissociation of the assembly [24,25]. This raises questions about what changes of the capsid protein occur during purification and whether calcium ions influence the heparin-binding affinity of the capsid proteins.

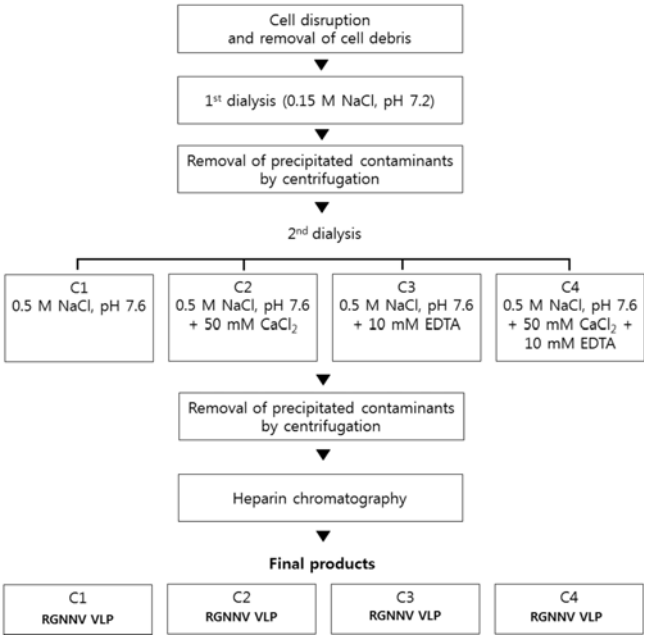

Fig. 1. Binding conditions for heparin chromatography. Supernatants of cell lysates were dialyzed against the 1st dialysis buffer, and precipitated contaminating proteins were removed by centrifugation. The supernatant after the centrifugation was divided into four parts that were dialyzed against four types of binding buffer (2nd dialysis) prior to heparin chromatography. The heparin chromatography was conducted after removing contaminating proteins precipitated during the 2nd dialysis.

In the present study, we used four binding conditions for heparin chromatography (Fig. 1) to investigate the effects of $CaCl_2$, EDTA and $CaCl_2$ + EDTA on the heparin-binding affinity of the capsid protein during heparin chromatography. Moreover, we tried to monitor changes in the size of the RGNNV capsid proteins during purification using size-exclusion chromatography (SEC) to investigate what aspect of the purification process facilitates capsid assembly.

2. Materials and Methods

2.1. Expression and purification of RGNNV capsid proteins

RGNNV capsid proteins were expressed and purified as reported previously [22]. *S. cerevisiae* Y2805 was transformed with YEGα-MCS-opt-RGNNV-CP and cultured in flasks

at 30°C for 72 h with shaking at 230 rpm. A 500 mL culture of cells was disrupted by vortexing with glass beads (Biospec Products, USA), and cell debris was removed by centrifugation at 12,000 *g* for 10 min at 4°C. The supernatant was dialyzed against a buffer (10 mM Tris–Cl pH 7.2, 0.15 M NaCl, 5% glycerol, 50 mM L-glutamine, 50 mM L-arginine + 0.05% Tween 80) for 24 h at 4°C to induce precipitation of contaminating proteins. The precipitated contaminants were removed by centrifugation as described above.

The supernatant (100 mL) after the centrifugation was divided into four aliquots of 25 mL each dialyzed against a different binding buffer for 24 h at 4°C. The four buffers were as follows: **C1**, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.5 M NaCl, pH 7.6 + 0.05% Tween 80 + 5% glycerol; **C2**, C1 buffer + 50 mM $CaCl_2$; **C3**, C1 buffer + 10 mM EDTA-Na; **C4**, C1 buffer + 50 mM $CaCl_2$ + 10 mM EDTA-Na. The dialyzed samples were centrifuged as described above and the supernatants were loaded onto 1 mL of heparin-bound resin (POROS50 HE, Applied Biosystems, USA) pre-equilibrated with C1 binding buffer. The heparin-bound resin was washed with five column-volumes of C1 binding buffer, and bound proteins were eluted by successive addition of C1 buffer containing 0.65, 0.75, 0.85, 0.95, and 1.2 M NaCl. The elution buffers were prepared by adding 0.15, 0.25, 0.35, 0.45, and 0.7 M NaCl to the C1 binding buffer, respectively. Protein was determined with a Bradford protein assay kit (Bio-Rad, USA).

2.2. SDS-PAGE

Elutes of the heparin chromatography above were mixed with Laemmli's sample buffer, heated at 87°C for 10 min, and fractionated by 12% SDS–PAGE on. The separated proteins were visualized by silver staining.

2.3. Indirect enzyme-linked immunosorbent assay (ELISA) to detect RGNNV capsid protein

96-well microplates (Greiner, Germany) were coated overnight with loading samples, flow-through or eluates of heparin chromatography at 4°C using phosphate-buffered saline (PBS), and blocked with 3% bovine serum albumin (BSA) in PBS-T (PBS containing 0.05% Tween 20). The RGNNV capsid protein was detected using mouse anti-RGNNV capsid protein polyclonal antibody prepared previously [22], followed by HRP-conjugated anti-mouse IgG (Bethyl, USA). Color was developed with *o*-phenylenediamine (Sigma, USA) and measured at 492 nm.

2.4. Size-exclusion chromatography (SEC)

SEC was conducted as described previously with modifications [26]. Loading samples, flow-through or eluates from heparin chromatography were loaded onto Superose-6 resin (1.5 × 34 cm, GE Healthcare), equilibrated with C1 buffer. The loaded protein was eluted at a flow rate of 0.3 mL / min and elution profiles of the protein were acquired using Autochro 2000 software (Young Lin Instrument Co., South Korea). Twenty fractions were collected, and the distribution of RGNNV capsid protein was determined by ELISA as described above.

2.5. Transmission electron microscopy (TEM) analysis of RGNNV capsid protein

Purified RGNNV capsid protein was absorbed onto carbon-coated grids and negatively stained with 2% uranyl acetate. Electron microscopy was performed on an energy-filtering transmission electron microscope (LIBRA 120, Carl Zeiss, Germany) at a final magnification of 150,000 ×. To detect the capsid protein with immunogold, the coated capsid protein was incubated with mouse anti-RGNNV capsid protein followed by 10 nm gold-labeled goat anti-mouse IgG polyclonal antibody (Sigma, USA). Thereafter the protein was fixed with 1% glutaraldehyde (Sigma) and stained with 2% uranyl acetate. Electron microscopy was performed as described above.

3. Results

3.1. Comparison of yields of RGNNV capsid protein purified by heparin chromatography

Four binding conditions were designed to investigate the effects of calcium ions on the yield of the capsid protein from heparin chromatography (Fig. 1). A 125 mL culture of cells producing RGNNV capsid protein was used for each purification. There was no remarkable effect on the yield when EDTA was added to the binding buffer (C3, Fig. 2B). As shown in Figs. 2A and 2B, the yield of capsid protein was enhanced when $CaCl_2$ (C2) or $CaCl_2$ + EDTA (C4) was added to the binding buffer. The yields of capsid protein obtained from C1, C2, C3 and C4 were 0.78, 0.98, 0.76 and 1.1 mg, respectively.

3.2. The dissociated form of RGNNV capsid protein as well as the assembled form can bind to heparin resin

Loading samples and flow-through samples from each condition were analyzed by SEC to investigate what proportion of the assembled form binds to the heparin. Capsid assembly of betanodavirus is known to be calcium-ion mediated. Therefore, chelating agent such as EGTA or EDTA can induce dissociation of the assembled particles [25]. Fig. 3A shows that the SEC profile of EDTA-treated RGNNV VLPs contains two peaks. Fig. 3B shows TEM images of peaks 1 and 2, respectively. Peak 1 was found to consist of the assembled form while peak 2 consisted of the

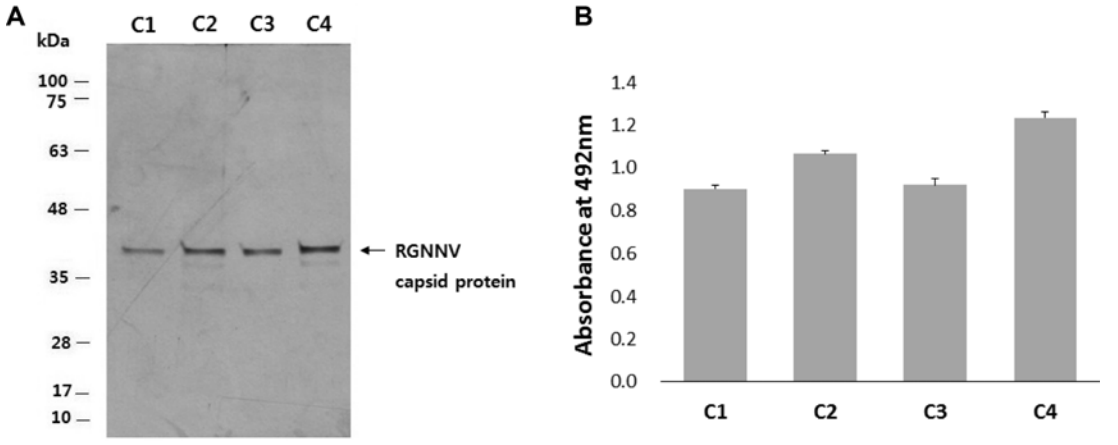

Fig. 2. Comparison of amounts of RGNNV capsid proteins purified by heparin chromatography. (A) The SDS-PAGE result for RGNNV capsid protein recovered from the heparin chromatography. (B) The ELISA result showing the yields of RGNNV capsid protein recovered from the heparin chromatography. Data are mean ± SD of triplicate.

dissociated from.

The loading samples from the C1 and C3 binding conditions appeared to contain major peaks corresponding to peak 2 (Figs. 3C and 3E) while those from conditions C2 and C4 contained significantly smaller peaks of this kind (Figs. 3D and 3F). Therefore, it seems that $CaCl_2$ increases the proportion of assembled particles in the loading sample. It was noted that the overall areas of the peaks of the flow-through fractions from conditions C2 and C4 were markedly reduced (Figs. 3D and 3F), compared to those from C1 and C3 (Figs. 3C and 3E), indicating that a greater proportions of the capsid protein contained in the C2 and C4 samples bound to the heparin resin, in agreement with the observation that the C2 and C4 conditions gave higher yields of the capsid protein after heparin chromatography (Fig. 2B). Moreover the lowest proportion of RGNNV capsid protein was found in the flow-through fraction from condition C4, indicating that the largest amount of capsid protein bound to the heparin resin in that situation.

It was found that the peaks corresponding to peaks 1 and 2 were absent from the flow-through fraction in all four conditions (Figs. 3C, 3D, 3E and 3F) indicating that not only the assembled but also the dissociated form of the capsid protein bound to the heparin resin.

3.3. Assembly of the capsid protein is induced during heparin chromatography

Fig. 4 shows SEC profiles and TEM images of the capsid protein eluted from the heparin column. As stated, both the assembled and dissociated form of the capsid protein bound to the heparin resin (Figs. 3C, 3D, 3E and 3F), but in all four situations the eluted capsid protein appeared to be in the form of VLPs of size corresponding to peak 1 in Fig. 3A (Fig. 4). These results suggest that the capsid protein may undergo assembly during the chromatography.

To investigate the reassembly of dissociated particles, the peak 2 fraction of Fig. 3A was loaded onto heparin-bound resin, and the SEC profiles of the loading sample, flow-through and elution fraction were analyzed. A large proportion of the heparin-bound dissociated input particles had a size corresponding to the assembled form (Fig. 5C) while that in the flow-through fraction had a size corresponding the dissociated form (Fig. 5B). There are two kinds of explanation for the size shift of the capsid protein. One is the interaction between it and the heparin resin, and the other is the increased NaCl concentration used to elute the capsid protein. However the mock treatment (Fig. 5D) shows that the increase of NaCl concentration compared with the loading sample was not responsible for the size increase. Thus it seems that the interaction between the capsid protein and heparin promotes capsid assembly, and the binding ability of the capsid protein is critical for capsid assembly. Therefore, we suggest that calcium ion treatment increases the proportion of capsid protein molecules that possess assembly potential.

4. Discussion

In the present study, calcium ions were found to increase the affinity of RGNNV capsid protein for heparin-bound resin (Figs. 3D and 3F), and to improve the final yield (Fig. 2B). Also, EDTA treatment in the presence of $CaCl_2$ further increased the heparin-binding affinity of the capsid protein (Fig. 2B). Moreover, the result of micro heparin-binding assay indicated that the heparin-binding affinity of

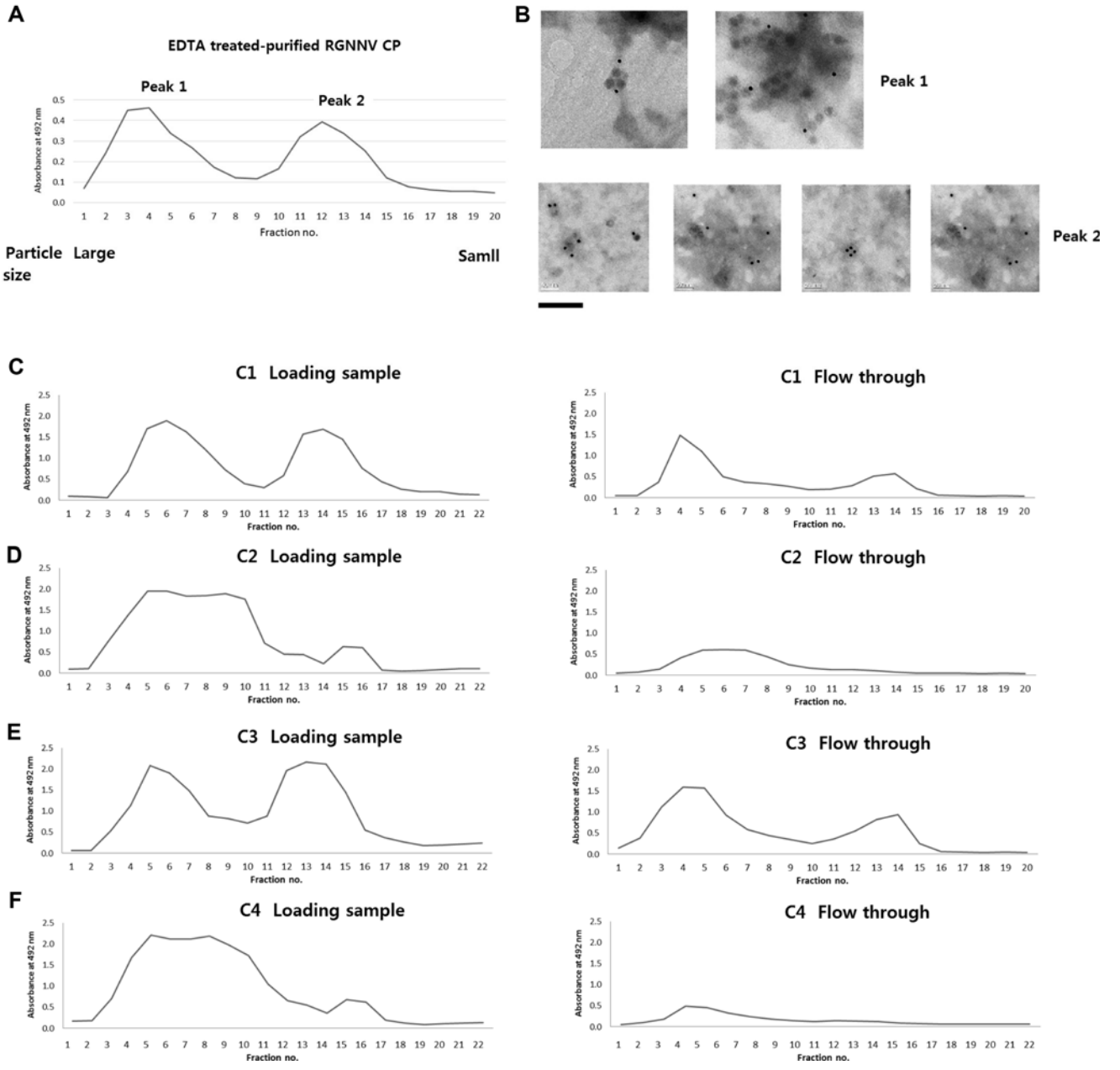

Fig. 3. SEC profiles of loading samples and flow-through fractions of heparin chromatography. A is the SEC profile of purified RGNNV capsid protein treated with 10 mM EDTA. B shows TEM images of peak 1 and peak 2 of panel A. Panel C, D, E, and F are SEC profiles of the loading sample and flow through in the C1, C2, C3, and C4 binding conditions, respectively. Bar in panel B, 100 nm.

the capsid protein is enhanced by calcium ions and that is further enhanced by EDTA treatment in the presence of $CaCl_2$ (Supplementary figure-1). Previously, it was suggested that EGTA can induce disassembly of dragon grouper NNV (DGNNV) VLPs, and the addition of calcium ions to the EGTA-treated capsid protein reassembled the capsid protein [25]. In this study, it was shown that not only $CaCl_2$ but also $CaCl_2$ + EDTA significantly reduced the proportion of dissociated capsid protein in the loading sample (Figs. 3D and 3F). Therefore it seems that the calcium ion-dependence of assembly of RGNNV capsid protein is similar to that of DGNNV capsid protein. Taken together, it seems that EDTA may facilitate exposure of heparin binding domains of the capsid proteins in assembly states.

As shown in Fig. 3A, EDTA induced partial dissociation of the purified RGNNV VLPs and EGTA was found to induce partial dissociation of RGNNV VLPs (data not shown). Previously, disulfide bonding between DGNNV capsid protein molecules was suggested to be important for the stability of the capsid protein [24]. Also, it was suggested

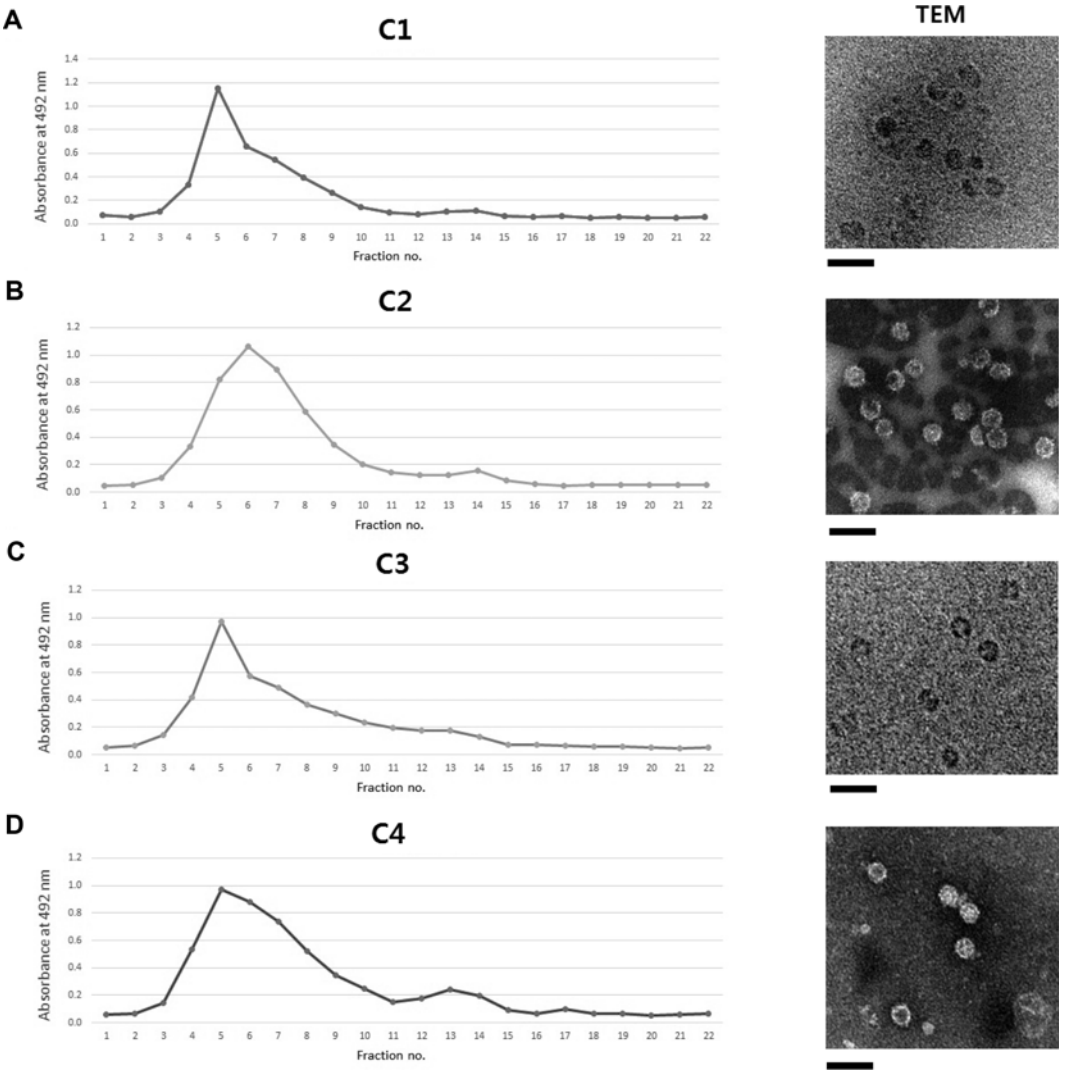

Fig. 4. SEC profiles and TEM images of elution fractions of heparin chromatography**.** A, B, C, and D are SEC profiles and TEM images of RGNNV capsid proteins purified in the C1, C2, C3, and C4 binding conditions for heparin chromatography, respectively. Bar, 50 nm.

that concentration of capsid protein, ionic strength and pH affect significantly physical integrities of VLPs [27,28]. Therefore, other factors such as disulfide bond formation between capsid proteins, buffer composition and pH are thought to be involved in the resistance of RGNNV capsid protein to dissociation by chelating agents.

Several types of viral capsid proteins have been successfully expressed in the *S. cerevisiae* expression system and in some cases recovered as VLPs by chromatography-based purification. In this study, the size of the capsid protein straight after cell disruption was found to be larger than that of dissociated form when examined by SEC and ultracentrifugation using a cesium chloride density gradient (Supplementary figure-2). Meanwhile, as shown in Fig. 3, the capsid protein in the four types of loading sample appeared to consist of dissociated particles, indicating that the structural integrity of the capsid protein was changed during dialysis. However, the capsid protein from heparin chromatography was found to be in the form of VLPs (Fig. 4). Also, the evidence indicates that the capsid assembly occurred during heparin chromatography (Fig. 5). Therefore, these results indicate that the capsid protein undergoes disassembly and reassembly during the purification process. Our observations about RGNNV capsid assembly may provide a good model for explaining VLP formation during chromatography-based purification processes.

Our results indicate that the calcium ion is critically involved in the binding of the capsid protein with heparin (Figs. 2 and 3), and the heparin-binding affinity is significantly linked to the potential for assembly of the capsid protein (Fig. 5). Recently, the crystal structure of grouper NNV (GNNV) was determined; it suggested that the protrusion

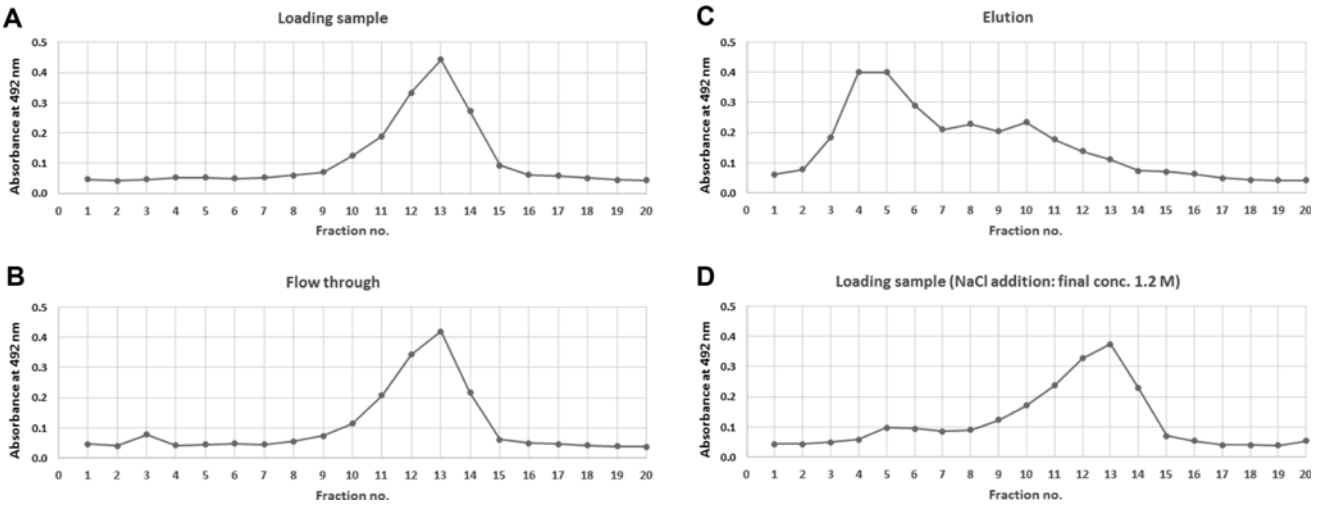

Fig. 5. Comparison of the SEC profiles of the loading samples, flow-through and elution fraction when the dissociated form of the capsid protein was applied in heparin chromatography. The peak 2 fraction of Fig. 3A was loaded onto heparin resin, and flow-through and elution fractions were collected. A, B, and C are SEC profiles of the loading sample, flow-through, and elution fraction (eluted by buffer containing 1.2 M NaCl) for the heparin chromatography, respectively. D is the SEC profile of the mock treatment: the loading sample was treated with 1.2 M NaCl.

domain formed by a trimeric interaction between capsid protein units is coordinated by two calcium ions that play a prominent role in the calcium-mediated trimerization process [29]. Therefore, the correlation between heparin-binding behavior and protrusion domain changes due to binding of calcium ions should be noted in future studies.

5. Conclusion

In conclusion, we found that calcium ions are important for binding of the RGNNV capsid protein to heparin and led to a 40% increase of the final yield of purified capsid protein when $CaCl_2$ plus EDTA was used for the heparin chromatography. Also heparin chromatography was essential for obtaining assembled particles of RGNNV capsid protein (Figs. 4 and 5). We anticipate that the increased understanding of the heparin binding behavior of RGNNV capsid protein will facilitate the development of downstream processes for purifying RGNNV VLPs.

Acknowledgements

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