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Immobilisation of a repressor protein for binding of plasmid DNA

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Abstract

The use of plasmid DNA in gene therapy and genetic vaccination has increased the need for scalable and sustainable production processes. One key challenge for bioprocess engineering is the separation of plasmid DNA from structurally related impurities. Affinity purification procedures allow a highly selective capturing of the target molecule. In this paper, we present the isolation of a his-tagged lac repressor, its non-covalent immobilisation to different matrices and binding of DNA, thus enabling us to screen for combinations of ligands and stationary phases by using a building block principle.

Keywords: Plasmid DNA; Gene therapy; Genetic vaccination; Protein–DNA interaction; Building block principle; Plasmid DNA purification

1. Introduction

As the identification of functional genes evolves so do prospective novel medical treatments such as genetic vaccination or gene therapy. Diseases such as cystic fibrosis, gaucher disease, ADA deficiency, etc. might be cured at the genetic level and some gene therapy products using viral and non-viral vector systems for gene delivery are already in clinical trial [1]. Non-viral vector systems like plasmid DNA are considered to be safer in terms of oncogene activation and unintended immunological reactions. Considering the production process plasmid DNA is also superior to viral delivery systems because the manufacture, storage, and application poses fewer process and quality control problems. An even higher potential for plasmid DNA is expected in genetic vaccination. In this case, the expression of a plasmid coded antigen leads to an immune response in the treated organism.

Clinical trials and future pharmaceuticals demand high quality plasmid DNA in multigram quantities. For this purpose, sustainable and scalable production processes have to be designed comprising cultivation of plasmid harboring *Escherichia coli*, cell disruption, removal of RNA, and concentration and purification of the supercoiled plasmid DNA. The production of high quality biomass for plasmid DNA production has thoroughly been researched [2]. However, the development of suitable purification processes poses several challenges in the field of bioprocess engineering.

Cell disruption is usually achieved by alkaline lysis followed by the removal of precipitated cell debris using centrifugation and filtration. To avoid shear forces on plasmid DNA and precipitated chromosomal DNA resulting in contamination of the subsequent product stream we applied a continuous lysis step followed by gentle flotation of cell debris (Voß and Flaschel, unpublished results). The produced lysate could be readily applied to subsequent purification steps without further clarification.

Most conventional anion exchangers used for plasmid purification have a low capacity because plasmid DNA is unable to access the pore volume of these stationary phases [3]. Higher capacities for plasmid DNA could be achieved by applying membrane adsorbers with large pores of 2 μm [4] or monolithic phases [5]. In both cases, transport through the resin is achieved by convective flow rather than slow diffusive transport into the pore volume of conventional media. The good access to functional groups in these stationary phases

Abbreviations: ADA, adenosine deaminase; LacI, lac repressor; LB, Luria Bertani; DTT, dithiothreitol; PMSF, phenylmethylsulfonylfluoride; SDS-PAGE, sodiumdodecylsulfate-polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactopyranoside

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results in an increased capacity for plasmid DNA. However, to avoid competition between plasmid DNA and structurally related molecules like RNA resulting in a significant decrease of capacity these contaminants have to be removed from the cleared lysate at first. Several strategies for RNA removal have been described. These include size-exclusion chromatography [6], precipitation [7–9] and aqueous two-phase extraction [10]. Most of these procedures are either tedious and time consuming [6] or afflicted with high product loss [10]. In the case of precipitation of plasmid DNA, no statement is made regarding the structural integrity of the product under high shear stress during subsequent separation of the precipitate by centrifugation or filtration. A selective capturing method would allow the separation of plasmid DNA from contaminants and concentration of the product. For this purpose, affinity procedures can be applied. The most common method for selective binding of double stranded DNA is the use of triple helix interaction [11]. However, protein–DNA interaction is seldom used for nucleic acid purification. Lundeberg et al. [12] purified an immobilised lac repressor fusion protein and used it for selective purification of short DNA sequences containing the lac operator. Kumar et al. [13] optimised the purification of the lac repressor by using diplacement chromatography and showed its retained biological activity after covalent immobilisation to a Sepharose matrix. The DNA binding activity of the lac repressor was also exploited in a novel biosensor [14] to monitor inducer molecules or DNA. Woodgate et al. [15] recently showed the use of a zinc finger protein for selective purification of plasmid DNA. In this paper, we present the non-covalent immobilisation of a his-tagged lac repressor to different types of stationary phases and subsequent examination of the DNA binding capabilities of these modified matrices. The application of this building block principle allows screening of different combinations of affinity ligands and supports for affinity purification. Furthermore, the use of a hexahistidine residue as an affinity linker allows us to control the orientation of the affinity ligand.

2. Experimental

2.1. Chemicals

Vegetable peptone was obtained from UD Chemie (Wörrstadt, Germany) and yeast extract from Oxoid (Wesel, Germany). RNA was obtained from Fluka (Buchs, Switzerland). All other chemicals were obtained from Sigma (Deisenhofen, Germany) or VWR (Darmstadt, Germany) and of analytical grade if not stated otherwise.

2.2. Stationary phases

For purification of proteins the following matrices were used.

Unosphere-S (Bio-Rad, Munich, Germany), Ni-Sepharose HP (Amersham Biosciences, Freiburg, Germany), VIVASPIN 20 concentrators (Vivascience, Hannover, Germany).

For non-covalent immobilisation of the his-tagged lac repressor we used Ni-Sepharose HP (Amersham Bioscience, Freiburg, Germany), paramagnetic Ni-beads (Promega, Mannheim, Germany), and metal chelate membrane adsorbers (Vivascience, Hannover, Germany).

2.3. Bacterial strains and plasmids

E. coli BL21(DE3) (Novagen, Madison, USA) harboring plasmid pJF118TacFus [16] was used for lac repressor production. Construction of a fusion protein comprising LacI and a C-terminal his-tag was achieved by cloning a PCR amplified repressor fragment from pJF118TacFus into pET20b(+) (Novagen, Madison, USA) using standard techniques [17] to obtain plasmid pLacIHis$_6$. Expression of the fusion protein was done in *E. coli* K12JM109(DE3) (Promega, Mannheim, Germany). The plasmids pQE30 (Qiagen, Hilden, Germany) and pET20b(+) (Novagen, Bad Soden, Germany) were applied to study the interaction of the repressor proteins with DNA.

2.4. Cultivation of bacteria

For production of lac repressor proteins batch cultivations were performed in a 30 L stirred tank reactor (MBR, Wetzikon, Switzerland) on semi defined glycerol medium comprising glycerol (15 g L^{-1}), yeast extract (7 g L^{-1}), vegetable peptone (13.5 g L^{-1}), KH_2PO_4 (1.5 g L^{-1}), K_2HPO_4 (2.3 g L^{-1}), NaCl (1.5 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.25 g L^{-1}), and ampicillin (100 mg L^{-1}). A volume of 20 L was inoculated with 200 mL of an LB culture. Cultivation was performed for 18 h at 37 °C and pH 7 until the culture reached a final OD_{600} of 23. Cell harvest was done by centrifugation in a Sigma 6K10 at 11800 × *g* and 4 °C. The cell pellet (632 g wet cell weight) was stored at −20 °C until further use.

2.5. Isolation of plasmid DNA

Essentially, pure plasmid DNA was used to examine the interaction of the repressor proteins with DNA. It was isolated with QIAGEN Maxi Kits (Qiagen, Hilden, Germany) according to the instructions of the manufacturer.

2.6. Isolation of lac repressor proteins

Cell pellets (60 g) were resuspended in 140 mL resuspension buffer (0.2 M Tris–HCl, pH 7.2, 0.2 M KCl, 10 mM $MgCl_2$, 5% (v/v) glycerol, 1 mM NaN_3, 0.3 mM DTT, 1 mM PMSF) and disrupted at 800 bar by using a Mini-Lab 8.30H high-pressure homogeniser (APV-Rannie, Copenhagen, Denmark). Cell debris was removed by sub-

sequent centrifugation at 27,000 × *g* at 4 °C in a Sigma 6K10 (Sigma, Deisenhofen, Germany). In case of the native lac repressor impurities were precipitated with 25% ammonium sulfate and LacI was concentrated by precipitation with 40% ammonium sulfate. Desalting of the resuspended protein pellet in 0.075 M potassium phosphate buffer (0.075 M potassium phosphate, pH 7.2, 5% (w/v) glucose, 0.3 mM DTT, 1 mM NaN_3) was achieved by dialysis over night against 0.045 M potassium phosphate buffer (0.045 M potassium phosphate buffer, pH 7.2, 5% (w/v) glucose, 0.3 mM DTT, 1 mM NaN_3). Further purification was achieved by cation exchange chromatography using a XK16-20 column (Amersham Biosciences, Freiburg, Germany) packed with 25 mL UnoSphere-S on a GradiFrac FPLC System (Amersham Biosciences, Freiburg, Germany). LacI was eluted from the column with a linear gradient of 0.075–0.4 M potassium phosphate buffer (0.4 M potassium phosphate buffer, pH 7.2, 5% (w/v) glucose, 0.3 mM DTT, 1 mM NaN_3) over 8 column volumes with a linear flow rate of 150 cm h^{-1}. Protein fractions were concentrated by precipitation with 40% ammonium sulfate, resuspended in resuspension buffer and desalted by diafiltration using a VIVASPIN 20 concentrator according to the manufacturer's instruction.

For purification of the his-tagged lac repressor 60 g of wet cell paste was resuspended in 140 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and disrupted by high-pressure homogenisation as described above. The recombinant repressor was isolated from the clarified lysate by applying it to a XK16-20 column (Amersham Biosciences, Freiburg, Germany) packed with 20 mL Ni-Sepharose HP. Washing was done with lysis buffer and elution achieved with a gradient from 10 to 250 mM imidazole (elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) over 9 column volumes with a linear flow rate of 90 cm h^{-1}. For non-covalent immobilisation imidazole was removed using VIVASPIN 20 concentrators as already described above.

2.7. Analytical methods

Protein fractions were analysed on precast 20% SDS-PAGE gels (Amersham Biosciences, Freiburg, Germany) using a PhastSystem (Amersham Biosciences, Freiburg, Germany). Protein concentration was determined by using a Bradford assay using bovine serum albumine as a standard.

Gel shift assays were performed to analyse protein–DNA interaction. For this purpose, 0.8 % agarose gels were cast. PeqGOLD 1 kb DNA ladder (Peqlab, Erlangen, Germany) was used as a marker with fragments of 10,000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, and 250 bp. Samples were applied as indicated in the results section and separated with 3.5 V cm^{-1}. Gels were stained with ethidium bromide and analysed using a Herolab E.A.S.Y. gel documentation system (Herolab, Wiesloch, Germany).

3. Results

3.1. Purification of lac repressor

In order to compare the DNA binding activity of the his-tagged lac repressor with the native repressor protein LacI was isolated from a cultivation of *E. coli* BL21DE3 harboring plasmid pJF118TacFus [16]. After cell disruption purification was achieved by ammonium sulfate precipitation and cation exchange chromatography. Protein fractions were analysed by SDS-PAGE. Fractions shown in lanes 6 and 7 contained the lac repressor in acceptable purity. Comparison with the marker showed a size of 38 kDa which is consistent with its theoretical size. These fractions were pooled, concentrated by a second ammonium sulfate precipitation, resuspended, and desalted by diafiltration. The concentrated protein is shown in lane 8 of Fig. 1. Part of the protein was sacrificed for purity reasons. Since native lac repressor was only needed for comparison with the his-tagged protein no attempt was made to optimise this purification.

The recombinant his-tagged lac repressor was isolated from *E. coli* K12JM109(DE3) harboring plasmid pLacIHis$_6$. After cell disruption the clarified lysate was applied to a XK16-20 column packed with Ni-Sepharose HP. Elution was performed by a linear imidazole gradient over nine-column volumes. The fractions were analysed on SDS-PAGE as shown in Fig. 2. The size of the isolated protein was 40 kDa which is consistent with its theoretical size. While the fraction eluting at imidazole concentrations between 114 and 160 mM imidazole contained most of the impurities the fractions eluting between 183 and 229 mM imidazole shown in lanes 5–7 contained LacI-His$_6$ in sufficient purity. They were pooled and desalted by diafiltration as described above.

3.2. Gel-shift assays

Before immobilisation on different stationary phases the produced lac repressor fractions had to be tested on their binding activity for nucleic acids. For this purpose, a simple and convenient method was necessary. Therefore, we examined the shift in electrophoretic mobility of nucleic acids interacting with the repressor molecule. Repressor proteins binding to nucleic acids decrease their specific charge and this results in a lower electrophoretic mobility of the complex [18]. Samples of the repressors were mixed with RNA, a lac operator fragment from of pUC18 cut out by suitable restriction enzymes, a plasmid containing the lac operon (pQE30) and one lacking it (pET20b(+)). As shown in Fig. 3, LacI and LacI-His$_6$ showed no interaction with RNA, but both molecules were clearly able to recognise the lac operator sequence. The results also indicated that the interaction of the DNA with LacI was stronger than the interaction with the recombinant LacI-His$_6$. However, interaction of the repressor is not limited to the operator sequence alone, but occurs also with plasmid DNA lacking this sequence as shown in Fig. 4. Good interaction could be achieved with LacI while LacI-His$_6$ showed a

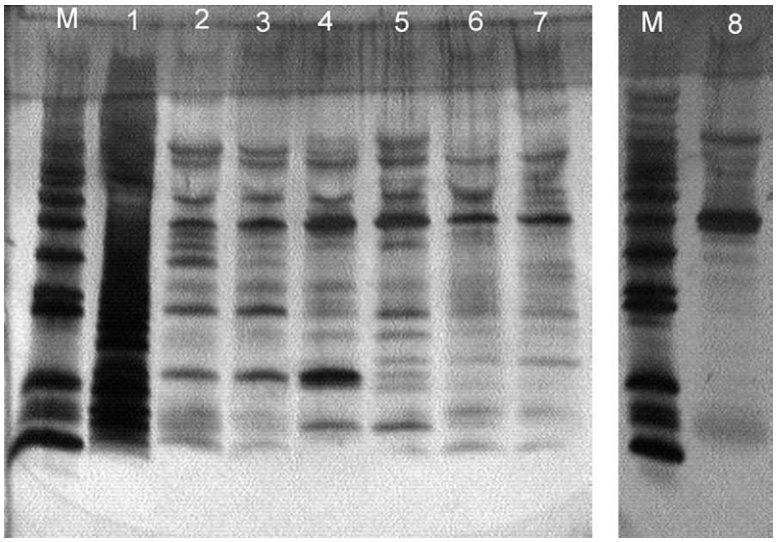

Fig. 1. SDS-PAGE analysis of lac repressor purification. Gradient was applied using 0.045 M potassium phosphate buffer (buffer A) and 0.4 M potassium phosphate buffer (buffer B). Lane M: PageRuler protein ladder (10–200 kDa) (Fermentas, St. Leon, Germany); 1: flow through; 2: 22% buffer B; 3: 24% buffer B; 4: 35% buffer B; 5: 41% buffer B; 6: 49% buffer B; 7: 55% buffer B; 8: resuspended protein fractions (lanes 6 and 7) after final ammonium sulfate precipitation.

weaker interaction in all cases. While a strong interaction was seen with plasmid pQE30 containing the operator sequence an interaction of comparable strength was also observed with pET20b(+) lacking the operator sequence. This indicates that unspecific DNA binding is observed in this gel-shift assay. Unspecific interaction is considered to be the first step and recognition of the operator sequence is achieved by one dimensional diffusion along the DNA double helix presumably being faster than a three dimensional diffusion to the operator sequence [19,20]. The results also show that DNA binding occurs with different plasmid isoforms like supercoiled and open circular plasmid DNA in the undigested sample as well as with linearised plasmid DNA. Interaction of the plasmid DNA with other DNA binding proteins is very unlikely

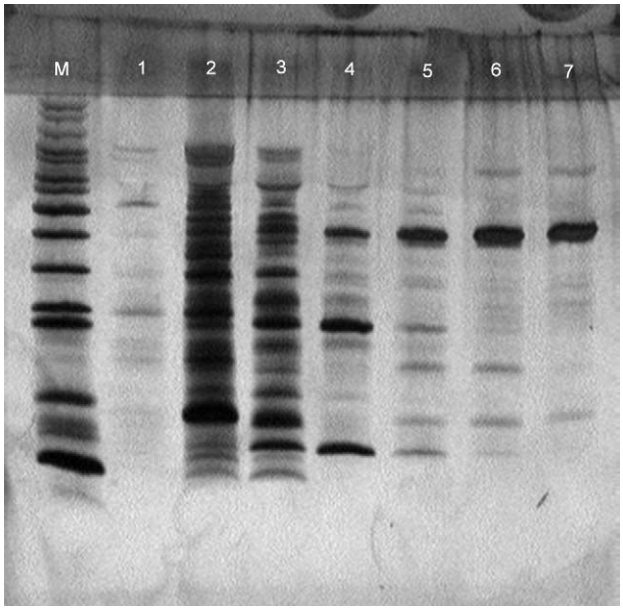

Fig. 2. Purification of LacI-His_6 on Ni-Sepharose HP. M: PageRuler protein ladder, 10–200 kDa (Fermentas, St. Leon, Germany). A linear gradient between 10 and 250 mM imidazole was applied over nine-column volumes. Eluted fractions are shown in lanes 1–7; 1: 90 mM imidazole; 2: 114 mM imidazole; 3: 137 mM imidazole; 4: 160 mM imidazole; 5: 183 mM imidazole; 6: 206 mM imidazole; 7: 229 mM imidazole.

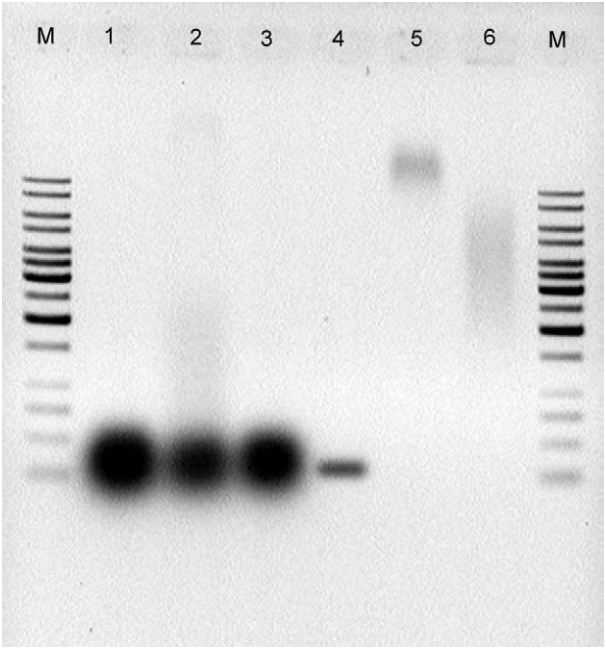

Fig. 3. Binding between lac repressor proteins and nucleic acids (negative agarose gel). M: 1 kb DNA marker; 1: RNA; 2: RNA and LacI; 3: RNA and LacI-His_6; 4: lac operator fragment; 5: lac operator fragment and LacI; 6: lac operator fragment and LacI-His_6.

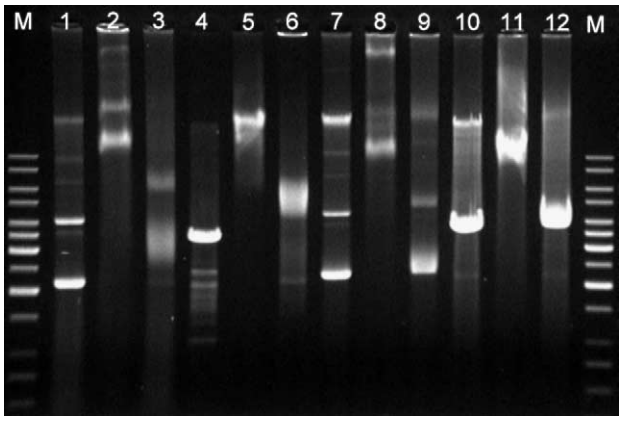

Fig. 4. Binding between lac repressor proteins and plasmid DNA. M: 1 kb DNA marker; 1: pQE30; 2: pQE30 with LacI; 3: pQE30 with LacI-His_6; 4: pQE30 after *Eco*RI digestion; 5: pQE30 after *Eco*RI digestion with LacI; 6: pQE30 after *Eco*RI digestion with LacI-His_6; 7: pET20b(+); 8: pET20b(+) with LacI; 9: pET20b(+) with LacI-His_6; 10: pET20b(+) after *Eco*RI digestion; 11: pET20b(+) after *Eco*RI digestion with LacI; 12: pET20b(+) after *Eco*RI digestion with LacI-His_6. Concentration of LacI: 14.2 mg mL^{-1}. Concentration of LacI-His_6: 1.35 mg mL^{-1}. Plasmid amount per lane: 300 ng. Protein concentrations were determined by using the Bradford assay.

because the isolated lac repressors were very homogenous as shown by SDS-PAGE analysis and therefore essentially pure.

3.3. Comparison of stationary phases

In order to find suitable combinations of ligands and stationary phases, a building block principle should be applied to analyse DNA binding capabilities. For this purpose, the his-tagged lac repressor was non-covalently immobilised on Ni-Sepharose as a porous material, paramagnetic Ni beads, and metal chelate membrane adsorber loaded with Ni. By using a C-terminally tagged lac repressor we were not only able to apply a building block principle but we could also control the orientation of the ligand between stationary phase and target molecules. This is of particular importance because the DNA binding domain of LacI is located at the N-terminal residue of the protein [21].

Stationary phases were loaded with excess LacI-His_6 in 0.01 M Tris–HCl, pH 8.0. After washing plasmid DNA was applied to the column followed by a washing step with 0.01 M Tris–HCl, pH 8.0 and subsequent elution of bound plasmid DNA with 10 mM IPTG in water. In case of Ni-Sepharose a linear flow rate of 30 cm h^{-1} was applied for all steps. An amount of 132 mg LacI-His_6 in 0.01 M Tris–HCl, pH 8.0 was immobilised on 9 mL gel. A volume of 12 mL plasmid DNA in water with a concentration of 129 μg mL^{-1} was applied to the column equillibrated with 0.01 M Tris–HCl, pH 8.0. Most of the plasmid DNA (1171 μg) was found in the flow through and the following washing step as presented in Fig. 5. Subsequent elution with IPTG resulted only in a small signal containing 380 μg plasmid DNA resulting in a dynamic capacity of 42 μg mL^{-1}. The results indicated that most of

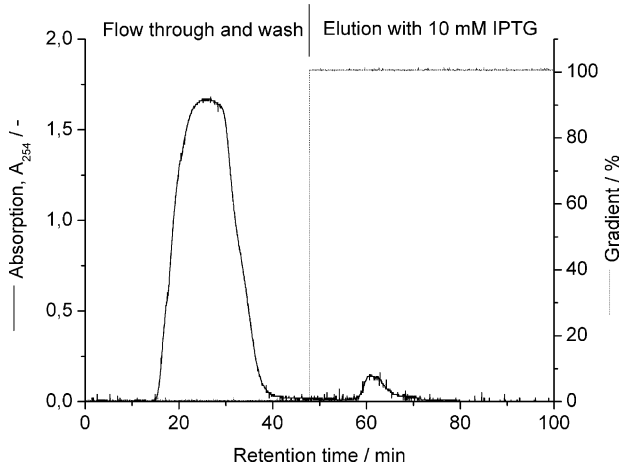

Fig. 5. Chromatogram of plasmid pQE30 loaded on LacI-His_6 after non-covalent immobilisation on Ni-Sepharose HP. Bound plasmid DNA was eluted using a 10 mM IPTG solution.

the repressor was located in the pore volume of the matrix thus being not accessible to the large plasmid DNA. Better accessibility of the immobilised ligand was expected to be achieved with paramagnetic Ni beads or membrane adsorbers. The results with these stationary phases are shown in Figs. 6 and 7.

In case of the paramagnetic Ni beads 819 μg lac repressor could be immobilised to a suspension of 30 μL of Ni beads. A volume of 500 μL plasmid DNA in water (con-

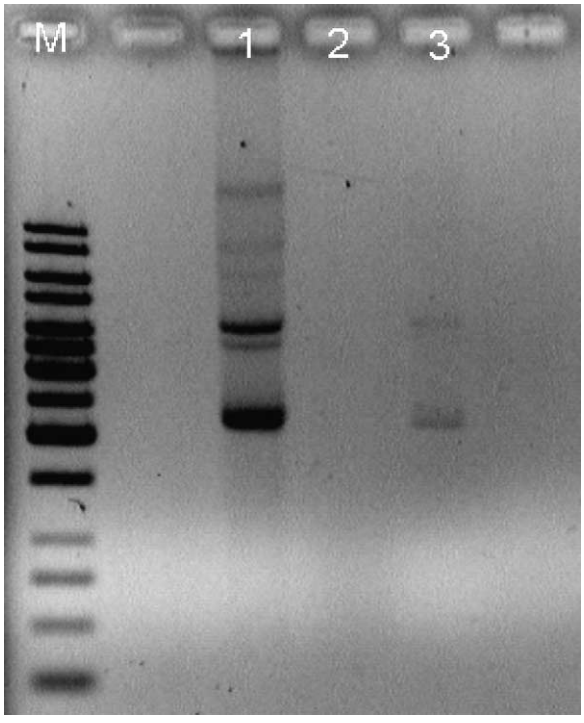

Fig. 6. Binding of plasmid DNA with LacI-His_6 after non-covalent immobilisation of the protein on paramagnetic Ni-beads (negative agarose gel). M: 1 kb DNA marker; 1: flow through; 2: washing with 100 mM Tris buffer, pH 7.2; 3: elution with 10 mM IPTG.

centration 17.4 μg mL^{-1}) was applied to the modified Ni beads. In this case, also most of the plasmid DNA was found in the flow through shown in lane 1 of Fig. 6. After washing (lane 2) a small amount of plasmid DNA could be eluted with IPTG. Comparable results were obtained in the case of metal chelate membrane adsorbers. An amount of 372 μg lac repressor was immobilised on the membrane and 200 μL plasmid DNA solution (44 μg mL^{-1}) were applied to the adsorber. Flow through was achieved by centrifugation according to the instructions of the manufacturer. In this case, the loaded plasmid sample is shown in lane 1 of Fig. 7. The flow through in lane 2 shows only smaller amounts of plasmid DNA. About the same amount of DNA could be eluted from the membrane with IPTG solution as shown in lane 3. However, in both cases the amount of bound DNA was still low when compared to other separation methods.

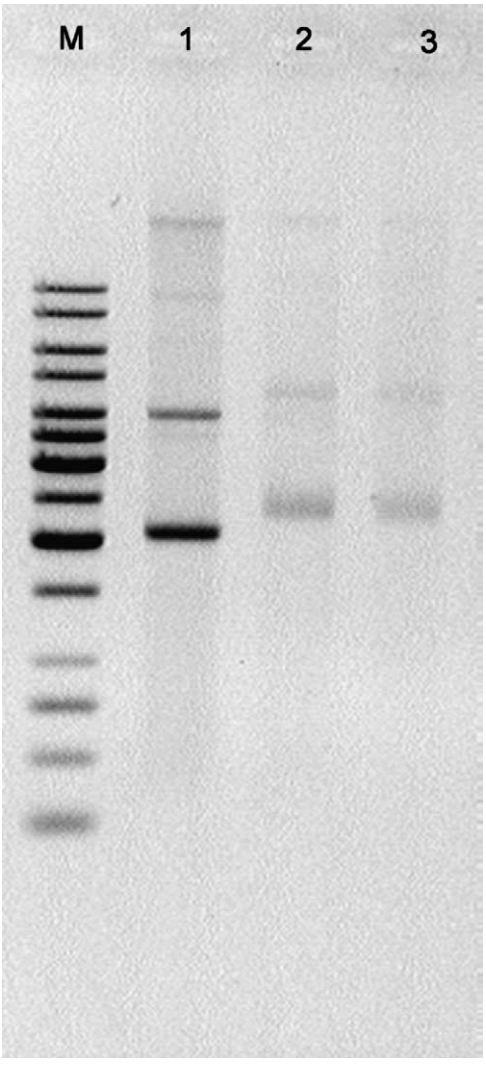

Fig. 7. Binding of plasmid DNA with LacI-His$_6$ after non-covalent immobilisation on a metal chelate membrane adsorber (negative agarose gel). M: 1 kb DNA marker; 1: loaded plasmid DNA sample; 2: flow through; 3: elution with 10 mM IPTG.

4. Discussion

Our studies enabled us to analyse the DNA binding capability of proteins by using a simple gel shift assay before immobilisation on stationary phases. By using this method we were able to show that interaction between repressor molecules and RNA was not detectable while interaction with double stranded DNA in form of a short operator sequence as well as plasmid DNA occurred. The use of a hexahistidine residue as an affinity linker between different matrices and ligands allows us to test different combinations of ligands and matrices during future research. In the case of LacI we were able to control the orientation of the ligand to its target molecule. Proof of principle was achieved by combining the his-tagged repressor with several stationary phases.

In the future, DNA binding capability of immobilised proteins shall be increased by reducing the size of the ligands to proteins only containing the DNA binding domain. Further research should also be directed on the influence of the affinity linker on protein–DNA interaction thus enabling us to establish reliable systems for analysing different matrix–ligand combinations.

Since DNA binding is also restricted by the accessibility of the ligands on the matrix further attention should be paid to non-porous materials, i.e. monolithic phases for this purpose.

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