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Purified curdlan and its hydroxyalkyl derivatives: preparation, properties and applications

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Curdlan, a β-1,3-glucan produced by fermentation of *Alkaligenes faecalis*, is a non-ionic gel-forming polysaccharide that is, along with its hydroxalkyl derivatives, a potentially important matrix for life science applications. The commercially available material contains residual nucleic acids, cellular debris and other contaminants that can interfere with electrophoretic separations and visualization procedures. Simple procedures have been developed for purification of the curdlan and coherent gel formation. Curdlan gels can be formed in a variety of chaotrope-containing, or chaotropic, solvents, including 40% formamide, 7 M urea. The chaotropes can be retained or subsequently removed by leaching. Heat treatment before or after leaching enables thermostable gel formation. Hydroxyethyl and glyceryl derivatives of curdlan have been prepared. Depending on the degree of substitution (DS), a whole spectrum of derivatives with a range of unique properties can be obtained. Lower DS hydroxyethyl derivatives form clear, elastic gels in 6 M urea, while the higher DS hydroxyethyl derivatives are soluble in hot water and gel on cooling to form clear, elastic, thermoreversible gels. Partial depolymerization of curdlan by γ-irradiation reduces the viscosity of subsequent preparations, enabling the preparation of higher concentration, more sieving gels. Use of selected preparations to form unique matrices for electrophoretic separations has been demonstrated.
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INTRODUCTION

Curdlan, an alkali-soluble β-1,3-glucan polysaccharide formed by fermentation of *Alkaligenes faecalis*, was first described by Harada *et al.* (1968). Because of the unique and useful properties associated with curdlan, it has been the subject of considerable investigation. This paper provides a summary of some work done at FMC Corporation on curdlan and its hydroxyalkyl derivatives for life science applications, particularly gel electrophoresis (Provonchee & Renn, 1988; Renn *et al.*, 1993; Harper *et al.*, 1995a, b).

Aqueous curdlan gels can be formed by a variety of methods, the simplest of which is heating an aqueous slurry to about 55°C and letting it cool to form a thermoreversible gel or by heating the slurry to >70°C to form a thermo-irreversible gel (Maeda *et al.*, 1967). These thermostable gels, whose melting temperatures are reportedly greater than 135°C (Kuge *et al.*, 1977), are not entirely coherent, but contain swollen gel particulates that can interfere with certain applications. Gels prepared by heating an aqueous suspension of curdlan in appropriate buffers have been used as matrices for electrophoresis (Hamase *et al.*, 1991, 1992, 1993a, b; Dong & Ohta, 1995).

Methods have been devised to enable formation of coherent curdlan gels (Towle, 1977; Hercules Inc., 1978; Kanzawa *et al.*, 1987) but these are laborious and/or time-consuming and do not assure homogeneous gel formation. This paper describes a simple method for preparing coherent, homogeneous, thermoreversible cold-set gels and thermoset heat-stable gels. The structure of the curdlan matrix depends on the method used to form the gel (Konno *et al.*, 1979; Takahashi *et al.*, 1986: Kanzawa *et al.*, 1987; Griess *et al.*, 1993).

Commercially available curdlan preparations contain cellular debris, nucleic acids and proteins that interfere with gel electrophoresis, specifically visualization of the separated nucleic acids and proteins. A simple method

was developed to remove the impurities that bind ethidium bromide and other stains to give an unacceptable background.

Stable, dry, water-soluble salts of the purified curdlan were prepared. When dissolved in water, the alkaline solutions could be used directly to prepare coherent gels by the critical temperature neutralization method.

Curdlan will form aqueous gels in the presence of hydrogen-bond-disrupting reagents, or chaotropes, including dimethyl sulfoxide (Aizawa *et al.*, 1974; Watase & Nishinari, 1992) and a variety of other chaotropic agents, including urea and various thiocyanates (Takeda Chemical Industries Ltd, 1975a). We found that the chaotropes used in gel formation could be extended to include formamide and aqueous guanidinium thiocyanate. These chaotropes can be removed by leaching and the gels can be further modified by heat treating, either before or after leaching.

Just as hydroxyalkylation imparts water solubility to cellulose, it also does this with curdlan, if sufficiently substituted. Depending on the degree of substitution, and the substituent introduced, hydroxyalkyl curdlans exhibit a wide variety of properties, including easily prepared, thermoreversible gels whose separation and resolution capabilities resemble those of cross-linked polyacrylamide. A number of derivatives of various β-1,3-glucans have been previously reported (Hamuro & Akiyama, 1974; Takeda Chemical Industries Ltd, 1975b; Takahashi *et al.*, 1978; Sasaki & Sugino, 1984; Miyashiro *et al.*, 1985; Matsuzaki *et al.*, 1986; Shibata *et al.*, 1989) but not the gelling, hydroxyalkyl derivatives of the native linear, non-branched curdlan.

MATERIALS

Commercially available curdlan (Lot #RF08) was obtained from Takeda Chemical Industries Ltd, Tokyo, Japan. An older preparation labelled "Takeda Polysaccharide 13140" was also used. The source and lot numbers of other reagents are indicated as appropriate.

EXPERIMENTAL

Coherent gel formation by critical temperature neutralization

Although curdlan will separate from an alkaline solution when brought to pH <8.5 at room temperature, there is a critical temperature range (50–55°C) where precipitation does not occur, even at pH values <4. Gelling can then be induced by cooling to <30°C to form a thermoreversible gel or heated to >70°C to form a thermo-irreversible gel. If heated to >70°C, the cold-set thermoreversible gel will be transformed into a heat-set, thermo-irreversible gel (Provonchee & Renn, 1988).

A 2% sol of curdlan was prepared in a 400 ml Erlenmeyer flask by dissolving 4.0 g of curdlan in 200 ml of 0.05% NaOH (aq). The flask containing the sol was placed in a 55°C water bath until the temperature of the sol was >50°C. While agitating and maintaining a temperature of 50–55°C, 2 ml of 0.5 N phosphoric acid was added. The resulting neutralized sol was poured into two gelling dishes, each containing approximately half of the sol. One was allowed to cool to room temperature, during which time it gelled. This gel was thermoreversible, melting at about 50°C. The gelling dish containing the other portion was covered and heated in a boiling water bath for about 15 min, during which time a thermo-irreversible gel was formed. Gels prepared this way were shown to be homogeneous by Aniline Blue staining (Nakanishi *et al.*, 1976). With this reagent, which binds to the curdlan, gels that are not coherent will exhibit internal variations in color distribution. Other acids could be used for pH adjustment and additional reagents could be added to the sol before gelling, including buffers, chaotropes, etc. In addition, other hydrocolloids, such as starch and locust bean gum, could be added to prevent or control curdlan gel syneresis, which can be significant. With appropriate reagents, the resulting gels were shown to have utility in nucleic acid and protein gel electrophoresis, as immunoassay gel media, and as microbial growth support matrices (Provonchee & Renn, 1988).

Curdlan purification

Curdlan gels were found to be unsatisfactory for DNA electrophoresis when ethidium bromide was used as the detection reagent. After removal of the excess stain by washing, considerable fluorescent background remained in the gel. This interfered with DNA detection of all but the strongest bands. In addition to residual nucleic acids, commercial curdlan was found to contain cellular debris.

Curdlan and nucleic acids have similar solubilities and a differential solubility technique exploiting minor differences was developed to remove, or at least minimize, the impurity content. Many variations were tried and the following simple, effective procedure evolved (Renn *et al.*, 1993). Ten grams of crude curdlan was suspended in 625 ml of 1% (w/v) aqueous sodium carbonate (anhydrous granular, Sigma Chemical Co., St Louis, MO). The pH was adjusted to pH 7.5 with 1.0 M HCl and the mixture stirred for 60 min. The suspension was vacuum filtered through a finely woven (about 200 mesh) polyester cloth to recover the curdlan. The collected cake was rinsed with

an additional 325 ml of the washing solution, then transferred to a beaker containing 625 ml of distilled water. This was stirred for 60 min then the curdlan was recovered as previously described, using an additional 350 ml of water to wash the cake. The partially purified curdlan, which at this stage contained small amounts of cellular debris, was suspended in 562 ml of distilled water. To this was added 62 ml of 1.0 M NaOH, to dissolve the curdlan, and 80 g of Hyflo SuperCel® filter aid (Manville Service Corporation, Lompoc, CA), and the slurry was mixed thoroughly by stirring. This slurry was poured into a 1 l pressure filter bomb and filtered through a piece of 10 cm diameter #54 Whatman® filter paper (Whatman LabSales, Hillsboro, OR) previously coated with 16 g of the filter aid. After completion of the filtration, the cake was rinsed using 50 ml of 1.0 M NaOH. This rinse was combined with the filtrate and recovered by coagulation in two volumes of acidified isopropyl alcohol (1400 ml containing 23 ml of 5 M acetic acid). The coagulum was washed in 1400 ml of distilled water, recovered on the polyester cloth (*c.* 200 mesh) and hardened in the same volume of 99% isopropyl alcohol. This was dried in a forced air oven at 55°C then ground through a 40 mesh screen. Gels made from this purified curdlan were essentially free of 260 nm-absorbing and ethidium bromide-retaining impurities.

UV absorption spectra for the commercial and the purified curdlans were obtained on 1% solutions of curdlan in 0.1 M NaOH) using a Beckman DU® 7 spectrophotometer. The following values were obtained:

Sample	A_{320}	A_{260}	A_{220}
Commercial curdlan	0.2775	0.8192	2.2482
Purified curdlan	0.0725	0.1434	0.5967

A 'gel-plug' assay was devised to examine the relative amount of ethidium bromide retention in 1% curdlan gels. To prepare a 1% (w/v) curdlan gel, 0.215 g of the curdlan sample was suspended in 22.5 ml of distilled water, and 2.5 ml of 1.0 M NaOH was added to dissolve the curdlan. The curdlan solution was heated to 55°C, using a hot plate, and 0.5 ml of 5M acetic acid was added with stirring. Next 1 ml of the neutralized curdlan solution was pipetted into a 10 ml beaker and allowed to gel at room temperature. The gel plug was removed and placed into an ethidium bromide solution (1 μg/ml). After staining for 30 min the gel was destained in distilled water for 30 min. The gel plugs were illuminated using a UV light (Fotodyne® Model 3-4400). Photographs were taken at F4.5, 1/2 second exposure, using a Polaroid® MP-4 Land camera containing Type 57 4″×5″ film. Visual comparisons were made. The commercial curdlan exhibited an unacceptably high background fluorescence, while the purified curdlan had essentially no background fluorescence.

Moisture and ash assays were done on the commercially available and purified curdlan preparations. Moisture was determined by the weight loss of the samples in a vacuum oven at 75°C overnight. Ash was determined by heating the samples from the moisture determination at 75°C in a platinum crucible until they became white and then determining the weight of the residues. The commercial curdlan contained 7.40% moisture and 3.05% ash, whereas the purified curdlan contained 4.44% moisture and only 0.38% ash.

To check the utility of the purified curdlan as an electrophoresis matrix, a 1% gel was formed using the critical temperature neutralization method described earlier. This was cast in an Aquebogue (Aquebogue, NY) model 750 mini-submarine gel electrophoresis chamber using a framing gel of 1% SeaKem® agarose (FMC BioProducts, Rockland, ME) prepared in 0.5X TBE buffer (49.5 mM Tris base, 49.5 mM boric acid, 1 mM EDTA, pH 8.0), then equilibrated overnight in the buffer. Before loading the samples, the buffer was changed. The wells were loaded with 5 μl (200 ng) of *Hind* III λ digest (Life Technologies, Grand Island, NY). Electrophoresis was performed at 60 V (5 V/cm) for 82 min, during which time the tracking dye migrated 4.5 cm in the agarose. The gel was removed and stained for 35 min in a 1 μg/ml ethidium bromide solution followed by destaining in distilled water for about 35 min. The gel was examined using a UV light source. In contrast to similar gels prepared previously from crude curdlan, where the intense residual background fluorescence obscured the separated nucleic acid fragments, they were readily visible. In addition, DNA migration was considerably faster in the curdlan than in the agarose framing, gel as seen in Table 1.

Using purified curdlan as the gel matrix for pulsed field gradient electrophoresis, and slices of *Schizosaccharomyces pombe* plugs (FMC BioProducts, Rockland, ME) as the samples, all three chromosomes (3.5, 4.7 and 5.7 Mb) were distinctly resolved (Renn *et al.*, 1993)

Preparation of water-soluble salts

The alkaline sodium salt of curdlan was prepared and isolated using the following procedure. 1 l of 0.1 N NaOH was used to dissolve 30 g of the purified curdlan. This sol was heated to 55°C using a microwave oven, then coagulated directly, without neutralizing, in 2 l of 99% isopropyl alcohol. The coagulum was recovered by vacuum filtration through a fine-weave polyester cloth, with excess fluid being removed by squeezing. The coagulum was washed by stirring with 2 l of 80% isopropyl alcohol for 30 min. Following recovery, the washed coagulum was hardened in 99% alcohol, then dried a forced-air oven

Table 1. Comparison of DNA migration in purified curdlan vs. agarose gels

DNA fragment size		Distance migrated (mm)	
(kb)	(kD)	1% agarose	1% curdlan
23.1	15000	10.7	20.0
9.4	6200	11.8	24.5
6.6	4300	13.2	27.6
4.3	2800	14.5	31.3
2.3	1500	19.5	38.6
2.0	1320	21.3	39.9
0.56	370	40.4	54.4

at 55°C. Recovery was 96% based on the initial weight of the curdlan, correcting for the difference in moisture content (7.40% for the curdlan and 9.80% for the sodium salt). This was ground through a 60 mesh screen. The resulting powder was readily soluble in distilled water, giving an alkaline solution which could be used to form gels by heating to 55°C then adjusting the pH of the sols followed by cooling or further heating to >70°C.

Gel formation in chaotropes with or without subsequent leaching

Two methods were used to prepare curdlan gels containing chaotropic, or hydrogen-bond-disrupting, reagents: (1) critical temperature neutralization (Provonchee & Renn, 1988) and (2) direct dissolution by heating, then cooling (Renn *et al.*, 1993). When the critical temperature neutralization method, described previously, was used, urea [5.2 M or 9 M, urea/formamide (9 M/25%)] or lithium bromide (9 M) was added to the alkaline solution of curdlan before heating to 50–55°C and neutralizing. The gels could be set by cooling or heating with the resulting gels being firm and coherent, exhibiting little syneresis.

When the direct dissolution method was used to form chaotrope-containing curdlan gels, the curdlan powder was added directly to the chaotrope (3 M lithium iodide (aq), 7 M and 8.5 M urea (aq), 4 M guanidine isothiocyanate (aq), formamide, 7 M urea/40% formamide), the slurry heated, with stirring, to about 55°C to dissolve the curdlan and then the sol was cooled or further heated to form the gel, whichever was appropriate. Gel strengths of those with the chaotropes remaining as part of the gel and those with the chaotropes removed by leaching with water were measured. Typically, leaching of the chaotrope was accomplished by stirring the gels overnight at room temperature in about 25 volumes of distilled water. On leaching the gels shrank significantly, increasing the effective concentration of the curdlan in the gel. A 1% gel prepared in 6 M urea and then leached with water contains 2.35% curdlan. To have a final curdlan concentration of 1%, the initial concentration of the curdlan must be about 0.4%.

Table 2 presents the gel strength values of selected gel preparations. Gel strengths were determined by preparing the gel in 70 cm×50 cm class crystallizing dishes (150 ml) and chilling the gel at 4°C for 2 h. The gel was then carefully inverted in the crystallizing dish. Using an FMC Marine Colloids® Gel Tester, Model GT-2 (FMC Corporation, Philadelphia, PA), in conjunction with a Model EW-3000A AND® digital balance, the average of four plunges was recorded as the gel strength value.

Table 2. Selected hydroxyalkyl curdlan derivatives and their properties

Derivative	Hydroxyalkylating reagent(ml/25 g curdlan, moles)	Water solubility	t_g(°C)	t_m(°C)
Hydroxyethyl				
	2-Chloroethanol			
a	(10.0, 0.149)	Autoclave	51	88
b	(12.5, 0.186)	Hot water	75	89
c	(15.0, 0.224)	Hot water	67	83
d	(20.0, 0.298)	Hot water	54.5	91
e	(30.0, 0.448)	Hot water	RT	81.5
Glyceryl				
	Glycidol			
a	(12.5, 0.188)	Insoluble	—	—
b	(25.0, 0.377)	Hot water	Ice bath	—

RT = room temperature.

Hydroxyalkyl derivatives of curdlan

Analogous to cellulose, which becomes water soluble when hydroxyalkylated, hydroxyethyl and glyceryl derivatives of curdlan are also water soluble in varying degrees, depending on the degree of substitution (Renn *et al.*, 1993). Depending on the specific derivative and degree of substitution, the hydroxyalkyl curdlans range from cold-water soluble to hot-water soluble to soluble at autoclave conditions to completely water insoluble. Some of the derivatives formed clear, thermoreversible gels when dissolved in hot water and then cooled. Some of the lower DS derivatives formed gels in the presence of urea. A representative preparation method similar to that used for aga and agarose derivatives (Guisely, 1976) follows, with variations and resulting properties of the derivatives presented in Table 3.

Hydroxyethyl curdlan, which contained 5.33% substituent as determined by a modified Zeisel analysis, was prepared by adding 25 g of the purified curdlan to 475 g of distilled water at room temperature. To this was added, with stirring, 4 ml of 4.4 M sodium borohydride in 14 M NaOH and 33.75 ml of 12 M NaOH. A few drops of an antifoam, 1-octanol, was added and this solution was heated to about 80°C in a hot water bath in an operating fume hood. Next 20 ml of 2-chloroethanol (lot #B17B, Eastman Kodak Company, Rochester, NY) was diluted with 50 ml of distilled water and added dropwise into the vortex of the stirred reaction mixture over a period of 25 min. This is about 3 ml/min. Stirring and heating were continued for 60 min. The final solution temperature was observed to be 84°C. Following the addition of 120 ml of cold distilled water (8°C), which lowered the solution temperature to 64°C, the alkaline solution was coagulated by stirring into 1400 ml of 99% isopropyl alcohol that had been acidified with 159 ml of 3 M acetic acid. The coagulum was recovered by vacuum filtration through finely woven polyester cloth, with the residual solvent removed by squeezing. This coagulum was washed in 1400 ml of 70% isopropyl alcohol for 30 min, recovered as originally described, then dried at 55°C in a forced-air oven for 18 h. The dried product (23.45 g, 93.8% yield) was ground through a 40 mesh screen. The hydroxyethyl curdlan prepared by this procedure was soluble in boiling water and, on cooling, formed a clear, very elastic, thermoreversible gel whose 1.5% gelling temperature was 54.5°C and gelling temperature was 91.0°C. In contrast to curdlan, its hydroxyalkyl derivatives exhibited little or no syneresis. The degree of elasticity was not measured quantitatively but was assigned by observation of the comparative flexibility of the gels.

Other ratios of 2-chloroethanol were used as the hydroxyalkylating agent in other experiments. Glycidol (lot #24F-3437, Sigma Chemical Company, St Louis, MO) was used to form the glyceryl curdlan derivative. The results of these experiments are found in Table 3.

The dynamic gelling and melting temperatures found in Table 3 were determined on 1.5% aqueous preparations contained in 1.5 cm × 10 cm glass test tubes suspended in a controlled-temperature water bath. For gelling temperature, the temperature of the surrounding water bath was lowered at the rate of about 0.5°C/min and the gelling point was taken as the value where an inserted and withdrawn thermometer would leave a visible well. For melting temperature, sols were poured into test tubes and gelled on a slant. A thermometer was inserted in the gel and the temperature of the water bath was raised at about 0.5°C/min. The melting temperature was taken as that temperature where the slanted gel surface began to level.

To check the utility of the hydroxylalkylated curdlan gels as electrophoresis matrices, the following experiment was run. A 2% SeaKem® LE agarose in 0.5X TBE buffer framing gel was cast in a horizontal submarine chamber (Model 850, Aquebogue Machine Shop. Aquebogue, NY) using a nine-lane slot-former template. The slot-former was removed from the framing gel and 3% sols of the b, c, d and e hydroxyethyl curdlan and b glyceryl curdlans (see Table 3) were prepared in 0.5X TBE buffer. These sols were poured into the lanes of the framing gel, with the

Table 3. Gel strengths (g/cm^2) of selected 1% curdlan gels prepared using chaotropes

Chaotrope	Cold-set gel	Leached	Heat-set gel	Leached
None (control)	106	74	155	186
3 M urea	242	357	424	471
6 M urea	218	523	168	394
9 M urea	95	382	152	458
0.5 M LiI	454	406	216	216
1.5 M LiI	346	345	230	330
3.0 M LiI	210	233	420	421
0.1 M guanidine-HSCN	244	276	184	247
0.275 M guanidine-HSCN	313	342	226	320
0.5 M guanidine-HSCN	186	359	310	315

remaining ones being filled with a 3% agarose sol. After all lanes had gelled, sufficient buffer was added to cover the gels and each lane loaded with 4 μl of 150 ng/ml *Hae* II φX174 (Life Technologies, Grand Island, NY). Voltage was applied 172 V or 5 V/cm) for 110 min. The bands were visualized using ethidium bromide, followed by water destaining. Results, as deduced from the migration rates, indicated that the sieving ability of the glyceryl derivative was equivalent to the agarose control. The sieving ability of the first three hydroxyethyl derivatives, as estimated by the relative positions of the DNA bands, increased with increasing levels of substitution. All were more sieving than the agarose control. In what appears to be an anomaly, the most highly substituted hydroxyethyl derivative was least sieving and was less sieving than the agarose. Band sharpness was superior in the derivatized curdlan gels. In another experiment, a 3.5% glyceryl curdlan gel was able to resolve the 238/242 base pair fragments of pBR322 *Msp* 1 digest (New England BioLabs, Beverly, MA), whereas the control 4% NuSieve® agarose was unable to do so. Hydroxyethyl curdlan gels, prepared in the presence of urea and urea/formamide as DNA sequencing gels (Harper *et al.*, 1995a) had a tendency to creep during electrophoresis. Hydroxyalkylated curdlans, partially depolymerized by being subjected to γ-irradiation from cobalt 60 at dosages of 200–800 Krad, enabled preparation of 6 and 8% gels that were very elastic and were highly sieving. DNA electrophoretic separation patterns on these gels resembled those obtained in polyacrylamide gels of equivalent concentrations (Renn *et al.*, 1993).

CONCLUSION

Although curdlan has been available since 1972, and has recently been approved for food applications in the USA, its utility in bioscience applications has been limited. Reasons for this include the previous difficulties in forming homogeneous, coherent gels from curdlan and the fact that commercially available material contains impurities that interfere with electrophoretic separations and subsequent visualization of nucleic acids and proteins. By disseminating the results of this initial research more widely, curdlan-based gelling agents may eventually find an important niche in specific life science applications. Curdlan and its derivatives are not seen as replacements for agaroses and/or polyacrylamides, but rather as complementary. The fact that curdlan is a fermentation polysaccharide confers on it potentially unlimited availability. With curdlan's unique properties, such as its ability to form thermostable gels by heating and useful chaotrope-containing gels, as well as its being amenable to preparing potentially useful derivatives, curdlan is assured of an interesting future.

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