

THE LOW TEMPERATURE, RAPID DISSOLUTION OF GELLAN AWAY FROM ROOT CULTURES

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SUMMARY

A buffer system consisting of 50 mM Tris-HCl-TRIZMA base plus 10 mM EDTA was used to rapidly dissolve gellan gels used for maintaining transformed carrot root cultures. The optimum conditions of pH 7.5 in the presence of 10 mM EDTA for dissolving gellan were first worked out on a model test system containing 0.4% gellan, 0.025% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and blue dye. The conditions were then tested on gellan gels (0.2% gellan plus nutrients) containing carrot roots. This gel dissolution system was rapid (18 to 20 min), did not require heating, and could also be efficiently performed at 4°C. Furthermore, the buffer system used for gel dissolution is a standard one used for plant cell fractionation studies.

INTRODUCTION

Gellan gum is frequently used as a solid support for nutrient media for plant tissue culture research. This polysaccharide has a repeating structure of two β -D-glucose, one β -D-glucuronic acid and one α -L-rhamnose residues. The presence of cations cross-link the polysaccharide to form a gel. Gellan gels provide an excellent way to grow transformed "hairy" root cultures and they grow more rapidly on this support medium compared to agar (Bécard and Piché, 1992). The gel is also clear and is the preferred method to study the interactions between mycorrhizal spores and cultured roots (Bécard and Fortin, 1988). Transformed root cultures growing on a solid support have also become a powerful tool for studying economically valuable natural plant products (Flores et al. 1987).

The physical removal of roots from the gellan gels can cause damage to the roots and complete removal of the gel cannot be achieved. Damaged roots will frequently brown and the browning reaction is detrimental to enzymatic activity. A recent approach was to dissolve gellan by chelating the cations (such as Ca^{+2} and Mg^{+2}) from the gellan matrix thereby minimizing root damage by simply reversing the gelation process (Doner and

Bécard 1991). To solubilize the gel, a 10 mM sodium citrate-citric acid buffer system at pH 6.0 was used at room temperature or heated at temperatures greater than 30°C to rapidly chelate cations. For biochemical studies on secondary plant metabolism (Flores et al., 1987), subcellular fractionation of membranes, and especially the isolation of cell walls, roots are kept on ice and acidic pH is normally avoided (Nagahashi, 1985). The purpose of this study was to develop a gel-solubilization system which was rapid, performed at room temperature or in the cold, and was feasible for future biochemical studies involving the identification of chemical signals that trigger the germination and stimulation of mycorrhizal growth.

MATERIALS AND METHODS

Preparation and dissolution of model gels. Model gels were prepared using 0.4% or 0.75% gellan gum (Gel-Gro, ICN Biochemicals, Cleveland, OH) crossed-linked with 0.025% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The model gels were used to mimic firm gels which are also used to grow root cultures. The gels were prepared as previously reported (Doner and Bécard, 1991) and mixed with methylene blue or trypan blue (25 ppm) before gelation to expedite visual observations during the dissolution process. The firm gels were easy to cut with a cork borer and small samples and small buffer volumes were used for the large number of preliminary experiments performed. Disks, 14 mm/diam, were cut from 80 ml gel slabs poured and solidified in Petri dishes (80 x 10 mm). The average disk weighed 2.0 g. Gel disks were dissolved in various volumes of TRIS-HCl-TRIZMA base buffer (Good et al., 1966) system or HEPES-MES buffer (Good et al., 1966) system at molarities and pH values as indicated in the text. The pH was adjusted by mixing various amounts of the two component buffer system. EDTA was added at concentrations given in the text and the final pH of the buffer system was adjusted with concentrated sodium hydroxide at room temperature.

Dissolution experiments were performed in beakers with 1.1 cm stirring bars on a stirring plate at room temperature or at 4°C with the stirrer set at 180 rpm. Dissolution was measured by visually observing the disappearance of blue gel fragments. To insure complete dissolution, the samples were filtered over a 90 μm sieve. Undissolved particles were readily detected. Buffers used to dissolve gels were kept at either 4°C or room temperature when the final pH was determined. This was essential since the pH of Good buffers (HEPES and TRIS) are temperature sensitive (Good et al., 1966).

Root culture. Carrot roots were transformed with T-DNA which originated from the Ri plasmid of *Agrobacterium rhizogenes* (Bécard and Fortin, 1988). Roots tips from parental cultures were excised and transferred aseptically to large Petri dishes (150 mm x 15 mm) containing 0.2% gellan and M medium (Bécard and Fortin, 1988) which contains essential elements and vitamins. After three weeks growth, the cultured roots were harvested.

Preparation and dissolution of gellan medium used for growing root cultures. In contrast to the model gel test system, the biological test system contained 0.2% gellan prepared with M medium as previously reported (Bécard and Fortin, 1988). This softer gel is frequently used to study transformed roots in dual culture with mycorrhizal fungi. Dissolution experiments were performed on these gellan gels with or without roots. Large pieces of the gel were removed from large Petri dishes (150 mm x 15 mm) and weighed. Each dish contained 90-95 g of solid gel.

Predetermined amounts of the gel were dissolved in the same buffered EDTA solutions as mentioned above. In these experiments, the gellan medium was mixed with the buffer system and gently shaken on a gyrotory shaker with a speed setting of 180 rpm at room temperature (22-24°C). To aid the visual observations of gel dissolution, 0.1 mL of 1% Coomassie blue was added per 50 mL of buffer. The darker background provided by this negative staining technique provided enough contrast to observe small pieces of clear gellan. Complete dissolution was verified by sieving as stated above. When biological gels containing carrot roots were used, the entire contents of each Petri dish was carefully removed and transferred to a 500 mL Erlenmeyer flask. Approximately 2-3 g fresh weight of roots was harvested for every 90 g of gellan gel. Typically, 180 mL of dissolution buffer/90 g of gel was used for each experiment.

RESULTS

pH dependent gel dissolution. The chelation of cations by EDTA was most effective at pH 7.5 or greater as indicated by the gel dissolution time (Fig. 1). In the model gel system, at pH 6.5, 30 min at room temperature was necessary to dissolve the gel. At pH 6.0, the gel was not dissolved after 60 min compared to complete dissolution at pH 7.5 in 10 min. Similar results were obtained with the biological gels used to grow cultured roots. With these gels, the time for complete dissolution was 18 min at pH 7.5 (Fig. 1).

The time to dissolve the biological gel was longer than the time needed to dissolve the model gels at alkaline pH (Fig. 1). This difference in time was due to the method of swirling the gels during dissolution. The model gels were stirred with a magnetic stirrer and the magnetic bar mechanically broke up large pieces of the gel allowing rapid dissolution. The gentle swirling method without a stirring bar was normally used for the biological gels since this prevented any damage to the growing roots.

Effect of EDTA concentration on the gel dissolution time. The gels did not dissolve in buffer alone indicating the necessity of the chelator. The TRIS HCl-TRIZMA buffer concentration used in these experiments was determined by varying the molarity while holding EDTA at 10 mM. The molarity of the buffer between 50 to 150 mM had no effect on the time needed to dissolve 0.4% or 0.75% gellan gels (data not shown). 50 mM buffer was then used for determining the optimum EDTA concentration to use for gel dissolution. In these experiments, the model gels (0.4%) were dissolved using a 2:1 and 5:1 ratio (v/w) of buffer to weight of gel. EDTA levels at 7.5 mM or higher gave the fastest gel dissolution time (Fig. 2). Identical results were obtained with 0.75% gellan gels (data not shown).

The softer biological growth gels were also dissolved with various concentrations of EDTA. The gels dissolved very rapidly at a concentration at or greater than 7.5 mM. For the experiments described in Fig. 2, a magnetic stirrer was used and the dissolution of

biological gels was considerably faster than dissolution of similar gels with a gyrotory shaker (Fig. 1).

Dissolution of gellan carrot root cultures. These dissolution experiments were performed with actual root cultures at room temperature with a gyrotory shaker. Buffer to gel ratios (v/w) of 10:1 down to 2:1 produced similar dissolution times (Fig. 3). When the ratio of buffer to gel was 1:1, the dissolution time was significantly increased. To insure that the gel dissolved, the roots were collected over a 90 μm sieve. Undissolved pieces of gellan could readily be detected by this sieving step. Within 15 to 20 min at room temperature, a 2:1 ratio provided complete dissolution of the gel. This experiment showed that a considerably large volume of buffer (10:1 or 4:1) was not necessary to efficiently dissolve gellan away from root cultures.

From a plant cell fractionation standpoint, it may be beneficial to dissolve gellan in the cold at 4°C. As expected, this could be done with a buffered EDTA solution after a longer dissolution period (Fig. 4). The EDTA dependent dissolution at 4°C was performed with the TRIS HCl-TRIZMA buffer or with 75 mM HEPES titrated with 75 mM MES to obtain a buffer at pH 7.5. These buffers are typically used in cell fractionation procedures for isolating and purifying membranes, soluble enzymes and cell walls. Both buffers completely dissolved the gellan culture medium in 30 to 35 min at a 2:1 ratio. Increasing the ratio provided only a slight decrease in the dissolution time. To eliminate wasteful use of buffer, a 2:1 ratio for 30 to 35 min at 4°C will completely dissolve the gellan medium away from the root cultures.

CONCLUSIONS

Roots can be easily recovered from gellan cultures by rapidly dissolving the gel with a cation chelator. EDTA concentrations at 7.5 to 10 mM produced the fastest dissolution times under all temperatures tested. Dissolution by EDTA is pH dependent and is most effective at pH 7.5 or greater. The time of the dissolution process is also affected by the type of system used to mix the buffer and gels. A magnetic stirring bar apparatus provides rapid dissolution of the culture gel (5 min) at room temperature while a gyrotory shaker (no mechanical shear) required 18 min for complete dissolution of the gel. The less turbulent process will not damage intact roots and will be the most desirable for physiological and biochemical studies.

The procedure developed here has an advantage over the recently reported process (Doner and Bécarré, 1991) in that gels can be readily dissolved at 4°C, unlike the citrate buffer system. Furthermore, the gels can be dissolved directly in buffer systems typically

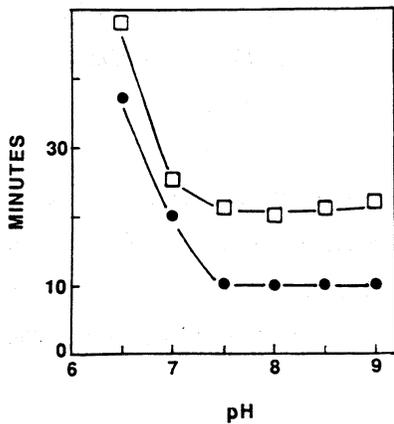


Figure 1. The pH dependent dissolution of gellan gels in the presence of 10mM EDTA at room temperature. Model gels(0.4% gellan) were stirred on a magnetic stirring plate and buffered with 50mM TRIS HCL-TRIZMA base at various pH(●). Biological gels(0.2% gellan) were dissolved with the same buffer system and gently shaken on a gyrotory shaker(□).The buffer to gel ratio(ml/g gel) was 2:1. See text for details.

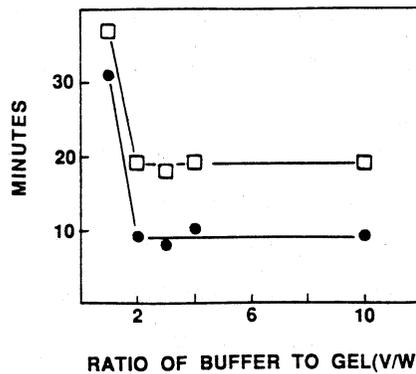


Figure 3. The effect of increasing the buffer to gel ratio on the dissolution time of gellan gels at room temperature. Model gels(●) were dissolved with a magnetic stirrer in the buffer system with 10 mM EDTA at pH 7.5 described in Figure 1. Biological gels containing transformed carrot roots were gently shaken in the same buffer system with a gyrotory shaker(□). Complete dissolution of the gel was monitored as described in the text.

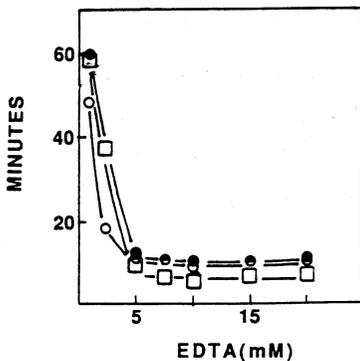


Figure 2. EDTA dependent dissolution of gellan gels at room temperature. Gels were dissolved in the buffer system described in Figure 1 at pH 7.5. Model gels were tested with a 2:1(●) or 5:1(○) ratio(V/W). Biological gels were tested at a 2:1 ratio(□). A magnetic stirrer was used for these experiments.

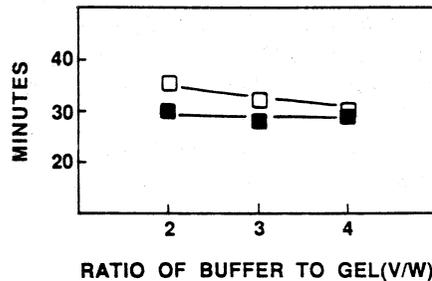


Figure 4. The dissolution of biological gels with increasing buffer to gel ratio(V/W) at 4 C. The gels contained transformed carrot roots and were gently shaken in a gyrotory shaker in either 50 mM TRIS HCL-TRIZMA base(□) or 75 mM HEPES-MES buffer(■) at pH 7.5 in the presence of 10 mM EDTA.

employed in plant cell fractionation studies. These slightly alkaline buffer systems (Fig. 4) are necessary to maintain the integrity of membranes (Nagahashi, 1985) and minimize contamination of cell walls during cell fractionation procedures (Nagahashi and Garzella, 1986).

Finally, the dissolution procedure developed here has particular value in studies involving the early events of VAM formation on transformed roots grown on solid medium in dual culture (Bécard and Fortin, 1988). It is also ideal for studies on secondary plant metabolism requiring small quantities of cultured roots or for studies on roots which cannot be grown in liquid culture.

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