EFFECTS OF BACTERIAL AGARASE ON AGAROSE GEL IN CELL CULTURE

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SUMMARY

Bacterial agarase, concentrated and purified from culture filtrate of agar-degrading bacteria, has been used to clean cells cultured in soft agarose from gel residues. The enzyme also has been used to liquefy the gel directly in the dishes to facilitate the removal of cells. The surfaces of glioma cells from agarase-treated colonies could not be distinguished in the scanning electron microscope from surfaces of cells which had never been in contact with agarose or agarase. This implies that most agarose residues had been removed, and also that the treatment did not seriously alter the cell surfaces. The influence of the agarase treatment also was tested by comparison of the mitotic index and the incorporation of [3H]thymidine in agarase-treated and untreated cells. No effects of the treatment could be seen in these tests.

Key words: agarase; agarose gel; cell culture; gel degradation.

INTRODUCTION

Agarose is a galactan with an alternating sequence of (1→4)-linked 3,6-anhydro-α-L-galactose and (1→3)-linked β-D-galactose residues. Some residues are replaced by methyl, sulphate or pyruvate groups (1). According to a model proposed by Arnott et al. (2), the polysaccharide chains are twisted into double helices which interact with each other to form large aggregates. Each chain takes part in many aggregates and this keeps the structure together. Large cavities are formed between the aggregates and, in gels of low concentration, most proteins may diffuse freely.

Agarose or agar gel is used commonly as a matrix in cultures of mammalian cells. The gel method has been used in tests of virus transformation of diploid cells (3, 4), in tests of cellular anchorage dependence (5), for cloning of hematopoietic cells (6) and for isolation of single cells (7). In our laboratory, three-dimensional cell colonies cultured in soft agarose gel (8) are used for radiobiological investigations.

If cell colonies cultivated in agarose are to be isolated, and perhaps also subcultured, it is important to have a technique by which they can be efficiently removed and cleaned from the matrix.

Some attempts have been made to solve this problem by using agarase (6) to degrade the agarose gel. We have tried currently available agarase preparations (culture filtrate from Vibrio agarlifaciens (6) and a commercial agarase preparation from Calbiochemie) and found them to be insufficient, probably because of a low concentration of agarase.

Now, however, it is possible to concentrate and purify agarase by affinity chromatography directly from culture filtrate of an agar-degrading bacterium. This bacterium and its gel-degrading properties have been described previously (9, 10). The concentrated and purified agarase preparation can efficiently liquefy gels of low concentration and also cause holes in harder gels. We used such a concentrated agarase preparation to isolate and clean colonies of glioma cells grown in agarose.

MATERIALS AND METHODS

Preparation of bacteria-free culture filtrate. The agar-degrading bacterium NCMB 1914 (National Collection of Marine Bacteria, Torry Research Station, Aberdeen, Scotland) was grown in shaken flasks at 30°C. The salt medium (9), supplemented with 0.25% w/v Difco Bacto Agar, was sterilized in 30-cm Fernback flasks. After gelation of the agar the medium was inoculated with 20 ml outgrown culture. The culture then was allowed to grow until it reached a stationary...
growth phase after 17 to 19 hr. The bacteria-free culture filtrate was obtained through centrifugation (twice) of the culture at 20,000 x g for 30 min. The methods of concentration and purification of agarase, which are briefly described below, will be discussed in detail by Malmqvist (11). Preparative adsorbent for affinity chromatography. Divinyl sulphone cross-linked Sepharose 4B (DVS-Sepharose) was prepared with 6% divinyl sulphone as described by Porath, Låås and Janson (12). This cross-linked agarose derivative is not degraded by agarase and its mechanical stability is raised, so that high flow rates can be used in chromatographic experiments.

Preparation of hydrolyzed agarose. A nongelling mixture of oligosaccharides from agarose was prepared for specific elution of agarase adsorbed to DVS-Sepharose. Agarose 1% w/v was melted in 25 mM Tris-acetate buffer at pH 7.8, placed in a water bath at 50°C, and concentrated culture filtrate or purified agarase was added. When a marked change in the viscosity of the solution became apparent, the reaction was stopped by boiling. If cloudy precipitate or gel occurred in the sample upon cooling, the polysaccharide chains had not been degraded sufficiently and the procedure had to be repeated. The reducing power of the degraded sample is equivalent to 5/~mol galactose per ml. The proteins finally were separated from the soluble oligosaccharides by ultrafiltration through an Amicon PM 10 membrane.

Affinity chromatography. Tris-acetate buffer (pH 7.8) and NaCl were added to the bacteria-free culture filtrate to final concentrations of 25 mM and 100 mM, respectively. To prevent bacterial growth, all buffers and the sample contained 0.02% NaN3. The solution was pumped through the DVS-Sepharose 4B column at a speed of up to 100 ml per cm2 per hr at 4°C. After passage of the sample, the column was washed with several column volumes of buffer, 25 mM Tris-acetate, pH 7.8. Specific elution of agarase from the column then was performed with four column volumes of 25 mM Tris-acetate, pH 7.8, containing 1% hydrolyzed agarose. The agarase was concentrated further by ultrafiltration through an Amicon PM 10 membrane to absorbance 3.1 at 280 nm (1-cm thick cuvette). The final concentration of agarase in the sample was then about 1000 times that in the initial culture filtrate.

Cell culture. The studied permanent cell line (118 MG) from a human malignant glioma has been described previously by Westermark, Pontén and Hugosson (13). The cells were grown, at 37°C in humid atmosphere, in Eagle’s Minimum Essential Medium (MEM) supplemented with 10% bovine calf serum and antibiotics (100 IU per ml penicillin, 50 μg per ml streptomycin and 1.25 μg per ml amphotericin B). Cultivation of monolayers was done in 5-cm Nunclon® plastic dishes. The medium (5 ml) was renewed three times a week.

Cultivation in the semisolid medium was performed as described by Macpherson (7), with the difference that agarose (Indubiose® A37, L’Industrie Biologique Française) was employed instead of agar. Five ml of 0.5% agarose medium, containing 10% bovine calf serum and 10% trypsin-phosphate broth in Eagle’s MEM, was poured into a 5-cm plastic dish and allowed to solidify at room temperature. Above this layer, 1.5 ml of 0.33% agarose medium containing 104 cells was added. The dishes were incubated at 37°C in humid atmosphere containing 5% CO2. Fresh medium (0.5 ml, Eagle’s MEM + 10% calf serum) was added on the top of the cultures three times a week.

Treatment with agarase. Twenty large colonies were picked out of the agarose gel, together with gel residues and about 100 μl medium, with a Pasteur pipette and placed on the bottom of a plastic dish. To each sample was added about 200 μl Eagle’s MEM as a large drop. In addition 10 μl of the agarase solution was added to 10 samples and the other 10 kept as controls. All colonies were incubated for 24 hr at 37°C in humid atmosphere containing 5% CO2.

Fifty μl agarase solution was given directly above colonies which remained in the gel. These gel plates then were incubated for 24 hr as described above. Glioma cells allowed to aggregate in suspension for 24 hr served as controls.

Scanning electron microscopy. After incubation as described, the isolated colonies which visually seemed to be devoid of gel residues were washed carefully in phosphate-buffered solution. They were fixed in 2% glutaraldehyde in 0.1 M Na cacodylate-HCl buffer with 0.1 M sucrose (pH 7.2, 510 mOsm) for 15 min at 37°C during gentle agitation to rinse away serum proteins with the fixative. The fixation then was continued at 4°C for another 24 hr. The colonies finally were attached to thin cover slips, using a thin albumin layer, and postfixed in 1% OsO4 in 0.15 cacodylate buffer, pH 7.2, at room temperature for 90 min.

The cells were dehydrated in a graded series of ethanol, transferred to water-free acetone and