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High-performance catalytic chromatography The adapter approach

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Abstract

A sequence-specific DNA that binds *Eco*RI endonuclease was immobilized on glycidioloxypropyl-silica and Sepharose by cyanogen bromide (CNBr)-activated coupling. Elution of bound enzyme by conventional affinity strategies (increase of salt concentration) or by catalysis-induced elution (adding a Mg^{2+} cofactor required for catalysis) was compared. Greater yield and fold-purification was obtained with catalysis-induced elution for both DNA-silica and DNA-Sepharose columns, and silica gives higher performance than Sepharose. Sodium dodecylsulfate polyacrylamide gel electrophoresis showed primarily a single band for *Eco*RI endonuclease for catalysis-induced elution from DNA-silica columns. Since catalysis-induced elution decreases the lifetime of DNA affinity columns, an alternative approach for preparing re-usable DNA columns was also developed. In this approach, a single stranded adapter DNA sequence is first coupled to silica or Sepharose and then annealed with another DNA sequence that contains a complementary, single stranded tail and the duplex binding site for *Eco*RI endonuclease. After use, replacing the hydrolyzed DNA regenerates the column. For this adapter approach, Sepharose gives better purity than silica and comparable yields and catalytic based elution gave the highest purity and yield, regardless of support. Substrate DNA with either a tail (for annealing to the column) at one end or both ends were compared and the former gave higher purity. Finally, enzyme binding to the substrate in solution ("trapping") or on a pre-bound substrate column was compared and trapping gave higher yield and similar purity to the alternative. Thus, trapping with a single tailed substrate oligonucleotide on a Sepharose adapter column and using catalytic elution gave the highest performance.

Keywords: DNA; *Eco*RI restriction endonuclease; Cyanogen bromide; Diol-silica; HPLC; Catalytic chromatography

1. Introduction

Specific sequence DNA, immobilized on low-pressure supports such as Sepharose, has been used for the isolation of a large number of proteins, e.g., transcription factors, restriction enzymes, DNA and RNA polymerases, etc. [1–6]. Since restriction enzymes recognize a specific sequence of DNA with high affinity, these proteins can be isolated using DNA affinity adsorbents containing the appropriate sequence.

Catalytic chromatography is a variant of enzyme affinity chromatography but different from it. Catalytic chromatography uses the specific biological affinity for substrate, as does affinity chromatography and but relies upon catalytic specificity since catalysis of substrate to product is used for elution [7]. Here, we focus on the catalytic chromatography of *Eco*RI as a model for this new mode. In the absence of Mg^{2+}, *Eco*RI endonuclease binds its cognate DNA restriction sequence. After washing the column to remove contaminant proteins, addition of required cofactor Mg^{2+} results in the specific and selective elution of bound *Eco*RI endonuclease. Elution is dependent on enzyme catalysis of the bound immobilized substrate. This single step purification approach resulted in a homogenous protein with a higher yield and fold-purification than conventional affinity chromatography [7]. Thus, by combining specific biological affinity with catalytic specificity, catalytic chromatography could significantly improve purification of enzymes. Elution

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of bound enzymes by catalysis-induced elution has also occasionally been reported for the specific and selective purification of other enzymes [8,9]. In fact, catalytic chromatography actually pre-dates affinity chromatography [10]. However, the greater purity and yield obtainable with catalytic chromatography when compared to conventional affinity chromatography was only recently shown [7].

The catalytic approach is broadly applicable. Most enzymes require one or more cofactors and approximately one-thirds of all enzymes require a metal ion, as does *Eco*RI. While we have used DNA substrates as the basis for these studies, other substrates could be easily used for this approach. Indeed the first uses of catalytic elution methods involved collagenase bound to collagen [10], purification of SNAP receptors by ATP hydrolysis-dependent elution [8], and plant ribulose bisphosphate carboxylase (Rubisco) large subunit *N*-methyl transferase elution with *S*-adenosylmethionine [9], all non-DNA binding proteins. Even if catalysis cannot be paused by leaving out a cofactor, other approaches can be envisioned. These would include using inhibitors, especially uncompetitive inhibitors, or adding product to the mobile phase for readily reversible reactions to favor the enzyme–substrate complex. Furthermore, most enzyme reactions involve multiple substrates and leaving out one or more of these could serve the same role as leaving out a cofactor to pause catalysis, depending on the mechanism. Elution in these other cases would be by adding the missing components or by removing factors (inhibitors or products) impeding catalysis in the forward direction. In our previous study, we also showed that DNA polymerase can be retained on a DNA template column by using a futile cycle in which the exonuclease and polymerase activities participate and then using the polymerase activity for elution. The method is clearly not limited to DNA-binding enzymes or *Eco*RI restriction endonuclease we used here as a model but can be adapted to virtually any enzyme.

*Eco*RI purification provides a good example of the changing strategies in purifying DNA-binding proteins. *Eco*RI purification originally was accomplished with fives steps including chromatography using phosphocellulose, hydroxyapatite and finally DNA-cellulose chromatography [11]. The DNA-cellulose was heterogeneous, fragmented nuclear DNA. Later, purification with only two chromatographic steps was developed using ion-exchange (phospho-cellulose) followed by gel filtration chromatography (Sephadex G-150) [12]. The same group also demonstrated a single step purification employing immunoaffinity chromatography. Another one step purification using sequence-specific oligonucleotide affinity chromatography with a double stranded eicosomer containing the recognition site for *Eco*RI was only partially successful, yielding 75–85% purity [13]. Apparently homogeneous *Eco*RI was obtained by combining oligodeoxynucleotide affinity chromatography with DEAE-cellulose chromatography in a two-step procedure [4]. All of these earlier studies had used salt gradient elution from DNA columns. Recently, Jurado et al. showed that single step purification of *Eco*RI to apparent homogeneity could be obtained by catalysis-based elution from sequence-specific oligodeoxynucleotide columns [7]. This catalytic chromatography has been shown to give higher purity and yield than conventional salt elution.

While giving higher purity and yield [7], catalytic elution has the disadvantage of short column lifetime; i.e., the column substrate is converted to product, often irreversibly. To remove this limitation, a new approach is here explored in which an adapter column is prepared to reversibly bind substrate. After chromatography, the product can be stripped away and replaced with fresh substrate for subsequent uses. This approach also has the advantage that a single adapter column can be used for multiple, different substrates or other uses.

Biomolecular HPLC of proteins has progressed from the conventional soft gels used for low-pressure liquid chromatography to silica-based supports, which often have greater resolution and speed of separation. Combining conventional affinity chromatography with HPLC to yield high-performance affinity chromatography (HPAC) should result in improved selectivity [14]. However, a limitation of HPAC is that most coupling procedures for silica supports require complex chemistries and are not widely used. Recently, we modified the most common coupling chemistry in low-pressure chromatography (i.e., cyanogen bromide (CNBr) activation) to diol-silica HPLC supports [15–17]. These developments have now allowed a direct comparison of silica and Sepharose using the same DNA coupling chemistry, and mobile phases to determine which perform best for conventional affinity chromatography and the catalytic approach. The results show that a single support does not always give the best performance by all criteria but that catalytic elution surpasses conventional methods regardless of support. The adapter approach also allows two alternatives where enzyme binding occurs in solution or on a column. Solution binding gives higher yield and is technically easier.

2. Experimental procedures

2.1. Materials

Unless stated otherwise, all reagents were of the highest-grade purity available. Growth media were from BIO 101 (Vista, CA, USA) and molecular biology reagents from New England Biolabs (Beverly, MA, USA) and Gibco BRL (Life Technologies, NY, USA). Acetone (HPLC grade, Fisher, St. Louis, MO, USA) was used and stored over 3 Å pore molecular sieves. Sepharose 4B and CNBr were obtained from Sigma (St. Louis, MO, USA), purified *Eco*RI was from New England Biolabs, diol-silica was Nucleosil 300–70 OH from Alltech Associates Inc. (Dearfield, IL, USA, Macrosphere GPC, 300 Å pore, 7 μm beads) and triethylamine (TEA) was from

Fisher (St. Louis, MO, USA). Anhydrous solutions of CNBr (1.0 M) and TEA (1.5 M) were prepared in anhydrous acetone for silica. For activation of Sepharose beads, CNBr and TEA solutions were prepared using 60% acetone. Synthetic DNA sequences used were ethanol-precipitated from TE (10 mM Tris, 1 mM EDTA, pH 7.5) and washed with 70% ethanol immediately prior to coupling. For coupling to diol-silica or to Sepharose, purified DNA was dissolved in coupling buffer (0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl).

2.2. Oligonucleotides used

Name	Sequence
EcoSph	5′-NH_2-GCATGC**GAATTC**GCAT-3′
$(AC)_5$	5′-NH_2-ACACACACAC-3′
EcoGT	5′-GCATGC**GAATTC**GCATGTGTGTGTGT-3′
αEcoGT	5′-ATGC**GAATTC**GCATGC-3′
EcoD	5′-CATGCATGC**GAATTC**GCATGCATGGTGTGTGTGT-3′
EcoGT-1	5′-GCATGC**GAATTC**GCATGCATGCATGCTGTGTGTGTG-3′
αEcoGT-1	5′-GCATGCATGCATGC**GAATTC**GCATGC-3′

"NH_2" in these DNA sequences refers to the 5′-aminohexyl moiety added on the last cycle of DNA synthesis (Integrated DNA Technologies, IA, USA). The *Eco*RI restriction sequence (GAATTC) is shown in bold. The first two of these were covalently coupled to supports in the various experiment, and the last five are used to charge the $(AC)_5$ adapter supports.

2.3. Annealing oligonucleotides

EcoSph and EcoD are self-complementary (i.e., anneals with itself). Other double stranded DNAs were made by mixing EcoGT with its complementary strand, αEcoGT; similarly EcoGT-1 and αEcoGT-1 are complementary. In all cases, annealing of oligonucleotides was by heating to 95 °C for 5 min, cooling slowly to 4 °C over an hour in a thermalcycler.

2.4. CNBr activation and DNA coupling

For activation of diol-silica with CNBr and TEA and coupling to DNA we followed the published procedure [15–17]. Briefly, 1.5 g diol-silica was suspended in 2 ml anhydrous acetone and bath sonicated under vacuum for 5 min. After centrifugation, the supernatant was removed and the support transferred to a small beaker to which sufficient anhydrous acetone was added to yield an approximately 50% slurry and cooled to −15 °C. For activation, 1 ml anhydrous CNBr (1.0 M) was added and 1 ml anhydrous TEA (1.5 M) was added dropwise over 3 min. The temperature of the activation reaction was maintained at −15 °C throughout. After activation, excess reagent was removed by filtration and the support washed with ice-cold water (50 ml) and coupling buffer (50 ml). Activated resin was transferred to a solution that contained 120 nmoles of the self-complementary, annealed DNA sequence (EcoSph) that binds the *Eco*RI endonuclease. The mixture was placed on a wheel rotator overnight at 4 °C. For coupling this DNA to Sepharose, the cyanoethyl transfer procedure that uses 60% acetone was used [18]. Uncoupled DNA recovered after washing the supports with coupling buffer was used for absorption measurements at 260 nm and by difference the amount that coupled was determined. A total of 105 nmoles DNA coupled/ml of Sepharose and 95 nmoles coupled/ml of packed silica beads. Remaining reactive groups were consumed with blocking buffer (0.1 M Tris, pH 8.0, 0.5 M NaCl). Similarly, 50 nmoles of the $(AC)_5$ oligonucleotide was coupled yielding 19.6 nmoles/ml Sepharose and 20.4 nmoles/ml packed silica beads. It should be noted that *Eco*RI, when loaded onto the columns, is in the sub-nanomole range and so there is enough DNA attached to the column for trapping the entire amount loaded. Columns were stored at 4 °C in TE containing 10 mM NaN_3 when not in use.

For one variant of the adapter approach, activation, coupling, annealing, and regeneration procedures were performed by an "in flow procedure" using an HPLC system [17]. For these procedures, 1.5 g diol-silica resuspended in anhydrous acetone was sonicated under vacuum for 5 min. After centrifugation, the supernatant was removed and the support resuspended in anhydrous acetone (2 ml) and packed into a stainless-steel column (5 cm × 0.46 cm) at 2 ml/min using anhydrous acetone as the mobile phase. For activation, 1 ml CNBr (1.0 M) and 1 ml TEA (1.5 M) anhydrous solutions were pumped at 1 ml/min to a mixer and then onto the prepacked diol-silica column. All steps were performed at room temperature (20 °C). Flow was stopped for 3 min to allow reaction with hydroxyl groups. Reagent excess was removed by washing with 50 ml of anhydrous acetone at 2 ml/min. The activated column was then washed with water (20 ml) and coupling buffer (50 ml). Then, 120 nmoles DNA was recirculated overnight at 0.1 ml/min for coupling. Uncoupled DNA was collected and absorption at 260 nm measured to determine, by difference, the amount of DNA coupled. Remaining reactive groups were consumed by overnight recirculation of blocking buffer at 0.1 ml/min.

2.5. "Charging" adapter columns

The annealed EcoGT/αEcoGT or EcoD DNA, with 3′ $(TG)_5$ tails (Fig. 4), was recirculated overnight at 0.1 ml/min through the complementary $(AC)_5$ adapter DNA column with the column being immersed in a water bath maintained at 4 °C. The amount of double strand DNA used for annealing was double the amount of adapter DNA present on the column. Non-annealed DNA was collected and used for absorption measurements at 260 nm to determine the amount of DNA loaded onto the column.

2.6. Preparation of crude bacterial extracts

Escherichia coli (strain RY13), an over-expressing strain for *Eco*RI endonuclease [4], was grown at 37 °C in rich media (Superbroth). A crude bacterial extract, prepared as described in [7], was dialyzed against 0.1 M KCl in buffer B (25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), pH 7.8, 7 mM β-mercaptoethanol, 1 mM EDTA, 0.1% igepal (v/v), and 10% glycerol (v/v)) and stored in −85 °C.

2.7. EcoRI endonuclease assay

For this assay, 3 μl of each fraction was added into a 50 μl reaction mixture that contains 1 μg of λ phage DNA as the substrate and 50 mM NaCl, 100 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, and 0.025% Triton X-100 [4,7]. Reaction was performed at 37 °C for 60 min and was stopped by the addition of 12.5 μl of 50% glycerol, 100 mM EDTA, pH 7.5, 1 mg/ml bromophenol blue. The reaction products were analyzed on agarose electrophoresis gel containing ethidium bromide. The bands were visualized by ultraviolet light with an Alpha Innotech camera system and active fractions were pooled. Total *Eco*RI endonuclease units present in pooled fractions from affinity, catalytic, or the RY13 crude extract, were assayed by using 3 μl of serial dilutions of each enzyme. To account for differences in the buffers used as the mobile elution phase for each chromatographic strategy (affinity and catalytic), pooled fractions were diluted in buffer containing the remaining component required to equal the composition of the other enzyme to be tested. Serial dilution of commercially supplied *Eco*RI enzyme (New England Biolabs, 20 units/μl) provided a standard. After digestion, samples were applied to the gel and densitometry of the restricted fragments was determined by using the Alpha Innotech camera system and NIH Image software. One unit of *Eco*RI enzyme activity was defined as the amount of enzyme required for the digestion of 1 μg of λ DNA during a 60 min reaction at 37 °C.

2.8. Protein concentration

Protein concentration was measured, following precipitation with trichloroacetic acid, by the bicinchoninic acid microassay procedure provided by Pierce Chemical Co. Bovine serum albumin was the standard. Alternatively, for proteins present in very low concentration, the concentration was measured by loading the samples on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining (Bio-Rad Laboratories kit). Serially diluted equal amounts of three standard proteins (bovine serum albumin, egg albumin and carbonic anhydrase) were used. The NIH Image software was again used to determine the density of the standard protein bands and the concentration of unknowns. This method was also compared to the BCA method and a conversion factor derived so that all protein concentrations are comparable regardless of the method used.

2.9. Purification of EcoRI endonuclease

For these experiments, the resulting DNA-silica and -Sepharose were slurry-packed into stainless-steel columns (5 cm × 0.46 cm) at 0.5 ml/min using buffer B containing 0.1 M KCl as the mobile phase. A crude bacterial extract from *E. coli* strain RY13 was loaded (typically 40 μl), the column was washed with 10 ml of buffer B containing 0.1 M KCl, and 10 ml buffer B containing 0.25 M KCl [7]. Bound *Eco*RI endonuclease was eluted either by a linear increase in salt concentration (0.25–0.5 M KCl) for a typical affinity chromatography experiment or with a linear $MgCl_2$ gradient (0 to 50 mM Mg^{2+}) for a catalytic chromatography experiment. Flow rate was 0.5 ml/min throughout. One ml fractions were collected and assayed for *Eco*RI endonuclease activity as described above [4,7].

After using the double-stranded adapter DNA-silica or -Sepharose columns for catalytic chromatography, the cleaved, annealed DNA (Fig. 4) was removed with 70 °C water. Alternatively, the DNA column was placed in a water bath equilibrated at 70 °C and washed with buffer B containing 0.1 M KCl to remove remaining DNA. Absorption (260 nm) was used to record the elution of the remainder DNA sequence. Further, for reusing the column, fresh DNA (either EcoGT/αEcoGT or EcoD) was used again to charge the column as described above, again monitoring absorption 260 nm to follow the course of annealing. The resulting regenerated double-stranded DNA column was used for purification of *Eco*RI endonuclease by affinity followed by catalytic chromatography as described above. Chromatography was using a Rainin ternary HPLC system unless otherwise stated.

3. Results and discussion

In this study, two different purification procedures (i.e., affinity and catalytic chromatography) were applied to sequence-specific DNA-Sepharose and -silica columns to study the purification of *Eco*RI endonuclease as a model for catalytic chromatography. For these initial experiments, the self-complementary DNA sequence 5′-NH_2-GCATGC**GAATTC**GCAT-3′ (EcoSph) was directly coupled using CNBr-activated supports. For Sepharose, CNBr-activation was by the method of Kohn and Wilchek [18] and for diol-silica the new, anhydrous cyanoethyl transfer method [15–17] was used. Both supports, packed into stainless steel columns (5 cm × 0.46 cm), were used for the purification of *Eco*RI endonuclease on an HPLC system. The strategy for binding the enzyme from a crude bacterial extract (RY13) was similar to that used previously and relies on the fact that *Eco*RI binds DNA in the absence of Mg^{2+} but is catalytically inactive [7]. Bound enzyme was eluted by

either a salt gradient (for a typical affinity chromatography experiment) or by adding Mg^{2+} (for a catalytic chromatography experiment). This last procedure will cause elution of the bound enzyme after hydrolysis of its column–DNA substrate [7].

In the original report, the column was washed with 33 column volumes of buffer containing 0.1 M KCl, followed by 33 column volumes containing 0.25 M KCl, prior to elution with a step gradient to either 0.4 M KCl or to 50 mM $MgCl_2$, for affinity and catalytic elution, respectively. Under these conditions, apparently (by SDS-PAGE) homogenous *Eco*RI was obtained by catalytic elution. Here, we used less extensive column washing (12 column volumes for each buffer) and elution with a linear gradient to either 0.5 M KCl or 50 mM $MgCl_2$. Under these conditions, lower purity is obtained but this allows us to observe differences in the purity more readily and to also detect differences in the elution peak width which reflects chromatographic performance. Even though these conditions are less than optimal, quite high purity was observed under some chromatographic conditions which will be described.

3.1. Direct coupling: catalytic chromatography on silica gives higher performance

The purification of *Eco*RI endonuclease by affinity (panels A and B) and catalytic chromatography (panels C and D) on Sepharose and silica under these conditions is shown in Fig. 1. Elution of *Eco*RI endonuclease is observed by its characteristic cleavage pattern of λ phage DNA. For DNA-silica columns (Fig. 1B and D), as expected for its small, homogeneous bead size ($7 \pm 1.5\,\mu m$), a sharper peak is obtained in contrast to Sepharose (45–120 μm) (Fig. 1A and C). This is especially noticeable for affinity chromatography (contrast panels A and B). The activity also elutes earlier in the gradients from the silica columns. The cause of this was not investigated but probably arises from different void (excluded) volumes for the supports.

Catalytic chromatography for these columns (Fig. 1C and D) was performed by a $MgCl_2$ gradient to induce catalytic elution [7]. Again, a sharper peak for *Eco*RI endonuclease activity is observed with DNA-silica (contrast panels C and D), however, the difference is not great suggesting that catalytic rate, in addition to column characteristics, may be an important factor governing peak width.

To investigate the yield and purity obtained by affinity and catalytic chromatography on both DNA-Sepharose and -silica columns, active fractions from three separate experiments were collected and assayed for *Eco*RI activity and protein concentration. The results, shown in Table 1, demonstrate that a significantly higher yield and fold-purification were obtained for catalytic chromatography on either support and these differences have a high statistical significance. The higher purity presumably results from the specificity of catalysis-induced elution previously reported [7]. Silica gave better performance than Sepharose.

These experiments also show that CNBr coupling strategies previously available only for soft gels like Sepharose can be directly applied, without modification, to the new CNBr silica method [15–17]. The amounts coupled are similar (see Section 2.4), and performance and flow rate can be increased by moving to the uniform, small-diameter porous silica, allowing high-performance affinity chromatography and high-performance catalytic chromatography (HPCC) variations of existing soft gel methods.

The purified proteins were also examined by sodium dodecylsulfate-polyacrylamide gel electrophoresis as shown in Fig. 2. The position of *Eco*RI (arrow) was confirmed by electrophoresis of commercially obtained *Eco*RI (data not shown). DNA-silica columns gave higher purity for both affinity and catalytic chromatography than DNA-Sepharose. This is in substantial agreement with Table 1, though the table would predict slightly higher purity for catalytic/Sepharose than affinity/silica. Also, the affinity-Sepharose suffered from both low yield and low purity and because of the extra proteins present, it is hard to locate the faint *Eco*RI band in this lane. Nevertheless, it is clear that highest purity was obtained by catalytic elution on the homogenous, small bead diameter silica, a procedure we call high-performance catalytic chromatography.

3.2. The adapter approach: catalytic chromatography on Sepharose surpasses silica

Previous results from our laboratory have indicated that the first use of catalysis-induced elution on sequence-specific DNA-Sepharose columns is accompanied by 80% hydrolysis of the bound DNA. For those experiments, 3 ml DNA-Sepharose columns that contain 8.3 nmoles/ml were used for purification of 0.3 ml of RY13 crude extract. On the second use, a further 8% of the DNA substrate was digested when the same column was re-used and yield suffered. Thus, catalysis-induced elution rapidly diminishes column capacity and eventually the column must be discarded [7]. In contrast, DNA-silica columns used in this study containing 95 nmoles DNA/ml were re-usable for at least seven times of catalysis-induced elution of *Eco*RI endonuclease. However, the eluted *Eco*RI endonuclease activity decreases for each successive catalytic elution (data not shown), and the column must eventually be replaced. To overcome this unique disadvantage a re-usable, sequence-specific DNA column was developed.

This approach is depicted in Fig. 3. A single stranded oligonucleotide is coupled to the column. The same $(AC)_5$ oligonucleotide we had used previously for transcription factor trapping [19] was used. The adapter can anneal a $(GT)_5$-tailed, double-stranded oligonucleotide which will serve as substrate for *Eco*RI. The annealed column can then be used for either affinity or catalytic chromatography and when performance decays, simply washing with hot water will release the substrate oligonucleotide which can then be replaced with fresh oligonucleotide to regenerate the column. A similar strategy of annealing a single stranded tail to a single

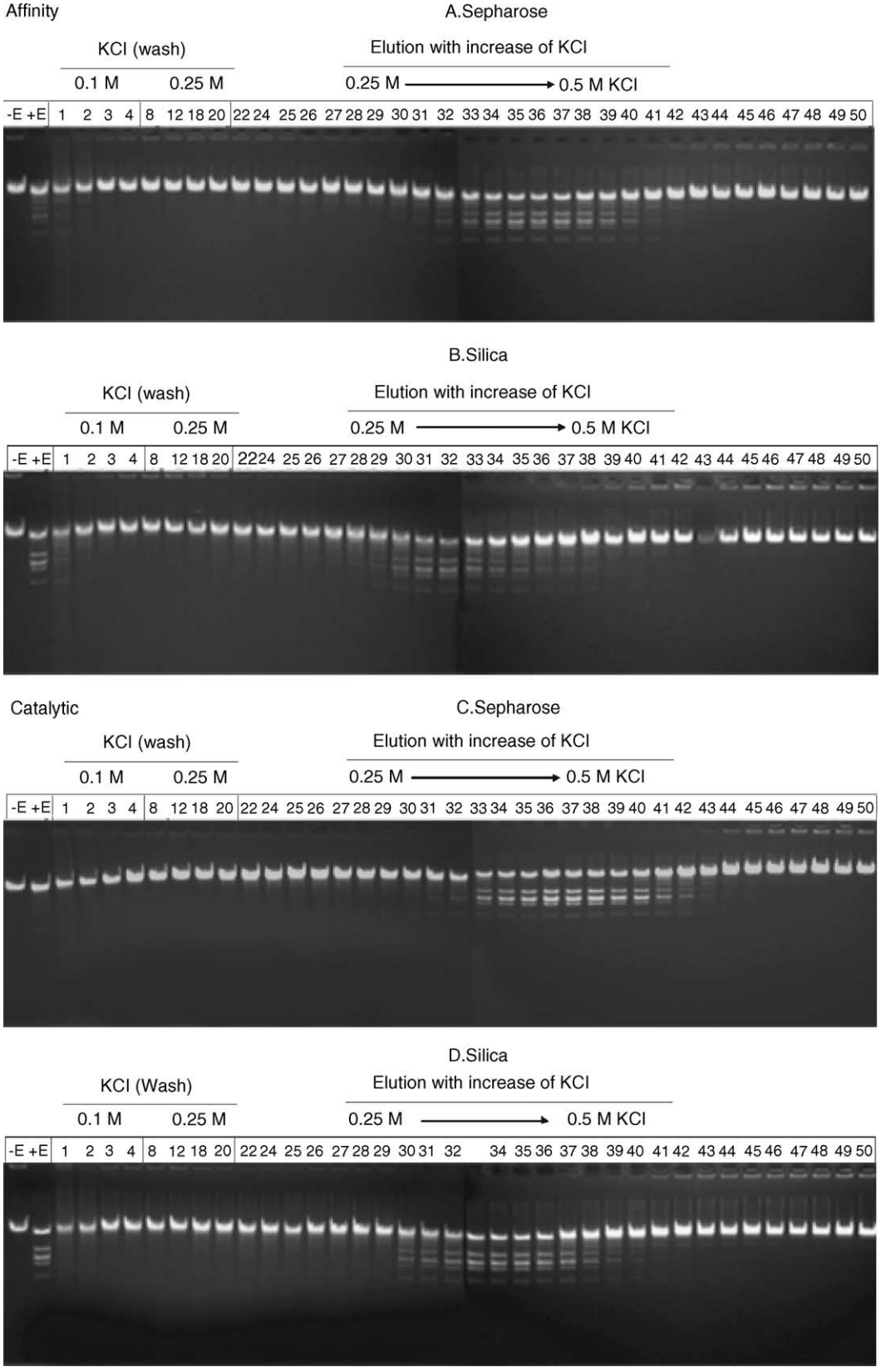
Affinity
A.Sepharose
KCl (wash)
Elution with increase of KCl
0.1 M
0.25 M
0.25 M
0.5 M KCl
-E +E 1 2 3 4 8 12 18 20 22 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
B.Silica
KCl (wash)
Elution with increase of KCl
0.1 M
0.25 M
0.25 M
0.5 M KCl
-E +E 1 2 3 4 8 12 18 20 2224 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
Catalytic
C.Sepharose
KCl (wash)
Elution with increase of KCl
0.1 M
0.25 M
0.25 M
0.5 M KCl
-E +E 1 2 3 4 8 12 18 20 22 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
D.Silica
KCl (Wash)
Elution with increase of KCl
0.1 M
0.25 M
0.25 M
0.5 M KCl
-E +E 1 2 3 4 8 12 18 20 22 24 25 26 27 28 29 30 31 32 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50

Table 1
Summary of *Eco*RI purification (using direct coupling of EcoSph oligonucleotide)[a]

Sample	Total protein (mg)	Total units	Specific activity (units/mg)	Yield (%)	Fold-purification
Crude	0.324	279	861	–	–
Sepharose					
Affinity chromatography	0.00366	166	45,355	59 ± 8[b]	53 ± 7[c]
Catalytic chromatography	0.00345	238	68,986	85 ± 9	80 ± 8
Silica					
Affinity chromatography	0.00306	193	63,072	69 ± 14[d]	73 ± 15[e]
Catalytic chromatography	0.00292	272	93,151	97 ± 1.3	108 ± 1.4

[a] The details of the purification condition are described in Section 2 and the legend of Fig. 1. Total units of *Eco*RI endonuclease activity and protein concentration were determined as described in Section 2. Statistical analysis is Student's *t*-test with two-tailed distribution and two-sample, unequal variance) of three separate experiments ($n = 3$) to compare yield and fold-purification.

[b] The results footnoted by b–d are all statistically different. For yield, affinity and catalytic chromatography on Sepharose have probability, $P = 0.02062$ that they are the same.

[c] Purity, Sepharose, affinity compared to catalytic, $P = 0.01216$.

[d] Yield, silica, affinity compared to catalytic, $P = 0.07295$.

[e] Purity, silica, affinity compared to catalytic, $P = 0.05496$.

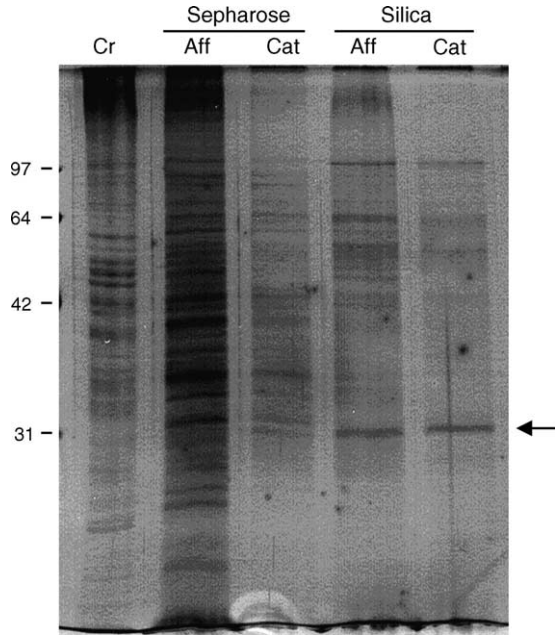

Fig. 2. SDS-PAGE of the proteins purified by affinity and catalytic chromatography on both DNA-Sepharose and DNA-silica columns using EcoSph oligonucleotide. The active pooled fractions eluted by affinity (Aff) or catalytic (Cat) chromatography on sequence-specific DNA-Sepharose and -silica columns were separately pooled. Polyacrylamide (12%) gel electrophoresis [23] and staining with silver is shown. Crude bacterial extract (Cr) is also shown for comparison, using 1/100th as much as used for the actual purification of the fractions shown. The mobility (Mr) of standards is shown in kDa. The arrow shows the mobility of *Eco*RI endonuclease.

stranded DNA adapter has been reported to result in useful DNA columns for the purification of other DNA binding proteins [20].

As shown in Fig. 4, *Eco*RI binds to the freshly charged (substrate annealed) column (panel A), no binding was found once the hydrolysed DNA was stripped from the column with hot water (panel B), and upon recharging with fresh substrate DNA, *Eco*RI again bound and eluted. Thus, the $(AC)_5$ adapter approach functions as expected.

The peak of *Eco*RI elutes slightly earlier on the recharged column. This is likely due to changes in the column bed resulting from the temperature and mobile phase changes used during recycling. This does not affect the utility of the approach.

Recycling a single $(AC)_5$-Sepharose or -silica column through eight cycles of charging, use, and regeneration showed no deteriation of capacity or performance showing these adapter columns are also durable as expected (data not shown).

Adapter affinity and catalytic chromatography on Sepharose and silica were compared in experiments analogous to Fig. 1 (data not shown but submitted for review). Again, silica gave narrower peaks and higher resolution. Overall, adapter columns and columns prepared by direct coupling gave remarkably similar chromatography; however, the balance sheet did reveal differences.

The balance sheet (Table 2) shows the comparison of Sepharose, silica, affinity, and catalytic chromatography for the EcoGT/αEcoGT charged adapter columns. Again catalytic chromatography always gave the higher purity and

Fig. 1. *Eco*RI endonuclease purification by affinity chromatography on sequence-specific DNA-Sepharose and DNA-silica columns using EcoSph oligonucleotide. 40 μl of crude bacterial extract was loaded onto the 5 cm × 0.46 cm column equilibrated in buffer B containing 0.1 M KCl. After washing the column with low salt concentrations, bound proteins was eluted with a linear salt gradient (0.25–0.5 M KCl) for 100 min (panels A and B) or a linear $MgCl_2$ gradient (0–50 mM) for 100 min (panels C and D). One millilitre of fractions was collected and the flow rate was 0.5 ml/min throughout. For the *Eco*RI assay, 3 μl of each fraction was added to a 50 μl assay mixture. The reaction products were analyzed on a 1% agarose electrophoresis gel stained with ethidium bromide. "−E" is assay mixture without *Eco*RI added and "+E" is the same with New England Biolabs *Eco*RI added and serves as a positive control. The positive control of panel C is absent. The numbers in the figure refer to the column fractions assayed.

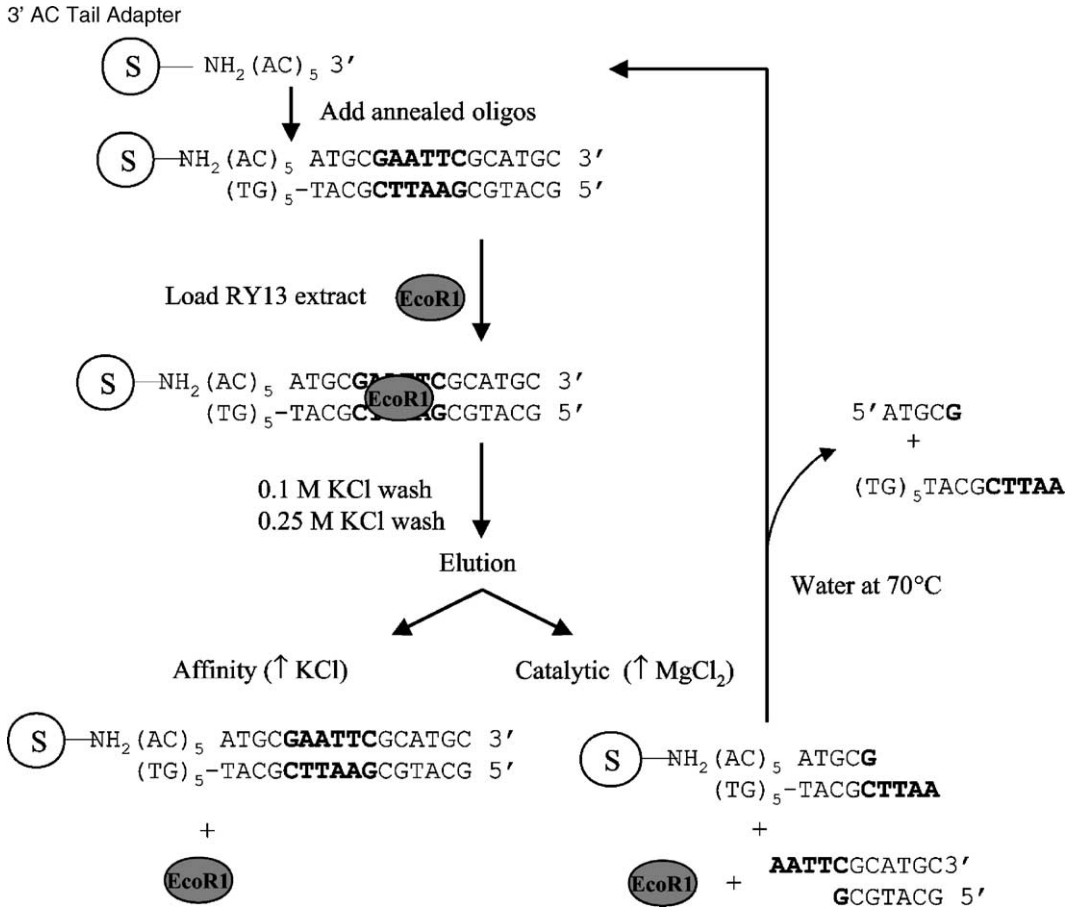

Fig. 3. Schematic representation of the affinity and catalytic chromatography with $(AC)_5$ adapter approach for the purification of *Eco*RI.

yield when compared to affinity chromatography regardless of which support was used. Interestingly though, for the adapter approach, Sepharose gave greater purity and comparable yield to that obtained with silica. The highest purity was obtained with catalytic chromatography using $(AC)_5$ adapter Sepharose and Fig. 5 shows that this *Eco*RI obtained is in a very high state of purity. Affinity chromatography on the same support yields less pure enzyme.

The most likely cause of this disparity in which support functions best for direct-coupling and adapter is that the latter must use a more complex DNA (the annealed oligonucleotide is 26-mer for the $(AC)_5$ adapter approach versus 16-mer used for direct coupling) and the 300 Å pore silica may be inadequate for the longer DNA. Clearly, the pore size needs to be optimized for adapter silica but until then, Sepharose provides a very workable alternative.

Table 2
Summary of *Eco*RI purification (using the $(AC)_5$ adapter approach)[a]

Sample	Total protein (mg)	Total units	Specific activity (units/mg)	Yield (%)	Fold-purification
Crude	0.219	698	3181	–	–
Sepharose					
Affinity chromatography	0.00153	406	266,568	58 ± 5.8	84 ± 11.8[b]
Catalytic chromatography	0.00135	461	341,448	66 ± 7.2	107 ± 10.7
Silica					
Affinity chromatography	0.00328	338	108,821	48.3 ± 12.8	34.3 ± 11.5
Catalytic chromatography	0.00345	498	139,541	71.3 ± 39.6	43.6 ± 16.5

[a] The details of purification conditions are described in the legend of Fig. 5. Total units of *Eco*RI endonuclease activity and protein concentration were determined as described in Section 2. Statistical analysis (Student's *t*-test with two-tailed distribution and two-sample, unequal variance) of three separate experiments ($n = 3$) to compare yield and fold-purification.

[b] Fold-purification is statistically different when comparing Sepharose to silica, for either affinity or catalytic chromatography. No other differences are significant.

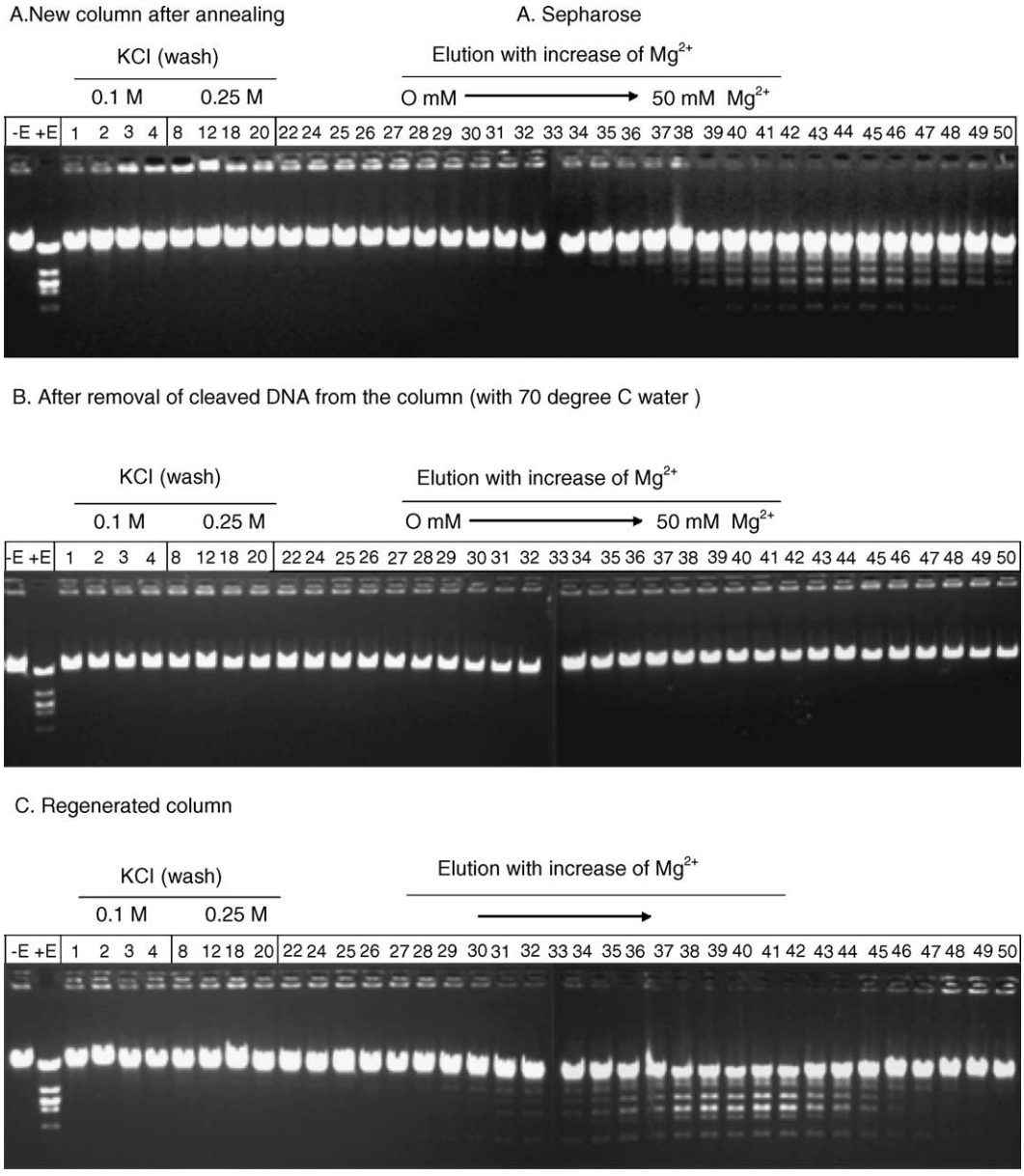

Fig. 4. Catalytic chromatography of *Eco*RI endonuclease on regenerative DNA-Sepharose column using $(AC)_5$ adapter oligonucleotide. A crude bacterial extract from *E. coli* strain RY13 (400 μl) was loaded on the charged (with EcoGT/αEcoGT annealed duplex) column, the column was washed with 8 ml of buffer B containing 0.1 M KCl, and 8 ml buffer B containing 0.25 M KCl [7]. Bound *Eco*RI endonuclease was eluted with a linear $MgCl_2$ gradient (0 to 50 mM Mg^{2+} for 30 min followed by another 30 min wash with 50 mM) for catalytic chromatography. Flow rate was 0.2 ml/min throughout. Fractions of 0.4 ml were collected and assayed for *Eco*RI endonuclease activity. The run was peformed using the Gilson 9000 HPLC system. For the *Eco*RI assay, 5 μl of each fraction was added into a 20 μl assay mixture. The reaction products were analyzed on a 1% agarose electrophoresis gel stained with ethidium bromide. (A) Initial binding and elution by catalytic chromatography from charged, double-stranded DNA-silica columns. (B) After stripping the column of annealed DNA with hot water, chromatography is repeated and *Eco*RI flows through the column and appears in the first two fractions only (data not shown). (C) Binding and elution of bound proteins after recharging the DNA-silica column with EcoGT/αEcoGT.

3.3. A single tail functions better than a two-tailed substrate

The adapter approach using $(AC)_5$-Sepharose was further investigated to learn what substrate DNA and enzyme binding methods function best. Preparing single tailed (S) substrate DNA, such as the EcoGT/αEcoGT duplex requires two complementary oligonucleotides, one containing the tail. Alternatively, a single, self-complementary DNA, in this case EcoD, could be used but this would produce a duplex containing a double (D) 3′ tail, one on both strands. Fig. 6 shows that either the double or single tailed approach retain similar amounts of *Eco*RI activity during loading and washing and a similar amount elutes with Mg^{2+}. However, when the same amount of each eluted protein is compared by silver stained SDS-PAGE, the single tail approach yields a sample

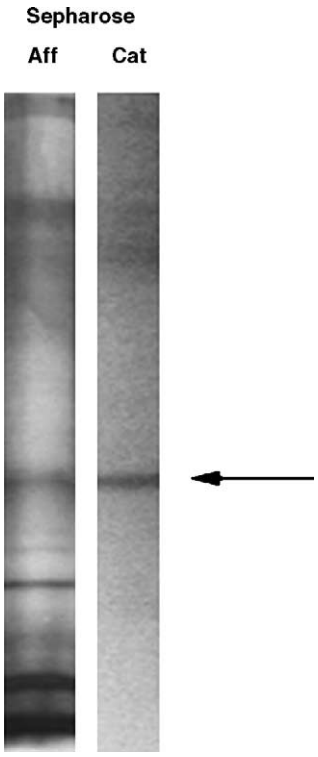

Fig. 5. SDS-PAGE of the protein purified by Sepharose affinity and catalytic chromatography on the DNA-Sepharose column using the $(AC)_5$ adapter. The method used for purification is described in the legend of Fig. 1. The active pooled fractions eluted by affinity (Aff) and catalytic (Cat) chromatography were pooled, separated by SDS-PAGE [23], and staining with silver. The arrow shows the mobility of *Eco*RI endonuclease.

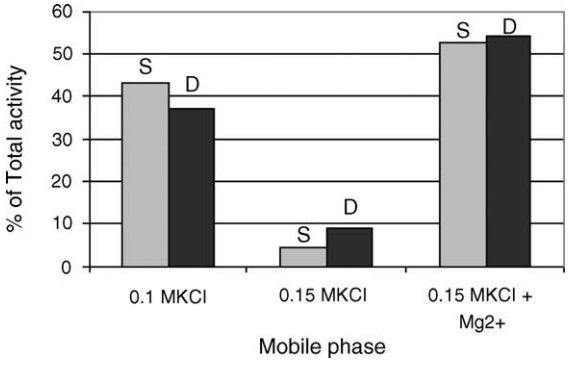

Fig. 6. Single and doubled tailed substrate DNA give similar yield but different purity. A 1 ml $(AC)_5$-Sepharose column was charged with 660 pmoles of either annealed EcoGT/αEcoGT or the self complimentary EcoD substrate DNA, yielding DNA containing either a single $(GT)_5$ tail (S), or DNA containing a double (D) tail, one at each end of the duplex, respectively. RY13 extract (100 μl) was applied to each column and the column was then washed with 10 ml of buffer B containing 0.1 M KCl, 10 ml buffer B containing 0.15 M KCl, and eluted by 15 ml of buffer B containing 0.15 M KCl and 50 mM $MgCl_2$. Fractions from each were pooled and assayed for *Eco*RI activity. *Eco*RI units were measured for only one run so standard deviation was not calculated. Figs. 6 and 7 were performed using the Bio-Rad BioLogic DuoFlow metal-free chromatograph and 1 ml plastic syringe columns.

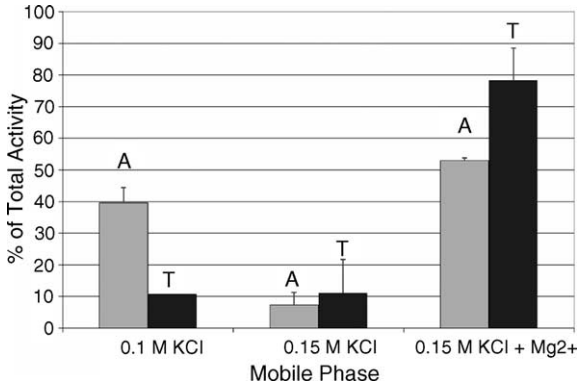

Fig. 7. Trapping gives higher yield than the adapter approach. A 1 ml column of $(AC)_5$-Sepharose column was used for adapter (A) chromatography by precharging it with 10 ml of Buffer B with 0.1 M KCl containing 660 pmoles (66 nM) of annealed EcoGT-1/αEcoGT-1 at a flow rate of 0.2 ml/min (annealing to the column was not by overnight recycling as was done for Figs. 1, 4 and 5) followed by washing with 10 ml of buffer B containing 0.1 M KCl, and then applying 100 μl of RY13 extract to bind to the column. Alternatively, trapping (T) was performed by mixing 10 ml of the 66 nM EcoGT-1/αEcoGT-1 with 100 μl of RY13 extract, and this mixture was applied to the $(AC)_5$-Sepharose. Both columns were then washed and eluted as described in Fig. 7. The experiments were performed in duplicates and the bars show the standard deviation. The *P*-value (two tail Student's *t*-test) gives a value of 0.177.

with higher amounts of *Eco*RI and less impurities (data not shown but submitted for review). This may be because the additional tail, present in the doubled tailed substrate is capable of binding other proteins not bound by the simpler, single tailed oligonucleotide. Thus, a single tailed oligonucleotide is preferable and was used for all further experiments.

3.4. Substrate binding in solution followed by trapping gives similar results to column binding

Finally, the adapter approach allows two alternatives for enzyme–substrate binding. The substrate oligonucleotide can be used to charge a column and the enzyme can then be applied to the charged column and allowed to bind there. This has been the approach used in all experiments up to this point and has been referred to as the adapter (A) approach. Alternatively, the substrate DNA can be mixed with the enzyme in solution and allowed to bind there. The mixture can then be applied to the column and allowed to anneal under flow, trapping (T) the enzyme–substrate complex. In either case, the column can then be eluted catalytically. These alternatives are compared in Fig. 7. Trapping retains greater amounts of the enzyme during washing and higher amounts of the enzyme subsequently elute in Mg^{2+}, though the difference was not statistically different ($P = 0.18$, $n = 2$). SDS-PAGE however showed that the two approaches yield enzyme of about the same high purity (data not shown). Since trapping, forming the enzyme–substrate complex in solution is the easier approach and may give higher yield, it would be preferred.

This experiment was preformed using 66 nM substrate DNA (660 pmol) either mixed with 100 μl bacterial extract

before chromatography or the same amount used to precharge the column. The yield (78 ± 10%) of enzyme activity recovered in Fig. 7 is similar or somewhat higher than that obtained in Table 2 (catalytic, Sepharose, yield = 66 ± 7.2%) where columns (5 cm × 0.46 cm, 0.83 ml) were fully charged using 40 nmol of DNA and 40 μl bacterial extract was used; the experiment in Fig. 7 used 60-fold lower amounts of DNA than that in Table 2. Thus, low amounts of DNA can be used with adapter columns, conserving substrate DNA. These lower amounts can be used for trapping at low DNA concentrations to promote specific enzyme–substrate binding and still retain high yield.

The main shortcoming of catalytic chromatography in the past was the short lifetime inherent in converting a column substrate into product. This shortcoming was effectively removed by using an adapter to bind the substrate reversibly. For other applications though, different adapter technologies would need to be developed or new applications of old supports would have to be tried. For example, nitrilotriacetic–agarose binds transition metals and could be used as an adapter support for some enzymes with metal ion cofactors. Substrates which contain a sulfhydryl or into which a sulfhydryl could be introduced, can be immobilized reversibly by disulfide formation [21] with thiopropyl-Sepharose or other supports. Similarly, silver ions can also be reversibly immobilized on thiol-supports and bind DNA reversibly with high affinity [22]. While the adapter approach may not always be feasible, it is likely it too could be applied in most cases.

The adapter approach removes the limitation of short column lifetime previously inherent to the catalytic approach by allowing columns to be recycled rather than discarded. Since catalysis-induced elution has the disadvantage of being potentially destructive to column lifetime, development of re-usable DNA columns removes the only significant disadvantage of catalytic chromatography. These re-usable adapter DNA columns make DNA a consumable reagent, which can be replaced instead of column supports, which are more expensive and tedious to make.

It also has the further advantage that a single adapter sequence could be used to make different DNA-sequence columns by simply charging with other oligonucleotides, allowing a single adapter column to be used for purifying multiple DNA-binding proteins. Indeed, we used $(AC)_5$-Sepharose along with $(GT)_5$-tailed response elements to purify transcription factors by the oligonucleotide trapping method [19]. Thus, $(AC)_5$-supports have wide application for purification of enzymes, transcription factors, and potentially other DNA-binding proteins.

4. Conclusions

For direct coupling procedures, silica surpassed Sepharose but the reverse is true for the adapter approach. The new method for CNBr activation of silica gives results comparable to the widely used CNBr activation of Sepharose. The adapter approach circumvents the column destruction, inherent in utilizing substrate hydrolysis for elution, by allowing used columns to be regenerate. In this approach, the substrate DNA is never attached to the stationary phase and becomes instead just another addition to the mobile phase. The single strand $(GT)_5$ sequence used to bind the adapter column $(AC)_5$ sequence should be present on only one strand of the duplex substrate sequence. Substrate binding in solution is somewhat easier than the alternative and trapping the enzyme–substrate complex under flow through the adapter column maintains high yield. Perhaps most remarkable of all is that catalytic elution gives better performance under a variety of conditions when compared to affinity chromatography. The net result of these studies is that using trapping, with a single tailed substrate DNA, on $(AC)_5$-Sepharose with catalytic elution yields the highest performance chromatography of all the options tested. The adapter approach used could be readily extended to other polynucleotide utilizing enzymes, which is a large and diverse group including DNA polymerases, exo- and endonucleases, topoisomerases, kinases and ligases. With the development of further adapter technology, adapter chromatography could be extended to virtually any enzyme and catalytic elution would likely give high purity and yield.

Acknowledgement

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