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A Method for Removing Contaminating Protein during Purification of Human Papillomavirus Type 18 L1 Protein from *Saccharomyces cerevisiae*

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Human papillomavirus (HPV) types 16 and 18 are the main targets in the field of prophylactic vaccines for preventing cervical cancer. L1 protein, the major capsid protein of HPV, self-assembles into virus-like particles (VLP), which are the major component of prophylactic vaccines. To obtain highly purified L1 protein, contaminants must be removed by several chromatography steps. However, this requires a great deal of time and labor, and results in loss of large amounts of the target protein. Therefore, we have sought to develop an efficient method for removing contaminants prior to chromatography during the purification of HPV18 L1 protein from *Saccharomyces cerevisiae*. For this purpose the contaminating proteins were removed by an ammonium sulfate precipitation step and further removed by a removal of precipitated contaminants step. Purification of the L1 protein by chromatography was significantly improved by the removal of precipitated contaminants step. In the present work we developed two one-step chromatography methods (heparin and cation-exchange chromatography), and HPV18 L1 proteins purified by both methods self-assembled into VLP. The two chromatographic purification methods are simpler and more convenient than previous methods and are widely applicable to work with VLPs.

Key words: Cervical cancer, Papillomavirus, Virus-like particles, Heparin, Cation-exchange

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INTRODUCTION

Human papillomavirus (HPV) is a double-stranded DNA virus, and over 100 types of HPV have been identified (Bosch et al., 1995; Jeong et al., 2009). Most cervical cancer is caused by HPV infection, and about 70% of all cervical cancer cases worldwide are associated with infections by HPV types 16 and 18 (Jeong et al., 2009; Moscicki, 2008; Schadlich et al., 2009). Therefore, these two types of HPV are the main targets in the development of vaccines to prevent cervical cancer.

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The capsid of HPV is composed of L1 and L2 protein. The L1 protein has a molecular weight of 55 kDa and can self-assemble into virus-like particle (VLPs), which are structurally and immunologically similar to native HPV (Buck et al., 2005). The VLP is composed of 72 L1 pentamers (capsomers), each consisting of five L1 monomers, and the L1 pentamer is established by hydrophobic interactions between the L1 monomers (Bishop et al., 2007). The HPV L1 and L2 proteins are not exposed to dendritic cells because HPV infection is nonlytic (Moscicki, 2008). Therefore, HPV infection does not induce the pro-inflammatory signals that activate dendritic cells. In addition, the highly immunogenic L1 and L2 proteins do not interact much with immune responses in the epithelium because the two capsid proteins are only produced late in infection (Moscicki, 2008). This mechanism for evading the immune response results in low neutralizing antibody titers in natural infections. However, recent clinical trials have shown that vaccination with HPV VLPs

can generate high neutralizing antibody titers and strong T cell responses against HPV (Garland et al., 2007).

The L1 protein has been expressed in *E. coli*, yeast, and insect and mammalian cell systems (Kirnbauer, 1996; Lowe et al., 1997; Park et al., 2008; Zhou et al., 1991). The yeast expression system has advantages in terms of cost-effectiveness, suitability for large-scale production, and low potential for contamination by toxins or viruses compared with bacterial and mammalian expression systems (Cook et al., 1999; Joyce et al., 1999; Neeper et al., 1996). Therefore, production of VLP-based vaccines via the yeast expression system has advantages over the other expression systems.

Infection by HPV essentially requires binding to heparan sulfate on the cell surface, and it is known that the HPV induces an immune response upon binding to heparan sulfate on dendritic cells, and that this step controls the immune response and is important for vaccine development (de Witte et al., 2007). Heparin, which is structurally similar to heparan sulfate, binds correctly folded HPV VLPs but not incorrectly folded HPV VLPs (Giroglou et al., 2001; Rommel et al., 2005; Wang et al., 2005). This suggests that it should be useful for selecting correctly folded VLPs. However, a heparin-based chromatography method has not been developed despite the fact that ten years have elapsed since the heparin-HPV VLP interaction was established.

Size-exclusion chromatography, ultracentrifugation on a sucrose cushion and cesium chloride density gradient centrifugation have all been used to purify HPV VLPs because the VLP is larger than most contaminants (Aires et al., 2006; Hofmann et al., 1995; Jeong et al., 2006; Kim et al., 2007; Park et al., 2008; Woo et al., 2008). However, these methods are limited to small scale purification because they cannot cope with large amounts of protein. In addition, further chromatography steps are required because the contaminants are not efficiently removed by these methods alone, and the additional chromatography steps result in large losses of the target protein. The demanding nature of the available methods for purifying HPV VLPs has been for a long time an obstacle to the study of HPV.

In the present work, we have developed a method that removes contaminating proteins efficiently prior to the chromatography step and have developed two one-step chromatography methods. One is heparin chromatography and the other, cation-exchange chromatography.

MATERIALS AND METHODS

Expression of HPV18 L1 protein

Vector YEGα-HPV18 L1 harboring the native HPV18 L1 gene was constructed and transformed into *Saccharomyces cerevisiae* (*S. cerevisiae*) Y2805 as described in Woo et al. (2008). To express HPV18 L1 protein, HPV18 L1-producing *S. cerevisiae* was cultured in YPDG medium (1% glucose, 3% galactose, 1% yeast extract and 2% peptone) at 30°C for 2 days. Growth was stopped when 1:10 dilutions in distilled water gave an OD of 1.4 at 600 nm.

Preparation of cell lysates

The yeast cells were harvested by centrifugation at 5000 g for 10 min, washed twice with phosphate-buffered saline (PBS) and frozen at -70°C. One hundred grams of wet cells were thawed at room temperature (RT) for 3 h and resuspended in 100 mL of cold break buffer (20 mM sodium phosphate, pH 7.2, 100 mM NaCl, 1.7 mM EDTA, 0.01% Tween 80). The resuspended cells were mixed 1:1 with glass beads (BioSpec Products) and broken in a Bead-Beater (BioSpec Products). Cell debris was removed by centrifugation at 12000 g for 10 min at 4°C, centrifugation being repeated until the debris was completely removed.

Ammonium sulfate precipitation

The HPV18 L1 protein in the clarified supernatant was recovered by ammonium sulfate precipitation (Fig. 1): the supernatant was slowly adjusted to 45% saturated ammonium sulfate at 4°C for 4 h, and precipitated protein was pelleted by centrifugation at 12000 g for 10 min at 4°C (Park et al., 2008). The pellet was resuspended in 16 mL PBS + 0.01% Tween 80, and the protein concentration was adjusted to 50 mg/mL. The suspension was then incubated at 4°C for 24 h and frozen at -20°C.

Removal of precipitated contaminants

Frozen sample was thawed at RT and dialyzed against PBS + 0.01% Tween 80 for 4 h at 4°C. The dialyzed sample was diluted 1:20 in incubation buffer (10 mM sodium phosphate, pH 7.2, 0.15 M NaCl + 0.01% Tween 80, the protein concentration of dilute was adjusted to 2 - 5 mg/mL) and incubated at RT for 24 h to induce precipitation of contaminants. The solution was clarified by centrifugation at 12000g for 10 min and the supernatant recovered. The HPV18 L1 protein in the supernatant was then purified by heparin chromatography or cation-exchange chromatography as described in Fig. 1. To evaluate the solubility of L1 protein and the fraction of the protein precipitated as

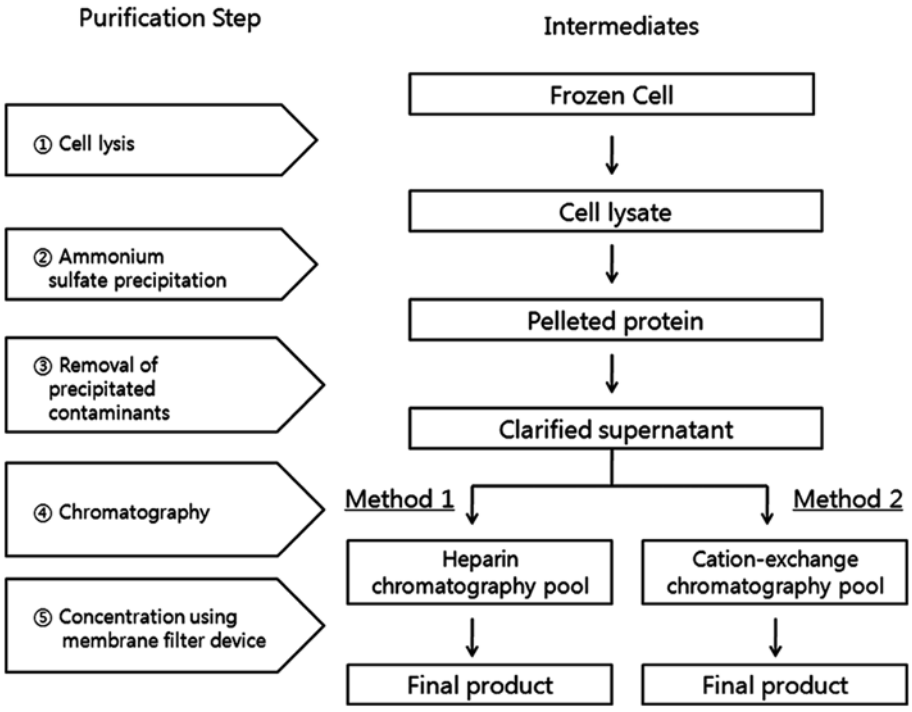

Fig. 1. Overview of the purification procedures

a function of NaCl concentration, we prepared 10 mM sodium phosphate, pH 7.2 + 0.01% Tween 80 containing 0, 0.15, 0.3, 0.6 and 1 M NaCl (Fig. 2). A sample dialyzed against PBS + 0.01% Tween 80 was diluted 1:20 into each of these incubation buffers and incubated at RT for 24 h. The precipitation ratio of protein and solubility of the L1 protein was determined by protein assay and Western blotting, respectively.

Heparin chromatography

The supernatant from removal of precipitated contaminants step was dialyzed against PBS + 0.2 M NaCl + 0.01% Tween 80 pH 7.0 (final NaCl concentration was adjusted to 0.33 M because PBS contained 0.13 M NaCl) for 3 h at 4°C, and a HiTrap™ Heparin HP column (GE Healthcare), prepacked with 5 mL of resin (10 mg heparin/1 mL resin), was equilibrated with five column volumes of the same buffer. The dialyzed sample was loaded on to the heparin column, and the column was washed until the OD of the flow-through was close to the base-line. The OD was monitored at 280 nm with a UV detector. The column was eluted with a linear gradient from 0.33 to 0.66 M NaCl for 35 min at a flow rate of 2 mL/min, followed by a linear gradient from 0.66 to 2 M NaCl for 15 min at a flow rate of 2 mL/min, and 2 mL fractions were collected. The purity of L1 protein was confirmed by SDS-PAGE and Western blotting, and fractions containing L1 protein were collected. The collected L1 protein was dialyzed against the binding buffer for heparin chromatography at 4°C for 3 h, and concentrated using a membrane filter device that has pores with a cut-off of 100 kDa (Amicon Ultra-4, Ultracel-100 k).

Cation-exchange chromatography

The supernatant was dialyzed against binding buffer (PBS + 0.37 M NaCl + 0.01% Tween 80 pH 7.2, final NaCl concentration was adjusted to 0.5 M because PBS contains 0.13 M NaCl) for 3 h at 4°C. The P-11 cationic phosphocellulose resin (Whatman) was packed in a 8 cm × 4 cm Poly-Prep column (Bio-rad Lab.), and equilibrated with the binding buffer. The dialyzed sample was loaded onto the column, and the column was washed with five column volumes of binding buffer. It was then eluted with elution buffers containing 0.6, 0.7, 0.8 and 1 M NaCl (the elution buffers were prepared by addition of 0.1, 0,2, 0.3 and 0.5 M NaCl, respectively, to the 0.5 M NaCl in binding buffer), and 4 mL fractions were collected. The purity of L1 protein was confirmed by SDS-PAGE and Western blotting, and fractions containing L1 protein were collected. The collected L1 protein was dialyzed against the binding buffer for heparin chromatography, and concentrated using a membrane filter device that has pores with a cut-off of 100 kDa (Amicon Ultra-4, Ultracel-100 k).

SDS-PAGE and Western blotting

For SDS-PAGE, each sample was mixed with sample buffer and heated at 75°C for 6 min, and then subjected to 12.5% PAGE in the presence of sodium dodecyl sulfate. Separated proteins were visualized with a silver staining kit (GE Healthcare). For Western blotting, each sample was separated by SDS-PAGE as described above, and then the proteins on the PAGE gel were transferred to a PVDF membrane (Q-Biogene) for 120 min at 200 mA. To detect L1 protein, rabbit anti-HPV18 L1 serum and goat anti-rabbit IgG-HRP (Zymed) were used as primary and secondary antibody, respectively. The L1 protein was visualized on X-ray film using ECL™ reagent (BD Bioscience). The density of the HPV18 L1 band was measured with Scion Image for Windows (Scion Corporation).

Determination of protein concentration

Protein concentration was determined using the Bio-Rad Bradford protein assay reagent (Bio-Rad Laboratories) with bovine serum albumin (PIERCE) as standard.

Transmission electron microscopy (TEM)

The concentrations of purified HPV 18 L1s purified by heparin or cation-exchange chromatography were adjusted to 120 μg/mL in the binding buffer for heparin chromatography, and samples were absorbed to carbon-coated grids for 5 min and negatively stained with 2% phosphotungstic acid for 1 min. The stained grids were washed with DW for 1 min and dried for 5 min. Transmission electron microscopy (TEM) was performed using a TEM200CX at a final magnification of 41,000× (Woo et al., 2008).

RESULTS

Optimum NaCl concentration for removing precipitated contaminants

As shown in Fig. 1, contaminating protein was removed by ammonium sulfate precipitation followed by removal of precipitated contaminants step. The HPV18 L1 protein was precipitated at 45% saturated ammonium sulfate as described in our previous report (Park et al., 2008). The fractions from chromatography contain many kinds of contaminant although about half of contaminating protein in cell lysate was removed by the ammonium sulfate precipitation step (Fig. 3, Fig. 4A and Fig. 5A). Therefore, we developed an efficient method for removing the contaminating protein. We discovered that the precipitation ratio of the contaminating protein and solubility of HPV18 L1 protein are different according to NaCl concentration. The precipitation ratio and solubility of L1 protein were analyzed on SDS-PAGE gel and Western blot, respectively, as described in Materials and methods. As shown in Fig. 2A, the precipitation ratio was

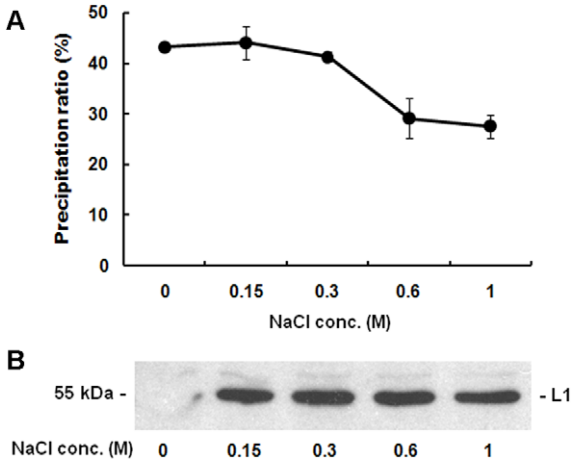

Fig. 2. The optimum NaCl concentration for precipitating contaminant in the removal of precipitated contaminants step. The pellet from ammonium sulfate precipitation was dialyzed and diluted in buffers containing 0, 0.15, 0.3, 0.6 and 1 M NaCl as described in Materials and methods. Each sample was incubated at RT for 24 h, and precipitated proteins were removed by centrifugation. The precipitation ratio and solubility of L1 protein were analyzed by protein assays and Western blotting, respectively. (**A**) shows the precipitation ratio determined by protein assay and B gives the results of Western blotting. The control value was set at zero percent in A, and the control sample was not incubated at RT. To investigate solubility of L1 protein according to NaCl concentration, equal volume of supernatants (3 μL) after removing precipitated contaminants were loaded for Western blotting (**B**). The L1 protein detected on Western blot is L1 protein maintaining solubility after removal of precipitated contaminants step.

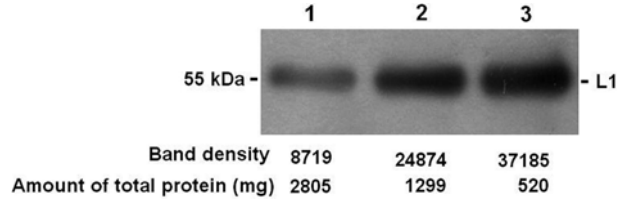

Fig. 3. Increasing the purity of L1 protein by ammonium sulfate precipitation and removal of precipitated contaminants step. Equal amount of proteins (3 μg) were loaded, and the amount of L1 protein was determined by Western blotting. Lane 1, 2 and 3 are samples after cell lysis, ammonium sulfate precipitation and removal of precipitated contaminants step. The amount of total protein refers to the amount of total protein after cell lysis, ammonium sulfate precipitation and the removal of precipitated contaminants step, and the amount of HPV18 L1 was determined by densitometry as described in Materials and methods.

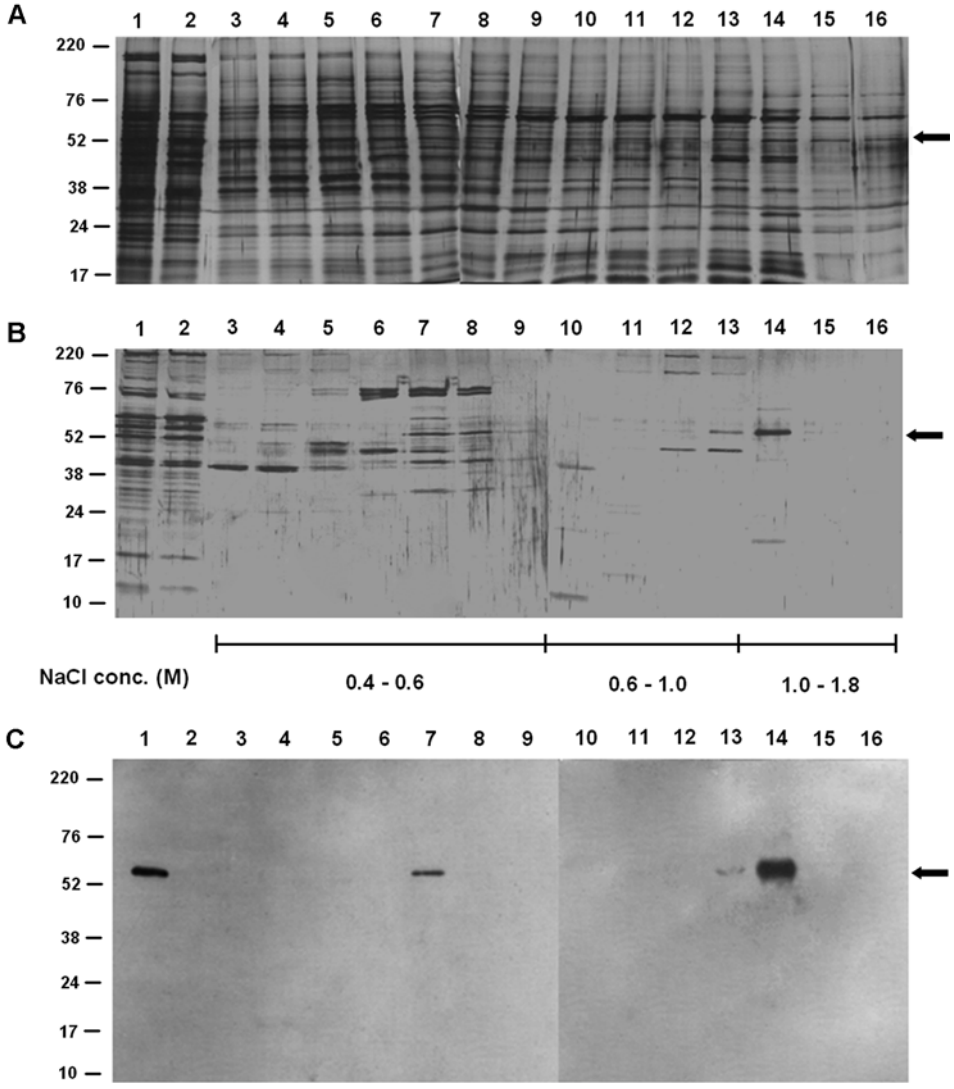

Fig. 4. Purification of HPV18 L1 by heparin chromatography. Heparin chromatography was performed as described in Materials and Methods. (**A**) The result of SDS-PAGE when the removal of precipitated contaminants step in Fig. 1 is omitted. (**B**) The result of SDS-PAGE when the removal of precipitated contaminants step is performed. (**C**) The result of Western blots of the heparin chromatography fractions in (**B**). Lane 1 is the loaded sample and lane 2, the flow-through. Lanes 3 - 16 correspond to fractions 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45 and 48. The arrow indicates 55 kDa, the molecular weight of HPV18 L1 protein.

highest in 0.15 M NaCl while the solubility of L1 protein was similar in 0.15 - 1 M NaCl, but was reduced in zero NaCl (Fig. 2B). This means that 0.15 M is the optimum NaCl concentration for precipitating contaminating proteins.

Increased purity of L1 protein after ammonium sulfate precipitation and removal of precipitated protein step

As shown in Fig. 3, total protein was reduced from 2805 to 520 mg by the ammonium sulfate and the removal of precipitated contaminants steps. Thus, 80% of total protein in cell lysate was removed by these two steps. To analyze the ratio of L1 protein to total protein in each step, equal amounts of protein were loaded, and the L1 protein was visualized by Western blotting (Fig. 3). The density of the L1 protein band increased about four fold after ammonium sulfate and removing precipitated contaminants. This result indicates that some contaminating proteins were selectively removed by these two steps.

Heparin and cation-exchange chromatography

We confirmed the effect of removal of precipitated contaminants step by heparin and cation-exchange chromatography. As shown in Fig. 4A and 5A, when

the removal of precipitated contaminants step was omitted the fractions from heparin and cation-exchange chromatography contained many kinds of contaminants, while most contaminants were removed when this step was performed (Fig. 4B and 5B). As shown in Fig. 4C, no L1 protein eluted in the flow-through or wash of the heparin column. Most was eluted by 1.2 M NaCl although some was eluted by 0.5 M NaCl (Fig. 4C). On the other hand, in the cation-exchange chromatography step some of the L1 protein appeared in the flow-through and wash (Fig. 5C), though most was eluted by 0.8 - 1 M NaCl. Therefore, it seems that some of the L1 protein molecules are differently charged from those that bind to the column; the former are found in the flow through and wash in the cation-exchange chromatography step but in the 0.5 M NaCl elution fraction of the heparin chromatography step because of differences between the binding conditions for the two kinds of chromatography (see Materials and methods).

The fractions eluted in 1.0 - 1.4 M NaCl by heparin chromatography and between 0.8 - 1 M NaCl by cation-exchange chromatography were collected and concentrated using a membrane filter device.

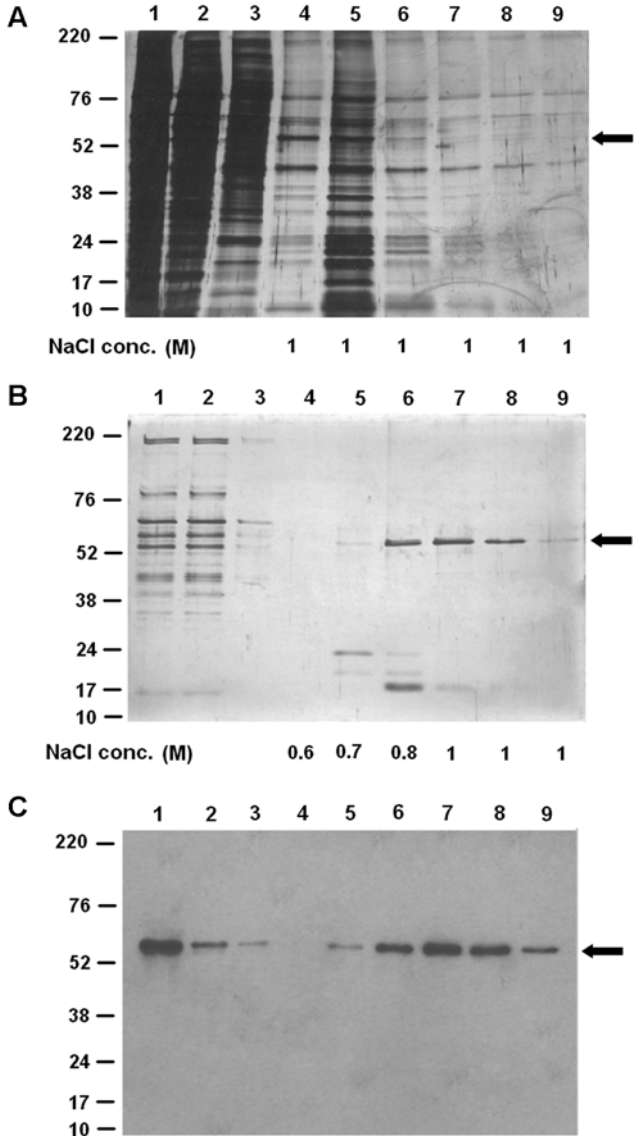

Fig. 5. Purification of HPV18 L1 by cation-exchange chromatography. (**A**) The result of SDS-PAGE when the removal of precipitated contaminants step in Fig. 1 is omitted. (**B**) The result of SDS-PAGE when it is performed. (**C**) shows Western blots of the cation-exchange chromatography in B. Lanes 1, 2 and 3 are the loaded sample, flow-through and wash, respectively. Lanes 4 - 9 correspond to fractions 1, 2, 3, 4, 5 and 6, respectively. The numbers below each figure are the NaCl concentrations of the elution buffer. The arrow indicates the molecular weight (55 kDa) of L1 protein.

Analysis of purified HPV18 L1 protein

The purity of the HPV18 L1 obtained by methods 1 and 2 was examined by SDS-PAGE and Western blotting (Fig. 6). In both cases L1 protein appeared as a strong single band of 55 kDa on SDS-PAGE and was also detected by Western blotting. 2.3 and 2.6 mg of L1 protein were obtained by methods 1 and 2, respectively from 2800 mg of protein in the initial cell lysate. Therefore, the purities and recoveries obtained by methods 1 and 2 were similar.

Conformation of purified HPV18 L1 protein

The conformation of the HPV18 L1 protein purified by heparin and cation-exchange chromatography was

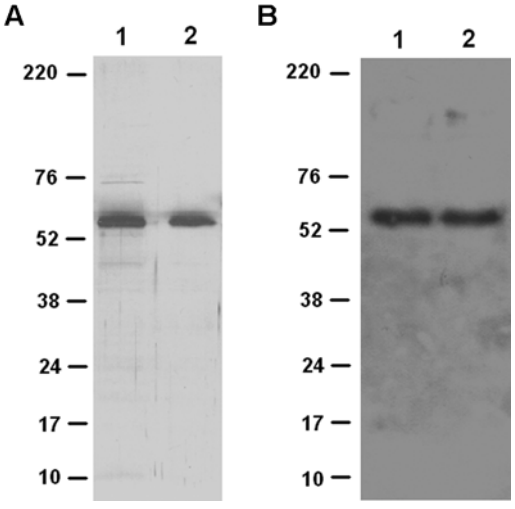

Fig. 6. SDS-PAGE and Western blot analysis of purified HPV18 L1. Equal amounts of purified HPV18 L1 protein (400 ng) were loaded. (**A**) SDS-PAGE, (**B**) Western blotting. Lanes 1 and 2 correspond to HPV18 L1 protein purified by heparin chromatography and HPV18 L1 protein purified by cation-exchange chromatography, respectively.

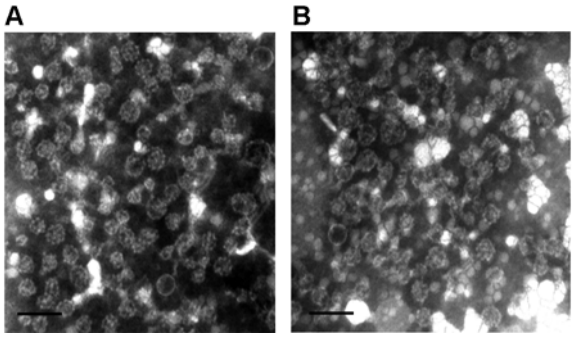

Fig. 7. Electron micrograph of HPV18 L1 protein demonstrating self-assembly into VLPs. (**A**) The conformation of HPV18 L1 purified by heparin chromatography, and (**B**) the conformation of HPV18 L1 purified by cation-exchange chromatography. Magnification is 41,000× (bars, 100 nm).

analyzed by TEM. As shown in Fig. 7, the HPV18 L1 protein obtained by both methods self-assembled into VLPs of 50 nm mean diameter, the same diameter as in the literature (Park et al., 2008; Woo et al., 2008).

DISCUSSION

Current purification methods for HPV VLP are time-consuming and not suitable for large scale purification although substantial amounts of purified HPV VLP are required for studying HPV and VLP-based vaccines. We sought a method for simplifying the chromatographic procedures for purifying HPV18 L1 protein. As shown in Fig. 3, about 80% of the protein in cell lysates was removed by ammonium sulfate precipitation and removal of precipitated contaminants step, and the ratio of L1 protein to total protein gradually increased during these two steps.

As shown in Fig. 4A and 5A, the fractions from heparin and cation-exchange chromatography contain many kinds of contaminating proteins when the removal of precipitated contaminants step in Fig. 1 is omitted. These contaminating proteins were not effectively removed even though additional chromatography was performed. Therefore, we needed to develop another method for removing these contaminants. We discovered that these contaminating proteins aggregated slowly at pH 7.2, and below 0.3 M NaCl and at 4°C during several repeat purifications. Conventionally, purification must be performed at low temperature to maintain the stability of the target protein. However, use of low temperature to maintain the VLP causes slow aggregation of the contaminants. Therefore, we induced aggregation of the contaminating proteins at RT in the presence of 0.15 M NaCl, and the solubility of HPV18 L1 was maintained under these conditions (Fig. 2B). The heparin and cation-exchange chromatography resulted in effective purification of the HPV18 L1 when the removal of precipitated contaminants step was performed whereas they were useless when the removal of precipitated contaminants step was omitted.

It has been experimentally confirmed that heparin only interacts with correctly folded HPV VLP, not incorrectly folded HPV VLP (Rommel et al., 2005; Wang et al., 2005). However, a procedure for obtaining highly purified HPV VLP using heparin chromatography had not previously been developed. In the present study, large numbers of contaminants were removed prior to the chromatography step, and this enabled one-step chromatographic purification of HPV18 VLP using heparin or cation-exchange chromatography. Recently, Knappe et al. showed that the interaction between HPV VLP and heparin was dependent on a positive charge distribution (Knappe et al., 2007). Both heparin and cation-exchange chromatography involve interactions with positively charged proteins. Therefore, we anticipate that our two chromatographic purification methods will be useful for studying the interaction between heparin and HPV, and that the heparin chromatography will be useful for selecting correctly folded HPV VLP if the L1 protein bound is further characterized. Our procedures reduce time, costs and labor, can be easily scaled up for industrial-scale purification, and should facilitate the study of prophylactic HPV vaccines.

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