

Characterization of human papillomavirus 6b L1 virus-like particles isolated from silkworms using capillary zone electrophoresis

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1 **Characterization of human papillomavirus 6b L1 virus-like**
2 **particles isolated from silkworms using capillary zone**
3 **electrophoresis**

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15 **Abstract**

16 **Human papillomavirus 6b L1 virus-like particles (VLPs) were successfully**
17 **expressed using *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid expression**
18 **system and rapidly purified using size exclusion chromatography (SEC) after**
19 **ultracentrifugation procedure and characterized by capillary zone electrophoresis**
20 **(CZE). The average capillary electrophoresis (CE) migration time was 11 min with**
21 **the relative standard deviation (RSD) of 0.3% of HPV 6b L1 VLPs. After this**
22 **threefold fractionation, the CZE samples were still further investigated by**
23 **dynamic light scattering (DLS) and immuno blotting. The versatile technique, CZE**
24 **not only proved to be a valuable tool for VLP characterization, but was also found**
25 **to be reliable and precise. Thus CZE will also be an important option for the**
26 **quality control of VLPs in pharmaceutical research level.**

27 [*Keywords:* Human papillomavirus 6b L1; Virus-like particles; Silkworm; Capillary
28 zone electrophoresis; Fractionation; Size exclusion chromatography]

29 **Introduction**

30 Subunit vaccines like virus-like particles (VLPs) are potent candidates for
31 immunization, since these VLPs are devoid of genomes but still mimics the shape of the
32 whole virus and they are efficacious to elicit immune response (1). Taking the
33 advantage of capsid protein, their autonomous folding to form structural conformation
34 and potential immune protection has drawn a lot of focuses on studies to improve their
35 ability in various applications like vaccines, drug delivery and protein trafficking
36 analysis (2). Several VLPs has been successfully developed using various expression
37 systems and analyzed by several analytical techniques to prove their conformation and
38 authenticity. Different expression systems like *E. coli*, yeast, transgenic plants,
39 mammalian cell line and insect cell lines has successively produced various VLPs and
40 several recombinant VLPs have been commercialized (3).

41 One of the contagious sexually transmitted diseases causing from genital warts to
42 cervical cancer is human papillomavirus (HPV), which is a non-enveloped DNA virus
43 with more than 100 different genotypes that infect humans. Ninety percent of HPV
44 infections causing genital infections are from HPV 6 and 11 (4). HPV are icosahedral
45 shaped viruses with 72 pentamers forming the capsid L1 proteins which function is to
46 adhere and enter into the squamous epithelial cells in the genital region forming warts

47 (5–6). Protection against HPV infection using vaccine has been successful to some
48 extent, commercially available vaccines like Cervarix[®] (Glaxosmithkline) and Gardasil[®]
49 (Merck), but these vaccines are not cost-effective to third world countries. A simple and
50 high-throughput production system from upstream and downstream processing of the
51 VLPs will be effective to reduce the cost burden of vaccines (7).

52 Baculovirus based expression system using silkworm has been competitively able
53 to express and produce recombinant proteins and VLPs. Much advancement has been
54 achieved in this system; especially, *Bombyx mori* nucleopolyhedrovirus (BmNPV)
55 bacmid-based expression is fast, inexpensive and non-laborious using silkworm larva
56 (8). Recently, we have expressed HPV 6b L1 capsid protein in silkworm expression
57 system, which successfully formed VLPs and manipulation of their epitopes (9–10).
58 However it is relatively difficult to purify VLPs from silkworm for pharmaceutical
59 research purpose, which is the key hindering step in downstream processing. The
60 method of its elimination should not pose a risk to the structure and integrity of the
61 target protein. Traditionally, protein isolation methods use combinations of filtrations
62 and chromatography-based purifications. Purifying and analyzing those recombinant
63 VLPs involves multiple steps, which in turn decreases the recovery ratio.

64 Though gel based electrophoresis is the widely used technique in laboratories

65 around the world to analyze proteins, the most common approach is sodium dodecyl
66 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Recently capillary
67 electrophoresis (CE) replaces traditional gel electrophoresis, because this technique has
68 been standardized to evaluate organic drug compounds, pharmaceutically important
69 proteins and peptides (11). In the CE, electrophoretic separations of analytes are
70 performed in a fused silica capillary tube of particular length. It is a very efficient
71 separation technique, also available for quantifying several viruses and VLPs (12). CE
72 replaces traditional gel electrophoresis step by step, as it is particularly non-laborious
73 and provides excellent separation capabilities for proteins. This flexibility makes CE a
74 very effectual alternative over the 1D-SDS-PAGE (13). Various proteins, peptides and
75 viruses were analyzed using CE as efficient electrophoretic technique (14, 21-23).
76 Improvements of capillary zone electrophoresis (CZE) as an analytical tool, are made
77 based by the prevention of denaturing or binding of analytes to the fused silica
78 capillaries using detergents and/or suitable buffers.

79 In this study, we tried to purify VLPs employing size exclusion chromatography
80 (SEC) and characterizing them by CZE, which could be a corroborated method in future
81 for high-throughput analysis of VLPs.

82

MATERIALS AND METHODS

83

Reagents and materials Sodium chloride, potassium chloride, disodium

84

hydrogen phosphate, potassium dihydrogen phosphate, sodium hydroxide, acetanilide

85

and Thesit[®] were obtained from Sigma-Aldrich (Steinheim, Germany) sodium borate

86

was purchased from Merck (Darmstadt, Germany). All samples and buffers were

87

prepared using double distilled water. Fifth-instar silkworm larva (Ehime Sansyu Co.

88

Ltd., Yahatahama, Ehime, Japan) was used for the expression of HPV L1VLPs. Fused

89

silica capillaries of 50- μ m internal diameter were purchased from Polymicro

90

Technologies (Phoenix, AZ, USA).

91

Preparation of samples and buffers Phosphate buffered saline (PBS, pH 7.4)

92

was used as lysis and elution buffer. This buffer solution was prepared by dissolving

93

137 mM of NaCl, 2.7 mM of KCl, 10 mM of NaH₂PO₄, 2 mM of KH₂PO₄ in 1000 ml

94

doubly distilled water. The background electrolyte (BGE) used for separation is 100

95

mM sodium borate which was adjusted to pH 8.3 with HCl, later 10 mM Thesit[®] was

96

added (15). Acetanilide as electro-osmotic flow marker was added into the SEC

97

fractions containing VLPs to a final concentration of 0.5 μ g/ml. All buffers and samples

98

were filtered through 0.45 μ m filter before use.

99 **Expression and purification of HPV 6b L1 VLPs** The VLPs were produced
100 from silkworm larvae using BmNPV bacmid-based expression system (9). In brief,
101 using standard molecular techniques the HPV 6b L1 gene was transformed into BmNPV
102 bacmid and the DNA of recombinant BmNPV bacmid was injected into silkworm.
103 Post-injection time of 7 days the fat body of silkworm larvae was collected and
104 sonicated.

105 **Sucrose gradient separation and size exclusion chromatography** Five
106 hundred microliters of fat body lysates were applied on to the 25–60% sucrose gradient
107 prepared in PBS (pH 7.4). Separation of contaminants was achieved by centrifugation at
108 $120,000 \times g$ for 3 hrs at 4°C. Centrifuged sample were aliquoted into 500- μ l fractions.
109 Fractions containing HPV 6b L1 VLPs were filtered through 0.45- μ m filters. Primary
110 purified HPV 6b L1 VLP fraction was applied to size exclusion chromatography (SEC).
111 Proteoma 300 A column (PSS, Mainz, Germany) packed with 5 μ m particle size of
112 porosity 300 Å (300 \times 8 mm), was equilibrated with 10 column volume of PBS (pH 7.4).
113 One hundred microliters of purified HPV L1 fractions of sucrose gradient separation
114 were injected and separated. Elution was carried out with PBS (eluent A) and PBS
115 containing 1 M sodium chloride (eluent B) using a linear gradient over 30 minutes

116 (0–100% B in 30 min) starting the gradient with the beginning of the separation. The
117 used flow rate during separation was 0.5 ml/min (16). The collected 1 ml size exclusion
118 fractions including the target protein were confirmed using western blot. The HPV 6b
119 L1 VLPs were further analyzed using CZE.

120 **Capillary zone electrophoresis** Agilent 7100 capillary electrophoresis system
121 with inbuilt diode array detector was used for the analysis (Agilent Technologies,
122 Waldbronn, Germany). Peaks were detected at 214 nm with a reference wavelength of
123 360 nm. Integration was done using in-house integration software corrected integration
124 software system (CISS, Würzburg, Germany) and data processing using Microsoft
125 EXCEL[™]. Before use, conditioning of fused capillaries was done by flushing at 1000
126 mbar of 1 M sodium hydroxide and subsequent with background electrolyte (BGE) for
127 15 min each. Preconditioning before each run was done using BGE for 2 min and
128 post-conditioning carried out using 1 M sodium hydroxide solution and rinsing with
129 double distilled water for 2 min. During preconditioning as well as post-conditioning a
130 pressure of 1.0 up to 2.0 bar was applied. The size exclusion fractions were injected
131 hydrodynamically with 100 mbar for 25 seconds into the capillary. Separation of
132 analytes was carried out at 25 kV and a mean current of 23 μ A using BGE in the inlet

133 and PBS in the outlet. Temperature was maintained at 25°C throughout the run. Samples
134 and running electrolytes were filtered and sonicated for 3 min before analyzing the
135 sample in CE system. The volumes of fifteen consecutive injections were collected in
136 one vial during the separations. During preconditioning and post-conditioning other
137 vials were used, which leads to separation from other components of the SEC fractions
138 which migrates slower than the VLP's

139 **Characterization of VLPs** Dynamic light scattering (DLS) and
140 immuno-analysis were carried out using Zetasizer nano ZS DLS analyzer fitted with
141 532 nm laser lamps (Malvern Instruments, UK), to prove that CE based separation and
142 analysis were authentic VLPs. Fractions (1 ml) of SEC and CZE were subjected to DLS
143 analysis at 25°C with 10 consecutive measurements. Western blot were carried out for
144 both SEC and CZE fractions, to confirm the presence of VLPs, the fractions were
145 subjected for SDS-PAGE and transferred to PVDF membrane using trans-blot[®] semi
146 electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA, USA). Blots were
147 developed by immuno staining with H6.J54 HPV L1 primary antibody (a kind gift from
148 Neil Christensen, Pennsylvania State University College of Medicine, USA) and
149 anti-mouse secondary antibody conjugated with HRP (GE Healthcare, Tokyo, Japan)

150 were used against VLPs. ECL plus detection reagents were used to detect HPV 6b L1
151 protein using Versa Doc Imager and analyzed using Quantity-One software (Bio-Rad).

152 **RESULTS AND DISCUSSION**

153 **Sample preparation** To achieve partial purity of the VLPs from cell lysates sucrose
154 gradient ultracentrifugation and SEC were performed. Sucrose gradient centrifugation
155 has been chosen for preliminary purification to obtain formed VLPs from lysates.
156 Fractionated sucrose gradient separated samples were re-clarified using SEC. The
157 separation was achieved by 1M NaCl, and the elution was approximately when 60–80%
158 of the NaCl gradient were performed (data not shown), which was later confirmed by
159 DLS and immunoblot detection.

160 **Characterization of VLPs using CZE** In order to perform an efficient CE
161 analysis the rinsing was initially done with the 1 M sodium hydroxide in combination
162 with the anionic surfactant SDS (17). However, even though the protein adsorption to
163 the capillary walls was reduced, the obtained relative standard deviation (RSD)
164 remained unsatisfactory. Weiss et al. employed non-ionic surfactants such as Thesit[®]
165 (15) which neutralize the anionic silanolate group on the inner capillary surface,
166 minimizing the protein adsorption to the capillary wall. Pressures from 1.0 bar up to 2.0

167 bar and further rinses with water and BGE containing Thesit[®] were employed (2 mins
168 each), which subsequently reduced the drift of the migration times to an RSD of less
169 than 1% (17–18). Note that the addition of non-ionic detergent pose a serious stability
170 problem with enveloped viruses which have lipid bilayer. However, HPV L1 is a
171 non-enveloped virus, which allows for analyzing and prevention of aggregation of VLPs
172 under the running conditions.

173 Analyzing the electropherogram of HPV 6b L1 VLPs using CISS, the migration
174 times of acetanilide and the HPV L1 VLPs were 2.5 and 11.0 min, n=10, with RSD of
175 0.3 and 0.6%, respectively (Fig. 1A), which was better than the generally accepted level
176 of 2%, proving the employed CZE method is precise. The corresponding RSD of the
177 peak areas was 6.2% (Fig. 1B). This remaining variability could be due to a still
178 persisting adsorption at the inner capillary surface. The signal to noise ratio (S/N) of
179 HPV L1 VLPs was calculated as 147 (mean of five consecutive runs) using the Agilent
180 chemstation software. This value is greater than 100, which is accepted as sufficient for
181 optimal precision of CE systems (19). If a scale up analysis to commercial scale of CZE
182 fractionation was desirable, the methods described here could be transferred to free flow
183 zone electrophoresis (FFZE), which provides very similar selectivity with much higher

184 sample loading capabilities.

185 **Hydrodynamic radius of HPV 6b L1 VLPs and immune analysis**

186 Hydrodynamic radius of VLPs in series purification of SEC and CZE was measured
187 (Fig. 2). In both samples, a peak at around 7 nm was mainly detected. This size
188 corresponds to HPV L1 capsomeres (11-12 nm) (20), indicating these peaks have HPV
189 6b L1 capsomeres and most of the purified L1 protein formed these ones. HPV 6b L1
190 proteins expressed in silkworm larvae formed various sizes of L1 VLPs (10 – over 100
191 nm) (9). In this study, samples of SEC contained mainly capsomeres and also VLPs
192 whose size ranged between 100 and 300 nm. Samples of CZE fractionation also mainly
193 show capsomeres and several sizes of VLPs, with additional species with sizes of
194 approximately 20, 50 and 700 nm. HPV 16 L1 protein purified from yeasts can be
195 disassembled into the L1 capsomeres under the low ionic strength and high pH in the
196 presence of a reducing agent (24). In addition, they formed in complete HPV VLPs and
197 uniform VLPs were obtained by dis- and re-assembling (25). This suggests that HPV 6b
198 L1 protein from silkworm formed or disassembled into some particles (the sizes of 20
199 and 50 nm) under the CZE condition. Particles with the size of 700 nm might be
200 aggregated forms because its size was too large compared to the HPV L1 particles.

201 L1 proteins purified by SEC and CZE were also detected by western blot (Fig. 3).
202 In both samples, two bands appeared. Based on the molecular weight of L1 protein, the
203 upper and lower bands correspond to full-length of L1 protein and partially degraded L1
204 protein, respectively, indicating that the main peak in CZE contains purified HPV L1
205 protein. This HPV L1 protein is prone to be degraded in the fat body of silkworm larvae
206 (9). Sometimes recombinant HPV 6b L1 proteins are detected in the fat body of
207 silkworm larvae by western blotting as several bands. Full-length of L1 protein can not
208 be separated from degraded L1 protein because full-length of L1 protein might form
209 into capsomeres and VLPs with degraded L1 protein (26).

210 CZE can also be applied to the investigation of the quality of purified
211 recombinant protein due to its reproducibility and reliability. However, individual CZE
212 condition has to be investigated in detail. For example, degraded L1 protein was not
213 completely separated from full-length L1 protein by CZE in this study, because fraction
214 collection was the main goal in the present work. It seems that several peaks in DLS
215 results might be caused by partial degradation of L1 proteins.

216 In this work we demonstrated the feasibility of CZE to analyze and quantify
217 VLPs, including fraction collection. CZE analysis fulfills the requirement of fewer
218 amounts of sample and high throughput. The achieved precision of CZE analysis is

219 essential, since VLPs e.g. for vaccines intended for human use require a high standard
220 for their quality and quantity. Even without a sophisticated protocol, analysis of CZE
221 fractions allows for further characterization by DLS and SDS-PAGE and subsequent
222 western blotting with sufficiently high sensitivity. A more elaborated fraction collection
223 protocol could further improve sensitivity and selectivity of this approach. Modifying
224 and even scaling up of this approach using other free zone and capillary
225 electromigration techniques will pave the way for new quality control standards of
226 pharmaceutical bioproducts.

227

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323

324 **Figure legends**

325 FIG. 1. Electropherogram of the HPV 6b L1 VLPs analyzed by CZE using acetanilide
326 as electroosmotic flow marker (A) and its stability during 10 CE runs (B).

327 FIG. 2. Dynamic light scattering analysis of HPV L1 VLPs. (A) Partially purified VLPs
328 after SEC. (B) Injected SEC fraction, further fractionated by CZE of HPV L1 VLPs.
329 Fraction (1 ml) was analyzed at 25°C.

330 FIG. 3. Immuno blot of HPV L1 VLPs. Lane M: Molecular weight marker, Lane 1:
331 Partially purified fraction after SEC, Lane 2: Fraction of capillary zone electrophoretic
332 analysis of HPV L1 VLPs. HPV L1 VLPs were detected against HPV L1 primary
333 antibody. Arrow indicates full-length of HPV L1 protein.





