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Reversed-phase high-performance liquid chromatography of virus-like particles

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Abstract

A quantitative reversed-phase high-performance liquid chromatography (RP-HPLC) method has been developed to detect the L1 subunit protein from virus-like particles (VLPs) of human papillomavirus (HPV). The method utilizes heat treatment with a buffer consisting of 50 m*M* Tris, pH 8.0 containing 8 *M* guanidine–HCl and 10% 2-mercaptoethanol to dissociate the VLPs into monomeric L1. Following dissociation, the sample is injected onto a C_4 or C_8 column. The L1 protein is eluted as a single, clearly resolved peak. Elution conditions have been optimized to enhance the separation of L1 from other contaminants. Based on spike recovery studies and sodium dodecyl sulfate–polyacrylamide gel electrophoretic analysis this method is suitable for quantitation of various partially purified in-process samples and can be used to facilitate purification process development.

Keywords: Virus-like particles; Papillomavirus; Proteins

1. Introduction

Papillomaviruses, a subfamily of papovaviruses, are small non-enveloped, double-stranded DNA viruses that induce genital and oral warts and play an important role in the development of certain cancers [1]. Over 70 different types of human papallomaviruses (HPVs) have been identified. A subset of these, most significantly HPV-16 and HPV-18, have been closely associated with cervical cancer [2,3].

Naturally-occurring papillomaviruses are composed of two major structural proteins, L1 and L2. The L1 protein ($M_r \approx 55\,000$) comprises 90–95% of HPV capsids and the L2 protein ($M_r \approx 72\,000$) represents the remaining 5–10%. The HPV capsid is typically composed of 72 pentameric capsomers of L1 arranged on a skewed icosahedral lattice [4–6]. L1 protein produced in a recombinant baculovirus expression system is capable of self-assembly in vivo into virus-like particles (VLPs) [7–9] which are 55 nm in diameter with a total molecular mass of approximately $20 \cdot 10^6$.

L1 is difficult to quantitate in crude extract preparations. Western blot analysis is time-consuming and only semi-quantitative. Enzyme-linked immunosorbent assay (ELISA) analysis often exhibits high backgrounds and is highly variable. For these reasons, a quantitative reversed-phase high-performance liquid chromatography (RP-HPLC) method has been developed. This method is suitable for quantitation of HPV-18 L1 protein derived from VLPs at various stages of purification and can be used to facilitate HPV purification process development.

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2. Experimental

2.1. Chemicals

Guanidine–HCl was purchased from Pierce (Rockford, IL, USA). 2-Mercaptoethanol was purchased from Research Organics (Cleveland, OH, USA). HPLC-grade water, acetonitrile and trifluoroacetic acid (TFA) were purchased from J.T. Baker (Phillipsburg, NJ, USA). BCA protein assay reagent kit was purchased from Pierce.

2.2. Sample preparation

HPV-18 samples are diluted 1:4 with dissociation buffer of 8 *M* guanidine–HCl/50 m*M* Tris, pH 8.0. After the addition of 10% 2-mercaptoethanol, samples are incubated at 55°C for 15 min.

2.3. HPLC

The HPLC analysis is performed on a Hewlett-Packard 1100 Series liquid chromatography system equipped with a G1322A vacuum degasser, a G1311A quaternary pump, a G1315A diode array detection (DAD) system, a G1313A autosampler and a G1316A thermostatted column compartment. All HPLC analyses are carried out on C_8 or C_4 reversed-phase column (25 cm×4.6 mm I.D., 5 μm particle size, 300 Å pore size) purchased from Vydac (Hesperia, CA, USA). Column temperature is set at 37°C. Solvent A is 0.1% TFA in water and solvent B is 0.075% TFA in acetonitrile. Gradient conditions used in the analysis are shown in Table 1. Flow-rate is at 1 ml/min and UV absorbance is monitored at 220 nm.

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE analysis of peak fractions was performed using 8–16% Tris–glycine gradient gel from Novex (San Diego, CA, USA) according to the method of Laemmli [10].

3. Results and discussion

3.1. Dissociation of virus-like particles

VLPs purified from recombinant baculovirus-infected insect cells are approximately 55 nm in diameter and contain a single capsid protein, L1 ($M_r \approx 55\,000$). The VLPs are typically composed of 72 pentameric capsomers of L1 arranged on a skewed icosahedral lattice. The molecular mass of the intact recombinant particle is approximately $20 \cdot 10^6$. In order to quantitate L1 protein, it is necessary to dissociate the VLPs prior to RP-HPLC analysis. A buffer comprised of 50 m*M* Tris buffer, pH 8.0 containing 8 *M* guanidine–HCl and 10% 2-mercaptoethanol was found to be effective in reducing disulfide bonds and destabilizing the VLPs to facilitate dissociation. Following a brief incubation at 55°C for 15 min, the VLP sample containing monomeric L1 protein is injected onto a Vydac C_4 or C_8 column. The L1 protein is eluted using an

Table 1
Slow and fast gradient methods used for RP-HPLC

Slow gradient method		Fast gradient method	
Time (min)	% Solvent B	Time (min)	% Solvent B
0	5	0	40
10	5	5	40
15	40	15	55
30	60	17	100
32	100	20	100
37	100		

This Table shows the gradient programs for the slow gradient and fast gradient methods, respectively. The slow gradient method is used to facilitate separation of L1 from contaminating proteins in cruder extract preparations. The fast gradient method is used for the analysis of highly purified preparations of L1.

acetonitrile–water/0.1% TFA gradient to obtain a single, clearly resolved peak (see Section 3.2).

3.2. Chromatography of HPV-18 and L1 protein identification

Examples of chromatograms obtained from various in-process samples are shown in Fig. 1. L1 protein eluted at approximately 24.3 min using a slow gradient method (Table 1) and 13.8 min using a fast gradient method (Table 1) on a Vydac C_8 column. The peak at 11.9 min under slow gradient conditions does not contain protein-like material by SDS–PAGE analysis, Pierce BCA protein assay and by protein-reactive staining using amido black. The slow gradient method was used to analyze cruder samples at earlier stages of purification to allow for better separation of L1 protein from other contaminants, while the fast gradient method was utilized to analyze samples at later stages of purification in a shorter period of time. The retention time of L1 protein on a C_4 column is 0.2 min shorter than the retention time on a C_8 column using the fast gradient method. Either the C_4 or C_8 column can be used with equal efficiency of separation providing calibration is carried out with purified reference standard material.

The L1 containing peak fractions from various samples were collected, dried down and resuspended in SDS–PAGE sample buffer. The identity of the L1 protein was confirmed by SDS–PAGE (Fig. 2) and Western blot analysis (data not shown). In Fig. 2, lanes 7 and 9, an extra band was observed with a molecular mass near 40 000 in the chromatography step 3 product. This extra band is a L1 degradation product. This band is reduced or eliminated after protease inhibitors were added to the early purification steps in the process.

3.3. Calibration curve and linearity

A calibration curve was generated using HPV-18 VLPs purified by sucrose gradient centrifugation. Peak area is linear over a range of approximately 2–30 μg of L1 protein. Two C_8 columns and one C_4 column were used for analysis and regression parameters are summarized in Table 2.

3.4. Comparison of L1 quantitation by RP-HPLC and Western blot analysis

Several HPV-18 in-process samples were analyzed and quantitated by both RP-HPLC assay and Western blot analysis. The data are summarized in Table 3. The L1 concentration for all samples is higher by RP-HPLC than by semi-quantitative Western blot analysis. The agreement is actually quite good considering that semi-quantitative Western blot analysis is based on detection by staining and quantitation by densitometry both of which exhibit greater inherent variability than UV detection.

3.5. Spiking studies

In order to determine whether the signal strength of L1 protein is comparable in crude and purified VLP preparations, spiking studies were carried out. For these studies, a known amount of a purified VLP preparation was spiked into various partially purified VLP preparations and spike recovery was calculated according to the following equation:

$$\%\ \text{spike recovery} = \frac{(\text{total sample L1} - \text{unspiked L1})}{\text{spiked L1}}$$

The total sample L1 is the amount of L1 in the sample plus the spike. The unspiked L1 is the amount of L1 in the sample. The spiked L1 is the amount of L1 spike alone. The data are summarized in Table 4. Spike recoveries in these preparations range from 92–99% suggesting that this RP-HPLC method is suitable for quantitation of partially purified samples and therefore can be used to facilitate HPV process development.

3.6. Quantitation of HPV production in bioreactors

The production of L1 VLPs following baculovirus infection of insect cells has previously been monitored by Western blot analysis, which is tedious, time-consuming and only semi-quantitative. In order to determine the feasibility of monitoring L1 content using the newly developed RP-HPLC method, a small amount of cell paste was homogenized with 20 m*M* sodium phosphate, 1% Triton X-100, pH 6.8. This sample was diluted 1:4 with the dissociation

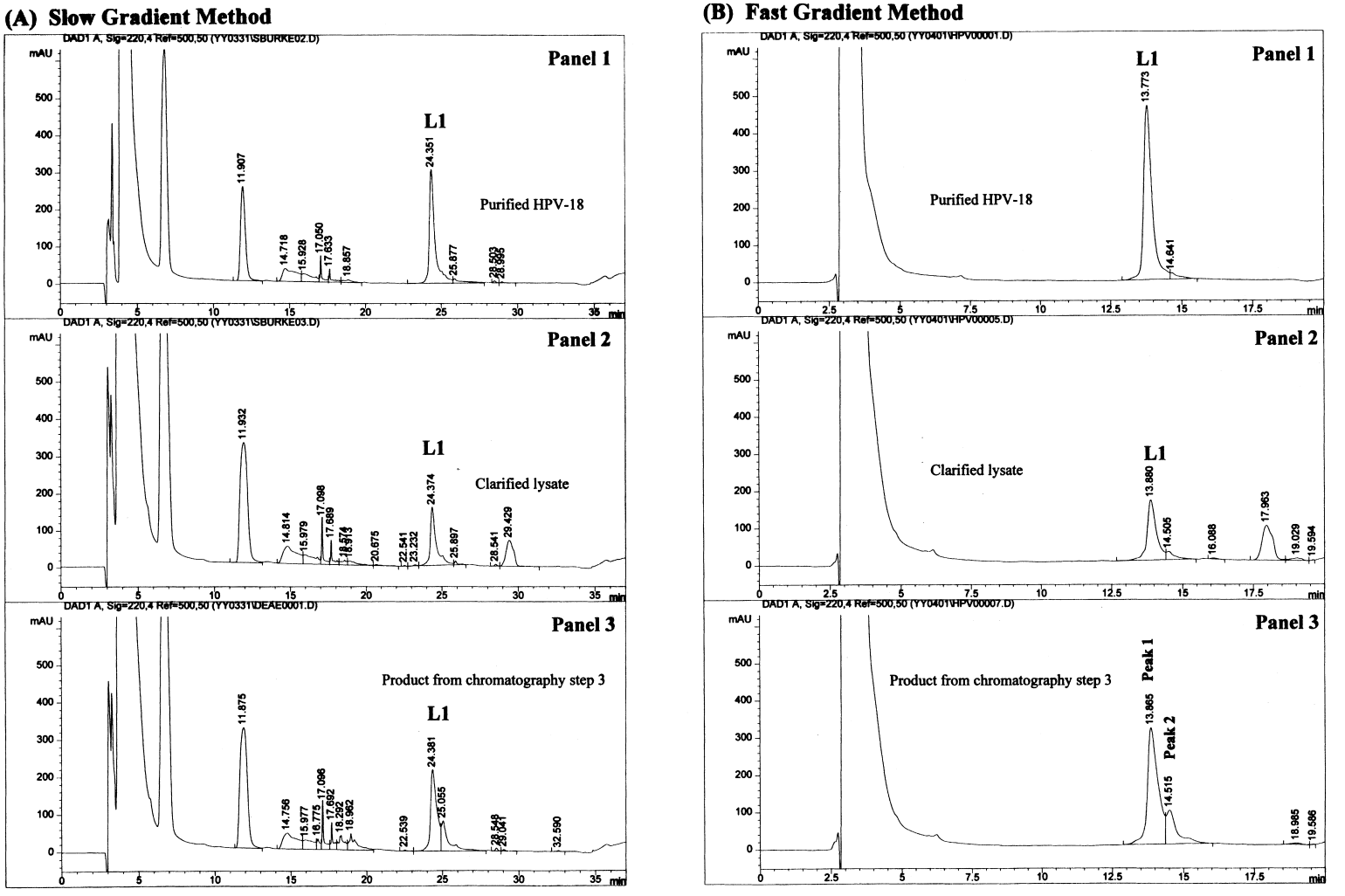

Fig. 1. RP-HPLC profiles of various HPV-18 samples on a Vydac C_8 column (25 cm×4.6 mm I.D., 5 μm particle size, 300 Å pore size). Panel A depicts the slow gradient method and panel B depicts the fast gradient method. Detailed gradient conditions are listed in Table 1. Samples analyzed were: (1) 50 μl of purified HPV-18, (2) 100 μl of clarified lysate and (3) 100 μl of chromatography step 3 product. Samples in panels A and B were prepared by the same purification procedure but at different times.

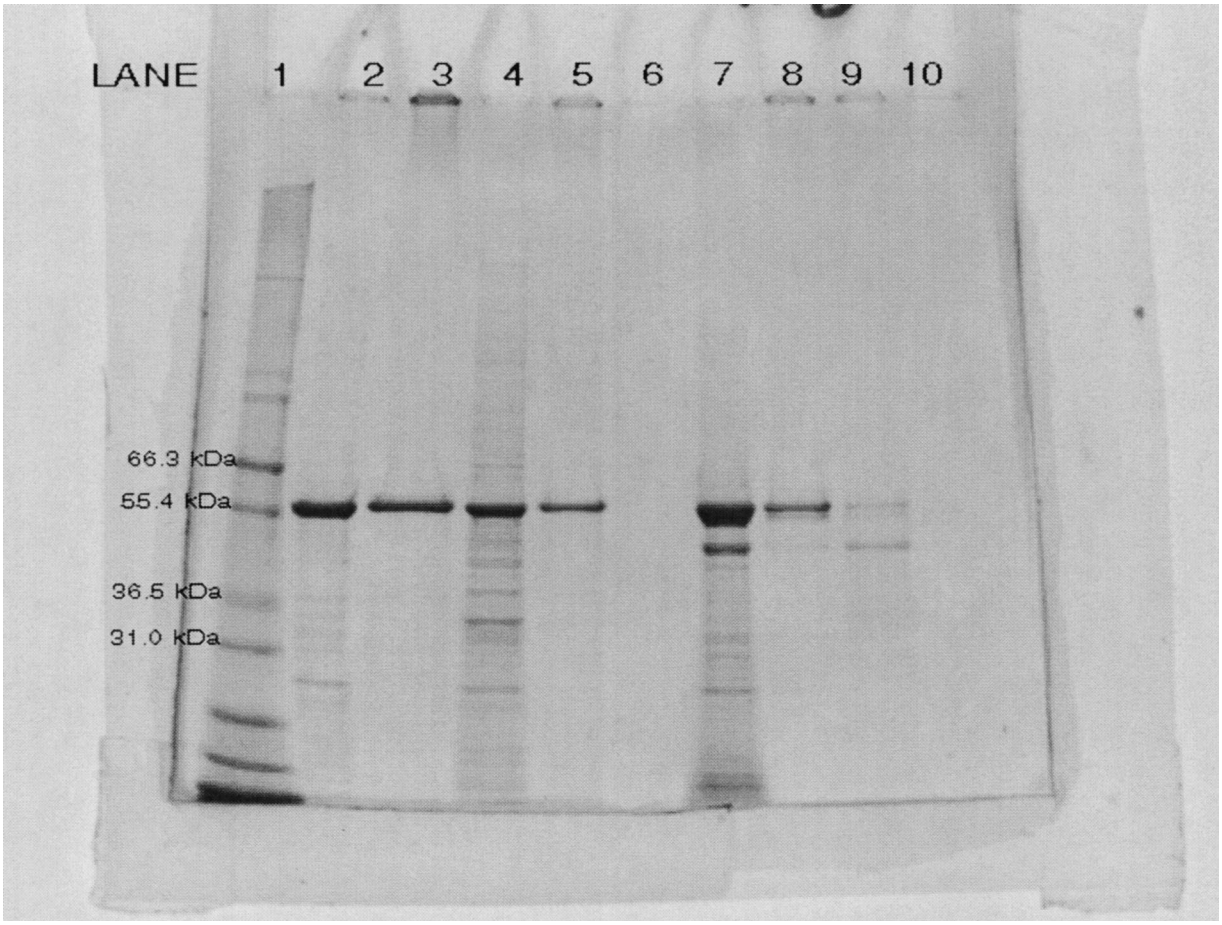

Fig. 2. SDS–PAGE analysis of HPV-18 L1 protein. Lane 1 is the molecular mass standards (200 000, 116 300, 97 400, 66 300, 55 400, 36 500, 31 000, 21 500, 14 400, 6000). The remaining lanes are as follows: purified HPV-18 (lane 2), L1 peak collected from Fig. 1/panel B1 (lane 3), clarified lysate (lane 4), L1 peak collected from Fig. 1/panel B2 (lane 5), chromatography step 3 product (lane 7), peak 1 collected from Fig. 1/panel B3 (lane 8), peak 2 collected from Fig. 1/panel B3 (lane 9). Peaks 1 and 2 are labeled in Fig. 1.

Table 2
Regression parameters of the calibration curves

Column/run	Slope	Correlation coefficient (R^2)
C_8 column I/run 1	0.001836	0.9991
C_8 column I/run 2	0.001824	0.9992
C_8 column I/run 3	0.001772	0.9900
C_8 column II	0.001933	0.9893
C_4 column	0.002326	0.9971

This Table shows the regression parameters for multiple runs and different C_8 reversed-phase columns as well as a comparison with C_4 reversed-phase column. Linear regression analysis was performed using Microsoft Excel. Column I was a Vydac C_8 column (lot No. 960424), column II was a Vydac C_8 column from a different lot (lot No. 961009) and the C_4 column was also a Vydac column (lot No. 960423).

buffer and heated for 15 min at 55°C. The sample was injected onto a C_8 column using the slow gradient method, and the chromatographic separation is shown in Fig. 3. Fractions of pre-L1 (22.3 to 23.8 min), L1 (23.8 to 25.0 min) and post-L1 (25.0 to 26.5 min) were collected, dried down and resuspended in SDS–PAGE sample buffer. SDS–PAGE analysis (Fig. 4) indicates that the L1 fraction exhibits the same molecular mass as purified L1 and there is no detectable L1 protein in the fractions preceding and following L1 peak. The lower-molecular-mass components of L1 peak in Fig. 4 are likely clipped forms of L1 generated during cell culture and early purifi-

Table 3
Comparison of L1 concentration by RP-HPLC and Western blot analysis

Sample	L1 concentration by RP-HPLC (mg/ml)	L1 concentration by Western blot (mg/ml)
Crude cell lysate	1.080	0.970
Clarified lysate	0.288	0.180
Chromatography step 1	0.264	0.173
Chromatography step 2	0.088	0.072
Chromatography step 4	0.226	0.195

Table 4
Spike recoveries

Sample	Spiked L1 (μg)	Unspiked L1 (μg)	Total L1 (μg)	Spike recovery (%)
Clarified lysate	6.98	5.18	11.69	93.3
Chromatography step 2	3.49	1.59	4.79	91.7
Chromatography step 3	5.81	4.14	9.90	99.1

This Table shows spike recovery calculated according to the following equation:% spike recovery $= \frac{(\text{total L1} - \text{unspiked L1})}{\text{spiked L1}}$
The total L1 is the amount of L1 in the sample plus spike. The unspiked L1 is the amount of L1 in the sample. The spiked L1 is the amount of L1 spike alone.

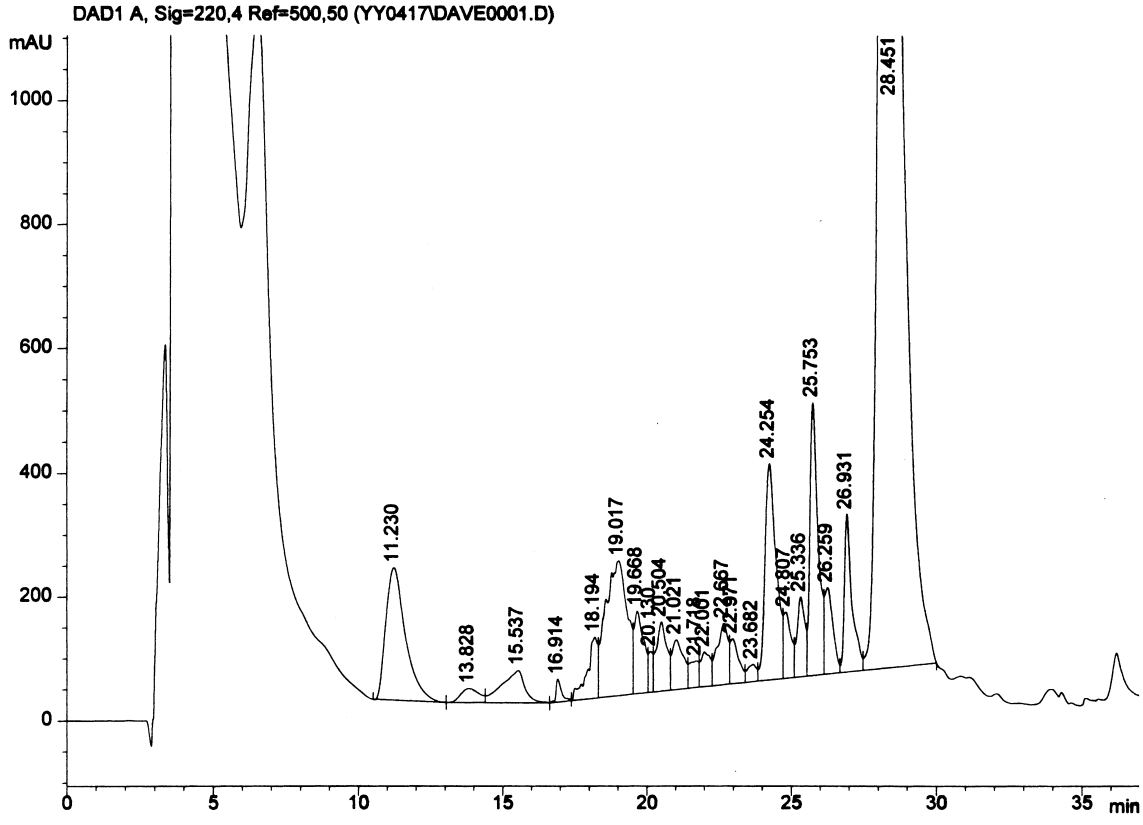

Fig. 3. RP-HPLC analysis of 100 μl HPV-18 crude cell lysate using slow gradient method on a Vydac C_8 column (25 cm×4.6 mm I.D., 5 μm particle size, 300 Å pore size).

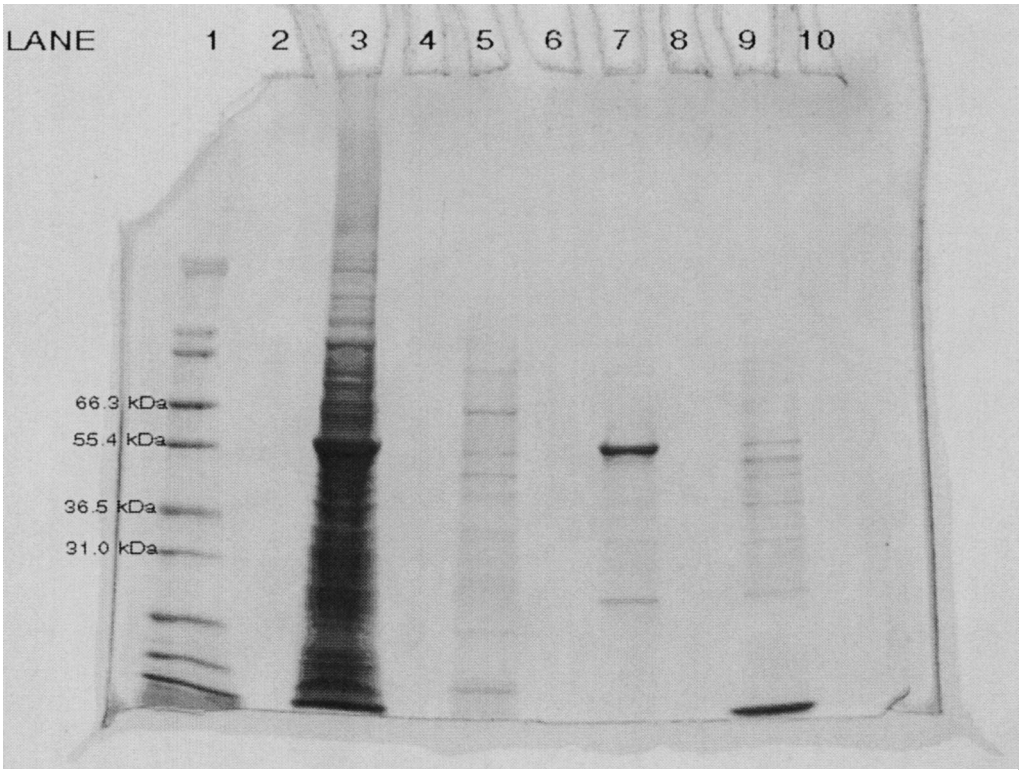

Fig. 4. SDS–PAGE analysis of HPV-18 crude cell lysate. Lane 1 is molecular mass standards (as in Fig. 2). The remaining lanes are as follows: HPV-18 crude cell lysate (lane 3), collected pre-L1 fractions 22.3 to 23.8 min (lane 5), collected L1 fractions 23.8 to 25.0 min (lane 7), collected post-L1 fractions 25.0 to 26.5 min (lane 9).

cation steps. The formation of these degradation products can be minimized by including protease inhibitors in the purification buffers. These results show that this RP-HPLC method could be used as a relatively rapid method to quantitate L1 production in bioreactors (less than 2 h compared to 2 days by Western blot analysis).

4. Conclusion

A quantitative RP-HPLC method has been developed to analyze L1 protein from HPV VLPs. Following initial heat treatment with dissociation buffer, the L1 containing sample is injected onto a C_4 or C_8 column and eluted in an acetonitrile–water/0.1% TFA gradient with detection at 220 nm. Gradient conditions can be manipulated to facilitate the L1 protein elution as a single, clearly resolved peak. The identity of L1 protein was confirmed by SDS–PAGE and Western blot analysis. Peak area is linear over a range of approximately 2–30 μg of L1 protein. Spiking studies using a purified VLP preparation in crude VLP preparations have demonstrated that signal strength is comparable in both preparations and spike recoveries in these preparations range from 92–99% suggesting that this method is suitable for quantitation of in-process samples including crude cell lysates.

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