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Aptamer affinity chromatography: combinatorial chemistry applied to protein purification

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Abstract

The systematic evolution of ligands by exponential enrichment process is a combinatorial chemistry method that allows the identification of specific oligonucleotide sequences, known as aptamers, that bind to a desired target molecule with high affinity and specificity. Here, a DNA-aptamer specific for human L-selectin was immobilized to a chromatography support to create an affinity column. This column was effectively applied as either the first or second step in the purification of a recombinant human L-selectin–Ig fusion protein from Chinese hamster ovary cell-conditioned medium. The fusion protein was efficiently bound to the column and efficiently eluted by gentle elution schemes. Application of the aptamer column as the initial purification step resulted in a 1500-fold purification with an 83% single step recovery. These results demonstrate that oligonucleotide aptamers can be effective affinity purification reagents.

Keywords: Aptamer affinity chromatography; Directed ligand evolution; Affinity chromatography; Proteins; L-Selectin

1. Introduction

The ability to generate antibodies to a large array of chemically distinct target molecules, coupled with the technology of monoclonal antibody production, has made immunoaffinity purification a powerful and routine laboratory technique [1,2]. With proper experimentation, 1000- to 10 000-fold purification can routinely be achieved from a complex mixture [3]. However, there are constraints that reduce the effectiveness of antibody based purification especially for large scale or industrial applications [3–7]. These constraints include: (1) antibody cross reactivity, especially with closely related molecules; (2) linkage of antibodies to columns that often result in couplings that are not uniform, leading to reduced capacity and/or affinity and that can allow leaching of the antibody from the column; (3) inability of antibodies to survive sanitation procedures common to manufacturing scale separations due to denaturation of the antibody; (4) elution conditions that can be harsh, requiring extremes of pH, detergents, organic solvents or chaotropic salts leading to denaturation of the target as well as the antibody. In fact some elution conditions, such as reducing agents, are not feasible because they cleave the disulfide bonds between the immunoglobulin heavy and light chains. Finally, some targets are toxic or are poorly immunogenic making the availability of the antibody problematic.

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The development of combinatorial chemistry methodologies that produce novel ligands to target molecules allows the creation of alternative affinity purification reagents [5–10]. In comparison to antibodies, these novel ligands could have superior performance characteristics. These new reagents bind to their targets with relatively high affinity and specificity, are generally smaller in size and complexity than antibodies, easy to manufacture, modify and uniformly link to matrices such as those used for column chromatography. By preselecting the desired buffer conditions for binding and eluting the target, as well as for column sanitation, these ligands can be specifically designed to conveniently fit into an optimum purification scheme. Also, ligands can be selected for their ability to discriminate between the target and closely related molecules.

One such combinatorial chemistry technique is the systematic evolution of ligands by exponential enrichment (SELEX) process. The SELEX process allows the rapid isolation of rare oligonucleotide sequences, from large random single-stranded sequence libraries, that recognize a target molecule with high affinity and specificity [11–34]. SELEX methodology has been used to identify oligonucleotide ligands, known as aptamers, to a wide array of chemically distinct targets (for reviews see Refs. [14–16]). Targets have included nucleic acid binding proteins [12,18] and nonnucleic acid binding proteins such as growth factors [19,20], antibodies [21], enzymes [22–24] and peptides [25]. Aptamers have also been identified that recognize small molecules such as dyes [17], amino acids [26–28], nucleotides [29,30], and drugs [31]. With the development of this target/aptamer repertoire, in vivo and in vitro applications are emerging [32–34]. However, no information regarding the practical performance characteristics for such ligands as affinity reagents has been reported.

In this study, we demonstrate the effectiveness of a human L-selectin specific, DNA-aptamer as an affinity reagent for the purification of L-selectin receptor globulin (LS-Rg). LS-Rg is a recombinant fusion protein consisting of the extracellular domain of human L-selectin fused to the amino-terminus of the hinge, CH2, and CH3 domains of human IgG1 heavy chain. L-selectin is a C-type (calcium dependent) lectin domain-containing protein expressed on the cell surface of lymphocytes, monocytes and granulocytes that is believed to mediate the binding of lymphocytes to high endothelial venules of peripheral lymph nodes (for reviews, see Refs. [35–37]).

The identification of aptamer antagonists specific for human L-selectin has been previously described [34,38]. The DNA-aptamer used in this study recognizes the lectin domain of L-selectin in a divalent cation-dependent manner and has been shown to inhibit L-selectin interaction with sialyl Lewis X in vitro and to inhibit L-selectin on human lymphocytes in vivo [34].

2. Experimental

2.1. Reagents

A cDNA clone for human L-selectin and the mammalian expression plasmid, pIG (pigtail expression system), were obtained from Research and Diagnostic Systems (R&D Systems, Minneapolis, MN, USA). L-Selectin specific DNA-aptamer, 5′-BGCGGTAACCAGTACAAGGTGCTAAACGTA-ATGGCGC-3′; scrambled control DNA, 5′-BAAGCGTTCACGGGACCTTCGAAGGTAT-GAAGAACGC, and polymerase chain reaction (PCR) primers were obtained from Operon (Alameda, CA, USA). B=biotin linked to the 5′-terminus. The aptamer was a modification of the previously described L-selectin aptamer, LD201 [34]. The human L-selectin specific monoclonal antibody, DREG56 (LECAM-1), was obtained from Endogen (Cambridge, MA, USA).

2.2. Plasmid construction

A vector for the mammalian cell expression of LS-Rg was constructed as follows: The Fc coding portion within the pIG vector was excised by digestion with BamHI and XbaI. This fragment was inserted into the corresponding BamHI, XbaI sites within the expression plasmid pCDNA3.1 (Invitrogen, San Diego, CA, USA) which contains the neomycin resistance gene. A DNA fragment coding for the leader sequence and extracellular domain of L-selectin was obtained by a PCR strategy with the

L-selectin cDNA clone as template. The 5′-primer, 5′ - GA CCAAGCTTAG CCA TGA TAT TTC CAT-GGAAA-3′; contained a HindIII site (underlined) while the 3′-primer; 5′-GCCAGGATCC**ACTT-ACCTGT**GTT ATA ATC ACCC TCC TTAATC - 3′, contained a BamHI site (underlined) followed by a splice donor site (bold text). After PCR, the 1 kilobase fragment was digested with BamHI and HindIII and inserted into the corresponding sites within the modified pCDNA.3.1 vector, downstream of the cytomegalovirus promoter, to create pCDNA–LS-Rg. The LS-Rg coding nucleotide sequence was confirmed by standard DNA-sequencing methods.

2.3. Cell culture

Stable LS-Rg-expressing Chinese hamster ovary (CHO) cell lines were produced by transfection of pCDNA–LS-Rg by standard methods [39]. The day after transfection, the cells were fed with Ham's F12 medium containing 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA). Thirty-six hours after transfection the cells were fed as before except the medium contained 300 μg/ml geneticin (Gibco-BRL, Gaithersberg, MD, USA). Selection proceeded until individual colonies could be isolated. Isolated colonies were expanded under selection conditions and analyzed for LS-Rg production.

For LS-Rg production, a CHO-cell clone was expanded until approximately 80% confluence. Cells were then fed with Ham's F12 medium containing 10% FBS. Four days later the medium was harvested and clarified by centrifugation at 2830 *g* for 20 min. Clarified supernatant was used for column chromatography.

2.4. Chromatography

Protein-A chromatography was performed at 4°C with 1-ml columns (Pharmacia Biotech, Piscataway, NJ, USA). Conditioned medium was loaded at 2 ml/min. After loading was completed, the column was washed with 20 column volumes of Dulbecco's phosphate buffered saline (PBS). Elution was performed with 0.1 *M* sodium citrate (pH 3.0). Eluted fractions were immediately neutralized by addition of 1 *M* Tris–HCL (pH 8.0). Neutralized fractions could be loaded onto the aptamer affinity chromatography (AAC) column directly or after dialysis against Dulbecco's PBS.

AAC columns were prepared by incubation in 1 ml of a buffer containing 25 m*M* sodium phosphate (pH 7.5), 500 m*M* NaCl and 1.6 mg of the biotinylated DNA-aptamer or scrambled control DNA with 1 ml of Ultralink immobilized streptavidin plus (Pierce, Rockford, IL, USA). Incubations were performed for 2 h at 4°C with continuous mixing. Unbound DNA was removed by washing with the same buffer. The amount of oligonucleotide coupled to the resin (1.1 mg) was determined by comparing the UV absorbance, at 260 nm, of the initial and unbound DNA fractions.

Chromatography was performed at 21°C with an HPLC system consisting of a Waters model 625 gradient pump, model 600E controller, and model 490 detector. Fractions were collected manually. Columns (0.5 cm I.D.×5 cm) were equilibrated and washed (7.5 column volumes) with Dulbecco's PBS. All steps were performed with the same flow-rate (0.75 ml/min) and the elution methods are described in Results and discussion.

2.5. Protein determinations and LS-Rg enzyme-linked immunosorbent assay (ELISA)

Protein concentrations were determined by a modification of the method of Bradford [40]. Soluble L-selectin ELISA was obtained from R&D Systems and performed according to the manufacturer's directions except that standard curves were obtained with highly purified LS-Rg fusion protein. Data were fitted to a sigmoidal dose–response curve, $Y = \text{Bottom} + [(\text{Top} - \text{Bottom})/1 + 10^{(\text{Log EC50}-X)(\text{Hillslope})}]$ by GraphPad Prism version 2.0 (GraphPad Software, San Diego, CA, USA). Standards were assayed in duplicate and each point was considered individually for the purpose of curve fitting. Convergence was obtained when two consecutive iterations varied the sum of squares by less than 0.01%.

2.6. Electrophoresis and protein blots

Standards (Novex, San Diego, CA, USA) and test samples were resolved on 1-mm thick Tris–glycine

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (4–12% acrylamide gradient gels, Novex). Electrophoresis was performed for 1.5 h at 150 V. For blots, resolved proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Transfer was for 1.5 h at 25 V on Novex western transfer apparatus according to the manufacturer's directions. Transfer buffer consisted of 25 m*M* Tris–HCl, 192 m*M* glycine, 20% methanol (pH 8.3). Membranes were blocked overnight at 21°C with Superblock Blocking buffer in Tris-buffered saline (Pierce). The next day the membrane was incubated for 2 h in SHMCK+ buffer [20 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (pH 7.35), 140 m*M* NaCl, 5 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$ 0.5g/l casein (I-block; Tropix) and 0.05% Tween-20] containing 1 μg/ml of the L-selectin specific antibody (DREG56) or 1 μg/ml of the biotinylated L-selectin specific aptamer or scrambled sequence control. For aptamer blots no special steps were taken other than to add 100 μg/ml of yeast tRNA to the SHMCK+ buffer. After incubating the detect reagent, membranes were washed three times (5 min each wash) with 10 ml of SHMCK+ buffer. The appropriate detect reagent, either a 1:1000 dilution in SHMCK+ buffer of alkaline phosphatase (AP)-conjugated rabbit antimouse antibody (Pierce) or a 1:1000 dilution in SHMCK+ buffer of AP-conjugated streptavidin (Tropix), was then added. After a 1-h incubation at 21°C, membranes were washed as before followed by two additional rapid water washes. Finally, 10 ml of Western Blue substrate was added (Promega, Madison, WI, USA). At the desired signal intensity, color development was stopped by washing the membrane with water.

3. Results

The L-selectin specific aptamer utilized for designing an AAC column was a DNA-oligonucleotide, 36 nucleotides in length that has a K_D for L-selectin of $2 \cdot 10^{-9}$ *M* [34]. To create an affinity column, this aptamer was biotinylated at the 5′-terminus and attached to a streptavidin-linked resin. The resulting 1 ml column contained 1.1 mg of aptamer.

To establish loading, washing and elution conditions for AAC, LS-Rg enriched fractions were utilized. Enriched fractions were obtained from CHO-cell conditioned medium by protein-A chromatography, a method that relies on the specific recognition of the immunoglobulin portion of LS-Rg. Following binding, the column was extensively washed and subsequently eluted with 0.1 *M* sodium citrate (pH 3.0). Eluted fractions were immediately neutralized and dialyzed against Dulbecco's PBS. At this stage the LS-Rg appeared to be approximately 25% pure as judged by protein SDS-PAGE analyses (see below). The major contaminant was bovine γ-globulin.

Optimal AAC methodology must include procedures for elution by methods that do not denature proteins. Because of the divalent cation dependence of aptamer binding, it was anticipated that addition of EDTA would be an effective and mild elution method. In addition, two other gentle elution methods, based on increasing ionic strength, were tested. For each of these three experiments, protein-A enriched fractions (220 μg of total protein) were applied to the AAC column under conditions for equilibration, loading and washing (see Experimental) previously determined to be effective. The three elution methods utilized were: (1) elution with a linear EDTA gradient (0 to 50 m*M* EDTA in 10 min); (2) elution with a linear NaCl gradient without the presence of divalent cations (150 m*M* to 1.15 *M* in 10 min); (3) elution with a linear NaCl gradient in the presence of 1 m*M* $MgCl_2$ and 1 m*M* $CaCl_2$.

The three elution methods produced very different peak shapes for the LS-Rg elution fraction (Fig. 1). The EDTA elution method gave the sharpest peak while the increasing NaCl gradient, in which divalent cations were present, gave the broadest peak. The area under the curves for the flow through peak [range; $18.8 \cdot 10^6$ to $19.2 \cdot 10^6$ microvolt seconds (μV·s)] and the elution peak (range; $5.3 \cdot 10^6$ to $6.1 \cdot 10^6$ μV·s) for each method were essentially the same. Such differences in peak shape are usually attributed to differences in off-rate created by the various elution methods.

The major contaminant present in the protein-A enriched fraction, bovine γ-globulin, had a molecular weight close to the approximately 160 kiloDaltons (kD) LS-Rg. However, by a combination of 4% to 12% SDS-PAGE run under nonreducing conditions

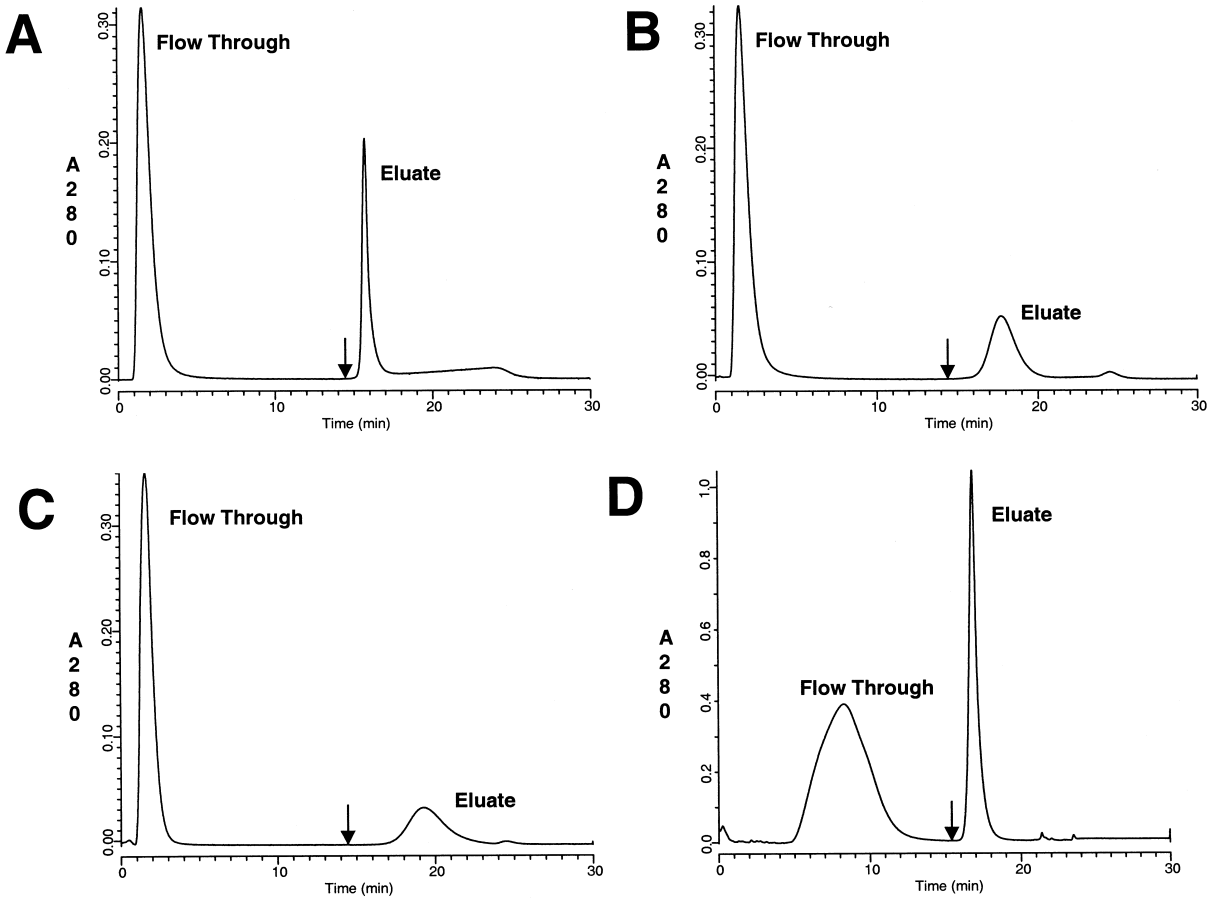

Fig. 1. AAC of partially purified LS-Rg fractions by multiple gentle elution methods. For panels A, B and C, approximately 220 μg of total protein was loaded on a 1 ml AAC column at a volumetric flow-rate of 0.75 ml/min. After washing the column with PBS until baseline was achieved, LS-Rg was eluted from the column by a linear EDTA gradient (A), a linear NaCl gradient containing no divalent cations (B) or containing 1 m*M* calcium and magnesium (C). Panel (D) is the same as (A) except that approximately 1.2 mg of total protein was loaded on the column. Because EDTA absorbs weakly at 280 nm, a slight rise in the baseline was observed for these elution profiles. Arrows indicate the initiation of the gradients.

and protein blot experiments, it was possible to analyze the fractions for each of the three purification trials. By protein blot analysis, no LS-Rg was detected in the column flow through for any of the three purification trials (Fig. 2C) while the γ-globulin fraction was separated (Fig. 2A). As determined by silver stain analyses of SDS-PAGE experiments, the elution fractions for all three conditions were identical and consisted of three distinct bands (Fig. 2B). All three protein bands for each elution condition were recognized by an L-selectin specific antibody (DREG56) in protein blot experiments indicating that all three bands share a common epitope and that the LS-Rg was highly purified (Fig. 2C). The band with the slowest mobility was the size expected for the glycosylated LS-Rg dimer. The identity of the other two bands is uncertain, although the one with the greater mobility may be the monomer.

Similar results were obtained by protein blot analysis using a novel detection system for nonnucleic acid binding proteins [32]. In this case a standard protein blot procedure was utilized (see Experimental) except that the detection probe was the same biotinylated aptamer as used for AAC. A streptavidin-linked AP detection system was used to

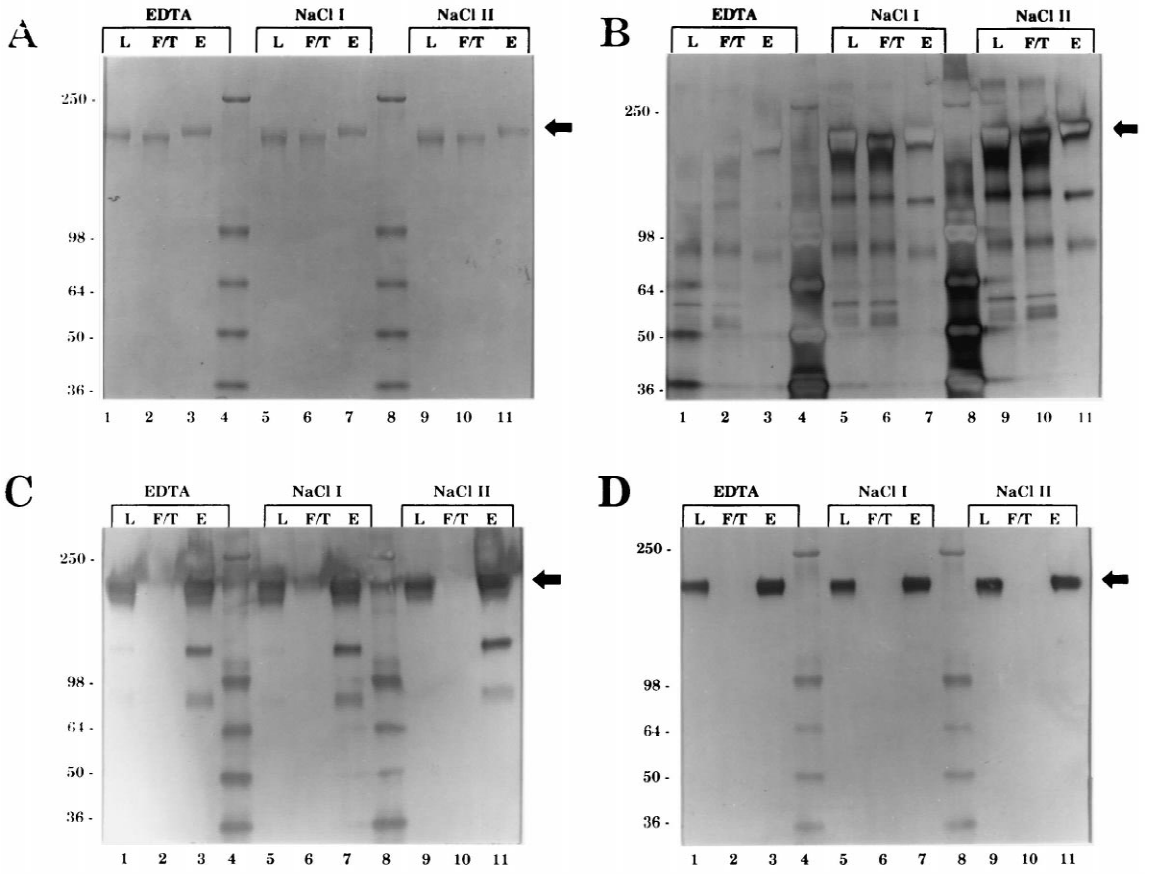

Fig. 2. SDS-PAGE and protein blot analyses of LS-Rg enriched fractions purified by AAC. (A) Coomassie stained SDS-PAGE analysis (4% to 12% polyacrylamide) of three AAC trials that differed by the method used to elute the LS-Rg fusion protein. Each lane contains 1 μg of total protein and the elution conditions [EDTA; NaCl without calcium and magnesium (NaCl I); or NaCl with calcium and magnesium present (NaCl II)] are shown at the top of the figure. The sizes of the molecular weight markers, lanes 4 and 8, in kD are shown at the left and the arrow indicates the position of the approximately 160 kD LS-Rg. Lanes 1–3 contain the column load (L), flow through (F/T) and EDTA elution (E) fractions respectively. Lanes 5–7 contain the column load, flow through and NaCl elution method I fractions respectively. Lanes 9–11 contain the column load, flow through and NaCl elution method II fractions respectively. (B) Identical to (A) except a silver stain analysis was performed. (C) Identical to (A) except that after transfer to Immobilon-P membrane, L-selectin was detected by immunoblot analysis with the antiL-selectin antibody, DREG56. (D) Identical to (A) except that after transfer to Immobilon-P membrane, LS-Rg was detected by aptamer blot analysis with the biotinylated L-selectin aptamer.

develop the blot. The results of this analysis are shown in Fig. 2D and again show no detectable LS-Rg in the flow through fractions for any of the trials. The aptamer blot did not detect the two faster mobility forms of LS-Rg in the elution fraction; possibly the result of reduced sensitivity compared to the antibody method or altered specificity of the aptamer for the immobilized low molecular weight forms of LS-Rg. In a similar protein blot experiment, no signal could be detected when using a sequence scrambled version of the DNA-aptamer (data not shown).

Because the EDTA elution scheme produced the sharpest peak, this method was repeated with a larger protein load (1.2 mg). The HPLC chromatogram (Fig. 1D) as well as the SDS-PAGE and protein blot analyses (data not shown) gave results very similar to the initial trial, demonstrating the reproducibility of the technique.

As a control, a similar DNA column was prepared

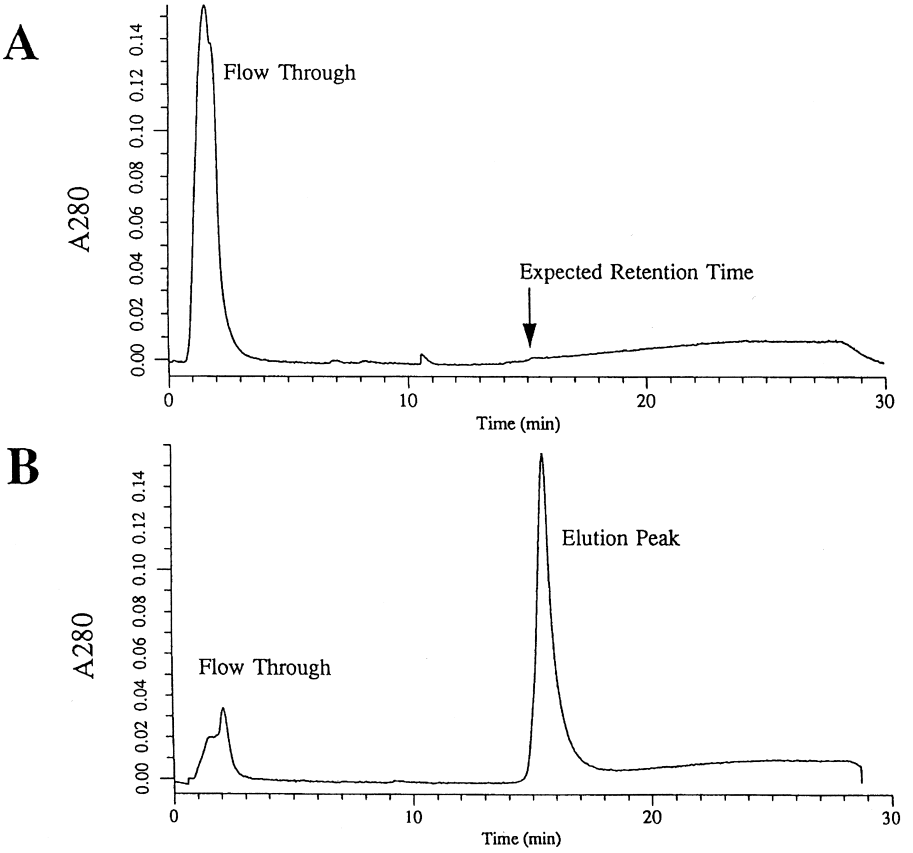

Fig. 3. LS-Rg did not bind to a scrambled sequence aptamer-DNA column. Purified LS-Rg (80 μg) was applied to a scrambled sequence version of the AAC column (A) or to the AAC column (B). The columns had identical dimensions (0.5 cm I.D.×5 cm) and similar DNA densities (1.1 mg/ml) and were run under identical conditions (see Experimental).

with a sequence scrambled version of the DNA-aptamer. Purified LS-Rg (80 μg in 200 μl) was applied to this column or to the AAC column under identical conditions. The scrambled sequence column failed to bind detectable levels of LS-Rg while the AAC column captured 80% of the total protein load (Fig. 3). The total integrated area for both chromatograms was nearly identical [$9.2 \cdot 10^6$ μV·s for (A) and $9.3 \cdot 10^6$ μV·s for (B)] indicating that the scrambled sequence column was incapable of binding LS-Rg. The inability of the AAC column to capture the entire applied protein under these conditions may reflect the presence of denatured protein in the sample or a kinetic limit for maximum binding.

To explore the full capabilities of AAC, the effectiveness of the AAC column when utilized as the initial purification step was examined. In this

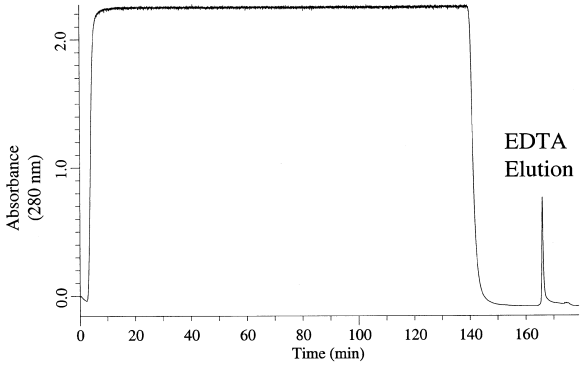

Fig. 4. HPLC chromatogram of LS-Rg purified directly from crude CHO-cell conditioned medium by AAC. CHO-cell conditioned medium was loaded on a 1-ml AAC column at a flow-rate of 0.75 ml/min. The column was washed with Dulbecco's PBS and eluted with a linear EDTA gradient (0–50 m*M* EDTA in 10 min). The EDTA elution peak is marked.

experiment the AAC column was loaded directly with 100 ml (694 mg total protein) of unfractionated CHO-cell conditioned medium. Equilibration, wash, load and elute steps were performed at room temperature and at a flow-rate of 0.75 ml/min. When the loading was completed, the column was washed with Dulbecco's PBS until the absorbance returned to baseline. The column was subsequently eluted with a linear EDTA gradient as before (Fig. 4).

Column fractions were analyzed by SDS-PAGE and protein blotting (Fig. 5). By Coomassie stained PAGE, only a single band of the expected molecular weight for LS-Rg could be detected in the elution fraction (Fig. 5A) while silver stain analysis, at high

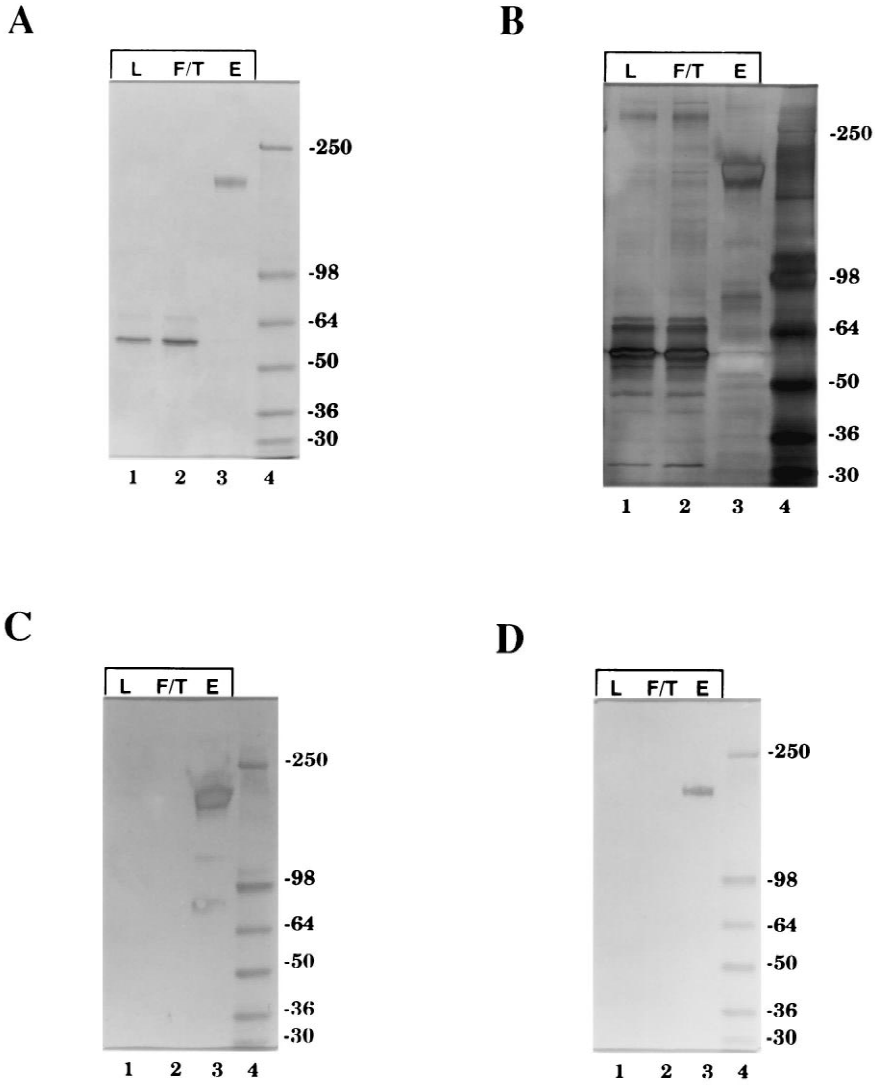

Fig. 5. SDS-PAGE and protein blot analyses of AAC fractions. Protein (1 μg) from the column load (L) (unfractionated CHO-cell conditioned media), flow through (F/T) and elution (E) fractions were resolved on 4–12% PAGE and total protein detected by Coomassie stain (A) or by silver stain (B). Alternatively, the resolved proteins were subjected to protein blot analyses with the L-selectin specific antibody, DREG56, as the detection probe (C) or with the L-selectin specific DNA-aptamer as the detection reagent (D). Lane 4 in all panels contains molecular weight markers with the size for each marker (in kD) indicated on the right. The major species in the elution fraction (Lane 3 in all panels) was the ~160 kD LS-Rg.

Table 1
Aptamer affinity chromatography of LS-Rg from CHO-cell conditioned medium

Fraction	LS-Rg[a] (mg)	Total protein[b] (mg)	Percent recovery	Percent purity	Fold-purification
Load	0.29	694	100	0.042	–
Flow through	0.003	674	1.1	–	–
Elute	0.24	0.38	83	63	1500

[a] Determined by ELISA with LS-Rg as standard.
[b] Determined by the method of Bradford [40] with bovine immunoglobulin as standard.

total protein load (1 μg), revealed numerous low level contaminants (Fig. 5B). Once again, protein blot analysis with DREG56 antibody demonstrated the presence of three specific bands in the elution fraction while no LS-Rg could be detected in the load and column flow through fractions (Fig. 5C). Protein blots with the biotinylated DNA-aptamer showed a similar result (Fig. 5D). The major band detected by both protein blot analyses corresponded to the major band identified by Coomassie stained PAGE.

To determine the purity, recovery and fold-purification of LS-Rg obtained by AAC, an ELISA was utilized. This ELISA was a modification of a commercially available soluble human L-selectin assay (R&D Systems) in which a highly purified and quantified LS-Rg fraction was used as a standard. ELISA analysis, in combination with assays for total protein, revealed a purity of 63% with a recovery of 83% (Table 1). The overall purification factor achieved by this single step was 1500-fold. The failure to account for 100% of the activity loaded onto the column may reflect losses during column washing or during sample handling.

4. Conclusions

DNA and RNA affinity chromatography for the purification of DNA or RNA binding proteins has been used successfully for a number of years. With the advent of SELEX technology, this technique can now be extended to targets that normally do not bind oligonucleotides. Because the interaction between target and aptamer is not dictated by biology, aptamers can be selected for optimal performance in the planned purification scheme. However, because a large portion of the interactions between target molecules and oligonucleotide-aptamers are often ionic, gentle elution by modest increases in ionic strength will likely result even without specific selection methods. The fast off-rates achieved with NaCl elution schemes presented here support this idea.

Although the complexity of the contaminating proteins in the experiments presented here was not high, the purification from cell culture supernatants demonstrates a practical example of the use of an AAC column. The same column also has been applied to the more complex purification of spiked L-selectin from unfractionated human serum. Here, a 4500-fold purification was achieved although with only 25% recovery (data not shown).

The DNA-aptamer used in these experiments was degraded by cell culture media and avoidance of nuclease contamination in buffers was important for all experiments. Because of this, applications to whole cell lysates have not been attempted with this aptamer although such experiments should be possible using nuclease resistant aptamers [20,41–43].

Finally, given that aptamers on a matrix can capture a specific protein target as shown here, it seems clear that high-density arrays of aptamers can be used to attack the ‘‘proteomics’’ question [44].

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