Some Comments Concerning Purification of Bovine Enterovirus

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Summary: There are various purification procedures for enteroviruses, depending upon the purpose of the experiment. HeLa S3 cells were grown at 37°C in a Roux bottle for virus multiplication. For the MZ-468 strain of bovine enterovirus, about 11 Roux bottles were sufficient for purification. 10 % polyethylene glycol 6000 was selected for the first virus precipitation step. This purification process gave a recovery of 43 %. A sucrose density gradient (SDG) was successfully formed with a hand-made apparatus, and was fractionated with a hand-made collecting system using a peristaltic pump. CsCl bouyant density gradient centrifugation was superior to SDG centrifugation. Even after one cycle of purification with CsCl, a comparatively pure virus preparation was obtained. The purity was checked by electron microscopy and SDS-PAGE analysis of virion proteins. Infectious virion RNA was obtained from the virions which were purified according to this method.

Key words: enterovirus—purification—CsCl bouyant density centrifugation—electron microscopy—SDS-polyacrylamide gel electrophoresis

Introduction

The virion of enterovirus is mainly composed of one single-stranded RNA and four capsid proteins. Because of this simple structure, it is easy to handle. Recently it has become a worthwhile target for molecular biology and immunology.

Purification procedures for this virus have been improved very much (e.g. Kew et al. 1984). The virus in the harvested fluid was directly precipitated using a large rotor. From the resulting pellets, the virus was banded on the CsCl gradient (Lee et al. 1979). Depending on the purpose, there are many other purification procedures (e.g. Nottay et al. 1981). In each case the most appropriate procedure was chosen. Each researcher often uses an easily available or hand-made apparatus.

The present paper first, describes the appropriate procedures for enterovirus purification; and secondly makes comments on the problems encountered during the purification processes.

Materials and Methods

Viruses and cell

HaLa S3 cells were usually grown at 37°C in Roux bottles in Eagle minimum essential medium (NISSUI SEIYAKU Co. Ltd.) containing 10 % bovine serum, and 0.292 g glutamine and 0.8 g sodium bicarbonate per liter. For the suspension culture an Eagle minimum essential medium described above, without CaCl2, was used. On the other hand the standard MEM described above supplemented with 0.1 % carboxymethyl cellulose was used. Dead cells were counted after staining with 0.3 %
trypan blue solution. Some characterizations and isolation procedures of bovine enteroviruses have been reported (Taguchi, 1981). In this report the MZ-468 strain of bovine enterovirus was preferentially used. Poliovirus sabin 1 and Mahoney strains were also used as a technical control.

The viruses were grown in HeLa S3 cells. These cells (approximately $2 \times 10^7$ cells/50 ml) were washed once with PBS (+), and inoculated with 2 plaque-forming units per cell of virus. When severe cell destruction was seen (usually 16 hours after infection), the cells and supernatant were frozen and thawed at least once. The harvested fluid was clarified by centrifugation at 8,000 × g for 20 minutes at 4°C. 20% polyethylene glycol (PEG) 6000 containing 1M NaCl was added to the supernatant, and stored overnight at 10°C. After centrifugation at 8,000 × g for 20 minutes, the pellet was dissolved in a small volume of PBS (-). After centrifugation at 10,000 × g for 10 minutes, the pellet was dissolved in PBS (-) and centrifuged again. For the dose response of PEG-6000 on the recovery of virus, different concentrations of PEG-6000 were used. Infectivity of the virus was assayed by plaque formation. Details of this technique have been described elsewhere (e.g. Yamamoto et al. 1968). HeLa S3 cells were used in this titration.

One-step growth of virus

HeLa S3 cells (approximately $4.4 \times 10^6$ cells/5 ml) were washed once with 5 ml PBS (+), and then inoculated with 5 plaque forming units per cell of the MZ-468 strain. After adsorption for 1 hour at room temperature, the cells were washed twice with 5 ml PBS (+) and incubated in fresh medium at 37°C. At various time intervals the infectivities were assayed by the plaque technique.

Sucrose density gradient centrifugation (SDG)

The sucrose gradients (15 to 35 %) were formed by a hand-made apparatus using an air pressure pump and two disposable syringes connected with a small silicon tube. Each solution in the syringe contained appropriate amounts of sucrose in tris-buffer (20 mM Tris-HCl, pH 7.8, 5 mM MgCl$_2$, 100 mM KCl). The virus pellets which were obtained from the harvested fluid by centrifugation at 40,000 rpm for 1 hour in a RP 55T rotor (Hitachi Co. Ltd.) were dissolved in 1 ml PBS (-), and centrifuged at 40,000 rpm for 10 minutes with a RPV 50T rotor (Hitachi Co. Ltd.). The gradients were fractionated into 50 fractions (0.6 ml or 23 drops per fraction) using a peristaltic pump and a collecting apparatus (Model 184, ISCO). At the same time A 260 nm was monitored using flow cell equipment (spectrophotometer 220A, Hitachi Co. Ltd), otherwise the fraction was diluted with 3 volumes of PBS (-), then monitored for A 260 and A 280 nm in a quartz cell. For virion RNA, centrifugation at 40,000 rpm for 30 minutes was performed in the RPV 50T rotor.

CsCl buoyant density centrifugation

The pellets described above were also dissolved in 8 ml PBS (-) containing 4.3 g CsCl (final density is 1.34 g/ml). The virus was banded on the CsCl gradient in a RP 55T rotor (Hitachi Co. Ltd.) at 40,000 rpm for 18 hours. After fractionation into 43 fractions using a peristaltic pump, the refractive index and A 260 (or A 280) nm were determined. On the other hand pre-formed CsCl gradient systems were also often used in the present experiments. The range of this gradient was wider than the above system. The sucrose or CsCl in the peak fraction was removed by ultrafiltration (Centriflo CF-25, Amicon), or dialysed against an appropriate buffer overnight.

Electron microscopy

A sample was applied on formvar coated grids and negatively stained with 1% po-
tassium phosphotungstate. The sample on these grids were observed with an electron microscope (H-500, Hitachi Co. Ltd.)

**SDS-polyacrylamide gel electrophoresis**

Details of this technique were reported previously (Hamada et al. 1984). The Gels were stained using a Silver Stain Kit (Bio-Rad Co. Ltd.).

**Acrylamide-agarose gel electrophoresis**

The techniques were the same as previously reported (Kurata et al. 1980).

**Results**

The titer of the harvested enterovirus (MZ-468 strain) was always near 10^7 pfu/ml when the virus was grown in a Roux bottle (about 127 cm²). According to the physical properties of the poliovirus, the amount of harvested virus fluid was estimated which would be sufficient for purification (Table 1) and it was concluded that at least 11 Roux bottles were needed for purification as long as we pursued the virus by spectrophotometry.

Fig. 1 illustrates the linear relationship between virus concentration and the number of plaques. It was concluded that one infectious particle was sufficient to produce one plaque. Therefore it was possible to quantify these virions by plaque formation. Fig. 2 shows the one-step growth curve of the MZ-468 virus in the HeLa S3 cell culture. This virus had a latent period of approximately 1 hour, and completed maturation within four to five hours after infection. The average virus yield per infected cell equals 126 pfu.

The growth curves for HeLa S3 cells in suspension culture (200 rpm) are represented in Fig. 3. With standard MEM plus 0.1% carboxymethyl cellulose (open circle), only maintenance of viable cells was possible. HeLa cells in standard MEM minus CaCl₂ grew normally for 3 days but abnormal cells (including fused cells) appeared after 4 days of incubation. As a result it is impossible to use this suspension system for virus multiplication at present.

Recovery of the viruses after sedimentation with different concentrations of

![Fig. 1. Relationship between the concentration of the MZ-468 strain and the number of plaques formed.](image)

**TABLE 1**

Properties of poliovirus

<table>
<thead>
<tr>
<th>MW of virion</th>
<th>E at 260 nm</th>
<th>physical particles/infected unit</th>
<th>A 260/280 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.5 × 10⁶</td>
<td>67</td>
<td>1,000 ± 500/pfu</td>
<td>1.69</td>
</tr>
</tbody>
</table>

*Refered from Schwerdt, C.E. and Schaffer, F.L. (1955)

polio virus solution (1 %)

0.01 g/(8.5 × 10⁶) × 6 × 10²³ = 7.1 × 10¹⁴ particles/ml

7.1 × 10¹⁴/67 = 1.1 × 10¹³ particles/A 260 nm

1.1 × 10³³/1,000/(5 × 10⁸)/0.5 A 260 nm = 11 Roux bottles
PEG-6000 is shown in Fig. 4. The plateau of efficient recovery was observed at 5 to 15% PEG-6000.

Table 2 represents the recoveries of the MZ-468 strain in each step of the purification process. The recovery of virus before applying it to the CsCl gradient was 43%. The half of the virus seemed to be lost during the earlier step.

Fig. 5 illustrates the SDG patterns for mouse liver ribosomes obtained in various systems which contained sedimentation and fractionation apparatus. a and d systems gave a better isolation of the peaks than b and c. Separation of the two peaks by a and d were the same. Therefore the simpler system, d, was adopted. Fig. 6 represents the SDG centrifugation of the MZ-468 virus. A single sharp peak was observed. The infectivity of the virus in this peak was accompanied by an absor-

<table>
<thead>
<tr>
<th>process</th>
<th>infectivity (pfu)</th>
</tr>
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<tbody>
<tr>
<td>Cultures</td>
<td>$150 \times 10^9$</td>
</tr>
<tr>
<td>Sup. after PEG-6000 tr.</td>
<td>$7 \times 10^5$</td>
</tr>
<tr>
<td>Before centrif. at 40,000 rpm 1 hr</td>
<td>$68 \times 10^9$</td>
</tr>
<tr>
<td>Sup. after centrif.</td>
<td>$36 \times 10^5$</td>
</tr>
<tr>
<td>Ppt. after centrif.</td>
<td>$64 \times 10^9$</td>
</tr>
<tr>
<td>recovery (%)</td>
<td>43</td>
</tr>
</tbody>
</table>
Fig. 5. Sucrose density gradient (SDG) centrifugation patterns with various kinds of apparatus. a. Gradient former made of an acryl resin and a hand-made collecting apparatus. b. Gradient former made of an acryl resin (GR-40, TOYO Co. Ltd.) and a commercial collecting system (ALC-2L, TOYO Co. Ltd.); c. Gradient former made of an acryl resin (see above) and a hand-made collecting apparatus. d. Hand-made gradient former (see MATERIALS AND METHODS) and a hand-made collecting apparatus.

Fig. 6. SDG centrifugation of the MZ-468 strain. Each bar on the abscissa represents the infectivity of the virus in each fraction.
absence of 260 nm. This peak was located slightly ahead of the 130S ribosome dimer (sedimentation marker). Results from buoyant density gradient centrifugation of the MZ-468 strain are shown in Fig. 7. A single sharp peak usually appeared around 1.34 (g/ml). The peak of infectivity for each fraction was banded at the peak absorbance of 260 nm. Fig. 8 shows the absorption spectrum of the virus. The maximum was at 257 nm and the minimum at 243 nm. The ratio of optical densities of virus purified by SDG at 260 and 280 nm was 1.41. The ratio of CsCl purified virus was 1.63. Therefore it was concluded that the purification using CsCl was superior to purification with SDG. The virion polypeptides of the MZ-468 strain were separated by SDS-PAGE (Fig. 9). Virion polypeptides VP1 to VP4 and VP0 were clearly shown. A trace of cell protein was seen above VP0. The relationship between the intensity of staining and the amount of protein is described in Fig. 10. Except for the low amounts of protein, linearity was observed. Fig. 11 represents the SDG pattern of virion RNA. A single 35S virion RNA was seen ahead of the 28S ribosomal RNA marker. Fig. 12 shows the dose response for infectivity of the 35S virion RNA. Table 3 summarizes the details. These results also demonstrated that no infectious virions had contaminated the sample. Fig. 13 shows the fine structure of the MZ-468 strain. The particle size was 27 nm. No cell derived debris was seen, indicating efficient purification of the bovine enterovirus.

Fig. 7. Bouyant density gradient centrifugation patterns of the MZ-468 strain. Each bar on the abscissa shows the infectivity of the virus in a given fraction. X represents the bouyant density evaluated from the optical density.

Fig. 8. Absorption spectra of purified viruses. The dotted line shows the spectrum of the sample before each centrifugation. a. SDG centrifugation (see the legend of Fig. 6). b. CsCl bouyant density gradient centrifugation.
Fig. 9. SDS-PAGE (12.5 %) of the MZ-468 strain. On the left side of the figure the molecular weight evaluated from marker proteins are indicated. A to E are the diluted viruses. F is the original purified virus.

Fig. 10. Relationship between the amount of virus protein and the absorption at 500 nm. The amount of virus protein was evaluated from the physical property table (Table. 1). Absorption at 500 nm was measured with a chromatoscaner (CS-930, Shimadzu Co.Ltd.). Width of 0.1mm was selected as the scanning steps along x axis and the reflection mode was adopted. The ordinate indicates the integration of absorbance along the lane at intervals of 0.1 mm.

Fig. 11. SDG centrifugation (see the legend of Fig. 5) patterns of the MZ-468 virion RNA. 28S and 18S mouse ribosomal RNAs were used as markers.
Fig. 12. The relationship between the concentration of virion RNA and the infectivity.

**TABLE 3**

Infectivity of the MZ-468 virion RNA on HeLa cells

<table>
<thead>
<tr>
<th>treatment</th>
<th>number of plaque</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^{-2}</td>
<td>10^{-3}</td>
<td>10^{-4}</td>
<td>10^{-5}</td>
</tr>
<tr>
<td>virion RNA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+RNase A</td>
<td>−*</td>
<td>122±15</td>
<td>9±1</td>
<td>0</td>
</tr>
<tr>
<td>virion RNA</td>
<td>−*</td>
<td>72±10</td>
<td>2±2</td>
<td>0</td>
</tr>
<tr>
<td>in Buffer</td>
<td>−*</td>
<td>50±5</td>
<td>6±2</td>
<td>0</td>
</tr>
<tr>
<td>virion/PBS (−)</td>
<td>−*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+RNase A</td>
<td></td>
<td></td>
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</tbody>
</table>

* Counting was not performed.
** Average ± SD

Fig. 13. The fine structure of the MZ-468 virus. The bar is 10 nm.

Discussion

Suspension culture of cells is considered to be a useful method for virus multiplication followed by purification. When using MEM minus CaCl₂, a significant number of abnormal cells appeared after 4 days of incubation. Consequently, a sufficient amount of cells could not be obtained. The cells may need sufficient time to adapt and
grow in the suspension culture.

The virus precipitation method with PEG-6000 was convenient. Only a centrifuge and a flask are necessary and moreover it gives high performance for the cost. 5 % PEG-6000 was sufficient for the precipitation of the virus. To account for a volumetric error and for easiness of handling, 10 % PEG was used. During the purification process unexpected amounts of virus (cf. Table 1) were often obtained. This may be partly due to a non-infectious virion in the sample. These non-infectious particles in the virus preparation must be accounted for especially in immunological studies.

Repeated cycles of the purification procedure usually reduced the recovery of the virus. For example, most of the virus was lost during recentrifugation (SDG) even when initiated with about 50 Roux bottles. This phenomenon may be due to adhesion of the virus to the centrifugation tube or loss during the concentrating process. Recentrifugation on a CsCl gradient also reduced the recovery. To minimize the loss of virus it may be necessary to use a buffer with a detergent (NP-40 or SDS) for centrifugation (Nottay et al. 1981).

Before centrifugation on CsCl it was necessary to centrifuge the sample at 15,000 rpm for 5 min. Without the centrifugation, it was difficult to clearly separate the peaks for the standard particles from the peaks for the empty capsids and cellular debris.

Silver staining of protein is highly sensitive. Even 200 ng of virus protein was easily detected on the slab gel. A linear relationship between the amount of virus protein and the absorbance at 500 nm was observed. The amount of virus protein can therefore be evaluated from the absorbance at 500 nm after electrophoresis.

When the virion RNA was incubated at 37°C, even without RNase, the infectivity was completely lost. Denoya et al. (1978) reported that a nuclease activity was located inside the FMDV particles. The loss of infectivity may be due to the trace of endogenous nuclease activity in the sample. However there was no problem with the RNase T1 oligonucleotide mapping study of this virus (Hamada et al. 1985).

References


