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Heparin elution of transcription factors from DNA-Sepharose columns

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Abstract

A novel method using heparin for eluting transcription factors from DNA-Sepharose columns was characterized. CAAT enhancer binding protein (C/EBP) or lac repressor fusion proteins were both eluted with heparin from columns containing specific DNA sequences coupled to cyanogen bromide activated Sepharose. The amount of the lac repressor chimera which eluted from the column was shown to increase with increases in the mobile phase heparin concentration. The elution of the protein was also shown to be dependent on the amount of DNA coupled to the column and more protein eluted from columns containing lesser amounts of DNA. These data suggest that heparin and DNA compete for binding to the protein; this competition causes elution. Comparison of heparin- and salt-eluted protein demonstrated the heparin-eluted fraction was significantly purer and comparable to that obtained by elution with isopropyl β-D-thiogalactopyranoside, a lactose analog. Heparin elution represents an important new tool in the purification of transcription factors and other DNA-binding proteins by DNA affinity chromatography.

Keywords: Heparin; Proteins; DNA

1. Introduction

DNA-binding proteins such as transcription factors, DNA and RNA polymerases and exo- and endonucleases play important roles in cellular function, differentiation and regulation. Hence, purification and characterization of these proteins is of great importance. The low amount of these proteins which frequently occur in cells makes their purification challenging. DNA affinity chromatography has been widely used for purification of DNA-binding proteins [1–3]. Specific or nonspecific DNA sequences are coupled to solid supports such as silica, Sepharose or cellulose. The different coupling procedures and supports have been previously reviewed [1,4]. After loading of proteins on these columns, the proteins are usually eluted with a salt gradient [1,4]. While DNA affinity chromatography is the most selective method currently available for the purification of these proteins, only rarely is a homogeneous transcription factor obtained using this method alone.

Heparin-Sepharose chromatography has also been widely used for purification of a large number of different proteins, including DNA-binding proteins [5,6]. For the latter proteins, the polyanionic structure of heparin presumably mimics the highly negatively charged backbone of DNA giving heparin the ability to bind the DNA-binding domains of protein

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[7]. Conversely, some DNA sequences are known to bind the heparin-binding motif in thrombin [8]. Heparin is typically coupled to Sepharose by cyanogen bromide activation [9] and a salt gradient is usually employed to elute proteins from heparin columns. Elution of proteins from heparin-Sepharose with heparin has also been reported [10]. Since heparin-Sepharose is also used in the purification of many other types of proteins, other protein types often co-eluted with DNA-binding proteins affecting purity.

Of all the methods which can be used for purification of DNA-binding proteins, specific sequence DNA affinity chromatography offers the highest selectivity. In spite of this high selectivity, purification to homogeneity is seldom obtained with this technique alone. The specificity of DNA affinity chromatography could be better exploited by using it repetitively [11]. However, repeating chromatography using the same or similar protocols would be of little use. What is needed is to elute the highly selective columns each time using different strategies based upon different principles. In practice, eluting protein from DNA affinity columns is currently limited to using high salt and, for a few proteins, elution by specific ligand [e.g., lac repressor elution with isopropyl β-D-thiogalactopyranoside (IPTG), or other lactose analogs]. This limits the number of times that DNA affinity chromatography can be effectively used in protein purification protocols. Hence new elution methods, based upon different principles, should facilitate their purification.

Lac repressor, which regulates the lac operon in *E. coli* [12] and CAAT enhancer binding protein (C/EBP) [13] which binds the CAAT element in eukaryotic promoters, are both well-characterized transcription factors. The DNA sequences, which are specifically bound by these proteins, have been identified [14,15]. In this paper we have used a lac repressor–β-galactosidase fusion protein (lacIZ) and a Green Fluorescent Protein–CAAT enhancer binding protein (GFP-C/EBP) fusion protein to characterize a new method for eluting DNA-binding proteins. Both LacIZ [16,17] and GFP-C/EBP [18] have DNA-binding properties comparable to lac repressor and C/EBP which justifies their use in our model study. Here, we show that these proteins elute from DNA columns with heparin in a highly purified state.

2. Methods

2.1. DNA Sepharose preparation

For studies with LacIZ, αOp1T6 oligonucleotide ($5'$-NH_2-$(T)_6$ - AATTGTTATCCGCTCACAATTC-CAC), containing the operator 1 (Op1) DNA sequence, was coupled to CNBr activated Sepharose. For studies with GFP-C/EBP, EP18 ($5'$-NH_2-GCAGATTGCGCAATCTGC) was coupled. The "$5'$-NH_2" represents the aminoethyl group added on the last synthesis cycle with the Amino Link reagent (Applied Biosystems). Op1- and EP18-Sepharose was prepared as described previously [19]. Two grams of Sepharose 4B was washed thoroughly with water. While stirring 0.4 g of cyanogen bromide was added and the mixture was maintained at pH 11 by addition of 5 *M* NaOH until the reaction slowed. The activated Sepharose was then washed rapidly under vacuum on a coarse sintered glass funnel with 100 ml ice-cold water and then with 200 ml ice-cold coupling buffer (100 m*M* $NaHCO_3$, pH 8, 500 m*M* NaCl). The Sepharose was then mixed with 100 nmol of $5'$-amino αOP1T6 or $5'$-amino EP18 to a final volume of 5 ml coupling buffer and mixed on a tube rotator overnight. The next day the DNA-Sepharose was washed with 15 ml 100 m*M* $NaHCO_3$ pH 8, 500 m*M* NaCl. The amount of DNA coupled, 22 nmol αOP1T6 and 29 nmol EP18 per gram of Sepharose was determined by UV absorption spectroscopy of the DNA before and recovered after coupling. The DNA-Sepharose was end-capped by adding 4 ml of blocking buffer (100 m*M* Tris, 500 m*M* NaCl, pH 8) and incubating on a tube rotator for 2 h at 4°C. After incubation, the Sepharose was washed two times with 4 ml of TE0.1 buffer (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, 0.1 *M* NaCl). $\alpha Op1T_6$ was made double stranded by adding 50 nmol of complementary strand ($5'$GTGGAATTGTGAG-CGGATAACAATTAAAAAA), the mixture was heated to 95°C, and allowed to cool slowly to room temperature. We refer to this double-stranded product here as Op1-Sepharose. The EP18 oligonucleotide is self-complementary and a double-stranded column results directly from coupling. All columns were stored at 4°C in TE0.1 buffer containing 50 m*M* NaN_3 when not in use. The DNA concentration for different columns of Op1-Sepharose was adjusted by diluting DNA-Sepharose with CNBr activated, and

end-capped Sepharose, which had be treated the same way except no DNA was used.

2.2. Production of proteins

Lac repressor-β-galactosidase fusion protein was produced by growing clone BMH-72-19-1 which was generous gift from Dr. David Levens (Laboratory of Pathology, National Cancer Institute, Bethesda, MD, USA). The clones were grown overnight in 2 l superbroth (1.2% bactotryptone, 2.4% yeast extract, 0.5% glycerol, 0.072 *M* K_2HPO_4 and 0.028 *M* KH_2PO_4) at 37°C and then induced for 4 h with 1 m*M* IPTG. The cells were pelleted by centrifugation at 8000 rpm for 30 min in the Sorvall GS-3 rotor. The pelleted cells were resuspended in 40 ml lysis buffer [4 mg/ml lysozyme (Boehringer Mannheim, Indianapolis, IN, USA), 5 m*M* NaH_2PO_4, 20 m*M*, Na_2HPO_4, 30 m*M* NaCl, 25 m*M* benzamidine, 10 m*M* 2-mercaptoethanol, 10 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride (PMSF) and 0.2% Tween 20]. The cells were then lysed by sonication on ice for 30 s, followed by 30 s incubation on ice without sonication; this was repeated three times at setting 12 using a VirSonic50 sonicator with a microprobe (Gardiner, NY, USA). Cellular debris was removed by centrifugation at 15 000 rpm for 30 min in the Sorvall SS-34 rotor. The protein was dialyzed against TE0.1 buffer and stored at −85°C until needed.

GFP-C/EBP was produced by growing *E. Coli* strain BL21 containing plasmid pJ22-GFPC/EBP as described previously [18].

2.3. Chromatography

All columns were 1 ml bed volume syringe columns initially equilibrated in TE0.1 buffer. Crude preparations of either LacIZ or GFP-C/EBP was loaded onto the column and eluted with a gradient of NaCl, heparin or IPTG. Details of the gradient are given in the figure legends.

2.4. Assay of fusion proteins

GFP-C/EBP was assayed by measuring fluorescence as described earlier [18]. LacIZ was assayed for β-galactosidase activity by mixing 150 μl of Buffer O (3 m*M* *o*-nitrophenyl-β-D-galactopyranoside, 0.1 *M* NaH_2PO_4, 1 m*M* $MgCl_2$ and 45 m*M* mercaptoethanol) with 50 μl of each fraction to be assayed. The reaction was done on microtiter plates and monitored continuously at 25°C for absorption at 405 nm.

2.5. Definition of enzyme units

One unit of LacIZ fusion protein was defined as that which gives a change in 1 absorption unit (at 405 nm) per min per ml of enzyme at 25°C.

2.6. Protein assay

Protein concentrations were determined by bicinchoninic acid method using the protocol provided by Pierce. All samples were precipitated with 10% ice-cold trichloroacetic acid (TCA) and redissolved in 2% Na_2CO_3, 0.1 *M* NaOH before assay.

2.7. Polyacrylamide gel electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis was on 12% gels using the method of Laemmli [20] and stained with silver using the Bio-Rad Labs. (Richmond, CA, USA) kit.

3. Results

Fig. 1 shows that GFP-C/EBP can be eluted from EP18 DNA-column with either a salt or heparin gradient. It was shown earlier that GFP alone does not bind to the DNA Sepharose column [18]. The fusion protein does not bind to Sepharose with no coupled DNA (data not shown). This indicates that the binding of this fusion protein to the column was due to the specific interaction between C/EBP and the specific DNA sequence coupled to the column, an interaction disrupted by high salt or heparin concentrations.

Since this is the first demonstration of heparin elution in DNA affinity chromatography, we next investigated whether other transcription factors can be eluted in the same way.

The elution of LacIZ from the Op1-Sepharose column is shown in Fig. 2. The amount of LacIZ eluted is dependent on the concentration of heparin in the mobile phase; however, concentrations of

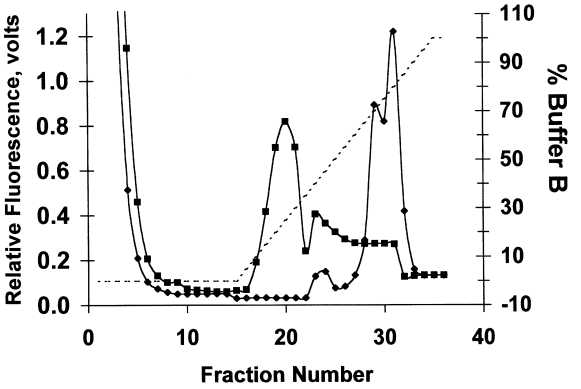

Fig. 1. Elution of GFP-C/EBP with heparin from EP18-Sepharose. A crude bacterial extract (500 μl) containing GFP-C/EBP was loaded on a 1 ml EP18-Sepharose column having 29 nmol DNA/g of resin. The column was washed with 16 ml TE0.1 and was eluted either with a 20 ml heparin gradient from 0 to 40 mg/ml heparin dissolved in TE0.1 or with a 20 ml salt gradient from TE0.1 to TE1.2 (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, 1.2 *M* NaCl). The flow rate was 0.5 ml/min and 1 ml fractions were collected.

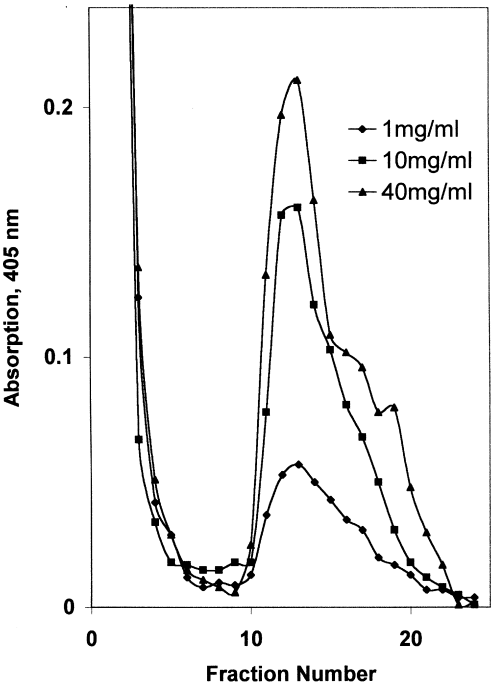

Fig. 2. Effect of heparin concentration on elution of LacIZ from Op1-Sepharose. Bacterial extract (100 μl) containing LacIZ was loaded onto a 1 ml Op1-Sepharose column having 4.4 nmol DNA/g of support. The column was washed with 10 ml of TE0.1 and eluted with either 1 mg/ml, 10 mg/ml or 40 mg/ml heparin dissolved in TE0.1. The flow rate was 0.5 ml/min min and 1 ml fractions were collected and assayed.

more than 40 mg/ml have a high viscosity and were not used routinely. A negative control, β-galactosidase, does not bind to the DNA-Sepharose column. LacIZ also failed to bind to the column containing only Sepharose indicating the specific interaction between LacIZ and lac operator sequence bound to the column provides the basis for this chromatography as well. Thus, elution is dependent upon the heparin concentration of the mobile phase.

Figs. 1 and 2 also show considerable material flowing through the column unretained. The columns in these experiments were intentionally overloaded so that we could gauge the ability of salt or heparin to elute all of the retained protein and to obtain large peaks which clearly show where elution occurred. However, the size of the unretained peak is also unrepresentative of the amount of the transcription factor which did not bind and overestimates it. Crude bacterial extracts contain multiple fluorescent compounds (Fig. 1) and glycosidases (Fig. 2; e.g., β-galactosidase) which are also present in these unretained fractions and increase the signal obtained. When these fractions are analysed by sodium dodecylsulfate–acrylamide gel electrophoresis and other methods (data not shown), we find that most of the DNA-binding protein binds to the column and is then eluted by either salt or heparin. After washing away these contaiminants, the signal (fluorescence or enzymatic activity) of the eluted peak does accurately reflect the amount of the fusion protein eluted.

Fig. 3 shows that more LacIZ elutes with 10 mg/ml heparin from columns having lower amounts of DNA. Columns with lesser amounts of DNA coupled also bound slightly less of the applied protein and yet, even though less bound, more total LacIZ is obtained from the 4.4 nmol column elution. This is because even though more LacIZ binds to high DNA columns, most of it cannot be eluted by the fixed, 10 mg/ml heparin concentration used in the figure. Higher concentrations of heparin or salt would be required for complete elution.

While LacIZ has its highest affinity for its specific operator sequence, the lac repressor (and many other transcription factors) will also bind to non-specific

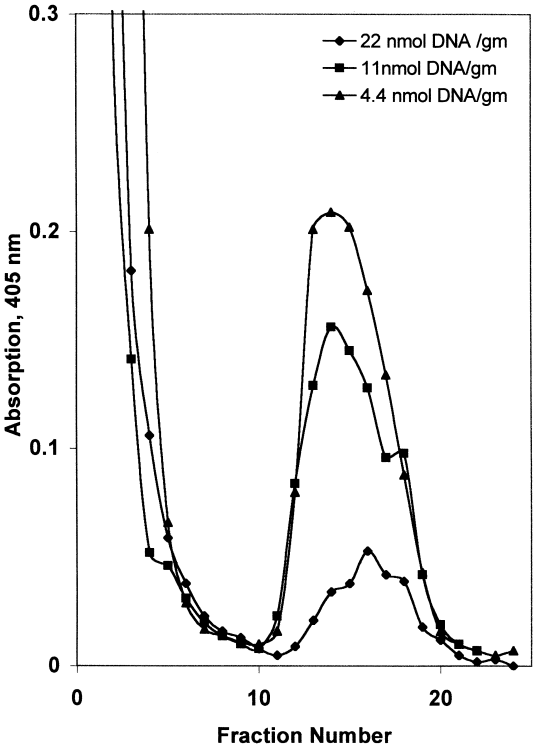

Fig. 3. The effect of DNA concentration on elution of LacIZ from Op1-Sepharose. Crude bacterial extract (100 μl) containing LacIZ was loaded onto 1 ml Op1-Sepharose columns having either 4.4 nmol, 11 nmol or 22 nmol DNA/g of Sepharose prepared by mixing 22 nmol/g DNA-Sepharose with control Sepharose. The columns were washed with 10 ml of TE0.1 and eluted with 10 mg/ml heparin dissolved in TE0.1. The flow rate was 0.5 ml/min min and 1 ml fractions were collected

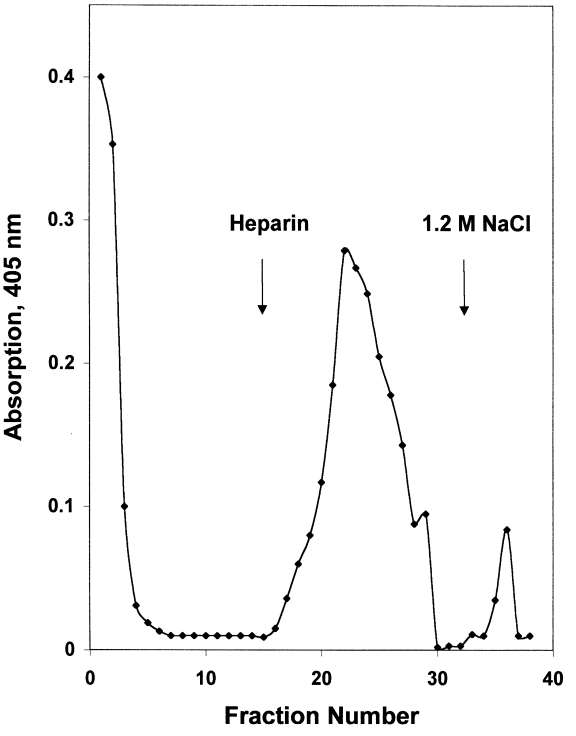

Fig. 4. Elution of LacIZ from the EP18 column. Crude bacterial extract (500 μl) containing LacIZ was loaded onto the EP18-Sepharose column having 29 nmol DNA/g of resin. The column was washed with 15 ml of TE0.1 and was eluted with a 20 ml heparin gradient from 0 to 40 mg/ml heparin in TE0.1. Any uneluted protein was then eluted with 5 ml of TE1.2. The flow rate was 0.5 ml/min min and 1 ml fractions were collected.

DNA sequences, albeit with lower affinity. Fig. 4 shows that LacIZ binds to EP18-Sepharose, a non-specific DNA in this context, and elutes with heparin. The EP18 sequence is not a normal consensus operator sequence for the lac repressor; the two sequences only have the CAAT element in common between EP18 and Op1 DNA. However, large amounts of LacIZ bind to the EP18-Sepharose column (Fig. 4). A negative control, β-galactosidase does not bind to EP18-Sepharose (data not included).

Fig. 4 demonstrates that most of the bound protein is eluted by heparin and very little additional LacIZ can be eluted with high salt. This EP18 column contains a relatively high amount of DNA (29 nmol/g Sepharose) and yet heparin elutes it completely. Even when lesser amount of a specific DNA were used in Fig. 3, complete elution was not obtained at these heparin concentrations. Thus, the specificity of the DNA-sequence used for the stationary phase also influences the heparin concentration required for elution. Thus, with two different transcription factors and with two different DNA-Sepharose columns, heparin is an effective eluent and elutes proteins which are specifically as well as nonspecifically bound. Heparin-elution is probably a general phenomenon which is widely applicable.

The purity and yield of protein obtained from the different elution methods is compared in Table 1. We found that the heparin pool was very pure as compared to the salt pool. The fold purification of the heparin pool was significantly greater than the salt pool with $P=0.014$ (i.e., significantly different at the 1% confidence level). The lower yields obtained

Table 1
Heparin eluted LacIZ is significantly purer than that eluted with salt[a]

Fraction	Total enzyme (U)	Total protein (mg)	Yield (%)	Fold purification
Crude	4.5	10.3	100	1
Heparin	0.56	0.006	10±6	212±84
Salt	0.87	0.040	16±9	48±11

[a] The results of three experiments were averaged ($n=3$) and averages are reported for all columns and with standard deviations for yield and purification.. For each experiment, 2 ml of a bacterial extract containing LacIZ was loaded onto a 1 ml Lac-Sepharose column having 11 nmol DNA/g of Sepharose. The column was washed with 10 ml of TE0.1 and then eluted either with a 20 ml heparin gradient from 0 to 40 mg/ml heparin in TE0.1 or with a 20 ml Salt gradient from TE0.1 toTE1.2 (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, 1.2 *M* NaCl). The flow rate was 0.5 ml/min. Active fractions were pooled together for assay.

for both heparin and salt were because of loading a large excess of protein onto the small columns so that sufficient protein eluted to determine its amount accurately.

We could not directly compare ligand specific elution using IPTG under the same conditions used in Table 1 because very high concentration of IPTG were required for elution in the TE0.1 buffer and these concentrations interfered with the β-galactosidase assay giving erroneous results. Dialysis to remove the IPTG was also unsuccessful since dialysing the LacIZ protein at these high dilutions led to its inactivation. It was found that less IPTG is required for elution when the NaCl concentration of the mobile phase is increased to 0.3 *M* and β-galactosidase activity could be accurately measured in the presence of these lower IPTG concentrations (data not shown). Under these conditions the purity of protein eluted with IPTG was also greater than that of salt eluted protein (Table 2). However, when heparin elution was attempted at 0.3 *M* NaCl so little protein eluted that, despite concentrating the eluate and trying two different sensitive protein assays, it was not possible to get an accurate measure of protein concentration and, hence, the purity of heparin eluted fraction. Thus, while heparin and IPTG could not be compared directly under the same conditions, the indirect comparison shown in Tables 1 and 2, show that IPTG elution yields purity which is similar to that obtained with heparin, and that any difference is not likely to be statistically significant.

Comparison of Tables 1 and 2 also demonstrates another important point: the purity of salt-eluted protein can be greatly improved by washing the column at an intermediate salt concentration. In Table 1, when the column was washed with TE0.1 prior to elution, the protein is only one third as pure as in Table 2 where washing in TE0.3 was performed. Washing with higher salt removes additional contaminants.

Fig. 5 shows the gel electrophoresis of IPTG, salt and heparin eluted fractions of LacIZ. It can be seen that the salt eluted fraction (in this case, from the column washed only with TE0.1) contains some contaminant proteins along with LacIZ fusion protein. However, the heparin fraction is very pure and only a single band corresponding to the LacIZ fusion protein (M_r 155 000) is seen. As expected the IPTG fraction is also very pure.

Table 2
Comparison of LacIZ eluted with salt and IPTG[a]

Fraction	Total Enzyme (U)	Total protein (mg)	Yield (%)	Fold purification
Crude	2	2.7	100	1
IPTG	0.0025	0.0025	24±11	254±38
Salt	0.67	0.007	35±50.007	148±66

[a] The results of the two experiments were averaged ($n=2$) and averages are reported for all columns and with standard deviations for yield and purification. For each experiment, 500 μl bacterial extract of LacIZ was loaded on 1 ml LacT6 having 11 nmol DNA/g of Sepharose. The column was washed with 10 ml of TE0.3 (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, 0.3 *M* NaCl) and eluted with 20 ml gradient of IPTG 0–0.1 *M* in TE0.3 or with a 20 ml salt gradient from TE0.1 to TE1.2. The flow rate was 0.5 ml/min. All the active fractions were pooled together for assay.

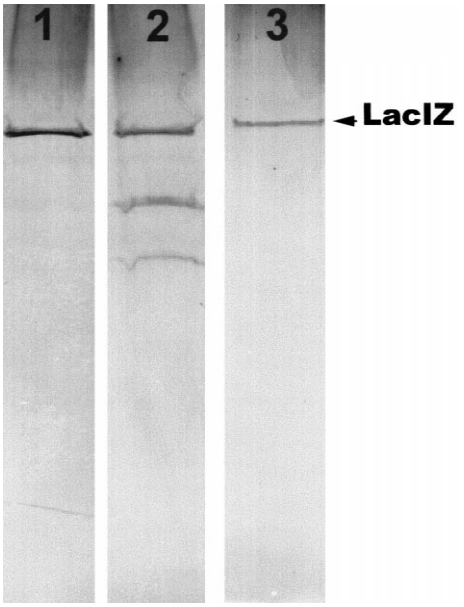

Fig. 5. Polyacrylamide gel of the salt-, IPTG- and heparin-eluted protein. Lanes 1, 2 and 3 represent heparin, salt, and IPTG eluted fractions, respectively. Lanes 1 and 2 are from the same gel; lane 3 was taken from a second gel and is shown for comparison.

4. Discussion

We have described an alternative method of eluting proteins from sequence specific DNA Sepharose columns. We have shown that GFP-C/EBP, a member of basic leucine zipper DNA-binding motif family of proteins [13,15], and lacIZ, having a helix-turn-helix motif [22], can both be eluted with heparin. It is very likely that protein having other DNA-binding motifs will also elute with heparin. We have also shown that protein (LacIZ) which is bound to a non specific sequence (EP18) can also be eluted with heparin (Fig. 4). Our hypothesis is that the elution with heparin results from a direct competition between heparin and DNA for the same site on the protein. During elution a dynamic equilibrium is established in which the protein shuttles between heparin in the mobile phase and DNA which is bound to the column. This equilibrium is affected by the concentration of DNA bound to the column, the amount of heparin in the mobile phase, and the affinity of protein for both DNA and heparin. Elution with salt, on the other hand, presumably results from salt shielding the charge on DNA and protein and hence disrupting ionic interaction between them. Ligand specific elution of lac repressor is caused by the ligand (IPTG) binding to a site other than the DNA-binding site leading to a conformational change and diminished DNA-binding properties. Thus elution with heparin is not just a new but probably a mechanistically different type of elution than either salt or ligand based elution.

Our hypothesis about competitive elution is supported since elution of LacIZ from the column is dependent upon the concentration of both column DNA and mobile phase heparin. This competitive elution probably accounts for the purer protein eluted by heparin relative to that obtained with salt. IPTG elution also yields highly purified protein but very few transcription factors have a specific ligand with which to elute them. In any case, our data suggest that heparin eluted protein is comparable in purity to IPTG eluted protein and purer than obtained with salt under the same conditions, the most commonly used strategy to elute transcription factors from DNA columns. Protein eluted with heparin can be diluted and reapplied to the same DNA column (data not shown) allowing repetitive uses of same DNA column with different elution strategies. Another interesting observation was that when heparin was dissolved in TE containing 0.3 *M* NaCl (TE0.3), the LacIZ activity eluted as a broader peak which contained so little protein it could not be accurately measured even after concentrating the protein and using sensitive protein assay techniques. This low protein concentration is also indicative of high purity but the purity could not be accurately measured. High salt concentrations have been shown to decrease the affinity of lac repressor for DNA [21] and we suspect high salt also decreases the affinity of this transcription factor for heparin. As both affinities are decreased, a broad peak results.

Heparin represents a very flexible and controllable method for eluting transcription factors from DNA columns. Elution is affected by the heparin and salt concentration of the mobile phase (Figs. 1 and 2, Table 1), the amount of DNA on the stationary phase (Fig. 3a and b), and its sequence (Fig. 4). The purification obtainable is several hundred-fold and comparable to that obtainable when ligand-specific

elution is feasible. In contrast, salt dependent elution is a much less flexible, cruder strategy. However, by washing columns at an intermediate salt concentration prior to elution, the method can be improved. By optimizing both salt and heparin elution of a particular protein, high purity of DNA-binding proteins should be more easily achieved.

A possible drawback of using heparin to elute was the lower yields obtained but this can be easily overcome by controlling the amount of DNA bound to the column. Another strategy to increase yield is to decrease the affinity of the column bound DNA sequence for the protein. This can be achieved by altering crucial base pairs of the DNA sequence or by using a nonspecific, or less specific, column. We have shown that LacIZ binds to EP18 column, a rather extreme case of an ''altered DNA'', and can be eluted with heparin. It was also observed that almost 90% of bound protein was eluted with heparin from this column while about 50% of bound protein is eluted from the specific Op1 column under similar conditions and when the columns have comparable amount of DNA (data not included).

In our previous paper we have discussed temperature-dependent elution of transcription factors [18]. In this paper we have demonstrated heparin as specific eluent of two different transcription factors. DNA affinity chromatography is the most selective of any known method for the purification of transcription factors. Alternative methods for eluting proteins from DNA columns such as temperature-dependent elution and heparin elution, allow this high selectivity to be applied repetitively to aid in the purification of DNA-binding proteins.

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