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HIGH-PRESSURE CHROMATOGRAPHY OF TOBACCO MOSAIC VIRUS ON SPHERON GELS

M. ČECH

Institute of Experimental Botany, Czechoslovak Academy of Sciences, Prague 6 (Czechoslovakia)

M. JELÍNKOVÁ

Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, 162 06 Prague 6 (Czechoslovakia)

and

J. ČOUPEK

Laboratory Instruments Works, 162 03 Prague 6 (Czechoslovakia)

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SUMMARY

High-pressure chromatography on Spheron 300 BTD and Spheron 1000 was used to separate tobacco mosaic virus from other components of an extract from infected tobacco leaves, including F_1, a protein having a molecular weight of 0.58×10^6. Comparative experiments have shown that the virus is not adsorbed by the carrier and is eluted in the free volume of the column; the virus is free from contamination by vegetable-juice components and is regained quantitatively. The virus can be separated partially, but distinctly, from the F_1 protein by using a column of microgranular Spheron 300. Polyhydric phenols in the vegetable extract are adsorbed on Spheron and eluted from the column after a delay.

INTRODUCTION

Owing to their mechanical and chemical resistance, hydroxyalkyl methacrylate gels of the Spheron type can be used as packings in high-pressure gel-permeation chromatography, providing optimal hydrodynamic conditions in addition to a constant flow through the column. Calibration experiments with fractionated dextran showed that gels could separate hydrophilic macromolecules within a broad range of molecular weights. Thus, Spheron 1000 separates them within a range of molecular weights of 3×10^3 to 10^6 (see ref. 1). Experiments have shown that Spheron 1000 can be used in the separation of artificial mixtures of up to eight proteins[2] within a molecular-weight range of 12,000 to 150,000. Good resolution in the high-molecular-weight range has permitted the distinct separation of a virus that enhances the activity of mouse-serum L-lactate:NAD-oxidoreductase from the accompanying enzyme proper[3]. These findings were an incentive for us to examine the upper region of the

separation range of some types of Spheron gels on a model system consisting of purified tobacco mosaic virus (TMV) and on protein-containing extracts from healthy and TMV-infected tobaccos.

EXPERIMENTAL

Materials and methods

Plant material, virus, infection tests. Tobacco plants (*Nicotiana tabacum* L. "*Samsun*"), some 6 weeks old, were inoculated with a purified vulgare stain of the TMV, and the leaves were collected 12–14 days after being infected. Leaf extracts from uninfected and infected plants were prepared as follows. Freshly collected leaves (100 g) were homogenized with 100 ml of extraction solution (0.01 *M* in Tris base, 0.001 *M* in disodium ethylenediaminetetra-acetate and 0.01 *M* in 2-mercaptoethanol), and the green juice obtained by pressing was subsequently emulsified in a mixer for 1 min with 10% (v/v) of octanol and for 1 min with 10% (v/v) of chloroform. The emulsion was then broken by centrifugation for 5 min at 5,000 *g*, and the green organic phase (with the intermediate phase) was not treated further. The clear and slightly brown aqueous phase was twice centrifuged for 20 min at 15,000 *g* to remove all cellular fragments. Purified TMV was obtained from the clarified extract of tobacco leaves after three cycles of differential centrifugation (90 min at 105,000 *g* and 20 min at 10,000 *g*).

Infectiousness was examined on the basis of formation of local necrotic lesions 3 days after inoculation of the leaves of a hypersensitive tobacco (*Nicotiana tabacum* cv. *Xanthi*) with the chromatographic fractions. Quantitative infection tests were performed only on fractions containing the virus peak. Each chromatographic fraction was inoculated into six leaves of the Xanthi tobacco in a Latin-square arrangement.

Preparation of carriers. Gels used as carriers in the separation of TMV were obtained by suspension copolymerization in the presence of an inert component[4]. After fractionation, each gel was swollen and packed into a column as described earlier[5]. The exclusion limits of the gels were determined by tests with standard polydextran fractions (Pharmacia, Uppsala, Sweden), the specific surface was determined by means of a sorptiometer based on the thermal-desorption principle[6]. The properties of the carriers are summarized in Table I.

Gel-chromatographic analysis. The analysis was performed on a chromatograph built at the Institute of Macromolecular Chemistry of the Czechoslovak Academy of Sciences. The eluent was supplied by a piston pump (type 6017 V, Development Works, Czechoslovak Academy of Sciences), and a differential refractometer (Knauer 2025) was used for detection. The fractions were collected, and the volume that had passed through the column was recorded, with an LKB fraction collector (S-161 25). The detector signal was recorded with an EZ 10 recorder (Laboratory Instruments Works, Prague, Czechoslovakia). The columns (1200 mm × 8 mm I.D.) were of stainless steel, and the samples were injected by means of an injection valve.

RESULTS AND DISCUSSION

Separation of virus on macrogranular Spheron gels

Clarified extracts from tobacco leaves infected with TMV, from which all cell

TABLE I

PROPERTIES OF CARRIERS

Gel	*Particle size (μm)*	*Exclusion limit*	*Specific surface (m^2/g)*
Spheron 300	200–300	300,000	77.7
Spheron 300	40– 80	300,000	77.7
Spheron 1000	25	$1 \cdot 10^6$	22.4
Spheron 300 BTD	40– 60	$2 \cdot 10^6$	9.3

fragments and organelles (including ribosomes) have been removed, are a complex mixture in which two kinds of molecules (differing from the other molecules in their molecular weight and in their amounts) are present. These molecules consist of rod-like TMV particles (300 × 15 nm in size) of molecular weight 39.4×10^6 (see ref. 7) and the so-called F_1 protein[8,9] (ribulose 1,5-diphosphate carboxylase)[10], of molecular weight *ca.* 0.5×10^6, the particles of which have a diameter of 10–12 nm[11–13].

Preliminary experiments in separating TMV from protein components of tobacco extracts were carried out in columns (100 × 1 cm) packed with various types of Spheron carriers (grain size 120–300 μm). In gels of relatively low porosity, such as Spheron 300, the high-molecular-weight F_1 protein was eluted, together with the virus, in the free volume of the column (V_0), as shown by control centrifugation analyses in a sucrose gradient. On the other hand, complete separation of the virus fraction was obtained in columns of Spheron 1000 and Spheron 300 BTD[14]. The yield obtained in the re-chromatography of this fraction, or in the chromatography of purified virus, varied from 88 to 94%; owing to the partial dissociation of the TMV particles that occurs even in neutral buffer solutions of low ionic strength (e.g., in dilution or purification), this yield can be regarded as almost quantitative.

The separation efficiency of Spheron 1000 and Spheron 300 BTD is roughly comparable with that of a similar column of Sepharose 2B according to the ratio of the elution volumes for F_1 protein and TMV $[(V_{F1})/(V_{TMV})]$, which for both types of Spherons is 1.8–2.0 and for Sepharose 2B is somewhat higher (3.0). The elution of TMV and F_1 protein from Spheron columns is independent of pH in the range 6.0–8.0 and of ionic strength in the range 0.005–0.2. The elution volumes are also independent of the composition of the elution buffer (Tris, borate, phosphate or citrate), even with a non-ionic detergent (Triton X-100) pre-adsorbed on the gel. All these findings suggest that the two proteins are not adsorbed on the Spheron carrier. On the other hand, considerable interaction takes place between the gel and polyhydric phenols in the plant extracts, and these compounds cannot be completely eluted merely by changing the ionic strength of the elution buffer. This interaction is due to the non-polar chain (backbone chain) of the basic gel matrix. Full desorption of compounds with an aromatic nucleus from macrogranular Spherons can be achieved only by raising the pH of the elution buffer to a value exceeding 9.0.

High-pressure chromatography on microgranular Spherons

Generally, columns packed with microgranular Spheron gels exhibit better resolution in the high-molecular-weight region than do columns of macrogranular carrier. Thus, on a simple column (0.8 × 120 cm) of Spheron 300 (grain size 40–80

μm), TMV is partly, but distinctly, separated from other plant proteins (see Fig. 1). Comparison of the chromatographic profiles of extracts from homogenized leaves from uninfected and TMV-infected tobaccos obtained under identical conditions clearly reveals quantitative and qualitative differences in the low-molecular-weight fractions, obviously induced by the virus infection. The experiment also shows that this gel in the microgranular form is able to separate proteins within a broader range of molecular weight than could be deduced from tests with defined dextran fractions. In contrast to macrogranular gels, polyhydric phenols and compounds of aromatic character can also be eluted with 0.01 *M* Tris hydrochloride buffer of pH 7.5. These adsorbed components are eluted, with delay, after fraction 24.

A remarkable separation has been achieved on the experimental gel Spheron 300 BTD (Fig. 2). On a single column (0.8 × 120 cm) of a gel of grain size of 40–60 μm, the virus was completely separated from other components of the vegetable juice. In this series of experiments, a purified 6-year-old TMV sample (greatly degraded during storage at −20°) was used as reference material. Besides low-molecular-weight degradation products, this sample contained particles of nominal length 280–320 nm,

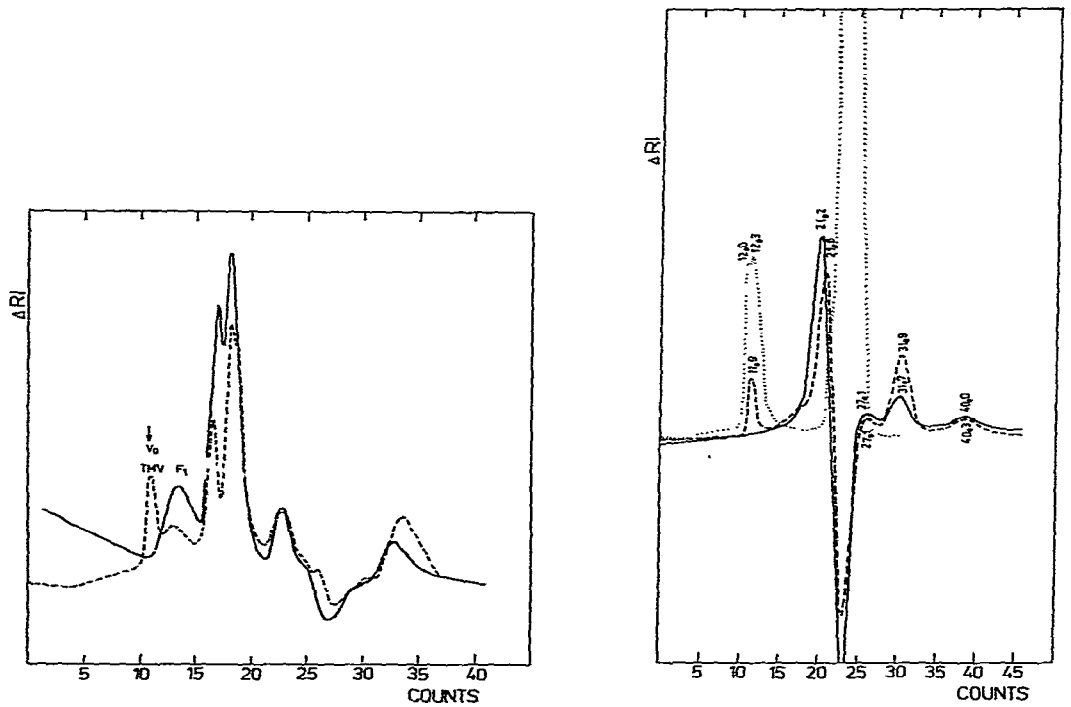

Fig. 1. Separation of TMV from accompanying plant proteins on a column (0.8 × 120 cm) of Spheron P-300 gel (particle size 40–80 μm), with 0.05 *M* Tris hydrochloride buffer (pH 7.51) as eluent (flow-rate 25 ml/h): ———, extract of uninfected tobacco; — — —, extract of tobacco infected with TMV.

Fig. 2. Separation of TMV from accompanying vegetable proteins on a column (0.8 × 120 cm) of Spheron 300 BTD gel (particle size 40–60 μm); eluent as in Fig. 1: ———, extract of uninfected tobacco; — — —, extract of tobacco infected with TMV; · · · · · ·, purified TMV.

aggregates of such particles and a large number of fragments. The inhomogeneity of the virus was reflected in this gel in the form of a double peak at 12.0 and 12.3 counts (1 count = 2.14 ml).

Complete separation of the virus nucleoproteide was also achieved on three columns (each 0.8 × 120 cm) connected in series and packed with Spheron 1000 (grain size below 25 μm) (see Fig. 3). The chromatogram of an extract of TMV-infected tobacco showed positive infection with fractions 36 to 51, the maximum infection (fractions 38–39) coinciding with the chromatographic peak of the virus. Extension of the infectiousness up to fraction 51 was not further investigated; it was not ascertained whether this infectiousness was due to tailing of the viral fraction in the column (which, because of the shape of the recorded peak of the virus is less likely) or to the presence of an infectious viral ribonucleic acid (molecular weight *ca.* 2 × 10^6) accompanying the virus in leaf extracts from infected tobacco.Preliminary experiments involving chromatography of the spherical cucumber mosaic virus (diameter of particles *ca.* 30 nm; molecular weight 5.8–6.8 × 10^6 daltons)[14] on a system of identical columns showed that the chromatographic distribution was always the same. Although a mixture of this virus and TMV could be completely separated from protein components of the plant extract, both viruses were eluted as one peak with the free volume of the column (V_0).

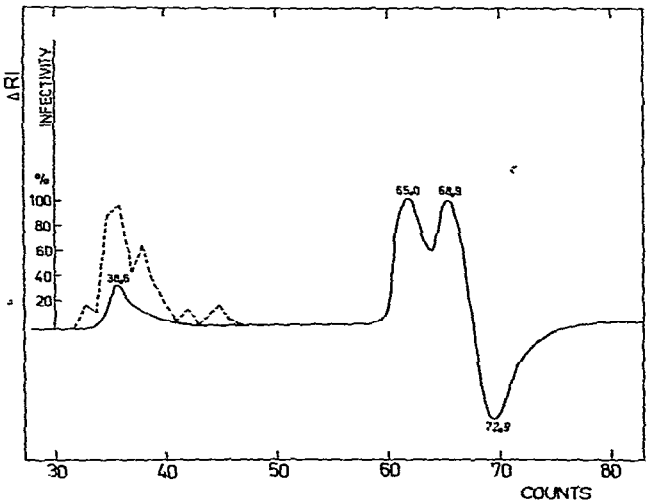

Fig. 3. Separation of virus nucleoproteide on microparticular Spheron 1000 gel (particle size 20–25 μm); three columns (each 0.8 × 120 cm) connected in series were used, and the eluent was as in Figs. 1 and 2: ———, extract of tobacco infected with TMV; — — —, infection curve of fractions 36–51.

CONCLUSIONS

The experiments described above and the isolation of a virus that enhances the activity of L-lactate:NAD-oxidoreductase in mice[3] indicate the possible application of high-pressure chromatography to provide rapid separation of virus particles from small samples and with minimal dilution.

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