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DETECTION OF SENDAI VIRUS PROTEIN BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COMBINED WITH IMMUNO-CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography (RP-HPLC) was applied to the detection of Sendai virus F_2 protein. Immunoaffinity chromatography was used prior to RP-HPLC to increase the specificity. With this combined method, the presence of F_2 protein in complex solutions could be demonstrated within 150 min. Using RP-HPLC, F_2 protein could be detected in amounts down to 20 ng, which could be reduced to 1 ng by application of a microbore column.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) for the separation and purification of proteins and peptides is well established[1]. Especially gradient elution on ion-exchange or reversed-phase (RP) columns is suitable for the separation and concentration of proteins from large volumes of dilute solutions. The combination of high resolution, rapidity and low detection limits makes RP-HPLC particularly suitable for the detection of small amounts of proteins.

The aim of this study was to investigate the application of RP-HPLC to the rapid detection of viral protein as a marker of virus infection. To increase the specificity, immunoaffinity chromatography was used for the pre-purification of viral protein prior to RP-HPLC. Sendai virus, a paramyxovirus of mice, was used as a model. It contains only a few structural proteins, of which the three envelope-associated proteins, the matrix protein (M), the haemagglutinin–neuraminidase protein (HN) and the fusion protein (F), can be selectively extracted with non-ionic detergents[2]. The F-protein consists of two subunits, F_1 and F_2, which are connected by disulphide bridges. The smaller one, F_2 (M_r = 13 500), was used in this study as the indicator protein.

Using the method described, the presence of this protein in complex solutions could be demonstrated unambiguously in less than 2.5 h.

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EXPERIMENTAL

Immunoaffinity chromatography

F_2 protein, purified with RP-HPLC from a 2% Triton extract of Sendai virus, as previously described[3], was used for the preparation of rabbit antiserum. Anti-F_2 IgG was covalently linked to protein A–Sepharose CL-4B (Pharmacia, Uppsala, Sweden) by cross-linking the IgG–protein A complex with dimethyl pimelimidate (Pierce, Rockford, IL, U.S.A.), as described by Schneider *et al.*[4]. The immuno-matrix was used as a column absorbent (35 × 5 mm I.D.). For convenience the column was used in a standard isocratic HPLC system, the injector of which was equipped with a 1.5-ml loop. Samples were passed through the column, which had been equilibrated with phosphate-buffered saline (pH 7.2) containing 0.05% Triton X-100, at a flow-rate of 0.2 ml/min. When not in use, the column was stored in this buffer, containing 0.02% of sodium azide, at 4°C. After washing with equilibration buffer, protein specifically bound to the immuno-matrix was eluted with 1.5 ml of 0.05 *M* glycine–HCl (pH 2.3), containing 0.05% of Triton X-100, at 1.0 ml/min. The eluted fractions were immediately neutralized with 1/10 volume of 0.5 *M* Na_2HPO_4.

RP-HPLC

Chromatography was performed with a system consisting of an LKB 2150 pump, a Rheodyne 7125 injector and a Waters 441 detector. Low-pressure gradients were generated by an Atom microcomputer (Acorn Computers, Cambridge, U.K.), interfaced with an LFYA three-way inert solenoid valve (Lee Company, Westbrook, CT, U.S.A.)[5].

Prior to analysis, samples containing F_2 protein were treated with Amberlite XAD-2 to remove detergent and with dithiothreitol to reduce protein disulphide bonds for 20 min at room temperature[3]. Proteins were chromatographed on a column (40 × 4.6 mm I.D.) packed with Supelcosil LC-318 (Supelco, Bellefonte, PA, U.S.A.) and detected at 205 nm. Elution was performed with a linear gradient from 12 m*M* hydrochloric acid in water–ethanol (15:10, v/v) to water–ethanol–*n*-butanol (15:8:2, v/v) in 5 min[3], at a flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

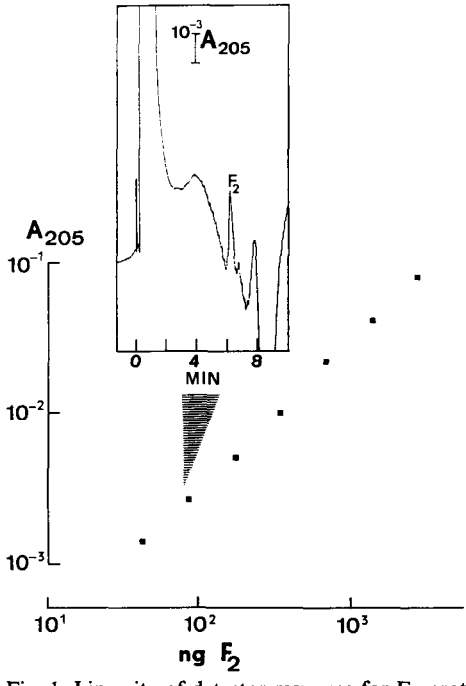
The purpose of this study was to apply a combination of immunoaffinity chromatography and RP-HPLC to the rapid detection of small amounts of viral protein. The first step identifies the viral protein immunologically and the second confirms the identity of the protein by its specific elution characteristics and allows quantification. The detection limit of the latter was determined with detergent extracts of Sendai virus, containing different amounts of F_2 protein. The amounts of F_2 protein applied to the reversed-phase column and to the immunoaffinity column were calculated from the known amount of the protein present in a more concentrated solution. The F_2 concentration of the latter was determined by amino acid analysis[3]. The linearity of the detector response and the detection limit were investigated by testing these samples with RP-HPLC alone, after removal of the detergent. The latter was necessary, because Triton X-100 was eluted close to the F_2 protein[3]. The identity of the F_2 protein was confirmed by sodium dodecylsulphate gel electrophoresis. From

Fig. 1 it can be seen that the detector response was linear over the range tested (40 ng to 5 μg of F_2 protein). The detection limit was about 20 ng of F_2 under these conditions.

To test the complete method, microgram amounts of detergent-extracted Sendai virus proteins were added to 1-ml samples that contained 100–200 times as much human serum albumin. This mixture was passed through the affinity column. Analysis of the collected fractions by RP-HPLC showed that the albumin was totally recovered in the unabsorbed fraction, by comparing the peak height with that obtained with an equal amount of albumin that had not been subjected to immunoaffinity chromatography. The F_2 protein was recovered in the fraction obtained after elution with glycine–HCl (Fig. 2).

Comparable results were obtained when extracted Sendai proteins (containing 100–200 ng of F_2 protein) were added to 200 μl of a supernatant of lung homogenate of non-infected mice. In both experiments, about 40% of F_2 was recovered in the glycine–HCl fraction. This immuno-matrix has now been used over 90 times without apparent loss of immuno-reactivity.

Recently, a great improvement in the method was achieved by the application of microbore columns. Microbore columns (50 × 1.0 mm I.D.) were used with other-



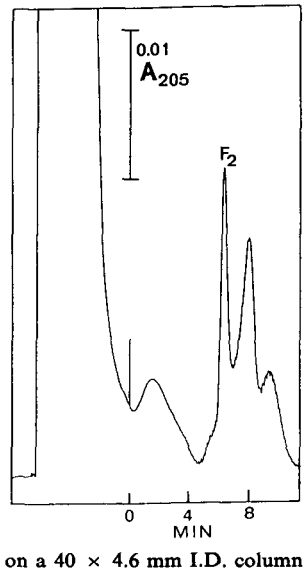


Fig. 1. Linearity of detector response for F_2 protein chromatographed on a 40 × 4.6 mm I.D. column packed with Supelcosil LC-318. The inserted chromatogram, corresponding with the indicated data point (85 ng of F_2), is shown for estimation of sensitivity.

Fig. 2. RP-HPLC analysis of the glycine–HCl fraction obtained after immunoaffinity chromatography of a 1-ml sample, containing 100 μg of human serum albumin and detergent-extracted Sendai virus proteins. The amount of F_2 protein in this sample was 1 μg. After injection the gradient was started at $t = 0$.

wise conventional gradient HPLC equipment at flow-rates of 0.1–0.2 ml/min. This resulted in a 20-fold increase in the sensitivity of detection and allowed the analysis of amounts of protein down to 1 ng, as will be described elsewhere[6].

In conclusion, with the method described, which combines immunoaffinity chromatography and RP-HPLC, the presence of the Sendai virus F_2 protein could be demonstrated unambiguously. The method is fast, as the whole procedure can be carried out within 2.5 h. Therefore, the method is promising for the rapid detection of nanogram amounts of viral or other specific proteins in complex solutions.

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