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Urea–polyacrylamide gel electrophoresis of alginic acid

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Alginic acid is a major component of brown algal cell walls. It is a linear polyanion, a co-polymer of β-D-mannuronate and α-L-guluronate, arranged in regions of homopolymeric blocks separated by regions approaching an alternating structure[1]. Homopolymeric alginate blocks are chemically produced from high-molecular-weight alginic acid by mild acid hydrolysis and selective precipitation in acid solutions[2]. Isolated blocks have been used as substrates in characterizing alginolytic enzymes[3], as well as for investigating solution behavior and physical structure of alginate[4,5].

A rapid method of analysis would be desirable to follow purification of alginate blocks and to determine their degradation by bacterial enzymes. Since algal alginates have a uniform charge to mass ratio, electrophoretic systems using polyacrylamide gels[6], agarose gels[7], and free-boundary methods[8] have been utilized. However, due to heterogeneity of chain length, analysis of alginate composition by electrophoresis has generally been less successful than other methods, such as nuclear magnetic resonance[4] or circular dichroism[5].

This report describes a urea–polyacrylamide gel electrophoresis (urea–PAGE) system to analyze acid degradation of high-molecular-weight alginate, to rapidly separate short alginate blocks, and to provide a technique that is rapid and sensitive for determining the specificity of enzymic degradation of alginate.

EXPERIMENTAL

Alginate

High-molecular-weight alginate from brown algae (*Laminaria hyperborea* and *Macrocystis porifera*) was obtained from Kelco (San Diego, U.S.A.). Alginate samples were converted to sodium salts, precipitated by addition of ethanol to 70% (v/v), and lyophilized. In all cases, alginate concentrations were given as the mass of sodium alginate per volume of buffer. Homopolymeric blocks of poly-D-mannuronate (M-blocks) and poly-L-guluronate (G-blocks) were obtained from high-molecular-weight alginate after mild heterogenous acid hydrolysis and selective acid precipitation[2]. The blocks had an average degree of polymerization of 24 and were approximately 97% M-blocks or G-blocks as determined by ^1H nuclear magnetic resonance[4] and circular dichroism[5]. Purified alginate blocks were used as electrophoretic standards or substrates for bacterial enzymes as previously described[3].

Electrophoresis

Separation of alginate blocks was performed using urea–PAGE. The stacking gel contained 6% (w/v) acrylamide, 6 *M* urea, and 50 m*M* Tris, pH 7. The separating gel contained 8 to 21% acrylamide, 4.5 to 6 *M* urea, and 50 m*M* Tris, pH 8.7. Where appropriate, separating gel composition is given in the text and figure legends. Both gels contained 1 m*M* calcium chloride, N,N′-methylene bis-acrylamide as 1% of the total acrylamide concentration, and were polymerized by addition of ammonium persulphate and N,N,N′,N′-tetramethylethylenediamine to a final concentration of 0.07%. The vertical slab gels (1.5 mm thick and 140 mm in length) were polymerized between glass plates enclosed by 6.4 mm thick plexiglass casting units and were run immediately at room temperature with a constant potential of 130 V. The upper-tray buffer was 400 m*M* glycine, 50 m*M* Tris (pH 8.7), while the lower-tray buffer was 50 m*M* Tris (pH 8.9) and 1 m*M* calcium chloride. The samples (0.5 to 30 μg alginate) were dissolved in 4 *M* urea, 10 m*M* ethyleneglycol-bis(β-aminoethyl ether) N,N′-tetraacetic acid (EGTA), and 50 m*M* Tris (pH 7.5), with 0.001% bromphenol blue and 0.005% xylene cyanole as tracking dyes. Electrophoresis continued for about 2 h or until bromphenol blue reached the end of the gel. Gels were stained for alginate by immersion for 15 min or longer in aqueous 0.1% toluidine blue O (TBO) and were destained in distilled water[7].

RESULTS

Acid hydrolysis of alginic acid

High-molecular-weight alginate from *L. hyperborea* was electrophoresed before and after partial acid hydrolysis to determine the electrophoretic behavior of alginate and the relationship between acid-insoluble hydrolysis products and chemically purified M-blocks and G-blocks. Alginate had low mobility prior to hydrolysis, since the majority of the stained material remained at or very near the origin (Fig. 1, lane D). The low electrophoretic mobility was due to the effect of calcium on the high-molecular-weight alginate, since when calcium was omitted from the gel, all of the high-molecular-weight alginate entered the stacking gel and formed a smear (results not shown). The effect of short, partial acid hydrolysis on alginate mobility was striking. After 2 h of hydrolysis (lane C), alginate disappeared from the origin (even with calcium in the gel), became highly mobile, and separated into three distinct staining bands near the electrophoretic front and one faint band midway through the gel. The mobility of the lead band was identical to M-blocks (lane B), with the following bands corresponding to G-blocks (lane A). More extensive acid degradation (up to 8 h, lane E) did not change this pattern.

Calcium effect on block separation

Urea–PAGE clearly separated M-blocks from G-blocks, only if a discontinuous gel system containing calcium was employed. When the separating gel was omitted (in effect a 140-mm stacking gel), both blocks co-migrated with bromphenol blue at the electrophoretic front. Even with a discontinuous gel, when calcium was omitted from the gels, all alginate blocks co-migrated with bromphenol blue. When a separating gel containing 8% acrylamide, 6 *M* urea, and 1 m*M* calcium was utilized, the majority of G-blocks (Fig. 1, lane A) migrated with xylene cyanole behind M-

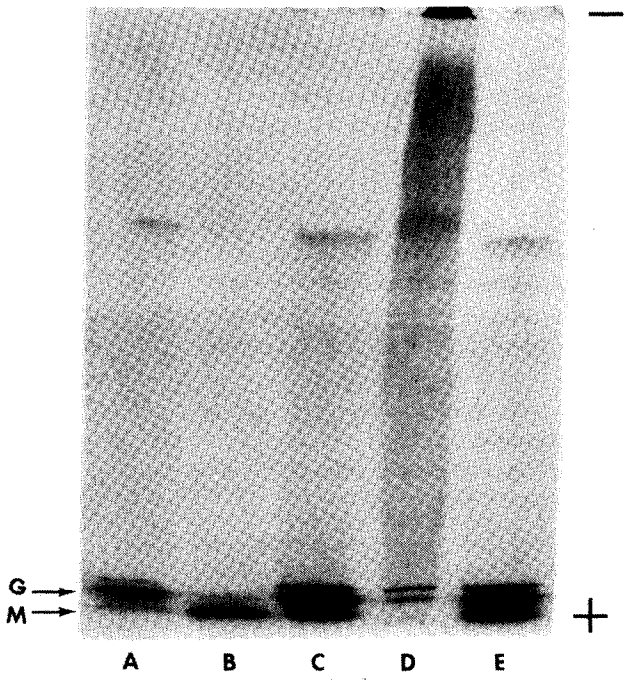

Fig. 1. Urea–PAGE (6 *M* urea, 8% acrylamide) of alginate. High-molecular-weight alginate from *L. hyperborea* was electrophoresed before (lane D), after 2 h (lane C), and after 8 h (lane E) of partial acid hydrolysis. For comparison, chemically purified G-blocks (lane A) and M-blocks (lane B) were also electrophoresed. Lanes A and B contained 5 μg alginate, while lanes C, D, and E contained 20 μg alginate. The location of the main bands of M-block and G-blocks near the electrophoretic front are indicated by arrows.

blocks (lane B), which migrated with bromphenol blue at the electrophoretic front. The separation of G-blocks from M-blocks by the addition of calcium was consistent with the results from free-boundary electrophoresis[8].

Enzymic degradation of alginate blocks

Urea–PAGE also proved to be a sensitive technique to visualize enzymatic degradation of alginate blocks. Two alginate lyase enzymes (one specific for M-blocks, another specific for G-blocks) were isolated from marine bacteria[3]. Purified homopolymeric alginate blocks were incubated with the enzymes, then electrophoresed (Fig. 2). The poly-D-mannuronate lyase degraded M-blocks (lane E) but not G-blocks (lane C), while the poly-L-guluronate lyase degraded G-blocks (lane D) but not M-blocks (lane F). Urea–PAGE allowed detection of alginolytic activity with concentrations of blocks as low as 0.05 to 0.1 μg per lane, due to the tight banding of alginate blocks and the sensitivity of the TBO stain. In contrast, determinations by colorimetric or UV absorbance measurements would have required considerably more substrate, a minimum of 50 to 100 μg, and viscometric analysis would have failed entirely, due to the inherently low viscosity of the blocks.

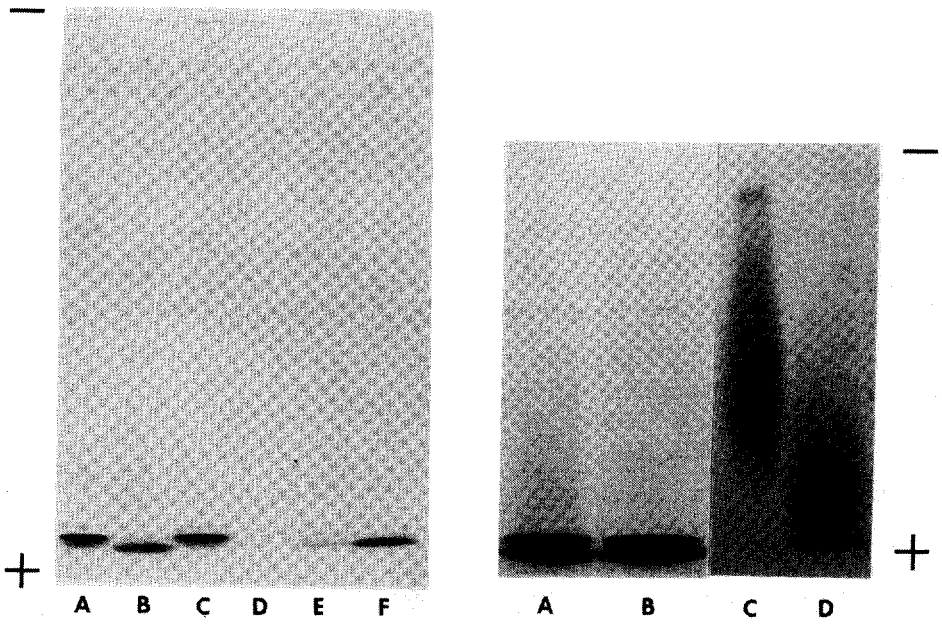

Fig. 2. Urea–PAGE (6 *M* urea, 8% acrylamide) of alginate blocks after incubation with alginate lyases. All lanes contained 10 μg alginate, and all enzyme incubations were for 120 min. Undegraded G-blocks (lane A) and M-blocks (lane B) were electrophoresed for comparison. G-blocks were incubated with poly-D-mannuronate lyase (lane C) and with poly-L-guluronate lyase (lane D). M-blocks were incubated with poly-D-mannuronate lyase (lane E) and poly-L-guluronate lyase (lane F).

Fig. 3. Urea–PAGE (4.5 *M* urea, 21% acrylamide) of purified alginate blocks. G-blocks (lane A) and M-blocks (lane B) were electrophoresed in a gel which contained no calcium. G-blocks (lane C) and M-blocks (lane D) were electrophoresed in a gel containing 1 m*M* calcium.

Alginate block size heterogeneity

Although urea–PAGE with low acrylamide concentrations (8%) and calcium in the gels successfully separated G-blocks from M-blocks, increasing the acrylamide concentration revealed size heterogeneity within the samples and indicated the important role of calcium in the separations. The effects occurred gradually as the acrylamide concentration was increased from 8 to 21% in steps of 2 to 3%. The results of urea–PAGE using 21% acrylamide gels with 4.5 *M* urea are presented in Fig. 3. In the absence of calcium (lanes A and B) both G-blocks and M-blocks migrated near the electrophoretic front; however, trailing behind the main bands of G-blocks were a series of evenly spaced, faint bands. When 1 m*M* calcium was present, G-blocks (lane C) were separated into a series of evenly spaced bands, well behind the electrophoretic front. M-blocks (lane D) migrated at the electrophoretic front with 8 to 10 trailing bands overlapping the G-blocks. According to the migration of DNA restriction fragments (HaeIII digests of φX-174 DNA, not shown), urea–PAGE separated polyanions on the basis of size.

DISCUSSION

The electrophoretic patterns of G-blocks and M-blocks can be attributed to both the gel composition and to the physical behavior of the blocks. Although the average polymer size of both blocks was similar, calcium–urea–PAGE was able to separate the blocks. The presence of sharp, separate, highly mobile bands (G-blocks and M-blocks) indicated that in gels with low (less than 10%) acrylamide concentrations block mobility was limited by ion effects near the electrophoretic front, since the gel pore-size was too large to cause sieving of the blocks. An increase in acrylamide concentration decreased the pore-size of the gel, and in the presence of calcium, G-blocks but not M-blocks separated completely into discrete bands well behind the electrophoretic front. Although calcium may have affected the structure of the acrylamide gel, the results from other studies suggest that the effect was probably directly on alginate. For instance, calcium-induced retardation of G-blocks but not M-blocks was reported[8] using free-boundary electrophoresis under conditions which would not have affected the stationary phase. Although the presence of urea may have precluded calcium-induced gel formation, ion pairing due to transient binding of calcium may have reduced the net charge sufficiently to cause retardation[9]. Hence, the tight bands of G-blocks were probably discrete size classes and not inter- or intra-molecularly cross-linked molecules.

Several reservations must be presented with respect to separations of alginates by urea–PAGE. High-molecular-weight poly-D-mannuronate (greater than 95% D-mannuronate residues) migrated as a smear behind G-blocks with some overlap. Therefore, high-molecular-weight alginates should be slightly depolymerized prior to electrophoresis. In addition, electrophoresis of bacterial alginates, with varying degrees of O-acetylation, may require de-acetylation to preserve a constant charge-to-mass ratio.

Urea–PAGE, with calcium present, can clearly and rapidly separate small quantities of alginate blocks in a large number of samples, typically 10 to 30 samples per gel. Unlike any other electrophoretic method yet reported, urea–PAGE allows one to make direct comparisons of enzymic or acid degradation of alginate. No other method has demonstrated separation of discrete populations of blocks on the basis of size, indicating that urea–PAGE may be valuable as a means of obtaining fine structure analysis of alginate during synthesis or degradation. Overall, it appears that urea–PAGE requires less time and smaller amounts of material to yield information not attainable by other techniques.

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