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Foot and Mouth Disease Virus Concentration and Purification by Affinity Chromatography

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ABSTRACT

Foot and mouth disease virus, (FMDV) from a crude cell lysate was purified in a single step by affinity chromatography with heparin as a ligand. The virus eluted from an Heparin-Ultrogel A_4R column at 1*M* sodium chloride in 10 m*M* sodium phosphate buffer, pH 7.0, while most cell protein and albumin did so at lower concentrations of sodium chloride in the same buffer. Purity of the eluted fraction containing the virus was assessed by SDS-PAGE, HPLC, ultracentrifugation, and UV absorption spectrum. With this method, intact viral particles are recovered in high yield (over 90%) and specific virus purity increases nearly 1000-fold. The capacity of the chromatographic matrix for the virus was found to be 1.1 mg viral mass per mL of hydrated gel.

Index Entries: Foot and mouth disease virus; purification; affinity chromatography.

INTRODUCTION

Abundant and very pure viral preparations, preferably with infective particles, are necessary for systematic biochemical and functional analysis of viruses (*1*). In addition, extensive purification is desirable when viruses

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are going to be used for vaccine preparation because of contaminants could raise indesirable hypersensitivity phenomena.

Several methods have been reported for concentration and purification of the foot and mouth disease virus (FMDV) such as poly(ethyleneglycol) precipitation (*2–4*), sodium sulphate precipitation (*3*), ultrafiltration (*3,5*), and aqueous two-phase partition (*3*). In general, they display poor specificity and the viral particle remains accompanied by a considerable amount of contaminants.

Einarsson et al. (*6,7*) utilized affinity chromatography with Heparin-Sepharose and sulfonated derivatives of polysaccharides for the purification of hepatitis B surface antigen (HBsAg). Baba et al. (*8*) showed that dextran sulfate, heparin and heparan sulfate interact with various viruses. In addition, several methods have been patented for purification of different viruses using a sulphuric acid ester of cellulose or a crosslinked polysaccharide as a gel for chromatography (*9–12*). Downing et al. (*1*) purified respiratory syncytial virus from crude cell lysate by a single pass through a Matrex Cellufine column, a cellulose support activated with a sulfate ester.

In this paper we report the concentration and purification of FMDV by affinity chromatography with heparin as a ligand. This method is simple, fast and easy-to-scale-up, giving high yields of intact viral particles, with high antigenic potency and essentially free of contaminants.

MATERIALS AND METHODS

Foot and Mouth Disease Virus

Type 0, Caseros FMDV was propagated for 48 h in a stirred system in baby hamster kidney cells BHK 21, clone 13 of Mac Pherson and Stocker (*13*), 2.5×10^6 cells/mL, suspended in an Eagles growth medium, containing 10% tryptose phosphate broth and either 1% or 5% normal bovine serum. The infected tissue fluids from these cultures were collected and stored in 500 mL amounts in sealed bottles at −40°C. Control virus was purified by zonal centrifugation through a preformed sucrose gradient (*14,15*).

Spectrophotometric Measurements

A Metrolab model 2500 spectrophotometer was used. The $E_{1cm1\%}$ of 76 for the whole virus, corrected by light scattering, was used according to Bachrach et al. (*16,17*).

UV spectra were recorded between 200 and 300 nm.

Prechromatographic Treatment

Virus-free cellular culture homogenate, obtained by freezing and thawing, or FMDV cell lysate were centrifuged at 3000 rpm for 10 min to

remove cell debris, and 2.5 mL of supernatant was applied to a Sephadex G-25 PD-10 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in 10 m*M* sodium phosphate buffer, 0.15*M* NaCl, pH 7.0, and eluted at a flow rate of 1 mL/min with 3.5 mL of the same buffer. This conditioning treatment provides samples suitable for the chromatographic step.

Ribonuclease-Treatment of Samples

Bovine pancreatic ribonuclease type I-A, dissolved in 10 m*M* sodium phosphate buffer, pH 7.0, was added to FMDV samples to a final concentration of 10 μg/mL, and incubated in a water bath at 26°C for 60 min. Digestion was terminated by passage through a PD-10 column equilibrated and eluted with 10 m*M* sodium phosphate buffer, 0.15*M* NaCl, pH 7.0.

Affinity Purification of FMDV

In a typical experiment, 3.5 mL of conditioned sample was applied to a 1 × 3 cm column of Heparin-Ultrogel A_4R (IBF Biotechniques, Villeneuve la Garenne, France) equilibrated in 10 m*M* sodium phosphate buffer, 0.15*M* NaCl, pH 7.0. After washing of the column with starting buffer to remove any unbound material (approx 5 mL), the adsorbed material was eluted with 5 mL of 10 m*M* sodium phosphate buffer, 1.25*M* NaCl, pH 7.0. Fractions of 1 mL of both eluates were collected and monitored at 259 nm. The flow rate was 0.5 mL/min.

The control virus (30 μg) was chromatographed under the same conditions.

In gradient-elution experiments, after the washing step, adsorbed components were eluted by successive steps of 5 mL of 10 m*M* sodium phosphate buffer, pH 7.0 with the addition of 0.3, 0.5, 1.0, and 1.25*M* NaCl. The elution was accomplished at a flow rate of 0.5 mL/min, and 1 mL fractions were collected and monitored at 259 nm.

Total Protein Determination

Total protein was measured by the method of Bradford (*18*).

Virus Infectivity Measurement

Viability of the virus was indicated by its infectivity. The number of plaque forming units (PFU) per mL in infective cell culture fluids and purified virus was determined in bovine kidney monolayer cultures by the plaque procedure described by Bachrach et al. (*19*).

SDS-Polyacrylamide Gel Electrophoresis

This was performed in a Pharmacia Phast System instrument with 12.5% homogeneous gels and Coomassie Blue staining, according to the instructions of the manufacturer.

Breakthrough Curve

The capacity of the column resin for the FMDV was determined by pumping the conditioned isodensity centrifugation purified virus through a 0.5 × 3 cm Heparin-Ultrogel A_4R column at a flow rate of 0.12 mL/min and noting the point where FMDV began to pass through the column. The virus was detected in the column effluent by a dot-blot assay using a Mab against FMDV protein and a peroxidase-conjugated anti-Mab.

Isodensity Ultracentrifugation

A Spinco model L ultracentrifuge (Beckman Instruments, Spinco Division, Palo Alto, CA) with a swinging bucket rotor SW-65, was used.

The nonlinear isodensity experiments started with 1.5 mL of chromatographic fractions layered over 3 mL of a preformed gradient of CsCl. This initial gradient was made in the cold by layering first 0.75 mL of 41.33% (w/w) CsCl having a density of 1.4514 g/mL, 0.75 mL of 39.67% (w/w) CsCl, 0.75 mL of 38.02% (w/w), and finally 0.75 mL 37.19% (w/w) with a density of 1.398 g/mL. The samples were ultracentrifuged at 40,000 rpm for 3 h, between 4°C and 13°C, to zone all the virus within the CsCl layer. The buffer solvent for CsCl was 0.5*M* Tris-HCl, pH 7.6.

Bottoms of the tubes were punctured to permit viral light-scattering zones to be separated dropwise from the overlaying protein.

HPLC Protein Measurements

Fractions from affinity chromatography were monitored for protein by RP-HPLC using a Kontron equipment with a Vydac 218TP54 column (25 cm height). One hundred microliters of each fraction was injected and, after 5 min of isocratic elution, a 30 min linear gradient from 10–60% acetonitrile-0.1% TFA was started. Flow rate was 1 mL/min and the effluent was monitored at 280 nm.

RESULTS AND DISCUSSION

It has been shown that FMDV is inactivated below pH 7.0, in an isotonic medium (*20*) and dissociates into protein and ribonucleic acid (RNA) at low ionic strength (μ = 0.002) at pH 7.5 (*16,17*). An inverse relationship exists between the pH and ionic strength required for stabilization (*21*). In accord with the above, in this work, the pH of the phosphate buffer was adjusted to 7.0 and NaCl was added to adjust the range of ionic strength between μ = 0.165 and μ = 1.0, in order to preserve the stability of the virus.

Figure 1 shows the affinity purification elution pattern from infective (1-A) and non-infective (1-B) cellular fluids. Two fractions were obtained, one in the washing step (I) and the other after elution with 1.25*M* NaCl in

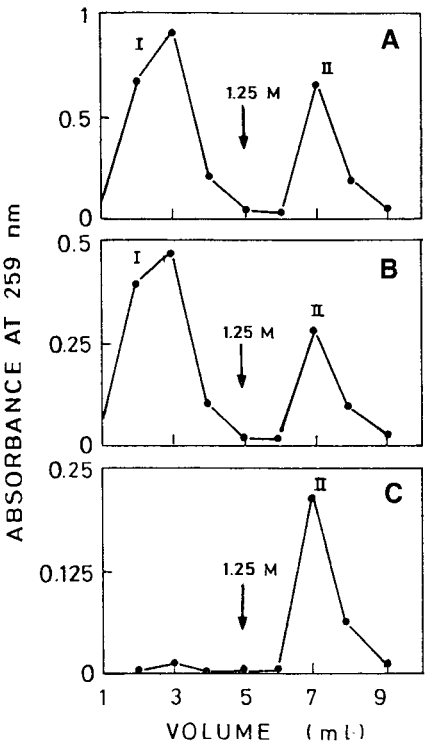

Fig. 1. Elution patterns from infective (**A**) and noninfective (**B**) cellular fluids, and control FMDV (**C**)—all of them chromatographed on Heparin-Ultrogel A_4R. After exchange to the equilibrating buffer (10 m*M* sodium phosphate buffer, 0.15*M* NaCl, pH 7.0) as described in Materials and Methods, 3.5 mL of conditioned samples (equivalent to 2.5 mL cell lysate) were loaded on to the affinity column (1 × 3 cm). It was washed with 5 mL of starting buffer and elution was accomplished by raising the concentration of NaCl to 1.25*M* in the starting buffer. Flow rate was 0.5 mL/min and 1 mL fractions were collected and their absorbance measured at 259 nm.

the equilibrating buffer (II). In both cases, Fraction I spectra resembles those of proteins, with a typical maximum at 280 nm. Virus control eluted under the same conditions (1-C) presents only one peak eluting at 1.25*M* NaCl in the equilibrating buffer (II), the spectrum having a maximum of absorbance at 259 nm (Fig. 2). The UV spectrum of Fraction II from noninfective cellular fluid is similar to those of Fraction I but, in the case of Fraction II from infective cellular fluid, the UV spectrum is different from the preceding ones; however, said spectrum shows a plateau between 259 and 280 nm, thus suggesting the coexistence of virus and proteins. The infectivity tests showed that active viral particles are only found in Fraction II from infective cellular fluid.

Protein determination of the material in peaks from Fig. 1A and B indicated that in both cases approx 15% of the total protein content was retained by the column and only eluted when the concentration of NaCl in the equilibrating buffer was increased to 1.25*M*.

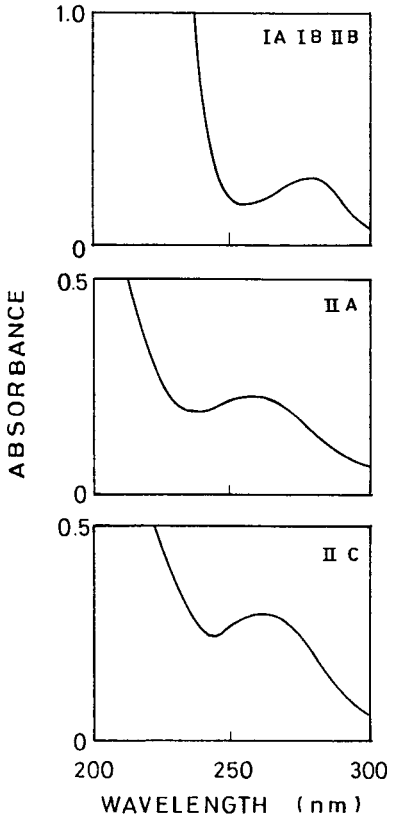

Fig. 2. UV spectra (200-300 nm) of the material in peaks I-A, II-A, I-B, II-B, and II-C from Fig. 1.

From 2.405 mg of total protein and $10^{8.5}$ PFU loaded into the column, 2.100 mg of protein and no infectivity were found in Fraction I-A, while, in Fraction II-A, the total protein content was 0.305 mg and the activity, $10^{8.5}$ PFU, thus indicating a very significative purification factor as well as a high yield.

In order to improve the selectivity of the chromatographic separation, a step gradient was utilized for elution. Figure 3 A and B shows the gradient elution pattern from the infective and noninfective cellular fluid respectively. Each different NaCl concentration in the elution buffer produced a peak of eluted material.

UV spectra of the materials in Peaks 1 and 2 (Fig. 3 A,B) indicated that both contained proteins, and Peaks 3 to 5 (Fig. 3A), are formed by ribonucleoproteins (not shown).

The total protein amount chromatographed was distributed as follows: Peak 1:88%, Peak 2:9.1%, and Peak 3:2.8%. Little protein amount was found in Peak 4 (0.1%) and Peak 5 (below the sensitivity of the assay method used), in spite of their high adsorption at 259 nm. Infectivity analysis showed that only the materials in Peaks 4 and 5 were infective (Table 1).

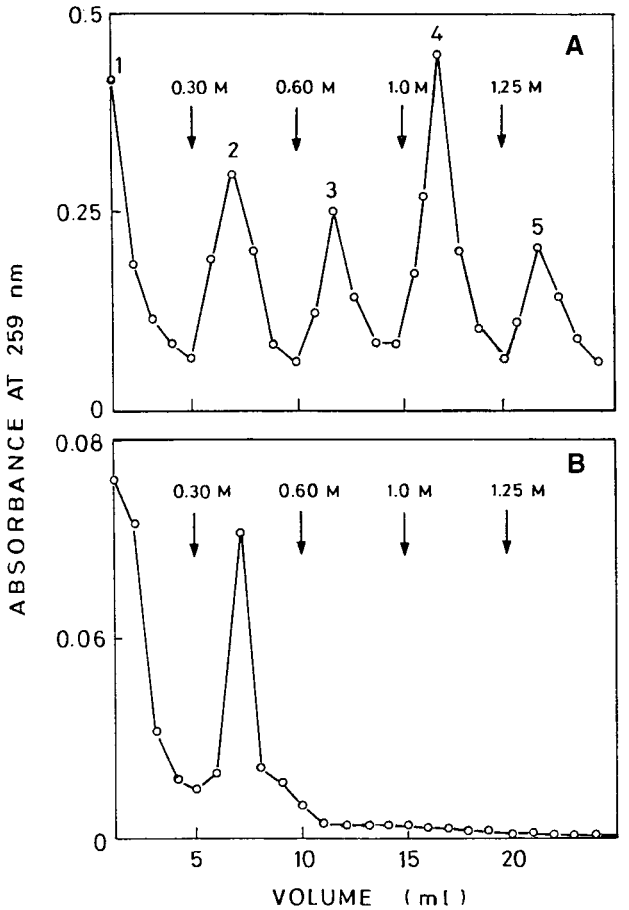

Fig. 3. Step gradient elution patterns from infective (**A**) and noninfective (**B**) cellular fluids chromatographed on Heparin-Ultrogel A_4R. Conditions were the same as in Fig. 1, but elution was performed in gradient with steps of 0.3, 0.6, 1.0, and 1.25*M* NaCl in the equilibrating buffer.

Table 1
Affinity Chromatography Purification
of Foot and Mouth Disease Virus from an Infective Cell Lysate

	Conditioned cell lysate	Chromatographic Fraction (Fig. 3)				
		1	2	3	4	5
Total protein, μg	2590	2268	236	71	2.3	—
Total protein, %	100.0	88.0	9.1	2.8	0.1	—
Infectivity, PFU	10^9	—	—	—	10^9	10^7

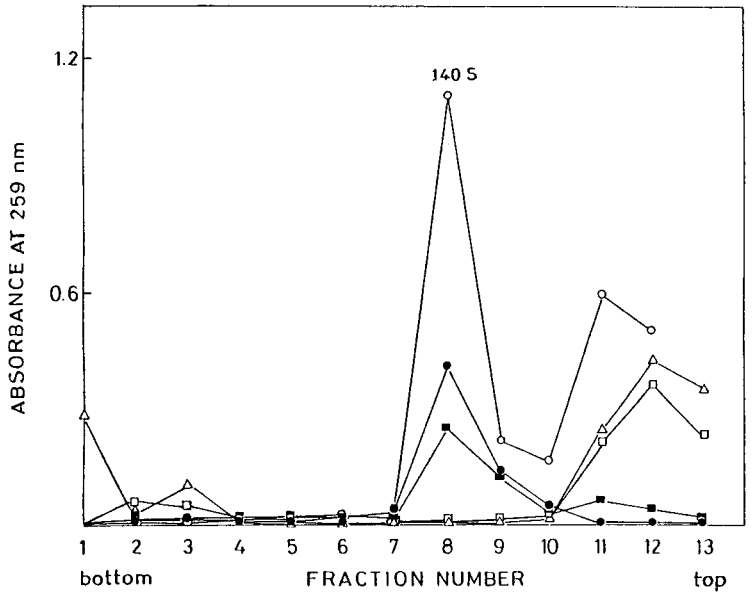

Fig. 4. Isodensity ultracentrifugation pattern of the fractions obtained by affinity chromatography (Fig. 3). 1.5 mL of each chromatographic fraction or control virus were layered over 3 mL of a preformed gradient of CsCl, as described in Materials and Methods. Ultracentrifugation was carried out at 40,000 rpm for 3 h, between 4°C and 13°C. (■) Control virus; (□) Fraction 2; (△) Fraction 3; (○) Fraction 4; (●) Fraction 5.

Previous RNAase treatment of the sample produced a 55–60% decrease in Peak 3 and a concomitant increase in Peaks 1 and 2, thus suggesting the presence of partially broken viral particles, or even nucleic acids which, on account of the enzymatic treatment, have lost their affinity for the chromatographic matrix (not shown).

The control virus was eluted in Peak 4, with NaCl 1.0*M* and was not affected either in infectivity or in elution position by the enzymatic treatment (not shown).

Further evidence on the peak composition was obtained by analytical ultracentrifugation in a CsCl gradient. Results are shown in Fig. 4. Only materials in Peaks 4 and 5 have a sedimentation coefficient of 140 S, fully agreeing with that of the control virus, thus indicating that both fractions show different affinity for the chromatographic matrix. Peak 3, with a UV-spectrum resembling that of a ribonucleoprotein but not being infective, have a sedimentation coefficient lower than 140 S, thus suggesting that it is composed of inactive nonintact virus or nucleic acids, retained by the chromatographic matrix but with a lower affinity. Peaks 1 and 2 are composed by proteins as judged by their UV-spectra and protein measurements.

In order to assess the purification obtained, samples from the affinity separation fractions were analyzed by SDS-PAGE and HPLC in comparison to cell lysate. SDS-PAGE of cell lysate revealed a lot of protein bands, mainly at molecular weight 120, 66, 58, 36, 31, 29, and 26 kDa. Material

from Peak 1 showed bands at molecular weight 120, 66, 58, and 29 kDa, from Peak 2 at 58 kDa, and from Peak 3 at 29 and 26 kDa. Material from Peaks 4 and 5, in contrast, did not reveal protein bands by Coomassie Blue staining, but viral proteins after transference to a nitrocellulose membrane and specific staining with a Mab conjugated to peroxidase.

The presence of proteins in material from Peaks 1, 2, and 3, and its absence in that from Peaks 4 and 5 was also demonstrated by HPLC (Fig.5).

These results, taken as a whole, evidence a high degree of FMDV purification by the affinity process.

Virus concentration, in relation to protein concentration, otherwise known as the specific virus purity, increased nearly 1000-fold, while the infectivity remained unchanged, thus indicating no inactivation of the viruses in the chromatographic process.

Breakthrough curve indicated that the capacity of the chromatographic matrix for FMDV is approx 1.1 mg viral mass per mL of hydrated gel, thus indicating that the purification process will also concentrate the virus.

Since the FMDV is harvested at low concentrations in the presence of large amounts of tissue debris, purification by differential centrifugation alone is very difficult. Although isodensity methods have been extensively reported for nucleic acids and virus purification (*22*), they are time consuming and lead to prohibitive losses of particle and infectivity (*23*). The same problems have been reported for influenza virus purified by zonal centrifugation, where this method yielded only 60% of the total virus loaded (*24*). Furthermore, Njayou and Quash (*25*) developed an affinity chromatographic purification procedure for the measles virus and compared the yield of the virus thus prepared with that from the ultracentrifugation method. The yield obtained with the affinity method was essentially 100%, whereas with the latter technique yield was of only 2.4%. These losses are probably because of irreversible aggregation and damage to the virus particle because of the high speed centrifugation. In the affinity chromatographic method proposed, processing times are short and denaturation losses are minimal, on account of it is being performed under selected conditions in order to preserve viral stability (*21*).

Ultrafiltration is a convenient method to concentrate the FMDV (*5*), but undesirable contaminating proteins are concentrated in the same way, thus making the process useful only as a previous step to further purification. The same is true in the poly(ethyleneglycol) precipitation method (*2*), where centrifugation or filtration is required to obtain the viral particle. In contrast, contaminating proteins are removed in the washing step in the technique herein described.

In conclusion, the method proposed allows concentration and purification of the intact viral particle from the cellular culture, and can be made available for the process scale in order to obtain a vaccine free of contaminating proteins and nucleoproteins or nucleic acids that could be responsible for hypersensivity problems (*26*).

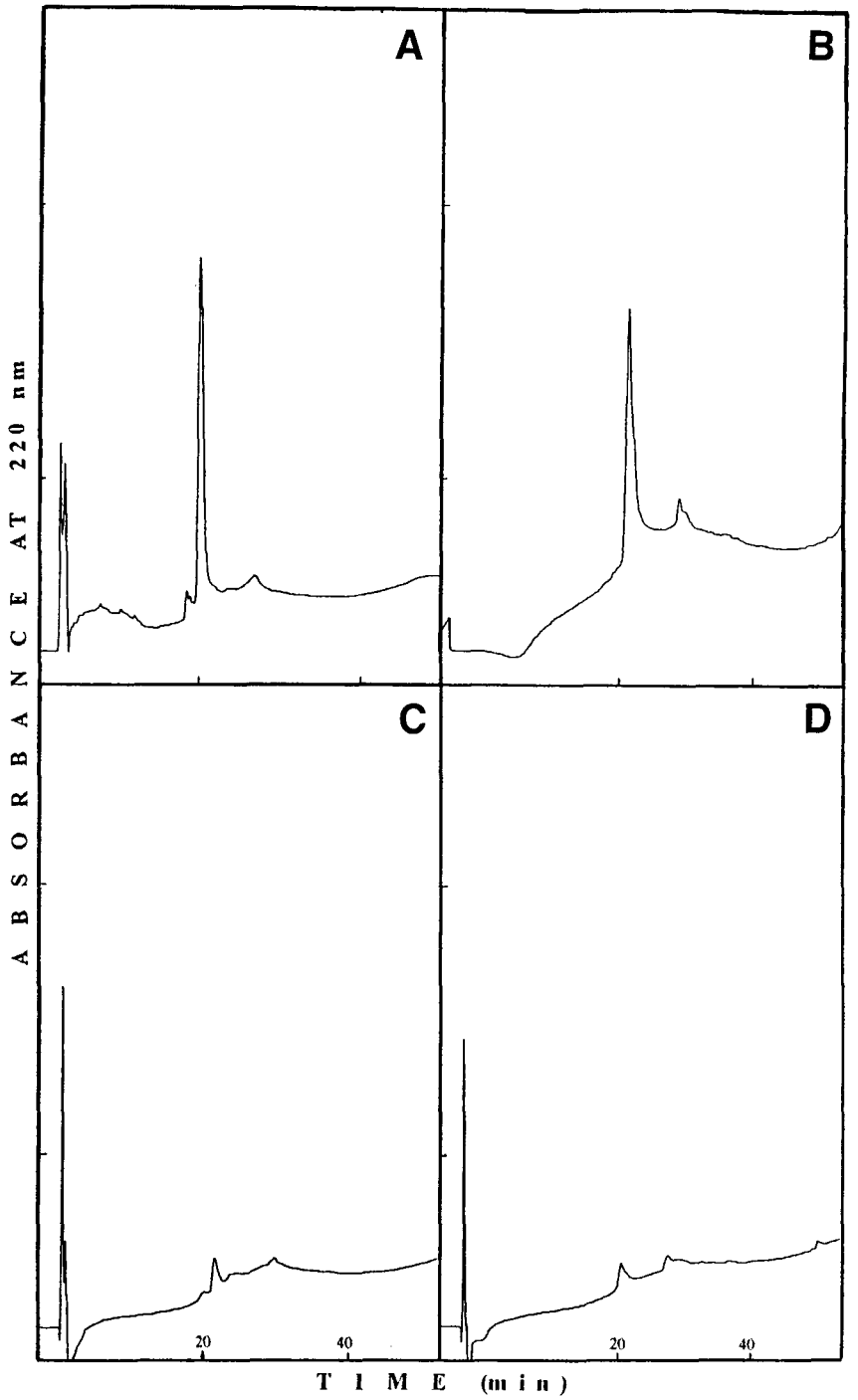

Fig. 5. RP-HPLC patterns of material in Peaks 1 to 5 from Fig. 3. One hundred microliters of each fraction was injected and, after 5 min of isocratic elution, a 30 min linear gradient from 10–60% acetonitrile-0.1% TFA was started. The effluent was monitored for its absorbance at 280 nm. Flow rate was 1 mL/min. (**A**) Conditioned infective cell lysate; (**B**), (**C**) and (**D**) Peaks 1, 2, and 3 from Fig. 3, respectively.

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