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Enhanced purification of plasmid DNA using Q-Sepharose by modulation of alcohol concentrations

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Abstract

Ion-exchange chromatography is one of the most commonly used methods for plasmid preparation. In this study a modified method was used to purify plasmid from bacterial lysate using Q-Sepharose. Incorporation of alcohols into the washing buffers enhanced the separation of plasmid from RNA and proteins. The use of isopropanol and ethanol achieved a high yield and purity whereas the use of methanol failed to improve the plasmid purification using Q-Sepharose by batch adsorption–desorption. Stepwise elution containing various concentrations of isopropanol and NaCl was used in preparative chromatography to enhance the plasmid purification. The same stepwise elution was applied to the chromatography columns packed with 0.5, 20, and 200 ml of Q-Sepharose for plasmid purification from 7.5, 300, and 3000 ml bacterial broth, respectively. Complete separation of DNA from RNA and proteins was achieved under gravity flow by modulation of the alcohol concentrations in the stepwise elution. These three scales of chromatography maintained an approximate plasmid yield and the purified plasmid contained undetectable levels of RNA and protein.

Keywords: Plasmid; DNA; Alcohols

1. Introduction

Plasmid DNA is an important element in genetic manipulation, and numerous methods have been documented for its preparation [1]. A typical process of plasmid production begins with the culture of transformed *Escherichia coli* followed by alkaline lysis of the harvested bacteria. After selective precipitation and concentration of DNA from the cell lysate, plasmid can be purified by different methods including cesium–chloride gradient ultracentrifugation, a membrane system [2,3], and chromatography. Many process improvements have been focused on chromatography due to its ease of operation and wide selection of resins. Currently several chromatography methods, such as ion-exchange [4,5], size-exclusion [6,7], affinity [8,9], and hydrophobic chromatography [10,11], have been demonstrated in plasmid purification.

Ion-exchange chromatography, a most widely used and economical method for plasmid purification, separates DNA from RNA and proteins based on the differences in their charge interactions with the resins in the presence of various ionic strengths and

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pH. The minimal differences in the charge interactions, however, result in either insufficient removal of RNA from the plasmid or severe yield loss. Two methods are usually adopted to improve the purification of plasmid using ion-exchange chromatography. One uses chemically modified resins to change the charge interactions. For example, the resins manufactured by Qiagen are silica gels of 100 μm in diameter, coated with a hydrophilic film before the high-density attachment of DEAE functional groups. Chromatography using the modified resins is simple to operate and allows the use of gravity flow. The other approach uses unmodified resins that are commercially available, such as Q-Sepharose, and controls the flow rates to create a continuous gradient for separation [12]. The buffers used in the chemically modified resins generally contain alcohols for plasmid purification; for example, the washing buffers employed in the use of Qiagen resins contain 15% isopropanol. The effect of alcohol on plasmid purification, however, has received less attention in ion-exchange chromatography that uses unmodified resins, especially as gravity flow is so simple.

In this study, Q-Sepharose was used for plasmid purification and complete separation of DNA from RNA was achieved under gravity flow by modulating the alcohol concentrations in the washing buffers. Different alcohols were examined for their effects on the yield and purity of the purified plasmid using batch adsorption–desorption. The scale-up of preparative chromatography was investigated in three different scales using stepwise elution under gravity flow.

2. Experimental

2.1. Materials

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

2.2. Plasmid amplification and preparation of standard plasmid

The pEGFP-C1 vector is 4.7 kb with a Cytomegalovirus promoter, a humanized and mutated green fluorescent protein gene, and a kanamycin resistant marker. The plasmid was transformed into competent *E. coli* DH5α by a standard procedure [1]. The selected transformed *E. coli* was preserved in LB medium plus 20% glycerol at −80 °C as a master cell bank. A transformed strain from the master cell bank was grown in LB medium containing kanamycin at 37 °C either by an orbital shaker for 14–16 h or by a 5-l fermenter (B. Braun Biotech, Melsungen, Germany) for 8 h.

Standard plasmid was obtained by using a Qiagen mini-prep kit and its concentration was determined by absorbance at 260 nm with one O.D. equal to 50 μg/ml of double stranded DNA.

2.3. Agarose gel electrophoresis

Since the high content of alcohol in the samples affected the movement of nucleic acid in gel electrophoresis, precipitation was used to remove the alcohol. Isopropanol was added into 0.5 ml of each sample to a final concentration of 50% (v/v). The samples were incubated at −20 °C for 15 min followed by centrifugation to obtain the precipitate of nucleic acid which was then dissolved in water and applied to 0.8% agarose gel. The bands on the gel were stained with ethidium bromide and visualized on a UV transilluminator. Photographs of the gel were taken with a Kodak Digital DC 4800 camera (Eastman Kodak, Rochester, NJ, USA) in black–white mode and the intensities of the digitalized photos were analyzed by Adobe Photoshop.

2.4. Protein measurement

The amount of protein in each sample was determined by a modified method using fluorescamine,

which can detect bovine serum albumin (BSA) as low as 0.2 μg/ml [13]. A 50-μl sample was added into 700 μl of reaction buffer (100 m*M* boric acid, 5 m*M* EDTA, pH 8.0) followed by addition of 250 μl of 0.01% fluorescamine in acetone. The mixture was incubated at room temperature for 10 min. The fluorescence intensity was measured at an emission wavelength of 380 nm and an excitation wavelength of 480 nm using an Hitachi fluorimeter F-2500 (Tokyo, Japan) with a slit setting of 5 nm. Various concentrations of BSA were used as standards in protein quantitation. In order to demonstrate that the presence of DNA or RNA hardly interfered with the protein measurement, 2 μg of plasmid DNA and 25 μg of yeast messenger RNA were added into the BSA standards, respectively, and the variations of measured intensities were found to be within 1% of the intensities of the standards containing BSA alone.

2.5. DNA measurement

Hoechst dye 33258 was used to quantitate the plasmid in the final product [14]. A 20-μl aliquot of each sample was mixed with 700 μl of Hoechst dye buffer (20 m*M* Hepes, 5 m*M* EDTA, 0.1 *M* NaCl, pH 7.5) followed by addition of 12 μl of Hoechst dye (0.01%). The mixture was allowed to reach equilibrium at room temperature for 5 min. The fluorescence intensity of each sample was measured at an emission wavelength of 352 nm and an excitation wavelength of 461 nm with an Hitachi fluorimeter F-2500. Because Hoechst dye exhibits high affinity with the AT base pairs of DNA, the fluorescence intensity of plasmid DNA was 50-fold the intensity measured from the same weight amount of yeast messenger RNA under the assay conditions of this study. The weight ratio of RNA/DNA was far less than 1 in all our final products as revealed by analysis of agarose gel electrophoresis. Therefore, the use of Hoechst dye can exclude the RNA interferences in DNA measurement. In order to further demonstrate the exclusion of protein interferences in the DNA measurement, 10 μg BSA was added into the plasmid DNA standards and the variations of measured intensities were found to be within 1% of the intensities of the standards containing plasmid DNA alone.

2.6. RNA measurement

The RNA of each sample was analyzed together with the standards containing various amounts of yeast messenger RNA by agarose gel electrophoresis. The amount of RNA was presumably proportional to its intensity on the gel. A calibration curve was constructed using the intensities of the known amounts of RNA and used to estimate the amount of RNA in each sample by interpolation of the measured intensity.

2.7. Preparation of cell lysate

Clear cell lysate was obtained by an alkaline lysis method. After cells were harvested by centrifugation from 150 ml bacterial broth, the cell pellets were resuspended in 10 ml resuspension buffer (50 m*M* Tris–HCl, 10 m*M* EDTA, 10 U/ml of RNase) followed by addition of 10 ml lysis buffer (0.2 N NaOH, 1% sodium dodecyl sulfate). After 5 min of incubation at room temperature, the mixture was neutralized with 10 ml of 3*M* potassium acetate for 15 min at 4 °C to precipitate the chromosomal DNA associated with the denatured proteins and lipids. The precipitates were then removed by filtration to obtain the clear cell lysate. The plasmid of the cell lysate was concentrated by precipitation in the presence of 2 *M* guanidine hydrochloride and 50% isopropanol at −20 °C for 30 min. After centrifugation the precipitate was rinsed with 70% ethanol twice to remove the residual guanidine hydrochloride before it was dissolved in a loading buffer (50 m*M* MOPS, pH 7.0).

2.8. Plasmid purification by batch adsorption–desorption

The anion-exchange resins, DEAE-Sepharose and Q-Sepharose, were equilibrated with the loading buffer. The adsorption of plasmid was performed by incubating 0.5 ml of the resin with the pretreated cell lysate of 7.5 ml bacterial broth for 5 min. The unbound plasmid was separated from the resins by centrifugation. The impurities on the resins were then removed by 0.5 ml of the washing buffer containing 50 m*M* MOPS, 1 N NaCl and various concentrations of different alcohols. The alcohols

included methanol, ethanol, and isopropanol and the volumetric concentrations of these alcohols were 0, 15, 30, 45, and 60% except that 60% isopropanol was not used due to the limitation of NaCl solubility. After washing four times, the desorption of plasmid was performed by using 0.5 ml of the batch elution buffer (50 m*M* MOPS, 2 *M* NaCl, pH 7.0) four times. The amounts of protein, RNA, and plasmid in the washing buffers and elution buffers were measured by methods described previously.

2.9. Preparative chromatography by gravity flow

Three different scales of chromatography were used in this study: small, medium, and large. The small scale used a column of 6.5 cm×0.8 cm I.D. packed with 0.5 ml Q-Sepharose for purification of 7.5 ml bacterial broth, the medium scale used a column of 9 cm×3.3 cm I.D. packed with 20 ml Q-Sepharose for purification of 300 ml bacterial broth, and the large scale used a column of 20 cm×6.5 cm I.D. packed with 200 ml Q-Sepharose for purification of 3 l bacterial broth. Ceramic nets were placed on the top of the resins. The same profile of stepwise elution driven by gravity flow was applied to each scale of chromatography. The unbound plasmid was first removed by the loading buffer. Then the step gradients were applied sequentially by: gradient 1: 0.5 *M* NaCl, 45% (v/v) isopropanol, pH 7.0; gradient 2: 1.5 *M* NaCl, 40% (v/v) isopropanol, pH 7.0; gradient 3: 1.5 *M*, 30% (v/v) isopropanol, pH 7.0; gradient 4: 1.5 *M*, 20% (v/v) isopropanol, pH 7.0. The volume of each gradient was four times the bed volume. The volume of each collected fraction was 0.25, 10, and, 100 ml for the small, medium, and large scale chromatography, respectively.

3. Results

3.1. Effect of isopropanol on the amount of plasmid in the elution buffer

When the washing buffer contained less than 30% isopropanol, analysis of agarose gel electrophoresis showed that both plasmid and RNA were detected in the washing and elution buffers by batch adsorption–desorption using Q-Sepharose, as shown in Fig. 1. The amount of plasmid in the washing buffer decreased with increased isopropanol concentration in the washing buffer. When the isopropanol concentration in the washing buffer reached 45%, the amount of plasmid in the washing buffer (lane 4W) and the amount of RNA in the elution buffer (lane 4E) became undetectable on the agarose gel, indicating that the purified plasmid was of high purity with minimal loss during the washing step.

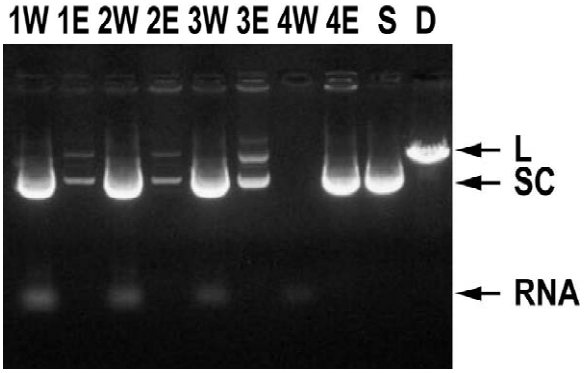

Fig. 1. Enhancement of amount of plasmid in the elution buffer by increasing isopropanol concentrations in the washing buffer. A 0.5-ml amount of Q-Sepharose was used to purify the clear lysate of 7.5 ml bacterial broth by batch adsorption–desorption. The washing buffers contained 1 *M* NaCl and various concentrations of isopropanol. The agarose gel showed the contents of washing buffer in the following lanes: 1W (0% isopropanol), 2W (15% isopropanol), 3W (30% isopropanol), and 4W (45% isopropanol). The elution buffers contained 2 *M* NaCl without any alcohol. Lanes 1E, 2E, 3E, and 4E showed the contents of the elution buffers corresponding to the washing buffers 1W, 2W, 3W, and 4W, respectively. Lane S showed the plasmid purified by Qiagen mini-prep kit. Lane D showed the product of the plasmid digested by EcoRI. L, linear form; SC, supercoiled form.

3.2. Effect of alcohol on the yield and purity of plasmid purification

Because isopropanol could promote the separation of plasmid and RNA as shown in Fig. 1, the effects of various alcohols on plasmid purification were examined on a quantitative basis. Fig. 2B shows that incorporation of alcohols into the washing buffers enhanced the purification yield of plasmid by batch adsorption–desorption using Q-Sepharose. The relative yield of plasmid was calculated as the total

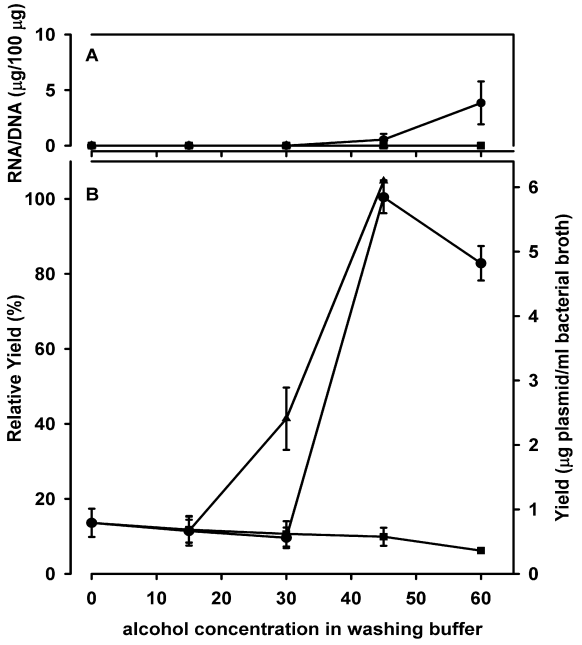

Fig. 2. Effects of alcohol on plasmid purification by batch adsorption–desorption using Q-Sepharose. The washing buffers contained 1 *M* NaCl and various concentrations of different alcohols (methanol: ■; ethanol: ●; isopropanol: ▲). The yield and purity calculated based on the amounts of plasmid in the elution buffers are shown in A and B, respectively ($n=3$, mean±S.E.).

amount of plasmid in the elution buffer divided by the total amount of plasmid, which was the multiplication of the broth volume and the amount of plasmid per ml obtained by Qiagen mini-prep kit. When the volumetric contents of alcohols in the washing buffers were below 15%, the improvements of yield were insignificant for isopropanol, ethanol, and methanol. When the washing buffer contained 30 and 45% of isopropanol, the relative yield increased from 18 to 40 and 100%, respectively. If ethanol was used instead of isopropanol, the relative yield increased from 18 to 100% as the ethanol content increased from 30 to 45%. A further increase of the ethanol content to 60% resulted in a decrease of the relative yield to 82%. The use of methanol was incapable of enhancing the yield and had the same yield as the use of aqueous phase alone.

The strong anion-exchange resin, Q-Sepharose, was replaced by a weak anion-exchange resin, DEAE-Sepharose, in the batch adsorption–desorption experiments to examine whether the alcohols could also enhance the yield and purity of plasmid purified by a weak anion-exchange resin. The incorporation of alcohols into the washing buffers improved the relative yield of plasmid purified by DEAE-Sepharose from 5 to 36%, as shown in Fig. 3B. Both the use of DEAE-Sepharose and the use of Q-Sepharose showed the same pattern of yield enhancement. Methanol still failed to enhance the yield. The critical concentrations of isopropanol and ethanol for the yield enhancement were 30 and 45%, respectively, which were the same values as observed when using Q-Sepharose and DEAE-Sepharose. But for DEAE-Sepharose the maxima of relative yields were limited below 36% even when the alcohol contents were further increased.

Since the protein level was undetectable in all eluted samples, protein contamination was excluded. Plasmid impurity was primarily due to residual RNA, and was expressed as the amount of residual RNA per 100 μg plasmid DNA. Figs. 2A and 3A

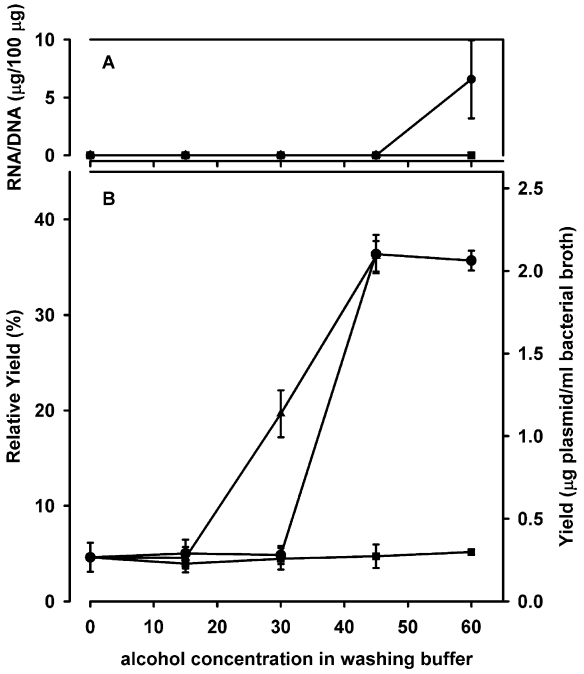

Fig. 3. Effects of alcohol on plasmid purification by batch adsorption–desorption using DEAE-Sepharose. The use of DEAE-Sepharose showed the same pattern of alcohol effect on the purity (A) and yield (B) when the washing buffers contained 1 *M* NaCl and various concentrations of different alcohols (methanol: ■; ethanol: ●; isopropanol: ▲) ($n=3$, mean±S.E.).

show the effects of various alcohol concentrations on the residual RNA level. Irrespective of the anion-exchange resins and alcohols, the purified plasmid in each elution buffer contained an undetectable level of RNA except for the samples eluted by 60% ethanol. Trace amounts of RNA were found in these samples, resulting in a reduction of plasmid purity.

3.3. Preparative chromatography by gravity flow

Q-Sepharose was used in the preparative chromatography by gravity flow as its binding capacity is higher than that of DEAE-Sepharose. When the buffers used in the batch adsorption–desorption process were applied to preparative chromatography, the eluted samples were found to contain both DNA and RNA, as shown in Fig. 4A. An optimization process was conducted by modulating the isopropanol and NaCl concentrations of washing buffer in small scale chromatography. The optimized condition consisting of the stepwise elution as described in the Experimental section, generated undetectable levels of protein and RNA in the purified plasmid, as shown in Fig. 4B. The stepwise elution was then applied to the medium and large scale chromatography under gravity flow in order to examine the possibility of scale-up.

The linear velocity of mobile phase varied with gravity flow but was controlled to between 0.15 and 0.35 cm/min by maintaining the hydraulic pressure at the appropriate level. Similar elution profiles were observed in the medium and large scale preparative chromatography, as shown in Fig. 5B,C, when the volume of each fraction was scaled proportionally to the bed volume of the resins. Most RNA and protein were removed when the buffer contained 0.5 *M* NaCl and 45% isopropanol as seen in the first peak. Some tightly bound RNA and proteins were then removed in a second gradient containing 1.5 *M* NaCl and 40% isopropanol. The remaining impurities were washed out by a further step gradient with a decreased isopropanol concentration. A major peak of DNA appeared when the buffer containing 1.5 *M* NaCl and 20% isopropanol was applied. The final product collected from the fractions of the last peak contained more than 90% supercoiled plasmid and undetectable levels of RNA and proteins. A tailing effect of plasmid elution was observed in the analysis of agarose gel electrophoresis but was not obvious in the measure of absorbance. Because the final product did not contain the fractions of eluted plasmid in the tail of the elution profile, the plasmid yields of preparative chromatography were lower than those of batch adsorption–desorption. In com-

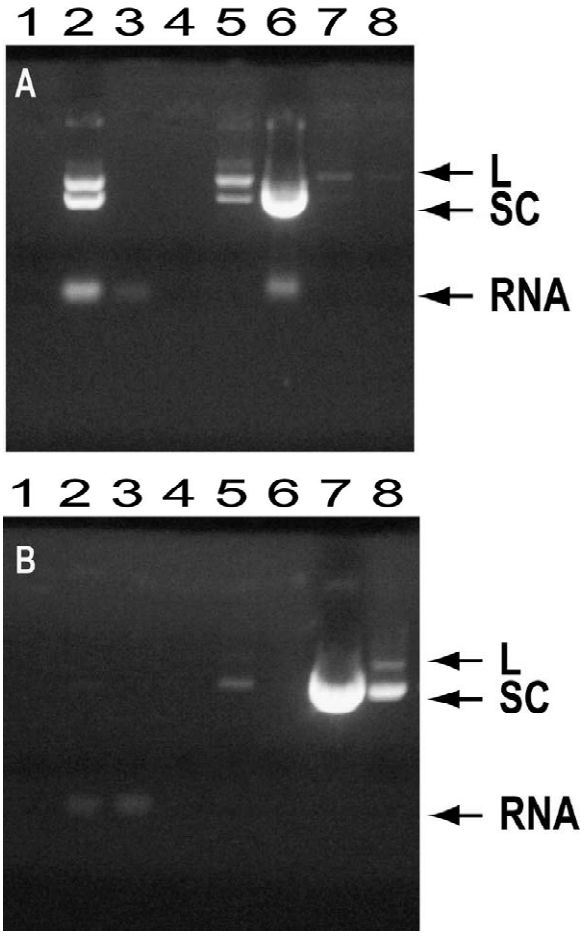

Fig. 4. Electrophoretic analysis of the RNA and DNA contents in the washing buffers of small-scale preparative chromatography. (A) Condition used in the batch adsorption–desorption process applied to the flow system. Lanes 1–4 were the contents in the washing buffer (1 *M* NaCl, 45% isopropanol, pH 7.0), and lanes 6–8 were the contents in the elution buffer (2 *M* NaCl, pH 7.0). (B) Optimized condition which consists of the following step gradients: gradient 1: 0.5*M* NaCl, 45% isopropanol, pH 7.0 (lanes 1 and 2); gradient 2: 1.5 *M* NaCl, 40% isopropanol, pH 7.0 (lanes 3 and 4); gradient 3: 1.5*M* NaCl, 30% isopropanol, pH 7.0 (lanes 5 and 6); gradient 4: 1.5*M* NaCl, 20% isopropanol, pH 7.0 (lanes 7 and 8). L, linear form; SC, supercoiled form.

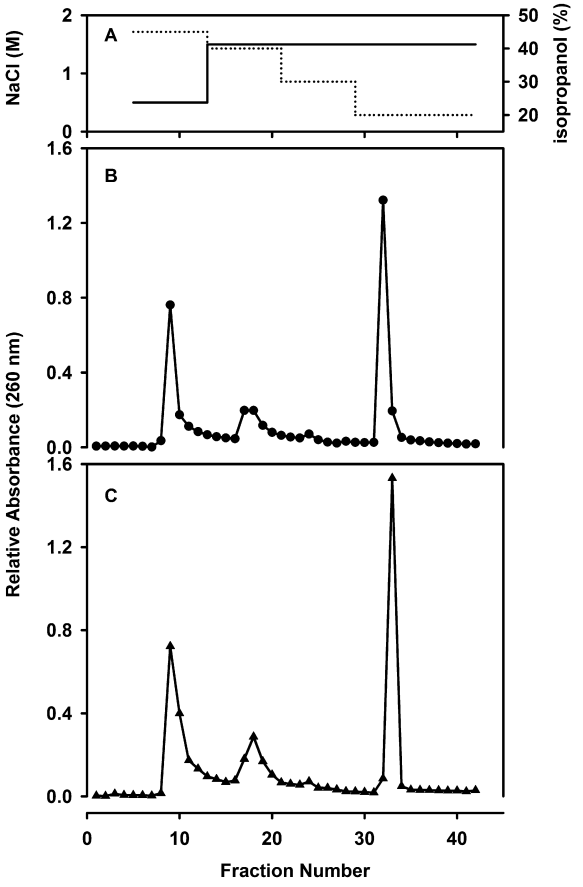

Fig. 5. Typical elution profiles of the medium scale and large scale preparative chromatography under gravity flow. The gradient profile (A) showed the isopropanol (- - -) and NaCl (—) concentrations in the buffers. The elution profiles showed the relative absorbance of each fraction of medium (B; –●–) and large scale (C; –▲–) preparative chromatography.

parison with the studies using continuous gradients, the use of stepwise elution was incapable of separating supercoiled and linear plasmids due to reduced chromatography resolution [15].

In order to examine the reusability of Q-Sepharose in the above process, the resins were cleaned sequentially with five bed volumes of 0.2 *M* NaOH and five bed volumes of 2 *M* NaCl, and then equilibrated with five bed volumes of loading buffer. The relative yields of the preparative chromatography which used the repeatedly cleaned resins were maintained at ~70% without any loss of purity for different scales as indicated in Table 1.

Table 1
Quality of the final product purified by three different scales of preparative chromatography using Q-Sepharose

	Small scale	Medium scale	Large scale
Residual protein[a]	N.D.	N.D.	N.D.
Residual RNA[a]	N.D.	N.D.	N.D.
Relative yield (%)	68±3	75±6	70±4
Yield (μg plasmid/ml bacterial broth)	4.0±0.1	5.4±0.5	4.2±0.2

The final products were collected from the fractions of the last peak in Fig. 5. Q-Sepharose was cleaned and repeatedly used (n=3, mean±S.E.). N.D., not detected.

[a] The detections of protein and RNA were based on a total amount of 10 μg purified plasmid.

4. Discussion

The interactions between the charged molecules can be described by Coulomb's law. Table 2 lists the dielectric constants of the buffers [16] used in this study. Incorporation of alcohols into the buffers promoted the interactions between charged molecules by reducing the dielectric constant. When the dielectric constant was decreased, both RNA and plasmid DNA became more tightly bound to the resins but to different extents. The binding strength between RNA and the resins increased less than that between DNA and the resins, resulting in the removal of RNA and retention of DNA at an elevated salt concentration. As shown in Figs. 2A and 3A, the purification yield increased when the dielectric constants of the buffers were decreased by the incorporation of isopropanol or ethanol.

There may be a critical dielectric constant value for the same alcohol to enhance the separation of RNA from DNA. For isopropanol, enhancement was not obvious until the dielectric constant was reduced from 80.1 (water) to 62.1 (30% isopropanol) and complete separation was observed when the dielec-

Table 2
Dielectric constants of the buffers used in this study

Volumetric content (%)	Methanol	Ethanol	Isopropanol
15	73.0	71.9	71.1
30	66.0	63.7	62.1
45	58.9	55.4	53.1
60	51.8	47.2	–

tric constant was further reduced to 53.1 (45% isopropanol) in the batch adsorption–desorption experiments using Q-Sepharose. For ethanol, an insignificant improvement of yield was observed when the dielectric constant of the buffer containing ethanol was above 55.4, as shown in Fig. 2B. Existence of the critical values was also observed when Q-Sepharose was replaced with DEAE-Sepharose for plasmid purification (Fig. 3A). When the dielectric constants were lower than the critical values, enhanced separation between RNA and DNA also occurred. But the lower yields were obtained due to the binding strength of the tertiary amine on DEAE-Sepharose being lower than that of the quaternary amine on Q-Sepharose. Both the use of Q-Sepharose and DEAE-Sepharose had the same critical values of dielectric constant, 62.1 and 55.4, for isopropanol and ethanol, respectively, suggesting that the critical values might be irrelevant to the types of anion-exchange resins.

The dielectric constant, however, was not the only factor that affected the interactions between the resins and the nucleic acids. Hydrophobic chromatography was reported to purify plasmid for therapeutic usage [11]. The aliphatic chains of alcohols in the solution might function like the hydrophobic ligands on the resins to create hydrophobic interactions with the nucleic acids. The 30% ethanol and 30% isopropanol have very close dielectric constants of 63.7 and 62.1, respectively, but the former had little effect on the purification process, whereas the latter doubled the relative yield from 18 to 40% (Fig. 2B). The 60% methanol has a dielectric constant of 51.8, lower than the 55.4 of 45% ethanol and the 53.1 of 45% isopropanol, but failed to improve the yield (Figs. 2B and 3B). These data suggest that the different hydrophobicities resulting from the aliphatic chains of alcohols might change the conformations of plasmid and RNA in different ways to affect the separation process. The base pairs of DNA are buried within the double helix, whereas the bases of RNA are exposed to the solution. The aliphatic chains of alcohols might interact with the exposed bases of RNA to alter the RNA conformation, whereas the minimal interactions with the unexposed bases of DNA might be incapable of altering the DNA conformation. In comparison with the low carbon number of aliphatic chain, the high carbon number of aliphatic chain has a high degree of hydrophobicity to interact with RNA and resulted in a high yield under the approximate values of dielectric constant. For example, the yield of 30% isopropanol was higher than that of 30% ethanol at the dielectric constant of ~63 (Figs. 2B and 3B).

A threshold of hydrophobicity might be required in order to alter the RNA conformation to enhance purification yield. Modulation of dielectric constant for process improvement is effective when using ethanol and isopropanol, but ineffective when using methanol. Hydration force plays an important role in changing the DNA conformations through the dehydration of DNA polar heads [17]. Lowering the water activity by alcohols changes the size and morphology of plasmid [18,19]. The single methyl group of methanol is incapable of creating sufficient dehydration for the conformational changes of nucleic acid to improve the separation process even under a dielectric constant value lower than those of ethanol and isopropanol.

Elevated salt concentration was required for the removal of RNA when the binding between the resins and the nucleic acids was enhanced by both the reduced dielectric constant and the increased hydrophobicity. As the bindings were enhanced to an extent, the removal of RNA in the washing step became incomplete under the same salt concentration. For example, the residual RNA level of the final plasmid product increased from 0 to 4 μg per 100 μg of plasmid DNA when 45% ethanol was replaced by 60% ethanol (Figs. 2A and 3A). A further elevation of the salt concentration to 2 *M* could restore the residual RNA level near 0 but resulted in reduced purification yield because the plasmid was washed out together with RNA at high salt concentration.

The preparative chromatography used stepwise elution to separate plasmid from RNA and proteins. Three peaks that corresponded to each gradient appeared before the elution of plasmid as observed in the elution profiles of Fig. 5B,C, suggesting that there existed at least three different binding strengths between the nucleic acids and the resins. Most RNA and proteins in the preparative chromatography had binding strengths less tight than those in batch adsorption–desorption. Therefore they could be removed under mild conditions (0.5*M* NaCl, 45% isopropanol, pH 7.0). Some RNA and proteins which

were more tightly bound were removed under an increased concentration of salt and a reduced concentration of isopropanol. The residual amount of RNA could be only removed by a further decrease of the isopropanol concentration. The different binding strengths might be due to the multiple configurations of nucleic acid or the orientations of nucleic acid upon its contact with the resins. Hydrophobicity might play an important role in altering the conformations of nucleic acid and lead to the multiple binding strengths. Such an effect might be also a major cause of the tailing of the eluted plasmid which had a high content of linear plasmid.

Preparative chromatography provided wide ranges of linear velocity and column dimensions. The gravity flow rates varied with hydraulic pressure and the viscosity of the buffer. The hydraulic pressure was maintained at an approximate liquid level. The linear velocity fluctuated in the range of 0.15–0.35 cm/min for the three different scales of preparative chromatography because the step gradients containing various concentrations of alcohols had different viscosities. Such variations, however, hardly affected the performance of chromatography. Even a change of linear velocity from 0.15 to 1.0 cm/min had no effect on the elution profile, purification yield, or purity for the medium scale chromatography (data not shown). When the column used in the medium scale was replaced with a column of dimensions 40 cm×1.6 cm I.D. (C16/40 column, Amersham-Pharmacia) with an adapter, the elution profile, purification yield, and purity obtained by these two different columns were similar when they were subjected to the same stepwise elution.

Although endotoxin level test was not performed in this study, incorporation of alcohols into the washing buffers has been reported to remove endotoxin effectively [20,21]. The purified plasmid was expected to contain only a minimal level of endotoxin after the washing steps with high alcohol concentration.

In conclusion, the process described in this study provides ease of scale-up plus a wide range of linear velocity operating conditions, and most importantly, generates high quality, purity and yield of plasmid under gravity flow by modulation of the alcohol concentrations in the washing and elution buffers. Both the dielectric constant and the length of aliphatic chain of the alcohols might play important roles in determining the purity and yield of plasmid. High yield and purity of plasmid could be obtained by using Q-Sepharose under either batch adsorption–desorption or gravity flow methods when the buffers contained alcohol with a low dielectric constant and long aliphatic chain.

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