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ABSTRACT

Ions influence the separation resolution and elution profile obtained by ion-exchange chromatography. This study investigates the effects of buffer ions on the purity and yield of the plasmid purified by Q-Sepharose in a batch adsorption–desorption process when the wash buffers include either 30% or 45% isopropanol. When NaCl, KCl, or $MgCl_2$ was used to remove RNA and proteins, the purified plasmid contained undetectable amounts of proteins and RNA at the salt concentrations that exceeded 1 M or 0.75 M in the presence of 30% and 45% isopropanol, respectively. Using $CaCl_2$ and Na_2SO_4 led to the incomplete separation of plasmid

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from RNA. When applied to chromatographic columns packed with 20 or 200 mL of Q-Sepharose to purify plasmid from 300 and 3000 mL bacterial broth, respectively, stepwise elution of various concentrations of isopropanol and $MgCl_2$ yielded a high quantity of plasmid with undetectable amounts of RNA and proteins.

Key Words: Ion-exchange; Alcohol; Ion; DNA.

INTRODUCTION

Plasmid DNA, an important element in genetic manipulation, is typically prepared from the cell lysate of transformed *Escherichia coli*.[1,2] Ion-exchange is currently the most commonly used technique in plasmid preparation[2–8] to separate DNA from RNA and proteins based on the differences between the interactions of their charges with resins at various ionic strengths and pH values.

The interactions of charges vary with pH because pH affects the net charges of proteins and the dissociation of functional groups on the resins as well as the extent of dissociation of proton from nucleic acid. Ions can modulate the interactions between charges on resins and those on nucleic acids. In anion-exchange chromatography, the anions in the buffers compete with the adsorbed components to bind to the resins, and displace the adsorbed components during washing or elution, while the cations in the buffers may interact with the adsorbed components to cause conformational changes. Ions can alter the elution profiles of adsorbed components, or influence the separation resolution of ion-exchange chromatography.[9] The effects of buffer ions on the purification of proteins have been extensively investigated.[9–13] However, the way in which buffer ions affect the purification of plasmid in anion-exchange chromatography has received little attention.

When ion-exchange chromatography is used to purify plasmid, insufficient removal of RNA from plasmid or a severe reduction in plasmid yield typically reduces the efficiency of separation. The incorporation of alcohols into the wash buffers, as in some commercial products, is normally used to increase purity. For example, the wash buffers in the Qiagen plasmid purification kit contain 15% isopropanol. One investigation also established that yield and purity can be increased using Q-Sepharose in either a batch adsorption–desorption process or in gravity flow when the buffers contain an alcohol of a low dielectric constant, such as isopropanol.[14]

This study investigates the effects of buffer ions on the purification of plasmid when Q-Sepharose is used and the buffers contain isopropanol. The yield and purity of plasmid depend on the isopropanol content,[14] so two

isopropanol contents were used—30% and 45%. The salts in the wash buffers were NaCl, KCl, $MgCl_2$, $CaCl_2$, NaOAc, and Na_2SO_4. Their effects on the yield and purity of the purified plasmid were studied by batch adsorption–desorption process. The salt $MgCl_2$, which led to a high yield and purity in the batch process, was further examined at different scales of preparative chromatography using stepwise elution under gravity.

EXPERIMENTAL

Materials

Ethidium bromide, fluorescamine, and Hoechst dye 33258 were from Molecular Probes (Eugene, OR). Yeast messenger RNA, RNase, bovine serum albumin, kanamycin, and 3-(*N*-morpholino)propanesulfonic acid (MOPS) were purchased from Sigma (St. Louis, MO). Agarose was from Amresco (Solon, OH). Plasmid pEGFP-C1 was from Clontech (Palo Alto, CA). *E. coli* DH5α was from Life Technologies (Gaithersburg, MD). Lysis buffer (LB) medium was from Becton–Dickinson (Sparks, MD). Q-Sepharose Fast Flow was from Amersham–Pharmacia (Piscataway, NJ). Organic solvents and other chemicals were obtained from Merck (Darmstadt, Germany).

Plasmid Amplification and Bacterial Culture

The pEGFP-C1 vector, 4.7 kb in size containing a kanamycin resistance marker and a mutated green fluorescent protein gene driven by a cytomegalovirus promoter, was transformed into competent *E. coli* DH5α. A transformed strain was grown in LB medium containing kanamycin at 37°C either by an orbital shaker for 14–16 hr or by a 5-L fermenter (B. Braun Biotech, Melsungen, Germany) for 8 hr. For each batch culture, 1 mL of the bacterial broth was purified by a Qiagen mini-prep kit (Qiagen, Hilden, Germany). The amount of plasmid obtained was used to calculate the relative yield, which was determined as the total amount of plasmid in the elution buffer divided by the multiplication of the broth volume and the amount of plasmid per mL broth obtained by the Qiagen mini-prep kit.

Agarose Gel Electrophoresis

Since the high content of isopropanol in the samples affected the migration of nucleic acid in gel electrophoresis, the isopropanol concentration was adjusted

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to 50% (v/v) to precipitate the nucleic acids at −20°C for 15 min followed by centrifugation at 12,000*g* for 5 min. The precipitates were then dissolved in water before application to 0.8% agarose gel. The bands on the agarose gel were stained with ethidium bromide and photographed by a Kodak digital camera DC 4800 (Eastman Kodak, Rochester, NY) in black–white mode. The intensities of the digitalized photos were analyzed by the Adobe Photoshop.

Protein, DNA, and RNA Measurements

The protein amount of each sample was determined by a modified method using fluorescamine as previously described.[14,15] Briefly, the sample was mixed with 0.01% fluorescamine followed by incubation at room temperature for 10 min. The fluorescence intensity was measured using bovine serum albumin (BSA) as standards at an excitation wavelength of 380 nm and emission wavelength of 480 nm by a Hitachi fluorimeter F-2500 (Tokyo, Japan) with a slit setting of 5 nm.

Because Hoechst dye exhibits high affinity with the AT base pairs of DNA, Hoechst dye 33258 was used to quantitate the plasmid in the final product as previously described.[14] After each sample was mixed with Hoechst dye, the fluorescence intensity of the mixture was measured at an emission wavelength of 352 nm and excitation wavelength of 461 nm by a Hitachi fluorimeter F-2500.

The RNA of each sample was analyzed by agarose gel electrophoresis using yeast messenger RNA as standards.[14] A calibration curve was constructed using the intensities of the known amounts of yeast messenger RNA and used to estimate the RNA amount in each sample by extrapolation of the measured intensity.

Preparation of Cell Lysate

The clear cell lysate was obtained by an alkaline lysis method,[1,2] and the outline of the whole procedure is shown in Fig. 1. The harvested cell pellets from 150 mL bacterial broth were resuspended in 10 mL suspension buffer (50 mM Tris–HCl, 10 mM EDTA, 10 units/mL of RNase) followed by addition of 10 mL lysis buffer (0.2 N NaOH, 1% sodium dodecyl sulfate). After 5 min incubation at room temperature, the mixture was neutralized with 10 mL of 3 M potassium acetate for 15 min at 4°C. The precipitates were then removed by filtration through layered cheesecloth to obtain the clear cell lysate. The plasmid of the cell lysate was further concentrated by precipitation in the presence of 2 M guanidine hydrochloride and 50%

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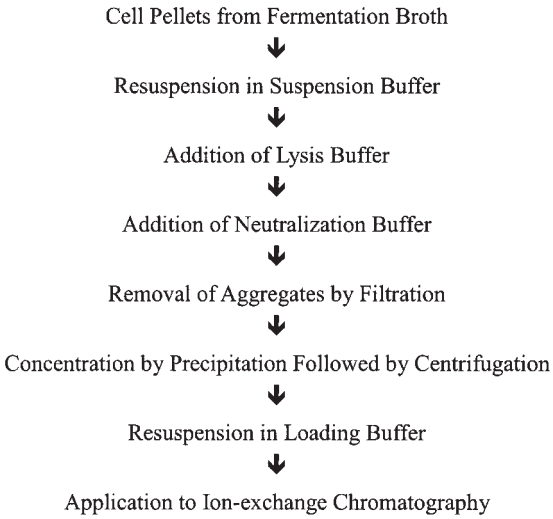

Figure 1. Process flow chart of preparation of clear cell lysate starting from cell pellets.

isopropanol at -20°C for 30 min followed by centrifugation at 12,000 *g* for 5 min. The precipitate, after being rinsed with 70% ethanol twice to remove the residual guanidine hydrochloride, was dissolved in a loading buffer (50 mM MOPS, pH = 7.0). After the above treatments the clear lysate contained plasmid, cellular proteins, and degraded RNA.

Plasmid Purification by Batch Adsorption–Desorption

The maximum binding capacity of Q-Sepharose Fast Flow was estimated around 0.1 mg/mL for the plasmid of 4.5 kb in our preliminary tests. The anion-exchange resin was equilibrated with the loading buffer, and 0.5 mL of the equilibrated resin was incubated with the pretreated cell lysate of 7.5 mL bacterial broth for 5 min under shaking conditions. After separation of the unbound plasmid by centrifugation, the impurities on the resins were removed by 0.5 mL of the wash buffer containing 50 mM MOPS and various concentrations of different salts plus either 30% or 45% isopropanol. The salt in the wash buffers included NaCl, KCl, $MgCl_2$, $CaCl_2$, NaOAc, and Na_2SO_4. After four cycles of washing, the resin-bound plasmid was eluted with 0.5 mL batch elution buffer (50 mM MOPS, 2 M NaCl, pH = 7.0) for four cycles. The amounts of protein, RNA, and plasmid in the wash buffers and elution buffers were measured by the methods described previously.

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Statistics

The results of relative plasmid yields at each salt concentration were examined for statistical significance by using a one-way factorial analysis of variance (ANOVA). Significant differences among the salts ($P < 0.05$) were determined using Tukey's modified *t*-test.

Preparative Chromatography by Gravity-Flow

Two different scales of preparative chromatography were used in this study: a column of 2.4 cm × 3.3 cm I.D. (inner diameter) packed with 20 mL Q-Sepharose for purification of 300 mL bacterial broth, and a column of 6.02 cm × 6.5 cm I.D. packed with 200 mL Q-Sepharose for purification of 3-L bacterial broth. The ceramic nets were placed on the top of the resins. Each scale of chromatography was subject to the same profile of stepwise elution driven by gravity flow.[14] After the unbound plasmid was removed by the loading buffer, the following elution steps were applied sequentially: the gradient 1: 0.5 M $MgCl_2$, 45% (v/v) isopropanol, pH 7.0; the gradient 2: 1.5 M $MgCl_2$, 40% (v/v) isopropanol, pH 7.0; the gradient 3: 1.5 M $MgCl_2$, 30% (v/v) isopropanol, pH 7.0; the gradient 4: 1.5 M $MgCl_2$, 20% (v/v) isopropanol, pH 7.0. The volume of each gradient was four times that of the bed volume. The volume of each collected fraction was 10 mL and 100 mL for the columns packed with 20 mL and 200 mL Q-Sepharose, respectively. The hydraulic pressure was used to control the linear velocity of mobile phase to be between 0.15 and 0.35 cm/min.

RESULTS

Effects of Buffer Cations on Plasmid Purification Using Q-Sepharose in the Presence of 30% Isopropanol

When the wash buffers contained 30% isopropanol, the relative plasmid yields decreased similarly as the concentrations of salts NaCl, KCl, and $MgCl_2$ increased. However, when the buffer that contained $CaCl_2$ was used, the relative yields of plasmid were maintained in the range 65–57%, independently of the concentrations of salt between 0.5 and 2.0 M (Fig. 2B). The falls in yield were attributable to the loss of plasmid, which was removed with RNA at high-salt concentrations during washing. When the concentrations of NaCl, $MgCl_2$, and KCl exceeded 1 M (Fig. 2A), the residual RNA in the eluted plasmid was reduced to undetectable levels, implying that such a salt

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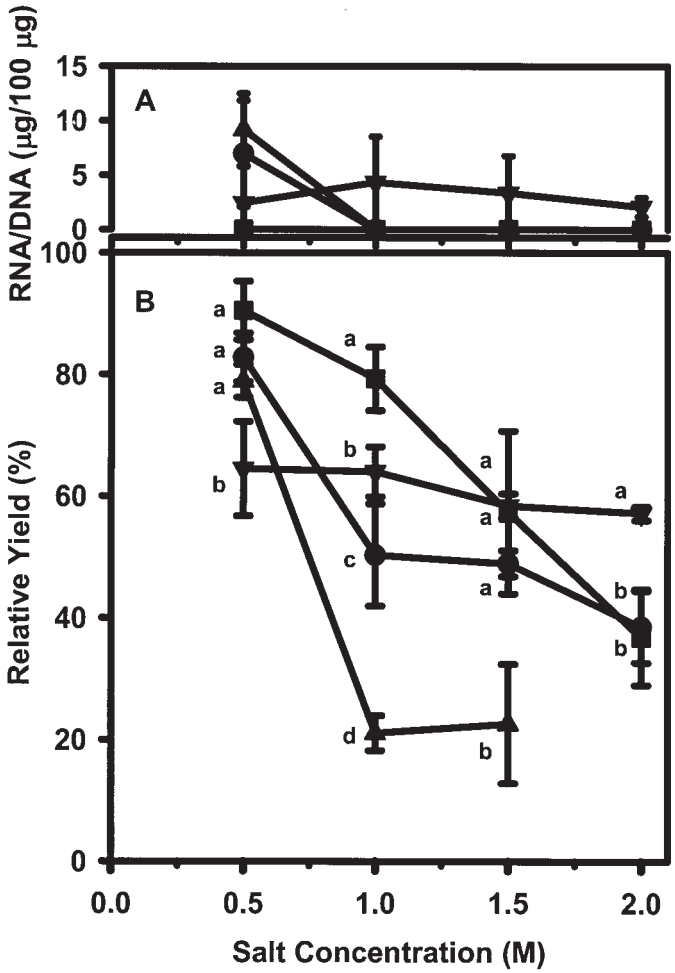

Figure 2. Effects of buffer cations on plasmid purification by batch adsorption–desorption using Q-Sepharose in the presence of 30% isopropanol. The wash buffers contained 30% isopropanol and various concentrations of different salts. (NaCl, ●; $MgCl_2$, ■; KCl, ▲; $CaCl_2$, ▼.) Panels A and B show the residual RNA amount and plasmid yield in the elution, respectively. Data bearing different letters at each salt concentration are significantly different ($p < 0.05$, $n = 3$, mean $\pm$ 95% CI).

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concentration was required to remove most RNA from DNA but at the expense of a fall in the relative yields of plasmid.

Effects of Buffer Cations on Plasmid Purification Using Q-Sepharose in the Presence of 45% Isopropanol

When the wash buffer contained 45% isopropanol and a salt concentration below 1 M, the relative yields were approximate when NaCl, KCl, $MgCl_2$, or $CaCl_2$ was used (Fig. 3). The relative yields were above 75% except when $CaCl_2$ was used. Increasing the concentration of salt from 1 to 1.25 M reduced the relative yields of plasmid. The yield fell abruptly from 98% to 56% when NaCl was used. Interestingly, the relative yields and residual RNA, when $CaCl_2$ was used, were in the ranges 65–50% and 1.8–4.3 μg RNA/100 μg DNA, respectively, similar to those obtained when the wash buffers contained 30% isopropanol (Fig. 3). The separation of plasmid from RNA was incomplete, probably because isopropanol failed to modulate the strengths of the charge interactions between nucleic acids and the resins when the buffers contained $CaCl_2$. In the presence of 45% isopropanol, the amounts of RNA and proteins were undetectable when 0.75 M of NaCl, KCl, or $MgCl_2$ was present; this concentration of salt was lower than that obtained in the presence of 30% isopropanol; the relative yield remained almost unchanged until the salt concentration was further increased to 1.25 M. The approximate relative yields of plasmid and reduced amounts of residual RNA implied that plasmid was retained on the resins, whereas RNA was washed out given salt concentrations between 0.5 and 1.0 M.

Effects of Buffer Anions on Plasmid Purification Using Q-Sepharose in the Presence of 30% Isopropanol

Various concentrations of NaCl, Na_2SO_4, and NaOAc were added to the wash buffers of 30% isopropanol to assess the effects of anions on the purification of plasmid. At salt concentrations below 1.0 M, the relative yields were the same as those obtained when NaOAc or Na_2SO_4 was used (Fig. 4B). However, the eluted plasmid contained higher levels of residual RNA when NaOAc or Na_2SO_4 was used than when NaCl was used (Fig. 4A). Increasing the concentration of NaOAc from 0.5 to 2.0 M gradually reduced the amounts of residual RNA from 55 μg RNA/100 μg plasmid to undetectable levels without reducing the relative yields of plasmid, and RNA was completely separated from plasmid with minimal loss of plasmid when the NaOAc concentration exceeded 1.5 M. However, increasing the concentration of Na_2SO_4 from 0.5

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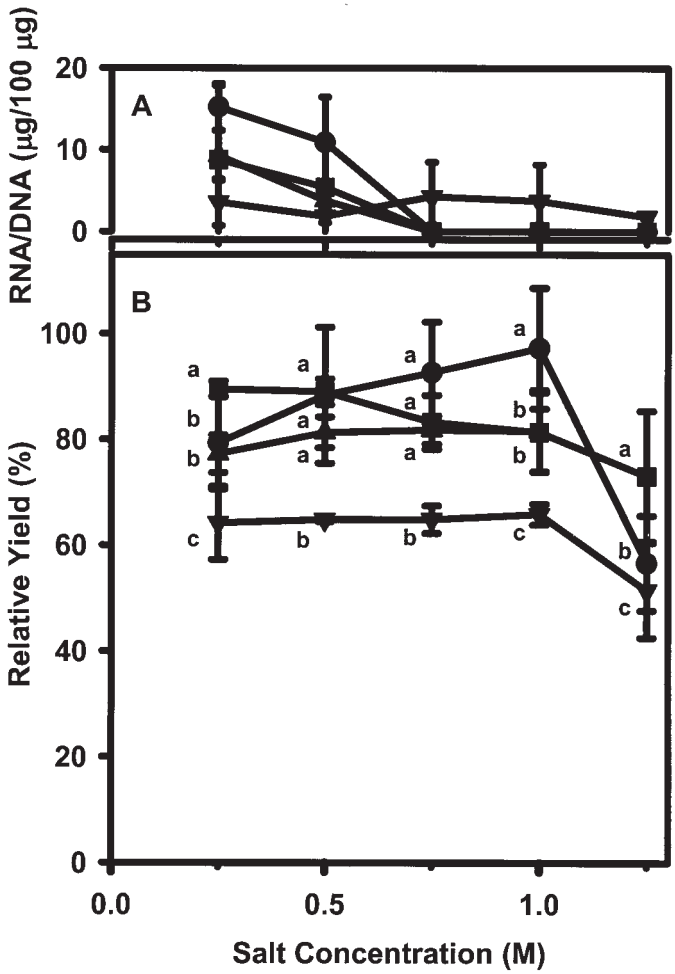

Figure 3. Effects of buffer cations on plasmid purification by batch adsorption–desorption using Q-Sepharose in the presence of 45% isopropanol. The wash buffers contained 45% isopropanol and various concentrations of different salts. (NaCl, ●; $MgCl_2$, ■; KCl, ▲; $CaCl_2$, ▼.) Panels A and B show the residual RNA amount and plasmid yield in the elution, respectively. Data bearing different letters at each salt concentration are significantly different ($p < 0.05$, $n = 3$, mean $\pm$ 95% CI).

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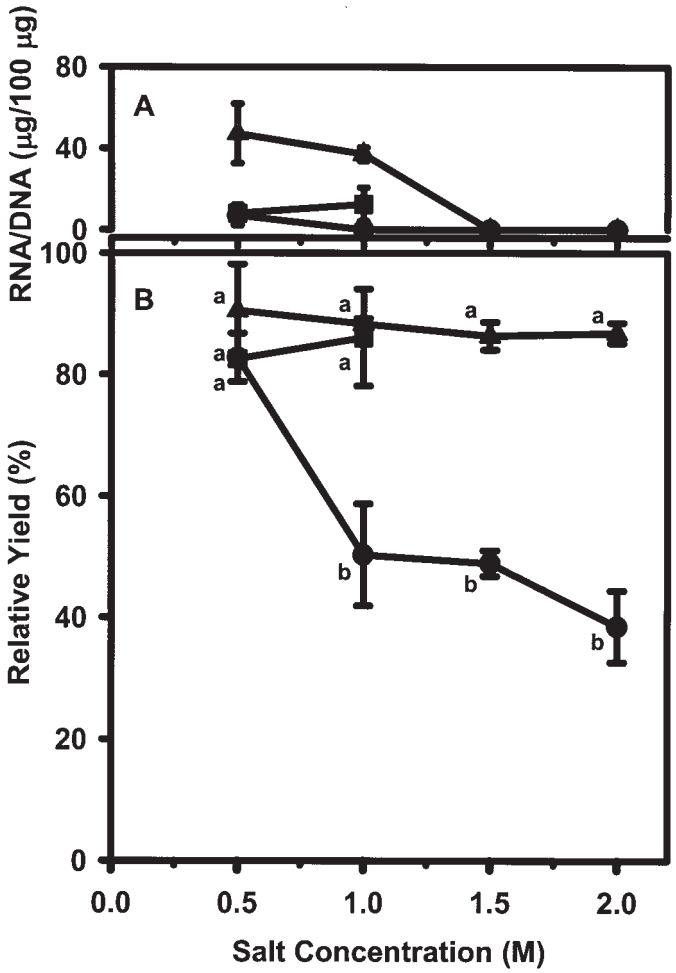

Figure 4. Effects of buffer anions on plasmid purification by batch adsorption–desorption using Q-Sepharose in the presence of 30% isopropanol. The wash buffers contained 30% isopropanol and various concentrations of different salts. (NaCl, ●; NaOAc, ▲; Na_2SO_4, ■.) Panels A and B show the residual RNA amount and plasmid yield in the elution, respectively. Data bearing different letters at each salt concentration are significantly different ($p < 0.05$, $n = 3$, mean $\pm$ 95% CI).

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to 1.0 M barely improved the purification, and further increases were limited by the relative insolubility of Na_2SO_4 in 30% isopropanol.

Effects of Buffer Anions on Plasmid Purification Using Q-Sepharose in the Presence of 45% Isopropanol

When the wash buffer contained 45% isopropanol and a salt at a concentration below 1.0 M, the relative yields of plasmid were maintained at 80–98% when NaOAc, Na_2SO_4, or NaCl was used (Fig. 5). This was similar to that obtained using buffer cations (Fig. 3). The amounts of residual RNA decreased as the salt concentrations increased when NaOAc or NaCl was used, but the complete separation of RNA from plasmid required a higher concentration when NaOAc (1.25 M) was used than when NaCl (0.75 M) was used. Although the relative yields were not reduced by increasing the salt concentrations, Na_2SO_4 still failed to remove the residual RNA from plasmid over the range of concentration used in this study.

Preparative Chromatography Under Gravity-Flow

In the batch adsorption–desorption process, NaCl or $MgCl_2$ separated RNA from plasmid with a minimal loss in yield. Although NaOAc could also completely remove RNA from plasmid, the required amount of NaOAc exceeded the required amounts of NaCl or $MgCl_2$, implying that the latter were better able to displace nucleic acids from the resins. Hence, these two salts were adopted in preparative chromatography. The eluted plasmid, however, was determined to contain trace amounts of RNA. Another investigation demonstrated that plasmid could be completely separated from RNA and proteins under gravity flow by modulating the concentrations of both NaCl and isopropanol. The approach of elution implemented in that other study[14] was used herein to purify plasmid from 3-L bacterial broth using 200 mL of Q-Sepharose, when the wash buffers contained $MgCl_2$ (Fig. 6).

Most of the protein (data not shown) and RNA were removed when washed with a buffer that contained 45% isopropanol and 0.5 M $MgCl_2$ (fractions 7–14). Some tightly bound proteins (data not shown) and RNA (fractions 15–22) were removed by increasing $MgCl_2$ to 1.5 M while lowering the concentration of isopropanol to 40%. Proteins (data not shown) and RNA were completely separated from plasmid after the second-step elution because a further drop of isopropanol concentration to 30% washed out undetectable amounts of protein (data not shown) and RNA (fractions 23–30), as revealed

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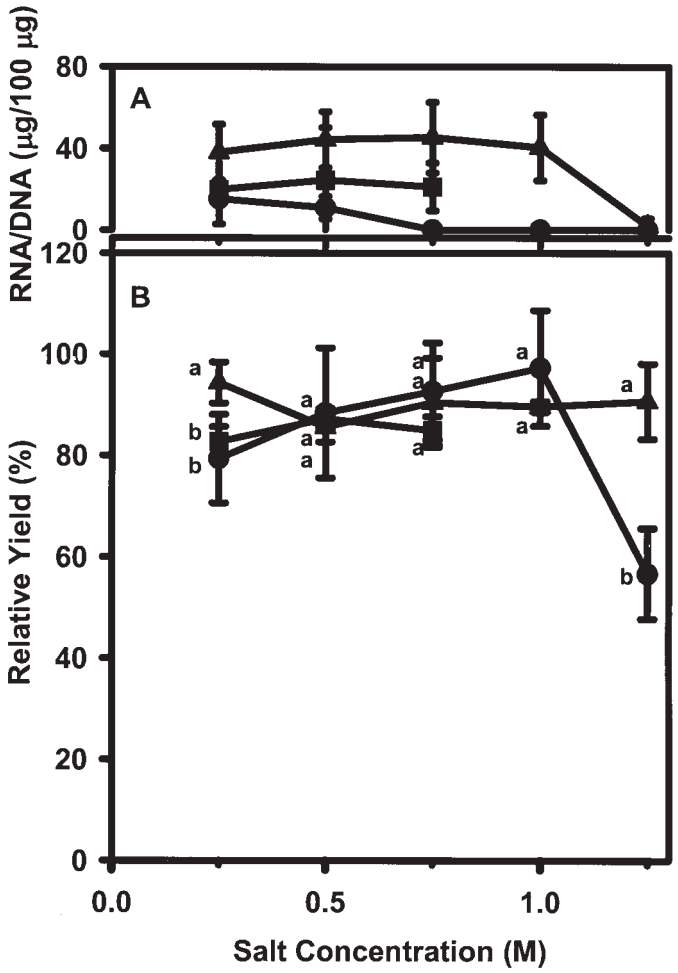

Figure 5. Effects of buffer anions on plasmid purification by batch adsorption–desorption using Q-Sepharose in the presence of 45% isopropanol. The wash buffers contained 45% isopropanol and various concentrations of different salts. (NaCl, ●; NaOAc, ▲; Na_2SO_4, ■.) Panels A and B show the residual RNA amount and plasmid yield in the elution, respectively. Data bearing different letters at each salt concentration are significantly different ($p < 0.05$, $n = 3$, mean $\pm$ 95% CI).

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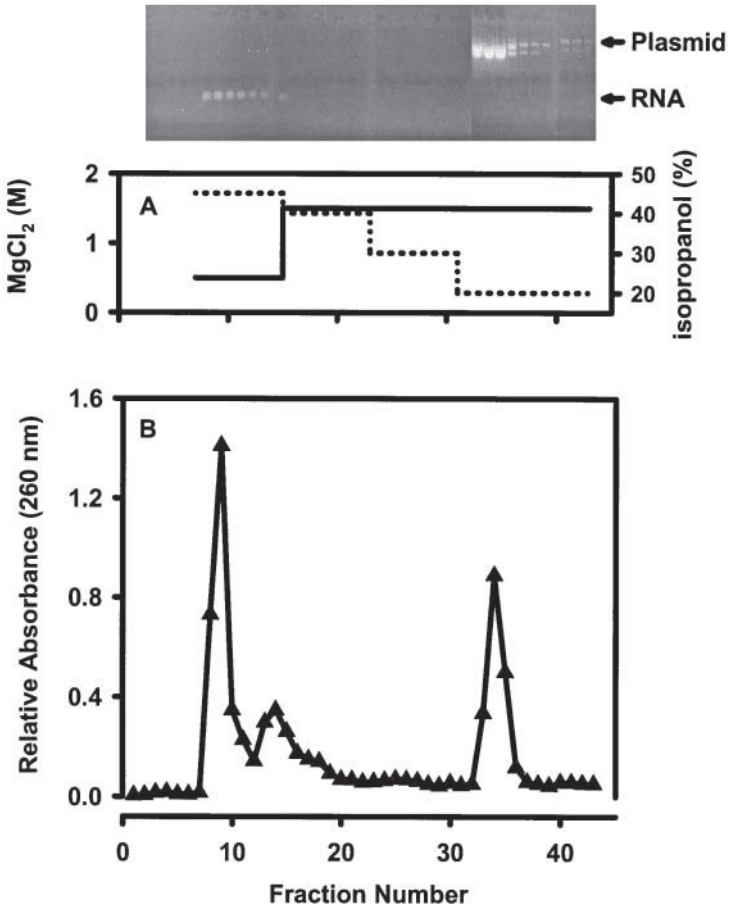

Figure 6. Typical elution profiles of preparative chromatography under gravity flow. The gradient profile (panel A) shows the isopropanol (···) and $MgCl_2$ (——) concentrations in the buffers. The elution profile shows the relative absorbance of each fraction (panel B; –▲–). Each lane of the top gel shows the electrophoretic analysis of the corresponding fraction in the elution profile.

by protein analysis and gel electrophoretic analysis. Eluting plasmid required a buffer that contained 20% isopropanol and 1.5 M $MgCl_2$. The purified plasmid in the eluates collected from fractions 32 to 36 contained undetectable amounts of RNA and proteins. Gel electrophoretic analysis revealed some degree of separation of the supercoiled plasmid from the open circular isoform during elution (fractions 32–34). A tailing effect of the eluted plasmid, in

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fractions 37–43, is evident in the top gel of Fig. 6 but is not clearly shown by the absorbance at 260 nm.

After each batch of plasmid was purified, the resins were regenerated by applying sequentially five bed volumes of 0.2 M NaOH, five bed volumes of distilled water, five bed volumes of 2 M NaCl, five bed volumes of distilled water, and then equilibrating by adding five bed volumes of loading buffer. Table 1 shows the relative yields obtained using the regenerated resins when the buffers contained $MgCl_2$. The purified plasmid contained undetectable amounts of RNA and proteins and had a relative yield of about 61%. When the same gradients of $MgCl_2$ were applied in preparative chromatography on a smaller scale, 20 mL of Q-Sepharose was used to purify plasmid from 300 mL of bacterial broth, and the same high quality of plasmid was obtained with a relative yield of about 71%, similar to that obtained in a previous report using NaCl.[14]

DISCUSSION

In batch adsorption–desorption, the concentration of isopropanol influenced the purification of plasmid according to the species of salt in the

Table 1. Quality of the plasmid purified by preparative chromatography when the wash buffers contained $MgCl_2$ and isopropanol or NaCl and isopropanol.

Salt	$MgCl_2$		NaCl[a]	
Q-Sepharose amount (mL)	20	200	20	200
Bacterial broth volume (mL)	300	3,000	300	3,000
Residual protein[b]	N.D.[c]	N.D.[c]	N.D.[c]	N.D.[c]
Residual RNA[b]	N.D.[c]	N.D.[c]	N.D.[c]	N.D.[c]
Relative yield (%)	71 ± 8	61 ± 1	75 ± 6	70 ± 4
Yield (μg plasmid/mL bacterial broth)	4.5 ± 0.5	3.1 ± 0.1	5.4 ± 0.5	4.2 ± 0.2

Note: The plasmid purified by the wash buffers that contained $MgCl_2$ and isopropanol was pooled from the fractions that correspond to the last peak in Fig. 6. Q-Sepharose was regenerated and used repeatedly ($n = 3$, mean $\pm$ S.E.).
[a]Data were obtained from Ref.[14].
[b]The amounts of protein and RNA were determined on the basis of a total amount of 10 μg purified plasmid.
[c]Not detected.

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wash buffers. Increasing the isopropanol content from 30% to 45% promoted the separation of plasmid from RNA and proteins using NaCl, KCl, $MgCl_2$, or NaOAc, but such an enhancement was not obtained when $CaCl_2$ or Na_2SO_4 was used. At 30% isopropanol and a high concentration of salt in the wash buffer, the resolution of separation of plasmid from RNA was increased but the plasmid yield was compromised. At 45% isopropanol, the high resolution of separation of plasmid from RNA was observed with a high plasmid yield at a salt concentration from 0.75 to 1.0 M. However, isopropanol did not affect the separation when the salt in the wash buffer was either $CaCl_2$ (Figs. 2 and 3) or Na_2SO_4 (Figs. 4 and 5) because the eluted plasmid was contaminated by a residual amount of RNA, despite the increases in the concentration of isopropanol or salt.

The monovalent anion, chloride, was more effective than the polyvalent anions, sulfate and acetate, in displacing nucleic acids from resins in washing during plasmid purification. The decrease in the relative yield with increasing salt concentration implied that the increased ionic strength promoted the removal of nucleic acid from the resins. The relative yield decreased as the NaCl concentration increased, but remained approximately constant when the concentration of Na_2SO_4 or NaOAc was increased (Figs. 4 and 5). Hence, NaCl removed more nucleic acids than did Na_2SO_4 or NaOAc at a given ionic strength or the same anionic concentration as depicted in Figs. 4 and 5. Such observations differed from those made of protein purification, during which polyvalent anions generally acted as stronger displacers than monovalent anions.[9] Another polyvalent anion, phosphate, was also examined for its ability to displace nucleic acids. The low solubility of sodium phosphate in the presence of isopropanol was such that the wash buffers contained 15% isopropanol and phosphate at a concentration from 0.25 to 0.75 M. Phosphate did not completely remove RNA from plasmid, and resulted in a relative yield of about 85–95% with 15–20 μg residual RNA per 100 μg plasmid (data not shown), a displacing ability approximately equal to that of sulfate under similar conditions.

Increasing the concentration of isopropanol was more effective in promoting plasmid purification when the buffer contained NaCl, KCl, or $MgCl_2$ than when they contained $CaCl_2$. Unlike proteins, nucleic acids are polyanionic, and so strongly interact with their electrolyte counter ions. Hence, cations are important in changing the conformations of nucleic acids. Many studies, using x-ray crystallography, have shown that monovalent and divalent cations can fit into the groove of the DNA double helix to cause DNA bending.[16,17] Ca^{2+} is generally localized more than Na^+, K^+, and Mg^{2+} ions, in the groove and is in direct contacts with DNA bases to cause conformational changes.[16,17]

An investigation, using synchrotron x-ray diffraction, established that after DNA was electronically associated with a cationic quaternary-amine

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lipid, dioleoy trimethylammonium propane, divalent cations, such as Co^{2+} and Mg^{2+}, condensed DNA on the lipid.[18] In the present study, when DNA became associated with Q-Sepahrose, the cations and isopropanol in the wash buffers might also have synergistically condensed DNA upon the resins during washing, promoting the separation of RNA from plasmid, by removing the uncondensed RNA. When DNA condensation did not occur, the plasmid adsorbed on the resins might have undergone conformational changes to enable the separation of RNA from plasmid. The strength of the charge interactions between RNA with Ca^{2+} might explain why Ca^{2+} failed to completely separate RNA from plasmid. In the presence of Ca^{2+} and isopropanol, RNA, along with plasmid, might have been condensed upon the resins such that the elute contained both RNA and plasmid.

In conclusion, ions determined whether the incorporation of isopropanol into wash buffers enhances plasmid purification. The interactions of ions with nucleic acids are important in determining the extent of such enhancements. The effectiveness of the anions in displacing nucleic acids from the resins followed the order, chloride > acetate > sulfate. When the wash buffers contained NaCl, KCl, or $MgCl_2$, plasmid was completely separated from RNA and proteins using Q-Sepharose in a batch adsorption–desorption process. The buffers that contained $MgCl_2$ and isopropanol produced a high yield of very pure plasmid using preparative chromatography under gravity flow. Isopropanol greatly enhanced the purification of plasmid when the wash buffer contained a salt, such as NaCl or $MgCl_2$, whose cation interacted weakly with DNA and whose anion was a strong displacer of DNA.

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