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Controlling Electrophoretic Trapping of Circular DNA by Addition of Starch Preparations to Agarose Gels

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Abstract

Starch preparations were added to agarose gels to enhance the electrophoretic trapping of circular plasmid DNA. The critical voltages required to trap the open circular (OC) and the supercoiled (SC) forms of a 13.1-kbp plasmid were measured in gels composed of agarose and added starch preparations. Modified starch preparations reduced the critical voltage required to trap the OC form of the plasmid to approximately one-third of the control value (in 1% agarose gels). Amylose (a fraction of starch with a low amount of branching) also reduced the critical voltage to trap the OC form in a similar manner. The critical voltage to trap the SC form of the plasmid was not significantly reduced by the starch preparations. The capacity to trap OC DNA was increased by the addition of higher amounts of the starch preparations added to the gels. Field inversion gel electrophoresis was used to characterize the length of the traps in the gels. The starch preparations and amylose increased the trap lengths approximately twofold. The increased trap length correlated with the decreased critical voltage required to trap the OC form of the 13.1-kbp plasmid.

Index Entries: Electrophoresis; DNA; agarose gel; trapping; starch; circular DNA; plasmid; separation.

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Certain commercial equipment, instruments, or materials are identified in this article to specify adequately the experimental procedures. Such identification does not imply recommendation by National Institute of Standards and Technology, nor does it imply that the materials or equipment are necessarily the best available for the purpose.

Introduction

We are interested in modifying the electrophoretic trapping of circular DNA in gels. Circular DNA can be trapped (immobilized by impalement) in gels during electrophoresis by becoming looped over a gel obstruction (the trap). Impaled circular DNA will be completely immobilized by an electric field, while linear DNA will be able to slip off such a structure. This mode of separation is very selective and can be taken advantage of for separating mixtures of circular and linear DNA. However, to be of practical use, several objectives need to be accomplished. It is important to determine the factors that influence the formation of intrinsic traps, and also to characterize the traps in gels. Controlling and enhancing electrophoretic trapping in gels would allow researchers to tune gels for specific separations of circular DNA. This study focuses on a method to enhance traps in agarose gels and the characterization of the resulting traps.

We identified the carbohydrate polymer starch as a promising additive to enhance electrophoretic trapping in agarose gels. Starch preparations are available with a wide variety of physical properties and have been used as substrates for electrophoresis *(1,2)*. Recent progress has been made in elucidating the complex structure of starch. Starch has traditionally been divided in two fractions: amylose (containing few branches) and amylopectin (the more highly branched fraction). Native (untreated) corn starch has from 72 to 75% amylopectin, with the balance being amylose *(3)*. Amylose and amylopectin are polymers of linked α-D-glucopyranosyl *(1–4)*, and the branch points are linked *(1–6)*. Estimates of the weight average molecular weight of potato amylose range from 7×10^5 to 9×10^5 (reviewed in ref. *3*). Amylopectin from corn is a much larger molecule with estimates of the weight average molecular weight ranging from approx 10^7 to 10^8 (reviewed in ref. *3*). Amylopectin is highly branched with a regular structure. The branched chains can be divided into three groups: short chains (14–18 glucose units), long chains (45–55 glucose units), and a few very long chains (>60 glucose units) *(3)*. Native starch granules have a semicrystalline structure. Starch granules are believed to be organized into regions of crystallinity (ordered according to the helix structures) and with amorphous regions. Starch chains can form three different helical structures termed A, B, and V. The A and B forms are parallel double-stranded helixes, and the V form is a left-handed helix with six residues per turn (reviewed in ref. *3–5*).

Gel electrophoresis of circular plasmid DNA is complicated by the presence of different topologic forms. A high-quality plasmid preparation will contain mainly the supercoiled (SC) form of the plasmid. Depending on the type of preparation and storage conditions, there may be significant amounts of the open circular (OC) form of the plasmid and sheared host genomic DNA (linear). The relative migration of the SC, OC, and linear forms of plasmid DNA in agarose gels varies with agarose concentration, electrophoresis buffer, and electric field strength *(6–8)*. Mickel et al. *(8)* investigated the electrophoretic mobility of a series of plasmids with a

range of sizes in agarose gels. They observed that when the size of OC plasmid DNA was increased, a threshold was reached, above which the DNA was trapped at the sample origin. They observed that the corresponding SC forms were not trapped in the agarose gels under the conditions used. The threshold size of OC plasmid trapping in agarose gels was dependent on the field strength and ionic strength of the buffer. Mickel et al. *(8)* proposed an impalement mechanism in which the OC DNA becomes looped on gel fibers to explain the arrest of the larger open circles. Support for the impalement mechanism was provided from the use of field inversion gel electrophoresis (FIGE) (periodically reversing the direction of the electric field) *(9)* and periodically turning the field off (allowing the DNA to diffuse off the traps) *(10)*.

Åkerman *(11)* studied the trapping of circular DNA in polyacrylamide gels and agarose gels. He showed that both OC and SC of plasmid DNA can be trapped in polyacrylamide gels, although the probability of trapping the more compact SC was significantly lower in polyacrylamide gels. Recently, we were able to significantly increase the density of traps in agarose gels by reducing the molecular weight of the agarose polymer used to form the gels *(12)*. We were able to trap the SC forms of larger plasmids at electric field strengths that were approximately two- to threefold higher than that required to trap the OC forms *(12)*.

In our previous studies of the trapping of plasmid DNA in agarose gels, we were able to increase the density of the traps (by reducing the molecular weight of the agarose polymer), but not the length of the traps. Enzymatic treatment of agarose to reduce the molecular weight results in gels that are fragile and difficult to use. The field strength required to trap the OC form of a 4.4-kbp plasmid was close to 100 V/cm. It is clear that the critical field strengths must be reduced to more practical levels in order to trap smaller plasmids. To reduce the critical voltage required to trap a given circular DNA, it is necessary to change the gel structure to form more effective traps (longer, stiffer, or more accessible).

We are interested in determining the factors that influence the enhancement of the electrophoretic trapping of circular DNA by starch. A few representative preparations of starch with different properties were selected to compare their effect on the electrophoretic trapping of circular DNA. We used a transverse gradient gel electrophoresis apparatus to determine the critical voltage required to trap the OC and SC forms of a 13.1-kbp plasmid. The capacity of the gels for trapping the OC form of the plasmid was determined using slab gels. FIGE was also used to characterize the length of the electrophoretic traps.

Materials and Methods

Starches and Enzymes

Unmodified corn starch (73% amylopectin, 27% amylose; no. S4126), and amylose (potato; no. A 0512) were obtained from Sigma (St. Louis,

MO). PC starch (Pure-cote B792) and PS starch (Pure-set B965) were samples from Grain Processing (Muscatine, IA). DNA standards and restriction enzymes were obtained from Life Technologies (Rockville, MD). DNase I (cat. no. 104 132) was from Boehringer Mannheim (Indianapolis, IN). Agarose (SeaKem LE) was from FMC (Rockland, ME).

Plasmid DNA Samples and DNase I Treatment

The plasmid preparations used were 8 (pDELTA) and 13.1 kbp (pYA101). The SC and OC forms of the plasmid DNA were determined by agarose gel electrophoresis (3 V/cm) for 4 h. The DNA concentrations were determined by fluorescent dye (Hoechst 33258) binding (Bio-Rad, Hercules, CA). The SC and OC bands were identified by treatment with DNase I. A solution of DNase was prepared by dissolving the contents of a bottle of lyophilized DNase I (20,000 U) in 1 mL of 20 m*M* Tris and 1 m*M* $MgCl_2$, pH 7.5. The solution was distributed into small quantities and quick frozen on dry ice and stored at –80°C. Individual tubes were thawed for use and then discarded. A 5X reaction buffer was prepared consisting of 250 m*M* Tris and 50 m*M* $MgCl_2$, pH 7.5. DNase was diluted by thawing a new tube and adding 10 µL (stock solution) to 10 mL of 1X reaction buffer (1:1000 dilution). Additional dilutions were also prepared. Typical reactions for each plasmid preparation contained 1.2 µg of the plasmid DNA, 1X reaction buffer, and the DNase I dilution in a total of 50 µL. Samples were gently mixed and kept at 21°C for 1 h. The reaction was stopped by adding 2 µL of 0.5 *M* EDTA, pH 8.0. Samples were dialyzed for 4 h against 10 m*M* Tris and 1 m*M* EDTA, pH 7.5.

Gel Formation

All gels, containing starch preparations, were cast as cogels containing 1% agarose. The gels were prepared by weighing agarose (1.5 g) and the starch preparation in a tared flask. Electrophoresis buffer was added (to a total weight of 150 g) and the solution brought to a boil with stirring. The heat was turned off and the solution stirred for an additional 20 min. The solution was cooled to approx 65°C, and water was added to replace that lost by evaporation. The gels were then poured into a gel tray and allowed to set for 1 h. Gels were run for at least 30 min before loading samples.

Gels containing amylose were cast with a different procedure to ensure that uniform gels resulted. To form a 2.5% amylose/1.0% agarose gel, amylose (3.75 g) was added to *n*-butanol (25 mL) in a beaker, and a stirring rod was used to make a slurry. Electrophoresis buffer (145 mL) was added, followed by agarose powder (1.5 g) while stirring. The uncovered solution was brought to a boil on a hot plate in a fume hood. The heating element was then turned off, and the solution was stirred for 15–20 min on the hot plate. The weight of the solution was checked to ensure that there was a weight loss of at least 35 g. Heated water (65°C) was used to bring the solution

weight back to 150 g, and the solution was mixed and poured into the gel tray. These gels were run overnight at 3 V/cm, and fresh buffer was added before the gel was used. Failure to disperse the amylose in butanol or to remove most of the butanol during the heating step resulted in gels that were not uniform and contained clumps of amylose.

Electrophoretic Velocity Measurements and FIGE

Commercial submarine gel chambers (CBS Scientific, VWR Scientific, Boston, MA) were used. These chambers have an internal cooling chamber, and the buffer solutions were circulated externally through heat exchangers in a water bath. The temperature of the water bath was maintained at 20.0°C. Gel trays 14 cm wide (12.7-cm id) by 20 cm long and 2-mm-thick combs to form the sample wells were used. The electrophoresis buffer used was TBE (45 mmol/L of Tris; 45 mmol/L of boric acid; and 1 mmol/L of EDTA, pH 8.3). Electric field strengths were measured by touching the probes of a voltmeter to the surface of the gel.

FIGE velocity was measured were done using a commercial pulsing instrument (PC 750 Hoefer; San Francisco, CA). DNA samples were run into the gel (approx 1 mm) using a direct current field (3.2 V/cm) for 25 min before beginning the pulsing step. The effective electrophoresis time was calculated by multiplying the actual time of electrophoresis by the factor: (forward pulse time – reverse pulse time)/(forward pulse time + reverse pulse time). The effective time corrected for the amount of time the field was in the reverse direction.

Apparatus for Transverse Electric Field Gradient Electrophoresis

An apparatus to generate a transverse electric field gradient was constructed by placing platinum wire electrodes into a gel-casting tray. One platinum wire (the negative electrode) was placed parallel to and just behind the sample wells (at a right angle to the sides of the tray). The other electrode (positive) was positioned 3 cm from the negative electrode at one side of the tray and 15 cm from the negative electrode at the other side of the gel tray (forming a diagonal along the gel tray). Gels were cast in an unmodified gel tray and cut into two wedge-shaped pieces. The wedge-shaped gel pieces were positioned in the modified gel tray between the electrodes. Samples were loaded into the wells, and the field was turned on. After the samples had moved into the gel (0.5–1 min) the buffer circulation pump was turned on. The probes (separated by 1.5 cm) of a voltmeter were touched to the gel surface just above the wells to determine the voltage gradient.

Results

Screening Starch Preparations and Their Effect on Linear DNA Mobility

All of the gels were cast as cogels containing 1% agarose. This was necessary because the starch preparations alone did not form strong gels at

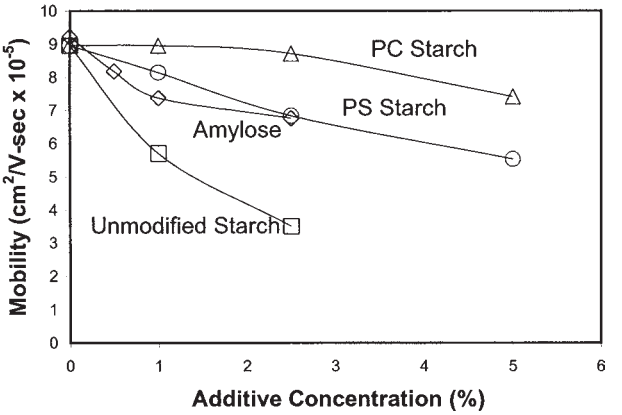

Fig. 1. Mobility of lambda DNA (48.5 kbp) in starch/agarose gels. The gels were formed using the indicated starch preparation concentrations and 1% agarose. The mobility of lambda DNA was measured after electrophoresis for 90–120 min (13 V/cm at 20°C).

the concentrations studied. Agarose was selected as a base gel because we had previously characterized its trapping behavior *(11,12)*. Unmodified corn starch was used in the initial screening experiments to determine the effect on trapping. Unmodified corn starch did enhance electrophoretic trapping, but it also had undesirable properties for use in gels. Solutions of unmodified corn starch were highly viscous, making it difficult to cast uniform gels. This high viscosity of unmodified starch resulted in the linear DNA traveling with a low mobility (Fig. 1). Unmodified starch caused severe smearing of the DNA (including linear DNA), making mobility measurements difficult. Because of these drawbacks, unmodified corn starch was not investigated further.

We used several commercial starch products modified to improve their characteristics to determine their effect on electrophoretic trapping. PS starch has been acid treated to reduce molecular weight and viscosity. PC starch has been treated to reduce viscosity and further modified to produce a product with enhanced film-forming and adhesive properties. The PC and PS starch products have lower viscosity, as shown by their lower reduction in electrophoretic mobility of the linear DNA marker compared to the results of unmodified starch (Fig. 1). An amylose preparation (that portion of starch with a low amount of branching) was also used. Amylose had an effect on the mobility of the linear DNA marker similar to the effect of PS starch (Fig. 1).

Amylose was limited to concentrations up to about 2.5%, because at higher concentrations the gels turned opaque, making it difficult to image the DNA. PS starch gels could be made up to about 5%, above which they also became opaque. PC starch gave the clearest gels and could be used at concentrations above 5%.

Use of Transverse Electric Field Gradient Apparatus to Estimate Critical Electric Field Strength Required to Trap Circular DNA in Gels

An electrophoresis apparatus designed to apply a transverse electric field gradient to a gel (described in Materials and Methods and in ref. *12*) was used to characterize the traps in the gels. The critical electric field strength required to immobilize the OC and SC forms of the 13.1-kbp plasmid was measured (Fig. 2A,B). This technique allowed the rapid comparison of a number of electric field strengths on DNA samples using a single gel. A gradient of about 2.5-fold in electric field strength was obtained with a single voltage applied to the apparatus (Fig. 2C). Two different preparations of the 13.1-kbp plasmid were used, one containing mainly the OC form and the other containing mainly the SC form. Sensitivity to DNase I and digestion with a restriction enzyme (as described in Materials and Methods) confirmed the identity of the SC, OC, and linear bands.

The onset of trapping was first seen as a characteristic smearing of the DNA (Fig. 2A). As the field strength was increased, the DNA became immobilized at the sample application point. The SC form required higher electric field strengths to trap compared with the OC form (Fig. 2B,C). The average values of the critical electric field strength obtained from the starch gels are shown in Fig. 3. The SC form required an electric field strength approximately threefold higher than the OC form (Fig. 3). All starch preparations showed significant reduction in the critical field strength required to trap the OC form of the 13.1-kbp plasmid. The critical field strength required to arrest the SC form was not significantly reduced by any of the preparations. The ratio of the critical electric field strengths to trap the SC form to the OC form is shown in Fig. 3. This ratio was increased approx 2.2-fold by 5% PS starch compared to the control gels. PC starch was not as effective in reducing the critical field strength compared to the reductions seen by PS starch and amylose.

Capacity of Starch Gels to Trap Circular DNA

The capacity of the starch gels to trap the 13.1-kbp OC DNA was determined by overloading the gel at a fixed voltage (14 V/cm). This voltage was set at just the onset of trapping for the gels, and increasing amounts of the 13.1-kbp OC DNA were loaded onto the gels (Fig. 4). When the capacity of the gel to trap circular DNA was overloaded, the DNA smeared and moved farther down the gel. The area of the smear (or length since the width was constant) was used as a measure of the capacity of the gels for trapping OC DNA. The lengths of the smears (normalized for time of electrophoresis) are shown in Fig. 4. Control gels (1% agarose) did not trap the 13.1-kbp OC DNA at this voltage. This finding shows that increasing the concentration of starch product increases the capacity of the gels to trap the OC DNA at a fixed voltage. Amylose and PS starch showed the highest capacity (Fig. 4B,C). The capacity of PC starch was significantly lower than that of amylose or PS starch (Fig. 4A).

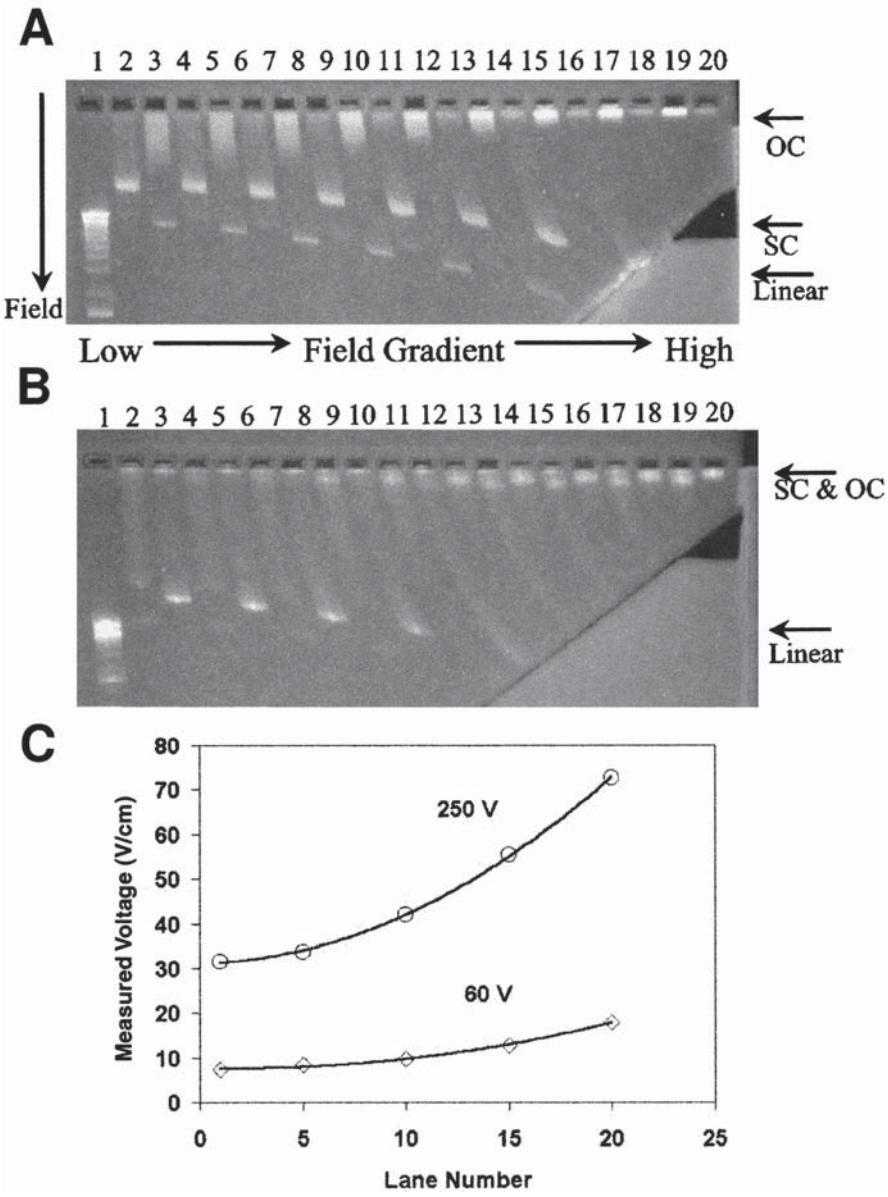

Fig. 2. Transverse electric field gradient electrophoresis for determination of critical field strength to trap circular DNA in a PS Starch (2.5%) and agarose (1.0%) Co-gel. The apparatus and conditions were as described in the Materials and Methods. **(A)** Trapping of the OC form of 13.1-kbp plasmid DNA with 60 V applied to apparatus. Samples applied were as follows: lane 1, lambda DNA and kb ladder (linear DNA); even lanes, 13.1-kbp plasmid prep (0.20 µg, mainly SC form); odd lanes, 13.1-kbp plasmid (0.24 µg, mainly OC form). **(B)** Trapping of both the SC and OC forms of the 8.0- and 13.1-kbp plasmid DNA with 250 V applied to apparatus. Samples applied were as follows: lane 1, lambda DNA and kb ladder (linear DNA); even lanes, 13.1-kbp plasmid prep (0.20 µg mainly SC form); odd lanes, 8-kbp plasmid (0.24 µg, mainly SC form). **(C)** Measured voltage gradients at the lanes for the gels shown in (A) (60 V applied) and (B) (250 V applied).

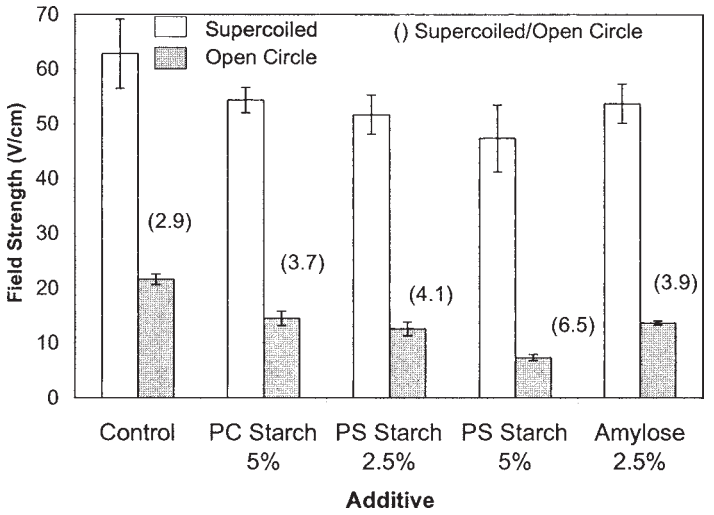

Fig. 3. Average critical field strength values for trapping 13.1-kbp plasmid in starch/agarose gels. The critical electric field strengths determined from transverse electric field gradient electrophoresis are shown (bars indicate the standard deviation of the mean of three to six values). The control values are from previous data *(12)* and additional measurements. The ratio of the voltage to trap the SC form over the voltage to trap the OC form is shown in parentheses.

Characterization of Electrophoretic Traps Using FIGE

FIGE was used to characterize the electrophoretic traps in the starch gels. An electric field strength (15 V/cm) was used that was sufficient to immobilize the OC form of the 13.1-kbp plasmid in the starch gels (Fig. 3). The forward pulse time was fixed at 1 s, and the reverse pulse time varied in each gel. The velocity of the DNA was corrected by using the effective time the DNA spent traveling in the forward direction (described in Materials and Methods). At short reverse pulse times, the OC remained trapped and did not move beyond the starting position (Fig. 5). When the reverse pulse times were increased, the OC form began to move (first seen as a smear). At longer reverse pulse times, the OC moved as a band and reached a plateau velocity. The velocities of the linear DNA marker (48.5 kbp) and the SC form of the 13.1-kbp plasmid were not changed by the pulsing electric field (Fig. 5).

The average time it takes to release a trap multiplied by the velocity of the DNA (through the gel) gives a measure of the trap length. The average untrapped velocity of the OC at longer pulse times was used as the velocity of the DNA through the gel. The average pulse time for releasing a trap was selected as the time corresponding to the midpoint of the velocity curve. The lengths of the traps were calculated from the data shown in Fig. 5. PC starch (5%) gave a value of 130 nm for the traps (Fig. 5A). PS starch at concentrations of 5 and 2.5% gave values of 240 and 200 nm, respectively (Fig. 5B,D). Amylose (2.5%) gave a value of 210 nm for the traps (Fig. 5C).

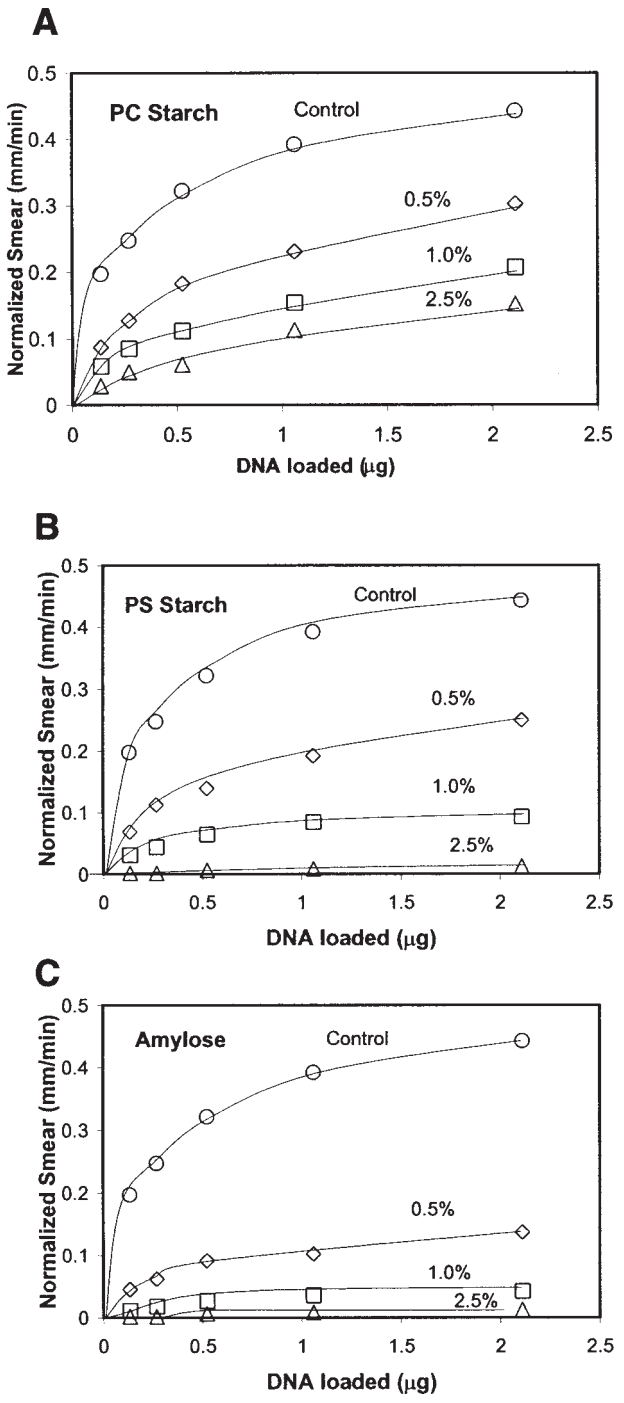

Fig. 4. Capacity of starch/agarose gels for the 13.1-kbp plasmid OC form. The indicated concentrations of the starch preparations were cast with agarose (1%). Electrophoresis was for 120–150 min (14 V/cm at 20°C). The front of the smear of the OC form was measured and normalized by the time of electrophoresis. Controls were 1% agarose gels. **(A)** Normalized smear (distance of the front of the smear divided by the time) of the 13.1-kbp plasmid in PC starch/agarose gels; **(B)** normalized smear (distance of the front of the smear divided by the time) of the 13.1-kbp plasmid in PS starch/agarose gels; **(C)** normalized smear (distance of the front of the smear divided by the time) of the 13.1-kbp plasmid in amylose/agarose gels.

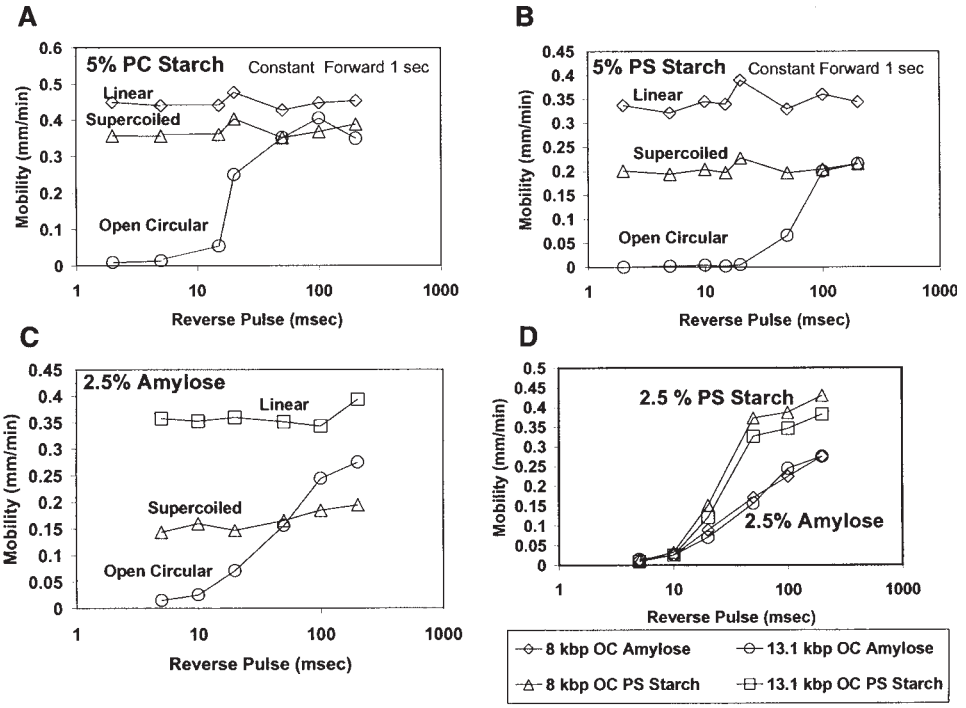

Fig. 5. FIGE of plasmid DNA to characterize trap length. An initial electrophoresis step (constant field of 3 V/cm for 20 min) was used to run the DNA into the gel prior to the FIGE step. FIGE was done for 90–120 min at 15 V/cm (20.0°C). The velocity of the indicated DNA was corrected for the reverse pulse times (described in Materials and Methods). The forward pulse time was 1 s and the indicated reverse time. **(A)** Velocity of the OC, SC, and linear forms of the 13.1-kbp plasmid in 5% PC starch/1% agarose; **(B)** velocity of the OC, SC, and linear forms of the 13.1-kbp plasmid in 5% PS starch/1% agarose; **(C)** velocity of the OC, SC, and linear forms of the 13.1-kbp plasmid in 2.5% amylose/1% agarose; **(D)** velocity of the OC form of the 8.0- and 13.1-kbp plasmid in 2.5% PC starch/1% agarose and 2.5% amylose/1% agarose.

Essentially the same results were obtained when the OC form of an 8.0-kbp plasmid was used to measure the traps in PS starch or amylose (Fig. 5D). Control gels (1% agarose) gave a value of 100 nm using the 13.1-kbp OC plasmid at 24 V/cm *(12)*.

Discussion

Agarose forms strong gels when two strands of the agarose polymer hydrogen bond to form a double helix, and aggregation of the helixes forms bundles (the gel fibers) with relatively large spaces (the pores) between the fibers *(13–15)*. The relatively large diameter of the fibers in agarose gels *(16)* explains the results that OC is relatively easily trapped, and SC forms are more difficult to trap *(11)*. The smaller effective diameter of the more tightly wound SC circles requires traps with smaller diameters. The higher fields

required to trap SC plasmid DNA in agarose gels indicate that the traps are less accessible to SC DNA, or that a set of traps with smaller diameters are shorter in length.

For traps to be effective, they must be sufficiently rigid enough to present a fixed obstacle to the DNA. The addition of the flexible polymer dextran (mainly an α-1,6 glucose polymer) to agarose gels did not increase electrophoretic trapping (results not shown). Based on the structural studies of starch, it is likely that starch forms helical structures in the agarose gels. The results in this article indicate that the effective traps (of sufficient rigidity) that starch forms in the agarose gels have a diameter that is too large to enhance the trapping of the SC plasmid DNA. When added to agarose, the PS starch and amylose (the fraction with a low amount of branching) were similar in their effectiveness at reducing the critical voltage. The similar results obtained with starch and amylose indicate that the presence of branches in the polymers did not increase or decrease the trapping efficiency.

Starch can conveniently be used to significantly enhance trapping of the OC form of circular DNA in gels. PS starch has desirable operating characteristics and was used at concentrations up to 5%. The starch preparations added to agarose gels reduced the critical voltage to trap the OC form of the plasmid DNA to levels that are more practical. The reduction in the voltage reduces the heating of the gel and allows the use of safer voltages. The trapping capacity of the OC DNA form was also significantly increased by the addition of starch to the gels.

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