Sodium Dodecyl Sulphate–Sodium Chloride Extraction of *Penicillium stoloniferum* Mycelium for Viral Double-stranded RNA

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SUMMARY

Incubation of mycelia of a virus-containing strain of *Penicillium stoloniferum* in a 1 % sodium dodecyl sulphate–4 % sodium chloride (SDS–NaCl) solution liberated protein and deoxyribonucleic acid within the first hours, whereas ribonucleic acid (RNA) release continued through an extended period. Although transfer RNA (tRNA) was readily solubilized by the treatment, viral double-stranded RNA (dsRNA) was not detected in the extracellular lysate during the initial 6 h of incubation. After 24 h of treatment, mechanical disruption of the mycelia liberated some dsRNA; this procedure demonstrated the incomplete solubilization of viral nucleic acid in SDS–NaCl. Scanning electron micrographs of mycelium treated with a salt–detergent mixture showed a progressive reduction in structural integrity of fungal hyphae. However, hyphal tips appeared to remain morphologically intact during incubation.

INTRODUCTION

The antiviral substance obtained from fungal fermentations has been identified as the double-stranded RNA (dsRNA) core of virus-like particles (VLP's) (Stark, Kleinschmidt & Probst, 1963; Lampson *et al.* 1967; Kleinschmidt, Ellis, Van Frank & Murphy, 1968). In initial studies, the component was obtained from the culture medium of a *Penicillium stoloniferum* fermentation (Kleinschmidt & Probst, 1962; Stark *et al.* 1963). Shope (1953) detected antiviral material in the culture broth of another fungus, *Penicillium funiculosum*, and in addition he observed that the active substance could be extracted from the mycelium. Later studies verified the presence of the antiviral component exclusively in the mycelium of VLP-containing fungi during the growth phase, with subsequent release into the culture medium (Banks *et al.* 1968; Ellis & Kleinschmidt, 1967). Several techniques have been employed for rupture of fungal mycelia and release of the VLP's (Shope, 1953; Banks *et al.* 1968; Cox, Kanagalingam & Sutherland, 1971). Procedures have also been described for direct extraction of dsRNA from fungi, including incubation of mycelia in detergent–salt solutions (Lemke & Ness, 1970; Sutherland, Heath & Bessell, 1971).

We have studied the detergent–salt-mediated release of nucleic acid and protein from mycelia of *Penicillium stoloniferum* NRRL 5267. The efficiency of extraction of dsRNA from mycelium incubated in sodium dodecyl sulphate (SDS) and sodium chloride has been determined. We have also examined the effect of SDS–NaCl incubation on the integrity of fungal mycelium.

METHODS

Production, harvest and disruption of mycelia. *Penicillium stoloniferum* Thom NRRL 5267 (ATCC 14586) mycelia were produced in 2.8 l Fernbach flasks containing 500 ml of a glycerol-glucose (0.5 %) medium (Stark, Kleinschmidt & Probst, 1963), inoculated with a spore suspension, and incubated on a rotary shaker at 28 °C. Mycelia were harvested by filtration and washed with an equal volume of 0.01 M-PO₄ buffer, pH 7.0. The filtered mould was resuspended in 0.01 M-PO₄ buffer, pH 7.0 (1 g wet wt/10 ml) before rupture. Mycelia were disrupted in a refrigerated Manton-Gaulin homogenizer at 5000 lb/in² pressure, with recycling of the slurry for 3 min. Cell debris was removed from the homogenate by centrifugation at 8000 g for 20 min.

Isolation and determination of RNA, DNA and protein. Harvested mycelia (48 h) were suspended in an aqueous solution of 1 % SDS and 4 % NaCl and gently stirred with periodic sampling and centrifugation of the lysate at 8000 g for 10 min. The clarified lysate supernatant was treated with 2 vols. of methanol and the precipitate collected by centrifugation at 3000 g for 10 min. The pelleted material was redissolved in 0.2 M-sodium acetate and an equal volume of aqueous 90 % phenol containing 0.1 % (w/v) 8-hydroxyquinoline was added (Cox *et al.* 1971). The mixture was stirred for 15 min at 25 °C before centrifuging at 4000 g for 20 min at 4 °C, and the RNA in the aqueous phase was freed from phenol by repeated precipitation (cold methanol, v/v) from 0.2 M-sodium acetate solution. The final precipitate was dissolved in a minimal volume of SSC (0.15 M-NaCl and 0.015 M-sodium citrate), pH 7.4. U.v. spectroscopy with a Beckman DB used a 1 cm light path. Isolated RNA was separated electrophoretically on polyacrylamide gels (2.4 %) for 2.5 h at 6 mA/tube (Bozarth, Wood & Mandelbrot, 1971). Gels were scanned on a Gilford linear transport system at 260 nm. The concentration of viral RNA and tRNA in the SSC preparations was determined from electrophoretograms by integrating peak areas and comparing values obtained from known quantities of dsRNA and tRNA similarly separated.

Release of protein and nucleic acid was determined in the aqueous solution remaining after cellular material had been sedimented by centrifugation at 8000 g for 20 min. Protein was determined by the Folin method (Lowry, Rosebrough, Farr & Randall, 1951), DNA by the diphenylamine technique (Burton, 1956) and RNA by the orcinol reaction (Mejbaum, 1939).

Scanning electron microscopy. Squares (10 × 10 mm) cut from glass microscope slides were placed on aluminium specimen stubs for mounting in the microscope. About 0.05 ml of a thoroughly washed, diluted mycelial suspension (48 h cells) was spread over the surface of the mount, dried and coated with aluminium to a thickness of 15 nm. Specimens were examined in a Cambridge Stereoscan mark II scanning electron microscope at an accelerating voltage of 20 kV; the final aperture was 200 µm and the beam specimen angle 45°.

RESULTS

Release of protein and nucleic acid from mycelia

Penicillium stoloniferum mycelia (48 h) were suspended (1 g wet wt/10 ml) in an aqueous solution of 1 % SDS and 4 % NaCl for extraction tests. The suspension was gently stirred at 25 °C with incremental removal of 100 ml samples, residual mycelium was sedimented by centrifugation, and methanol (2 × vol.) was added to the supernatant with subsequent centrifugal sedimentation of the precipitate and resuspension in 0.2 M-sodium acetate. The latter solution was tested for the presence of protein and nucleic acids (Fig. 1). Release of

Fig. 1. Time course of protein, DNA and RNA release from *Penicillium stoloniferum* mycelia during incubation in 1 % sodium dodecyl sulphate (SDS)–4 % NaCl solution. After 100 ml of mycelial (1 g wet wt/10 ml) suspension was removed it was sedimented by centrifugation. Methanol (2 × volume) was added to the supernatant and the precipitate was sedimented and resuspended in 0.2 M-sodium acetate. ○, Protein release determined by the Folin method; □, DNA release determined by the diphenylamine method; △, RNA release determined by the orcinol method.

protein from the mycelia was rapid (Fig. 1, curve ○), with essentially complete extraction of the solubilized fraction during the initial hour of the 24 h incubation. Slight reduction of the protein level during later stages of incubation probably represents gradual protein denaturation in the test solution with subsequent precipitation in the residual mycelium fraction. DNA was released from mycelia more slowly than protein, with the maximum level in the extracellular milieu occurring after 6 h of incubation (Fig. 1, curve □). RNA detected in solution did not reach a maximum during the 24 h treatment but at the end of the trial period was slowly increasing (Fig. 1, curve △).

Quantities of DNA, RNA and protein were determined in the aqueous phase and in mycelia after 24 h incubation in SDS–NaCl solution (Table 1). Slightly less than half the total protein and RNA were released during the procedure and three-quarters of the DNA was solubilized. Fig. 1 shows that most of the release of macromolecules during SDS–NaCl incubation occurred in the initial 24 h period. The nucleic acid and protein remaining in the mycelium after incubation were therefore resistant to further detergent–salt extraction.

Fig. 2. Qualitative electrophoretic characterization of phenol-treated RNA fraction obtained from SDS-NaCl lysate and Manton-Gaulin homogenization of *Penicillium stoloniferum* mycelia. The final precipitate from phenol treatment was solubilized in SSC and added to polyacrylamide gels (2.4 %). Electrophoresis was carried out for 2.5 h at 6 mA/tube. Developed gels were scanned on a Gilford linear transport at 260 nm. (a) Two-hour incubation in SDS-NaCl with subsequent phenol treatment of the supernatant from the lysate. (b) Manton-Gaulin homogenization of the mycelia treated for 2 h in SDS-NaCl with subsequent phenol treatment of the supernatant. (c) Incubation (24 h) in SDS-NaCl with subsequent phenol treatment of the supernatant from the lysate. (d) Manton-Gaulin homogenization of the mycelia treated for 24 h in SDS-NaCl with subsequent phenol treatment of the supernatant.

Table 1. Extraction of protein and nucleic acid from *Penicillium stoloniferum* mycelia during incubation in 1 % sodium dodecyl sulphate (SDS)-4 % NaCl solution and subsequent Manton-Gaulin homogenization

Mycelia (75 g wet wt) were incubated in 750 ml of SDS-NaCl for 24 h at 25 °C with subsequent filtration, determination of the macromolecular levels in the filtrate, resuspension of the treated mycelia in 0.01 M- PO_4 , pH 7.0 (1 l), Manton-Gaulin homogenization of the treated mycelia, sedimentation of cell debris, and measurement of macromolecular levels in the supernatant. Values are means of three replicates.

	Protein		RNA		DNA	
	mg	% of total	mg	% of total	mg	% of total
SDS-NaCl supernatant	405	46	230	42	7.8	77
Manton-Gaulin homogenate	480	54	313	58	2.4	23

Characterization of RNA released by SDS-NaCl

An electrophoretic examination was made of the RNA fraction released by SDS-NaCl or mechanical disruption (Fig. 2). After 2 h of SDS-NaCl treatment there appeared to be exclusive release of a single RNA entity; the substance(s) exhibited mobility essentially

Table 2. *Time course of dsRNA release from Penicillium stoloniferum mycelia during incubation in 1 % SDS-4 % NaCl*

Mycelia (100 g wet wt) were incubated in 1 l of SDS-NaCl at 25 °C with subsequent filtration, phenol treatment of the supernatant, solubilization of the nucleic acid in SSC, and electrophoretic separation of the RNA constituents on polyacrylamide gels. The AU units (absorbance units at 260 nm in a 1 cm cell) are composite values from each sample, obtained by computation of the area under the viral dsRNA electrophoretogram peaks from known volumes.

SDS treatment (h)	Viral dsRNA (AU units)
0.25	—
0.50	—
2	—
3	—
6	0.3
8	0.4
12	5.0
24	46.0

identical to the reference tRNA from yeast with a mol. wt of 35 000 (Fig. 2 *a*). Manton-Gaulin homogenization of the mycelia recovered after 2 h released an RNA fraction characterized by Fig. 2 (*b*). This fraction contained, in addition to tRNA, the dsRNA components identified as the nucleic acid cores of the two virus-like particles from the mould (Bozarth *et al.* 1971). The lowest mobility group of multiple peaks contained molecules ranging in mol. wt from 1.10×10^6 to 0.89×10^6 daltons, whereas the largest viral dsRNA band contained a molecule of 0.23×10^6 daltons. The nucleic acid fraction solubilized during 24 h SDS-NaCl incubation contained dsRNA viral constituents and tRNA (Fig. 2 *c*). Further disruption of the mycelia after the 24 h treatment released predominantly dsRNA with much less tRNA (Fig. 2 *d*). These tests demonstrated that tRNA was preferentially released from the mycelia early in the incubation with detergent and salt. After 24 h in SDS-NaCl some of the dsRNA remained associated with the cellular fraction.

The release of dsRNA from mycelia was further examined by determining the amount of the substance solubilized at various time intervals in detergent-salt solution (Table 2). No detectable quantity of dsRNA was released during the initial 3 h. Limited amounts of the nucleic acid were detected after 6 and 8 h incubation with about a tenfold increase in the solubilized viral RNA between 8 and 12 h and a similar multiple increase between 12 and 24 h. The total AU (absorbance units at 260 nm in a 1 cm cell) present in the SSC solution before electrophoretic separations was compared with the aggregate viral dsRNA AU values observed after resolution of the nucleic-acid fractions. This test showed that more than 80 % of total AU in SSC was released during the initial 6 h of SDS-NaCl incubation, but there was little viral RNA solubilized during the same test period. Of the total AU in SSC at the end of the experiment (24 h in SDS-NaCl), 12 % was viral dsRNA.

Changes in fungal cells induced by SDS-NaCl

The extent of mycelial lysis achieved during the 24 h SDS-NaCl treatment was examined by determination of viability at the end of the trial. Samples of extraction medium (sterile incubation) were transferred to nutrient agar plates; after 3 days at 28 °C abundant *Penicillium stoloniferum* growth appeared. Microscopic examination of mycelia before and after treatment revealed no characteristic conidial forms. Therefore, some of the mycelium maintained viability during salt-detergent incubation.

Fig. 3. Scanning electron micrographs of *Penicillium stoloniferum* mycelia incubated in 1 % SDS–4 % NaCl or 0.01 M- PO_4 , pH 7.0. (a) Untreated mycelia, (b) hyphal tip of untreated mycelia, (c) mycelia after 1 h in SDS–NaCl, (d) mycelia after 8 h in SDS–NaCl, (e) debris associated with hypha treated for 8 h in SDS–NaCl, (f) mycelia after 8 h in PO_4 buffer, (g, h) mycelia after 12 h in SDS–NaCl and (i) mycelia incubated for 12 h in PO_4 buffer.

Since a portion of the mycelium was apparently not losing critical macromolecules during the SDS–NaCl treatment, incubated mycelial fractions were examined in a scanning electron microscope to elucidate morphological variations. Fig. 3 (a) shows the typical morphology of untreated mycelium. Although high-vacuum fixation partially collapsed the mycelium, it is clear that cytoplasmic contents remained in the intact hyphae. Fig. 3 (b) is a slightly higher magnification of an untreated hyphal tip and reveals the apparent rigidity of the extremity. After 1 h in 1 % SDS–4 % NaCl (Fig. 3 c), a few of the hyphae appear weakened, and fixation produces some casing-like structures of mycelial wall seemingly devoid of protoplasm. After 8 h in SDS–NaCl there was a large increase in the number of empty hyphae (Fig. 3 d). However, hyphal tips appeared less liable to collapse than the mycelium behind the extremities. This micrograph also shows some amorphous debris that seemed to

be cellular material extruded during the high-vacuum fixation. Fig. 3 (e) presents a higher magnification of debris in close association with the mycelium. A control mycelial incubation was carried out in 0.01 M- PO_4 buffer, pH 7.0, to provide a comparison between the morphological variations. After 8 h in buffer, almost all the hyphae remained structurally rigid (Fig. 3f). Fig. 3 (g) and (h) shows the typical hyphal morphology after 12 h in SDS-NaCl; essentially all the mycelial strands appear empty, but the hyphal tips remain distended. Mycelia incubated in buffer for 12 h had few empty hyphae, and inflated mycelia (Fig. 3i) contrasted with the collapsed wall structures observed after SDS-NaCl treatment for a similar period (Fig. 3g, h).

DISCUSSION

Experiments were carried out to gain information on the selective release of viral dsRNA from *Penicillium stoloniferum* mycelia. Both protein and DNA were rapidly released when mycelia were incubated in 1 % SDS-4 % NaCl, whereas RNA release continued longer. Specific determination of RNA fractions solubilized during the SDS-NaCl treatment showed that tRNA was rapidly released, chiefly during the first few hours of incubation. The dsRNA, however, did not appear in the aqueous phase of the lysate before 6 h; after 24 h of incubation a significant portion of the viral RNA remained associated with the insoluble cell fraction. The failure to solubilize all the viral nucleic acid in a detergent-salt solution indicated that either the molecules were tightly bound to cytoplasmic structure(s) or they were retained in parts of the mycelium which were not being lysed by the treatment. Tests of the structural strength of the hyphal strand after detergent-salt treatment indicated that hyphal tips remained rigid, whereas older hyphae in the mycelium were readily emptied. In addition, after 24 h of SDS-NaCl incubation mycelia were viable, an observation which provides further evidence that parts of the mycelium retain their integrity. Some of the viral dsRNA located within the mycelium could be solubilized by a detergent-salt treatment, but a fraction of the viral nucleic acid appeared to be associated with hyphal components that were not disrupted by the procedure. Our scanning electron micrographs suggest that the resistant components were located at the hyphal tips. Therefore, we tentatively conclude that the tip regions of fungal hyphae contain viral nucleic acid.

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