

# Purification of commercial gellan to monovalent cation salts results in acute modification of solution and gel-forming properties <sup>☆</sup>

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## Abstract

Lithium, sodium, potassium, and ammonium salts of the industrial polysaccharide gellan were prepared. The salts were freely soluble in water at room temperature (25°C). The opinion had been generally held that heating to 100°C was necessary for gellan to achieve complete solubility in the presence of mono- or multivalent cations. Then, upon cooling, the solutions would form gels. These conclusions were based on the properties imposed upon gellan samples by the presence of contaminating divalent cations. Commercial gellan samples contain calcium and magnesium at levels exceeding 0.9%, sufficient for counterion formation with over one-third of gellan's carboxyl groups. Purification was rapid and included sequential treatments with a cation-exchange ( $H^+$ ) resin, LiOH, NaOH, KOH, or  $NH_4OH$ , and an anion-exchange ( $Cl^-$ ) resin. About 95% of the divalent cations and nearly 90% of the phosphate that contaminated commercial gellan were removed. The purified monovalent salts of gellan set in the presence of divalent cations and provide well-defined agents for gelling media used for propagation of microbes and plants. In a manner analogous to sodium alginate, solutions of lithium, sodium, potassium, or ammonium gellanate form beads when dropped into solutions of divalent cations. This property was exploited for entrapment of enzymes and cells in beads.

**Keywords:** Gellan; Phytigel<sup>®</sup>; Gel-Gro<sup>®</sup>; Cationic salts; Solution; Gel formation

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<sup>☆</sup> Mention of brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

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## 1. Introduction

Gellan is an extracellular anionic polysaccharide produced by the bacterium known earlier as *Pseudomonas elodea* [1]. More recently this bacterium has been classified as a member of the genera *Auromonas* [2] and then *Sphingomonas* [3]. It is composed of a linear sequence of tetrasaccharide repeating unit  $\rightarrow 3)\text{-}\beta\text{-D-Glc p-(1}\rightarrow 4)\text{-}\beta\text{-D-Glc pA-(1}\rightarrow 4)\text{-}\beta\text{-D-Glc p-(1}\rightarrow 4)\text{-}\alpha\text{-L-Rha p-(1}\rightarrow [4]$ . Native gellan is partially acylated with acetyl and L-glycerate groups [5]. The commercial gellan preparations are deacylated with alkali before being precipitated with 2-propanol from the fermentation medium [1]. In the presence of sufficient concentrations of divalent cations to neutralize and crosslink carboxyl groups in gellan, firm and exceedingly clear gels are formed at low concentrations. Much higher concentrations (about 30-fold) of monovalent cations are required for gel formation. Gellan gels provide several advantages over agar gels in addition to optical clarity (agar gels are cloudy and opaque). At the 0.5% level, they have the same strength as 1.5% agar gels [6]. In addition, they can be easily liquified, allowing recovery of viable tissue for further study [7,8]. For these reasons, gellan is increasingly being used as an agent for gelling microbiological and plant tissue culture media, and also as a support for plant micropropagation. A comprehensive review of the properties and uses of gellan has been published [9].

The major use of gellans is for foods, where Kelcogel® (Kelco, a unit of Monsanto Co.) is marketed as a thickener and stabilizer. This study focuses on non-food applications. Gellan is commercially available from Kelco as Gelrite® for cell and tissue culture applications and is also marketed with the brand names Phytigel® (Sigma), and Gel-Gro® (ICN). Gellan was shown to compare quite favorably with agar in several media used for bacterial growth [10]. In seed germination and root formation studies, both the number and length of roots were greater for seeds germinated in gellan than those germinated in agar [11]. The transparency of gellan gels greatly facilitates the observation and photography of root growth and determination of the presence of microbes. For example, gellan is routinely used to gel the medium used for dual cultures of roots and mycorrhizal fungi [12].

The levels of impurities in gelling agents such as gellan and agar are rarely considered when preparing media for the culture of microorganisms or plant tissues. Ash in commercial gellan and agar preparations, however, range up to 7% and 9.5%, respectively [13]. As a result, the levels of macro- and microelements in the widely used Murishige and Skoog [14] tissue culture medium are altered significantly by contributions from the gelling agent. The variable effects of agar brand and concentration on properties of tissue culture media have been described [15], and detailed comparisons of both agar and gellan in terms of elemental analyses and physical properties have been made [16]. Divalent cations are present in the fermentation medium used to produce gellan, and significant levels of these cations are precipitated with gellan as counterions. In reports [17] comparing plant callus tissue growth, gellan was superior to agar. In light of the qualitative and quantitative differences in levels of impurities in gellan and agar, explanations for such differences are unavailable.

Levels of phosphorus in commercial lots of gellan typically range from 0.08 to 0.21%. In our studies of root growth in 0.2% gellan gel-based media, more phosphorus

is provided by the gelling agent than by the media. The inability to control levels of this important nutrient severely handicaps such studies, particularly those in which dual cultures of vesicular-arbuscular mycorrhizal fungi and plant roots are examined [12].

The purpose of this study was to prepare a purified and well defined material, with diminished levels of contaminating divalent cations and phosphorus, from commercial gellan. The improved solution and gel properties of the purified gellan were applied to culturing root tissue and to immobilizing viable enzymes and cells.

## 2. Results and discussion

Calcium and magnesium account for nearly all of the multivalent cations present in a typical commercial gellan preparation. About 38% of the carboxyl sites on gellan are occupied by these two cations. Their presence prevents one from preparing aqueous solutions of these materials except at temperatures approaching 100°C. Their replacement with potassium or another monovalent cation results in a material with water solubility at room temperature. The levels of lesser contaminants, such as phosphorus, manganese and copper are also reduced in potassium gellanate. Commercial lots of gellan from different suppliers contained somewhat variable levels of impurities, but after purifying to potassium gellanate, all had similar composition and properties. Conditions for gellan purification were optimized using Phytigel®; subsequently, lots of Gel-Gro® were purified, yielding a similar product.

*The gellan purification procedure.*—Step I involved stirring at 60°C a turbid 1% aqueous solution of Phytigel® with a cation-exchange ( $H^+$ ) resin. The pH dropped from 6.1 to less than 3.0 within seconds of adding the resin; after stirring for a total of 15 min, the pH was 2.2. Replacement of the divalent cations was quite effective due to the selectivity of the cation-exchange resin for these cations. Phytigel® solutions were treated with varying proportions of the technical grade resin Dowex 50W-X8 ( $H^+$ ), and a ratio of 2.5:1 (resin: Phytigel, w/w) was found to be optimal. Solutions containing greater than 1% concentrations of commercial gellan at 60°C or less solidified when the divalent counterions were exchanged for resin protons. The purification procedure was tested at 70°C and 80°C, but final products did not gel with divalent cations. Depolymerization of the polysaccharide probably occurred at the higher temperatures. The 2-propanol precipitable product in the protonated form was insoluble under any conditions tested, so Step II, titration with hydroxides of monovalent cations, was mandatory.

In Step II the gellan solution in the protonated form was treated with either lithium, sodium, potassium or ammonium hydroxide while holding the temperature at 60°C. Potassium hydroxide was most often used in our study, since this cation is most compatible with the liquid media in our plant tissue cultures. Products with similar solution and gelling properties resulted, whichever monovalent cation was used. The titration curve is presented in Fig. 1. When the pH had risen to about 4.3, near the endpoint of the titration, the solution spontaneously changed from turbid to crystal clear. An additional increment of alkali increased the pH to between 7.0 and 8.0. The clear polysaccharide solution produced during the titration liberated particle bound contaminants such as phosphate, allowing their removal in Step III. The treatment with the  $H^+$

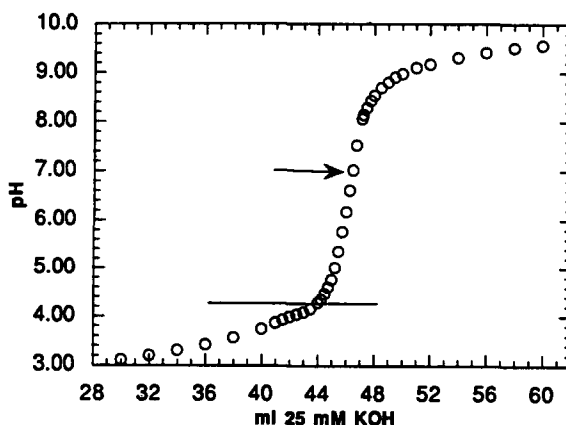


Fig. 1. Titration of Phytigel® (1.5 g) with 25 mM KOH after exchange of cations for protons with Dowex 50W-X8 ( $\text{H}^+$ ) resin. The line at pH 4.3 was the pH at which the turbid solution became clear; the arrow indicates the pH above which Dowex 1-X8 ( $\text{Cl}^-$ ) resin was added.

resin in Step I was a necessary prelude to Step II, since 1% suspensions of Phytigel® in water did not clarify after titration with KOH, even after prolonged stirring at 60°C at pH 10.0. Apparently, the divalent cation contaminants in commercial gellan preparations were very tightly bound.

Step III was an anion-exchange ( $\text{Cl}^-$ ) resin treatment. The solution remained clear during the 15 min, 60°C stirring period, and the pH dropped from between 7.0 and 8.0 to about 5.5. Phytigel® solutions were treated with varying proportions of the technical grade resin Dowex 1-X8 ( $\text{Cl}^-$ ) and a ratio of 5:1 (resin: Phytigel, w/w) was found to be optimal. This ratio could be halved when stirring period was increased to 1 h. The binding capacity of the resin (and that used in Step I) exceeded by several-fold that required for quantitative removal of ionic contaminants. Both resins displayed thermal stabilities well above 60°C. After removal of the resin, fibrous potassium gellanate was collected after precipitation with 2-propanol (Step IV). Yields of potassium gellanate were about 65%. The less than quantitative yield was the result of Step I [cation-exchange ( $\text{H}^+$ ) resin treatment]. A fraction of Phytigel® was insoluble at pH 2.3 and was filtered out with the resin. Higher yields would have likely resulted if initial Phytigel® concentrations of less than 1% were used.

Other approaches were tested for removing divalent cations and phosphate from Phytigel®. These included acid-washing Phytigel® before KOH addition, dialysis of turbid solutions against dilute HCl or KOH, treatments with mixed-bed ion-exchange resins, and centrifugation of Phytigel® suspensions. These approaches resulted in low yields and were ineffective in removing divalent cations, although typically more than one-half of the phosphate was removed.

**Composition and properties of potassium gellanate.**—Purification resulted in an overall  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  reduction of 95.1% (Table 1). Their original levels were sufficient to serve as counterions for 37.9% of the carboxyl groups in Phytigel. After purification this dropped to 1.77%. The remaining carboxyls in Phytigel® before purification were

Table 1

Levels (%) of major contaminants in Phytigel® and in purified potassium gellanate

Element	Phytigel®	K gellanate	Reduction (%)
P	0.099	0.011	88.8
Ca	0.707	0.039	94.5
Mg	0.225	0.007	96.9
Na	0.844	0.044	94.8
K	2.13	5.75	

neutralized mainly with  $\text{Na}^+$  and  $\text{K}^+$ , and in potassium gellanate almost exclusively with  $\text{K}^+$ . It was not surprising that the solution and gel-forming properties of the two materials were markedly different.

The purified salts of gellan with monovalent cations, such as lithium, sodium, potassium, and ammonium ions were extremely soluble in water at room temperature. Their maximum useful concentrations are determined by viscosity rather than solubility. This is contrary to the claim that “gellan requires heating to approximately 100°C to achieve complete solubility in the presence of ions” [18]. The presence of significant levels of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  had led to such claims and has resulted in limited non-food applications of commercial gellans.

For determination of setting temperatures, gels were prepared as described in the Materials and methods section.  $\text{Mg}^{2+}$  was used as the cation, and setting temperatures were found to increase with gum concentration. At concentrations in water of 0.6%, Phytigel® and potassium gellanate set at 33.5°C and 28.0°C, respectively. When 1.0 and 2.0% solutions of each material were prepared in water (no divalent cation added), Phytigel® set at 30°C (1.0%) and 38°C (2.0%). Aqueous solutions of potassium gellanate did not gel at either concentration, but solutions froze at  $< 0^\circ\text{C}$ . The same was true for lithium, sodium, and ammonium salts of gellan.

*Potassium gellanate as gelling agent for microbiological and tissue culture media.*—Detailed microanalyses of various lots of commercial gellan and agar have been published elsewhere [16]. Gellans were quite variable in elemental composition, but these were not as variable as different brands of agar. Levels of the major divalent cation contaminants in three lots of gellan ranged from 0.12 to 0.83% for calcium and 0.15 to 0.27% for magnesium. Agars generally contain much lower levels of these cations (and potassium). An agarose with a low gelling temperature is available with very low levels of elemental impurities, but it is very expensive. We calculated the excess over expected levels of some macronutrients when impure gellan (Table 1) was used to prepare 0.4% gels of minimal (M) media, a commonly used plant tissue culture media [12]. Calcium exceeded expected levels by 44%, magnesium by 11%, potassium by 91%, phosphorus by over 4-fold, and sodium by 75-fold. In potassium gellanate, the divalent cation contributions to their overall level in gelled media are very low, and the increased levels of potassium are compatible with plant tissues. Levels of chloride ion introduced in step III after removing phosphate and other anions with Dowex 1-X8 ( $\text{Cl}^-$ ) resin are low and compatible with media.

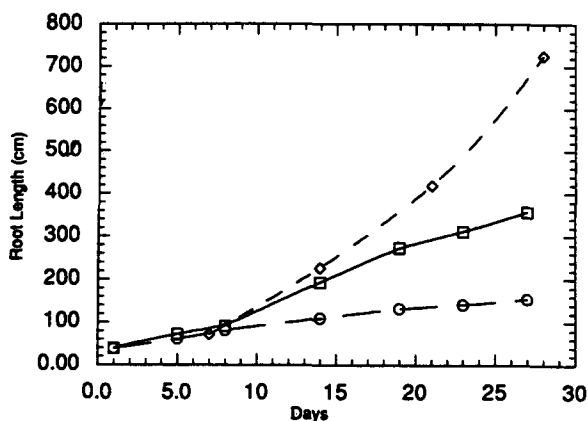


Fig. 2. Comparison of growth of transformed carrots in cultures of (◇) M medium gelled with Phytigel®, (□) M medium gelled with potassium gellanate, and (○) M medium (without phosphate) gelled with potassium gellanate.

Media gelled with potassium gellanate are well defined. The fact that phosphate levels were reduced by nearly 90% was important for our research on dual culture of vesicular-arbuscular mycorrhizal fungi and carrot roots, in that phosphate inhibits colonization of roots by the fungus [19]. The presence of high levels of phosphate in Phytigel® was noted when examining the growth of transformed carrot roots on M media. We found that roots accumulated more phosphate than would have been possible from that which had been provided by the M medium. The growth of carrot roots in (a) M medium gelled with Phytigel®, (b) M medium gelled with potassium gellanate, and (c) M medium (without phosphate) gelled with potassium gellanate were compared. Those grown as in (a) grew to twice the length as those grown as in (b) and more than four times the length of those grown as in (c) in 27 days (Fig. 2). Agar gels are not suitable for such studies. They lack optical clarity, and, unlike gellan gels, agar gels cannot be liquified [7,8], so recovery of viable tissues for further examination is not possible. Lithium, sodium, or ammonium salts of gellan can be added to media when the presence of these cations is preferred to potassium.

**Gellan beads and their uses.**—Rigid and clear beads were prepared by dropping 1.0% solutions of potassium gellanate into  $Mg^{2+}$  or  $Ca^{2+}$  solutions. More rigid beads resulted from using  $Ca^{2+}$  solutions, in which case solutions of less than 1.0% yielded quite firm beads. It was necessary to warm solutions of concentrations greater than 1.0% because of their high viscosities. Preliminary results with enzyme and bacterial immobilization in gellan beads were promising. Beads prepared after stirring invertase into a 1.0% potassium gellanate solution remained catalytically active after repeated uses. In addition, cells of *Bradyrhizobium* were entrapped in gellan beads and remained viable when perfused with essential nutrients. The high gel-setting temperatures (above 50°C) of adequate concentrations of commercial gellans are imposed by divalent cation contaminants, and this renders them unsuitable for enzyme or cellular immobilization.

Various chelators have been employed to lower gel-setting temperature [20] in attempts at immobilizing cells in commercial gellan.

Essentially the same procedure as described above is used for entrapment of enzymes and cells into alginate [21].  $\text{Mg}^{2+}$  is one divalent cation that does not allow alginate to form crosslinkages, so  $\text{Ca}^{2+}$  is generally used. Other possible advantages of potassium gellanate over alginate are optical clarity and the lower charge density (about one-fourth), and hence the requirement for lower divalent cation concentrations for crosslinking.

Gellan beads have promise for entrapment of enzymes, cells, and as inert supports for controlled drug-delivery systems. Since more common sugars constitute gellan, it is possible that immune responses elicited when uncoated alginate is used to entrap therapeutic agents [22] would not be encountered. As with alginate, gellan beads are unstable when they encounter chelators such as citrate. This problem can be minimized by supplementing media with divalent cations.

### 3. Materials and methods

*Purification of gellan.*—Purification steps were optimized using gellan marketed as Phytigel® (Sigma Chemical Co, St. Louis, MO). Gel-Gro® (ICN Biochemicals Cleveland, OH) yielded a product with similar composition and gelling properties.

*Step I. Replacement of cations with protons.* On a magnetic stirrer-hot plate, Phytigel® (10 g) was sprinkled gradually into rapidly stirring distilled, deionized water (1.0 L) at room temperature. The turbid mixture was heated to 60°C, and 25 g of the cation-exchange resin Dowex 50W-X8 ( $\text{H}^+$ , 20–50 mesh, Bio-Rad Laboratories Melville, NY) was added. After 15 min the resin was removed by vacuum filtration (12 cm Buchner funnel) through several layers of fine mesh (grade 60,  $32 \times 28$  threads/in<sup>2</sup>,) cheesecloth. The vacuum flask was held in a reservoir of hot water so the temperature of the filtrate remained above 50°C.

*Step II. Addition of hydroxides of monovalent cations.* The turbid filtrate was heated to and maintained at about 60°C while N KOH, LiOH, NaOH or  $\text{NH}_4\text{OH}$  were added. The initial pH was about 2.2, and, after addition of about 11.8 mL of N KOH, it had risen to pH 4.3. At that point the solution became very clear. Addition of N KOH was stopped when the pH was between 7 and 8 (addition of another 0.7 mL titrant), and the solution then was stirred another 5 min.

*Step III. Removal of contaminating anions.* Dowex 1-X8 anion-exchange resin (50 g,  $\text{Cl}^-$ , 20–50 mesh, Bio-Rad Laboratories, Melville, NY); was added, and the mixture was stirred 15 min while the temperature was maintained at 60°C. The resin was removed by vacuum filtration as described above, maintaining the filtrate above 50°C.

*Step IV. Isolation of potassium gellanate.* The filtrate was poured into 2 vol of a rapidly stirred solution of 2-propanol. A white fibrous precipitate formed immediately and was recovered on a no. 20 (850  $\mu\text{M}$ ) stainless steel sieve. (Cu was detectable in product isolated on brass sieves, so their use should be avoided). After excess 2-propanol–water was hand-squeezed from the purified gellan, the product was air dried for 30 min and then dried to constant weight in a vacuum oven at 40°C. The yield was 6.5 g (65%). The product was converted to a powder with a conventional chopper-grinder.

Elemental analyses by ICP were performed by Galbraith Laboratories, Inc. (Knoxville, TN).

*Preparation of gels with Phytigel® and potassium gellanate.*—Optimal conditions for preparing 0.5% gels were developed using salts of the divalent cations  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ . Gels (0.5%) of optimal strength and clarity resulted when 500 mg was stirred into 100 mL of 3.6–6.0 mM solutions of divalent cation salts ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  or  $\text{CaCl}_2$ ). The solutions were heated to boiling, allowed to cool to about  $50^\circ\text{C}$ , and then transferred to Petri plates. The 3.6 mM cation concentration was sufficient to serve as counterions to the carboxyl groups in gellan. Gels (0.5%) with divalent cation concentrations above 7.2 mM were somewhat cloudy, especially when  $\text{Ca}^{2+}$  salts were used. Cation concentrations were increased or decreased proportionally when preparing gels other than 0.5% potassium gellanate.

Appropriate levels of Phytigel® or Gel-Gro® were added to M media (composition below), and the mixtures were autoclaved for 16 min at  $121^\circ\text{C}$  [2]. The clear solutions were cooled to about  $50^\circ\text{C}$  and dispensed into Petri plates. Most media contain levels of divalent cations such as  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  that supplement those included in commercial gellans, and in total allow clear and firm gels to form upon cooling. When potassium gellanate gels were prepared, it was necessary to double the level of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  in order to produce firm gels. Gel setting temperatures of Phytigel® and potassium gellanate were determined using an established procedure [20].

*Growth of carrot roots in M media gelled with Phytigel® and potassium gellanate.*—Minimal (M) medium included the following components in 1.0 L of water:  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (731 mg);  $\text{KNO}_3$  (80 mg);  $\text{KCl}$  (65 mg);  $\text{KH}_2\text{PO}_4$  (4.8 mg);  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  (288 mg); sucrose (10 g);  $\text{NaFeEDTA}$  (8 mg);  $\text{KI}$  (0.75 mg);  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (6 mg);  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (2.65 mg);  $\text{H}_3\text{BO}_3$  (1.5 mg);  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.13 mg);  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (2.4  $\mu\text{g}$ ); glycine (3 mg); thiamine  $\cdot \text{HCl}$  (0.1 mg); pyridoxine  $\cdot \text{HCl}$  (0.1 mg); nicotinic acid (0.5 mg); and *myo*-inositol (50 mg). Gels (0.4%) of both Phytigel® and potassium gellanate in this medium were prepared by adding 4.0 g/L and autoclaving at  $121^\circ\text{C}$  for 16 min. M-media without  $\text{KH}_2\text{PO}_4$  was also prepared. After the media had gelled, transformed carrot roots were layered onto the gels [12] and their growth was monitored over a 4-week period.

*Preparation of gellan beads.*—A 1.0% solution of potassium gellanate was prepared by sprinkling the powder into stirred  $\text{H}_2\text{O}$  at the desired temperature and allowing a few min for complete dissolution. This solution was added dropwise (away from the vortex) into slowly stirred  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  or  $\text{CaCl}_2$  (40 mM, aq) solutions. Bead formation was spontaneous, and stirring was continued for 30 min in order to cure the beads, which were then rinsed several times with water. Beads were removed by sieving or decantation. From a 26 gauge needle, 75 beads/mL resulted, so the diameter was about 1.4 mm (assuming no swelling with  $\text{Mg}^{2+}$ ). From a 22 gauge needle, beads were about twice this size. The 1.0% potassium gellanate solutions were quite viscous and the resulting beads were firm. Stable beads were also prepared by using 0.5% potassium gellanate solutions, and the resulting beads were somewhat smaller. Beads with entrapped invertase or *Bradyrhizobium* were prepared using a 22 gauge needle by dispersing these into 1.0% potassium gellanate solutions, followed by dropping into  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  or  $\text{CaCl}_2$  (40 mM, aq).



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