Purification and Physical Properties of Mycophage PS I

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SUMMARY

Mycophage PS I, produced by the mould Penicillium stoloniferum, was concentrated and purified by zonal centrifugation or by a combination of isopropanol precipitation and zonal centrifugation. Continuous flow isopycnic banding was accomplished using the K-II zonal centrifuge. Isopropanol precipitates of fermentation broths were further concentrated by continuous flow isopycnic banding in the B-IX rotor followed by rate zonal separation and a second isopycnic banding in the B-XV rotor. Mycophage PS I was a potent inducer of interferon. Double-stranded RNA isolated from both the virus-like particle and from the rate zonal gradient also induced interferon. The characters of the double-stranded RNA from both sources were identical.

INTRODUCTION

Although the infection of moulds with viruses has been postulated, no virus particles have actually been isolated until recently. A virus-like particle has been detected, not through the production of a pathological manifestation, but through its capacity to induce interferon (Ellis & Kleinschmidt, 1967; Banks et al. 1968). Recently, Banks et al. (1970) have also reported the isolation of a virus from Aspergillus foetidus.

Statolon, a fermentation product of the mould Penicillium stoloniferum, was found in our laboratories to possess the property of inducing interferon both in animals and in tissue culture (Kleinschmidt, Cline & Murphy, 1964). Initially, the activity of statolon appeared to be associated with a predominantly polysaccharide moiety (Kleinschmidt & Probst, 1962). However, upon further purification by gradient centrifugation, the interferon-inducing activity was found to be associated with virus-like particles (Ellis & Kleinschmidt, 1967). The mould P. stoloniferum is the source of the virus-like particles, and we have termed the virus-like particle a mycophage (Mycophage PS I) (Kleinschmidt et al. 1968). Zonal centrifugation of statolon preparations revealed an active small molecular fraction, as well as the active particulate fraction in the gradients (Kleinschmidt et al. 1968). The small molecular fraction has been identified as a double-stranded RNA with properties identical to those of the double-stranded RNA of the mycophage. We describe here the purification and physicochemical properties of mycophage PS I.

METHODS

Mould culture. Penicillium stoloniferum M46-1758 was grown in stirred submerged culture at 25°C, employing a medium similar to that described by Stark, Kleinschmidt & Probst, 1963.

Preparation of isopropanol precipitate. Five-day-old P. stoloniferum cultures were filtered
through coarse filter paper to remove the mycelia. Cold isopropanol was added to cooled filtrates (4°) to a concentration of 33% in the presence of 1% Hyflo Supercel. After stirring for 4 to 6 hr, the material was filtered, the filter cake was resuspended in cold 1% (NH₄)₂CO₃, stirred for 2 hr and filtered again. The filtrate served as the starting material for further purification by zonal centrifugation.

Assay of interferon-inducing capacity. Mice were injected intraperitoneally with test materials. After 16 hr the mice were bled, and the interferon concentrations in the sera were determined by a tissue culture assay employing the mouse L-cell-vesicular stomatitis virus system. Interferon titres are reported as the reciprocal of the highest dilution giving a 50% plaque reduction.

Isolation of RNA. Purified virus fractions were dialysed against 0.01 M-phosphate buffer, pH 7.9, and were extracted for 30 min. at 35° with an equal volume of 88% phenol equilibrated at pH 7.9, followed by two more phenol extractions at 25°. Greater yields were obtained when the temperature of the initial extraction was increased to 40°. The RNA was isolated from the water by means of CF-11 cellulose (Franklin, 1966) or ECTEOLA cellulose (Lampson et al. 1967) columns.

Ribonuclease treatment. Sensitivity of the RNA to pancreatic ribonuclease (Sigma) was tested in 1 SSC (0.15 M-NaCl, 0.015 M-sodium citrate) at pH 7.0 with 0.2 µg./ml. for 30 min. at 25° or with 20 µg./ml. for 2 hr at 56°.

Zonal centrifugation. Two distinct classes of rotors were used in these experiments. The B-XV rotor, fabricated by the Oak Ridge Gaseous Diffusion Plant, was driven in a modified Spinco zonal ultracentrifuge (Anderson et al. 1967). This rotor was used for rate zonal centrifugation, which separates particles primarily on their rate of sedimentation.

The second class of rotors was used for the continuous flow isopycnic banding of virus from culture broths or isopropanol preparations. These rotors were the B-IX (Anderson et al. 1966a) driven in a modified Spinco zonal ultracentrifuge and the K-II rotor driven in a K-II B experimental zonal ultracentrifuge (Anderson et al. 1969).

Gradients in the rate zonal separations were generated using a Beckman Instruments, Inc, No. 141 gradient pump or the gradient generator developed by Anderson & Rutenberg (1967).

Gradient shape. Gradient shape was determined by measuring the refractive index of each fraction with a Bausch and Lomb Abbe refractometer.

Ultraviolet absorption spectra. Extinction between 250 and 350 nm. was determined for all fractions using a Cary 15 recording spectrophotometer.

Solutions. The gradient solutions contained 1.0% NaCl and 0.15 M-glycine. The pH was adjusted to 8.9 to 9.0 with saturated NaOH.

RESULTS

Studies with the K-II centrifuge

Earlier work showed that the isopropanol precipitation procedure would concentrate virus particles present in culture broth. In order to determine whether virus particles could be isolated from the broth, volumes of broth ranging from 13 to 42 l. were centrifuged in the K-II B centrifuge using the continuous-flow isopycnic banding rotor. The centrifuge was operated at 30,000 rev./min., and the flow rate through the rotor was 4 to 8 l./hr (Fig. 1). The majority of u.v.-absorbing material present was due to sedimentation of cell debris, components of the culture medium and other non-virus substances into the gradient. Ellis & Kleinschmidt (1967) reported that the mycophage bands isopycnically at a density of about 1.204 (45% w/v, sucrose). Electron microscopy showed the presence of virus-like
particles in this region of the gradient along with some subcellular debris. These data and u.v. spectra of individual fractions indicated that isopycnic banding alone would not produce mycophage of high purity and that further purification would be necessary.

Studies with the B-IX rotor

In order to obtain larger quantities of purified mycophage, continuous-flow isopycnic banding of isopropanol preparations was investigated. The isopropanol precipitation procedure resulted in a 10- to 20-fold volume concentration of the culture broth. The isopropanol preparation was run through the centrifuge at a flow rate of 1 l./hr at 40,000 rev./min. After all the sample had flowed into the centrifuge, centrifugation was continued for 1 hr to facilitate sharpening of the mycophage band (Fig. 2). Considerable variation in the u.v. absorption profiles of the various centrifuge runs was observed in the amount and location of u.v.-absorbing material present. However, all batches that had interferon-inducing activity also showed u.v. extinction in the region of the gradient containing 44 to 46 % sucrose.

Studies in the B-XV rotor

Earlier experiments indicated that two moieties in the isopropanol preparations, the particulate and the small molecular, were capable of inducing interferon (Kleinschmidt et al. 1968). In order to characterize these substances, the following series of experiments was

![Graph showing isopycnic banding of culture broth in the K-II zonal centrifuge rotor. The dashed line shows the shape of the gradient as determined by measurement of the refractive index of individual fractions. The solid line denotes the extinction at 280 nm. of the fractions.](image-url)
Fig. 2. Continuous flow isopycnic banding of isopropanol precipitated culture broth in the B-IX zonal centrifuge rotor. Dashed line (RI) is the refractive index of the gradient. Solid line is the extinction at 280 nm. of the fractions.

Fig. 3. Rate-zonal centrifugation of virus pool from B-IX rotor (see Fig. 2) in the B-XV zonal centrifuge rotor. Dotted line is refractive index. Dashed line is extinction at 260 nm., solid line extinction at 280 nm.
Purification of mycophage PS1

done. The fractions designated ‘pool’ in Fig. 2 were dialysed against the buffer used to prepare the gradients to reduce the sucrose concentration to that required for rate zonal separation. The conditions used for the rate zonal separation were optimum for the separa-

Fig. 4. Isopycnic banding of the virus zone from the rate-zonal separation in the B-XV rotor (see Fig. 3). RI is refractive index. Dashed bars are extinction at 260 nm., solid bars are extinction at 280 nm.

Fig. 5. PS1 virus from the peak virus fraction of a B-XV centrifugation. Fraction was diluted in 0·2 M-ammonium acetate and then centrifuged on to carbon-coated electron-microscope grids and negatively stained with 2% phosphotungstic acid.
tion of the virus-like particles from the large particulate material (Fig. 3). There were two extinction peaks. The first had a $E_{260}/E_{280}$ ratio $> 1$ and should have contained the myco-
phage according to a computer program for the B-XV rotor developed by Bishop (1966). The second had $E_{260}/E_{280} < 1$ and was composed of large particular material that had banded isopycnically in the same region as the mycophage during the initial continuous flow isopycnic separation. The fractions designated 'pool' in Fig. 3 were banded isopycnically in the B-XV rotor to concentrate and further purify the mycophage. There were two peaks (Fig. 4); the first (A) showed a typical ergosterol-like u.v. spectrum and the second was composed of the major peak (B) with a $E_{260}/E_{280}$ ratio of 1.48 and a shoulder (B') with a $E_{260}/E_{280}$ ratio of 0.77. The buoyant density of the peak and shoulder were 1.245 and 1.217, respectively. Material from both the peak and the shoulder induced interferon (Table 1). The mycophage particles contained in the major peak (B) are shown in Fig. 5.

Table 1. Interferon titres

<table>
<thead>
<tr>
<th>Fig.</th>
<th>Sample description</th>
<th>Interferon units/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Peak B</td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>Shoulder B'</td>
<td>400</td>
</tr>
<tr>
<td>6</td>
<td>Peak A</td>
<td>735</td>
</tr>
<tr>
<td></td>
<td>Peak B</td>
<td>735</td>
</tr>
<tr>
<td></td>
<td>Peak C</td>
<td>925</td>
</tr>
</tbody>
</table>

During the initial continuous flow isopycnic banding of the virus particles in the B-IX rotor the majority of a small molecular interferon-inducing component would not have sedi-
mANTED into the gradient but would have remained in the effluent. To determine the efficiency of separation of particles in the B-IX rotor and to characterize the small molecular compo-
nent further, a sample of the B-IX effluent was centrifuged in the B-XV rotor (Fig. 6). The major peak coincided with the region of the gradient into which the sample was introduced and was apparently composed of material with an S value so low that it did not move during

![Fig. 6. Rate-zonal centrifugation in the B-XV rotor of the effluent from the B-IX rotor. RI is refractive index. Dashed line is extinction at 260 nm., solid line is extinction at 280 nm. For interferon titres of A, B and C, see Table 1.](image-url)
Purification of mycophage PS I

39

centrifugation. The $E_{260}/E_{280}$ ratio of the descending limb of the peak decreased rapidly to $< 1$ and then rose to $> 1$ (Fig. 6, peak A). This region of the gradient contained the small molecular interferon-inducing moiety which has been identified as double-stranded RNA (Table 1).

The two peaks B and C, with densities of 1.202 and 1.220, had $E_{260}/E_{280}$ ratios of 0.8 and 1.14, respectively, and contained mycophage particles. The peak with the highest $E_{260}/E_{280}$ ratio possessed the most active interferon-inducing capacity (Table 1). In effect, this and the following centrifugation were combined rate zonal isopycnic separations. Because of their small size, small molecular components would have been separated according to their sedimentation rate, while the mycophage particles would have been isopycnically banded owing to their comparatively large size.

Fig. 7 shows the u.v. profile of the results of batch centrifugation of the isopropanol preparation in the B-XV rotor. This was a sample of the material that was centrifuged in the B-IX rotor (Fig. 2). These results illustrate the amounts of small molecular material, first peak, and large particulate material, second peak, that were present in the filtrate from the isopropanol precipitate. Centrifugation conditions were such that the material present in the first peak would have passed through the B-IX rotor into the effluent.

Characterization of RNA

Phenol extracts of purified mycophage were chromatographed on CF-11 cellulose or ECTEOLA cellulose, and yielded material that possessed maximum u.v. extinction at 257 nm. and a minimum at 235 nm. Sensitivity of this material to RNase was measured by the reduction of its capacity to induce interferon in mice. Resistance to pancreatic ribonuclease was observed at a concentration of 0.2 μg./ml. of enzyme at 25° for 30 min. in 1 SSC, pH 7.0. However, treatment with RNase (20 μg./ml.) for 2 hr at 56° inactivated the material. A titre of 1200 units of interferon/ml. of serum was induced with 10 μg. of the RNA/mouse before it was treated with RNase; less than 30 units/ml. were induced with the
same material after it was treated with the enzyme. Treatment with DNase (5 μg/ml for 1 hr) had no effect on the activity of the material. Exposure of this material to 56° for 2 hr without enzyme also failed to decrease its activity. The u.v. extinction characteristics and ribonuclease sensitivity identified the material as RNA. The differential sensitivity at higher enzyme concentration and temperature indicated that the RNA was double stranded.

Double-strandedness was confirmed by further tests. Double-stranded RNA is characterized by a hyperchromic effect as it is denatured by heat. The midpoint of the hyperchromicity ($T_m$) at 260 nm. of 27 to 28% occurred at 84° and 85° for two different preparations of RNA. The thermal denaturation of the RNA was done in 0.1 SSC (Fig. 8). Formaldehyde reduces the $T_m$ of hydrogen-bonded polynucleotide strands. The presence of 2.76% formaldehyde in 0.1 SSC produced an increase of 45% extinction at 260 nm. during thermal denaturation and reduced the $T_m$ to 65.5° (Fig. 9).

Lampson et al. (1967) established that double-stranded RNA obtained from helenine preparations of P. funiculosum could induce interferon in cells. They suggested that their double-stranded RNA was probably of virus origin. We found that double-stranded RNA from the mycophage particles of P. stoloniferum induced interferon in mice. A dose as small as 0.01 μg. of double-stranded RNA was capable of inducing a measurable quantity of interferon. One μg. of the double-stranded RNA was sufficient to protect a mouse against 3 LD 50 of MM virus (Kleinschmidt et al. 1968). These observed characteristics established the active components extracted from the mycophage particles of P. stoloniferum as a double-stranded RNA.

Studies on the active small molecular fraction obtained by sucrose gradient centrifugation
Purification of mycophare PSI revealed free double-stranded RNA present in isopropanol precipitated statolon preparations. This fraction collected from either the SW 25.1 swinging bucket rotor or from the B-XV rotor was further centrifuged in a CsCl gradient using the International SB 283 rotor for 68 hr at 35,000 rev./min. The double-stranded RNA possessed the following characters: (a) \( T_m = 83.5^\circ \), (b) resistance to 0.2 \( \mu \)g./ml. RNase at 25\(^\circ\) for 1 hr, (c) sedimentation coefficient of 10 to 12 S, and (d) capacity to induce interferon. These characters coincided with those of the double-stranded RNA derived from the virus particle.

**DISCUSSION**

Mycophage PS I, which has a diameter of about 30 nm., and contains double-stranded RNA, may be classified as a small RNA virus. Three other viruses are known to contain double-stranded RNA: reovirus, wound tumour virus, and rice dwarf virus. All these viruses are from 55 to 80 nm. in diameter, have icosahedral symmetry, and contain from 11\% to 20\% RNA. As yet, the RNA content of mycophare PS I is unknown. However, it is expected to be relatively low because of the low isopycnic density of this mycophare. Complete characterization of PS I has been prevented by lack of a satisfactory purification method. The isopropanol precipitation procedure results in an increased concentration of interferon-inducing activity but also concentrates extraneous materials present in the culture fluid. Rate zonal density-gradient centrifugation indicated that at least three major groups of sedimentable materials were present in isopropanol preparations. These were a small molecular fraction, the mycophare particles, and a great mass of cell debris and extraneous material. Both the small molecular fraction (double-stranded RNA) and the mycophare were capable of inducing interferon. Continuous-flow isopycnic zonal centrifugation effectively concentrates the mycophare either from culture broth or from isopropanol preparations; however, isopycnically banded fractions were not pure, and it was therefore necessary to purify the mycophare further by rate zonal centrifugation and then to band the mycophare zone isopycnically again. This is an example of a s-p separation originally proposed by Anderson et al. (1966b). It is doubtful whether this purification could have been achieved by any means other than zonal centrifugation.

The in vivo assay system presently used to determine the interferon-inducing activity of this mycophare is at best semi-quantitative. It was difficult to obtain satisfactory electron micrographs of highly purified mycophare preparations, probably because of aggregation of the particles.

The zonal centrifuge rotors used in these experiments were obtained under a co-operative agreement with the Molecular Anatomy Program of Oak Ridge National Laboratories, directed by Dr N. G. Anderson.

**REFERENCES**


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