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Capillary affinity chromatography and affinity capillary electrophoresis of heparin binding proteins

A new approach for separation, capillary affinity chromatography, is introduced for studying the interaction of heparin with antithrombin III and secretory leukocyte proteinase inhibitor. Heparin is covalently immobilized on the surface of an etched capillary through a silane spacer. The proteins are injected into the heparinized capillary, bound to the heparin, washed with buffer, eluted with sodium chloride in the same buffer using a pressure injection mode and eluting protein detected by absorbance. The resulting affinity separation is similar to that obtained from traditional affinity chromatography. The quantity of loaded protein in capillary affinity chromatography is at the nanogram level, offering an improvement over the milligram levels required for standard affinity chromatographic methods.

1 Introduction

Affinity interaction of protein with various ligands creates a basis of communication and regulation within and between different structures in living organisms. Many papers describe methodologies useful in affinity-based separations, including affinity chromatography [1–3], affinity electrophoresis [4–6], and affinity capillary electrophoresis (ACE) [7–15]. Among these methods, ACE has been the subject of much attention recently for the evaluation of affinity interactions because of both its high resolution and the extremely small amounts of sample required. This second advantage is particularly important because of the high cost of pure proteins and the difficulties to obtain sizable quantities of these molecules [12]. While ACE is certainly a valuable method, problems arise from the nonspecific adsorption of protein on the silica surface of the capillary, seriously limiting the number of proteins giving good results. Furthermore, strategies that reduce protein adsorption in CE, such as additives and extremely high or low pH buffers, often interfere with the affinity interaction of protein with ligand or represent a harmful environment destabilizing the native protein.

Well-known heparin-binding proteins, which show specific interaction with heparin, include the serine protease inhibitor antithrombin III (ATIII) [16]. The interaction of other protease inhibitors such as secretory leukocyte protease inhibitor (SLPI) with heparin is less understood and is currently the subject of study in our laboratory [17]. Heparin affinity chromatography has been extensively used to purify such heparin-binding proteins [18, 19]. While ATIII is an anionic protein, SLPI is a basic, cationic protein and, like most of the heparin-binding proteins, is susceptible to serious adsorption on a negatively charged silica surface. Matrices that are neutral and hydrophilic, such as polysaccharide (agarose and Sepharose) and polyacrylamide beads, are usually chosen for heparin affinity chromatography to reduce nonspecific adsorption of heparin-binding proteins.

Recently, our laboratory introduced the use of a capillary containing tightly, but not covalently, bound heparin for use in the affinity separation of heparin-binding peptides by capillary electrophoresis [20]. The limited surface area of the capillary used in CE provided a relatively low level of ligand coverage, only slightly retarding the movement of heparin-binding peptides through the capillary. In capillary electrochromatography [21], a capillary is etched with a fluoride compound at high temperature to increase its inner surface by 1000-fold [22, 23]. This results in almost the same specific surface area as found in a capillary packed with macroporous silica beads.

This manuscript introduces a new approach to affinity electrophoresis, capillary affinity chromatography (CAC), using an etched capillary to which heparin has been covalently immobilized. The use of CAC to study the affinity interaction of extremely small amounts of protein with heparin ligand is described.

2 Materials and methods

2.1 Materials

The SLPI, molecular weight 11 700 (10 mg/mL), >95% purity, was kindly provided by Amgen (Boulder, CO). The ATIII, molecular weight 58 000, 4 IU (4.5 mg/mL), purity >95%; low molecular weight (LMW) heparin, average molecular weight 4800; and semi-purified "raw" heparin (containing 18.8 μmol/g of unsubstituted amino groups, average molecular weight 12 000) were supplied by Celsus Laboratories (Cincinnati, OH). Bovine serum albumin was from Boehringer Mannheim (Indianapolis, IN). Ammonium hydrogen fluoride, 3-glycidoxypropyltrimethoxysilane, boron trifluoride diethyl etherate, hydroxypropylcellulose (molecular weight 80 000) and hydroxypropylmethylcellulose (molecular weight 10 000) were from Aldrich Chemical Company (Milwaukee, WI). All other chemicals were of analytical grade. Triply distilled water was used for CE and CAC buffers. Membrane filters (0.45 μm) were from Millipore (Bedford, MA). Capillaries (375 μm OD × 75 μm ID) were purchased from Polymicro Technologies (Phoenix, AZ) or Yongnian Capillary Factory (Yongnian, Hebei, China).

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Abbreviations: **ACE**, affinity capillary electrophoresis; **ATIII**, antithrombin III; **CAC**, capillary affinity chromatography; **LMW**, low molecular weight; **SLPI**, secretory leukocyte protease inhibitor

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2.2 Equipment

The capillary electrophoresis system (Dionex, Sunnyvale, CA) was controlled by an IBM PC interface. A Shimadzu (Tokyo, Japan) GC-14A gas chromatography oven was used for etching of capillaries.

2.3 Etching the capillary

The capillary etching procedure followed literature methods [22, 23]. Briefly, the bare capillary (length 55 cm) was filled with concentrated HCl solution using compressed N_2, sealed by bringing both ends of the capillary together with a 6 cm polyethylene tube and then heated overnight at 80°C. The capillary was next washed with 300 µL each of water and acetone, dried with N_2 for 0.5 h, and a 5% w/v methanol solution of ammonium hydrogen difluoride was forced through the dried capillary using compressed N_2 and allowed to react for 1 h at room temperature. The methanol solvent was removed by flowing N_2 through the capillary for 3 min, the capillary was sealed and heated for 5 h at 250°C in a gas chromatography oven. Finally, the capillary was washed with methanol and water, respectively, for 1 h and dried for 20 min with N_2 prior to use.

2.4 Immobilization of heparin

The etched capillary was washed for 0.5 h with 0.1 M HCl, water and acetone, dried with N_2, and a 300 µL 50% v/v toluene solution of 3-glycidoxypropyltrimethoxysilane containing 1 µL pyridine was forced into the capillary under N_2 pressure, and the capillary was sealed at both ends and heated at 80°C for 24 h. After cooling, the capillary was washed for 0.5 h with toluene, ethyl acetate, acetone, and water. Propionic acid solution (pH 2.6) was forced through the capillary under N_2 pressure, allowed to remain in the capillary for 2 h at room temperature before washing the capillary with water and acetone, and then drying it with N_2. Tresyl chloride was forced through the capillary under N_2 pressure for 5 min, after which pyridine was forced through the capillary. This process was repeated a second time. The capillary was filled with semipurified (raw) heparin (50 mg/mL) in 25 mM sodium phosphate buffer containing 0.1 M NaCl, pH 7.4, and allowed to react for 24 h at 4°C. Unreacted tresyl groups in the capillary were blocked by filling the capillary with 1 M of ethanolamine aqueous solution for 2 h at 4°C. The capillary was washed with water, sealed, and stored briefly at 4°C.

2.5 Capillary affinity chromatography

A detection window was prepared by burning the outside polymer layer of the capillary in the proper position for the Dionex capillary electrophoresis system. The capillary was rinsed for 4 min each with 25 mM sodium phosphate buffer containing 1 M sodium chloride (pH 7.4) and then 25 mM sodium phosphate buffer (pH 7.4). Gravity injection of the protein sample (1–10 mg/mL) was used with a 55 cm height for 15 s. After approximately 2 min, to allow the affinity interaction between heparin and protein to reach completion, 25 mM sodium phosphate buffer (pH 7.4) was pumped into the capillary using 2 psi pressure injection to wash away nonspecific binding protein. Sodium phosphate buffer (pH 7.4) containing NaCl (up to 1 M) was forced through the capillary using 2 psi pressure injection to elute the retained protein. The protein eluting in the wash was detected at UV 220 nm.

3 Results and discussion

3.1 ACE

ACE was first examined in coated capillaries to study the interaction of heparin with the serine protease inhibitors ATIII and SLPI. In preliminary experiments, the capillary inner surface was coated to prevent protein absorption using standard methods. For example, a capillary was treated with 3-glycidoxypropyltrimethoxysilane toluene solution and a diol layer was introduced by immobilizing hydroxypropylcellulose or hydroxypropylmethylcellulose [24] in dry dimethylformamide in presence of BF_3.etherate according to published methods [25]. Alternatively, the capillary was coated with linear polyacrylamide as described in the literature [26, 27] (polyacrylamide coating). Using such modified capillaries, ACE was performed on ATIII and SLPI at various concentrations (0.4–5 mM) of LMW heparin using benzyl alcohol or mesityl oxide as neutral markers. No changes in the retention of ATIII or SLPI was observed when the heparin concentration in the CE buffer was increased. One reason for the failure of ACE to resolve ATIII and SLPI may have been due to the adsorption of these heparin-binding proteins on the wall of the coated capillary. A second possibility is that the coated capillary resulted in low electroosmosis and, thus, the migration time for the protein was prolonged, resulting in a very broad peak (the peak baseline width was about 5 min on a 40–50 min separation). A flat protein peak, offering no improvement in the separation, was observed on ACE under the same conditions using reverse polarity.

ACE was next performed on ATIII (an anionic protein) in a bare capillary. When continuous injections were done in

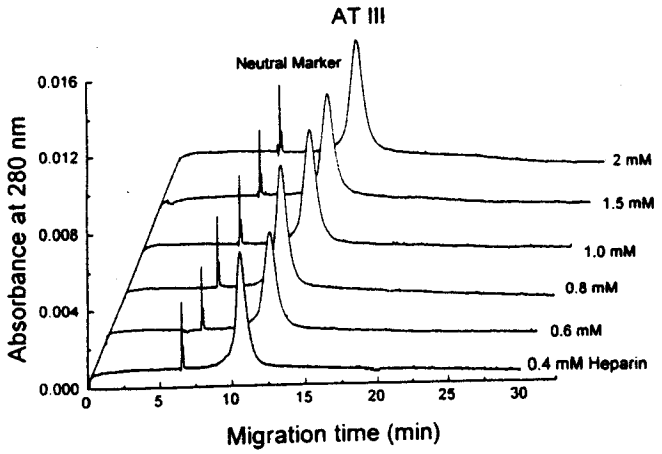

Figure 1. ACE of ATIII in presence of various concentrations of LMW heparin. ATIII, 4.5 mg/mL CE conditions, uncoated capillary 50 µm ID × 60 cm (52 cm from injection to detection window). Injection mode: gravity, height 55 cm, time 30 s. Running buffer, 50 mM sodium phosphate, pH 7.4. Neutral marker, benzyl alcohol. Voltage, 13 kV. Detection wavelength, 280 nm. Heparin added to buffer ranged from 0.4 mM to 2 mM. In the absence of added heparin an identical electropherogram was obtained.

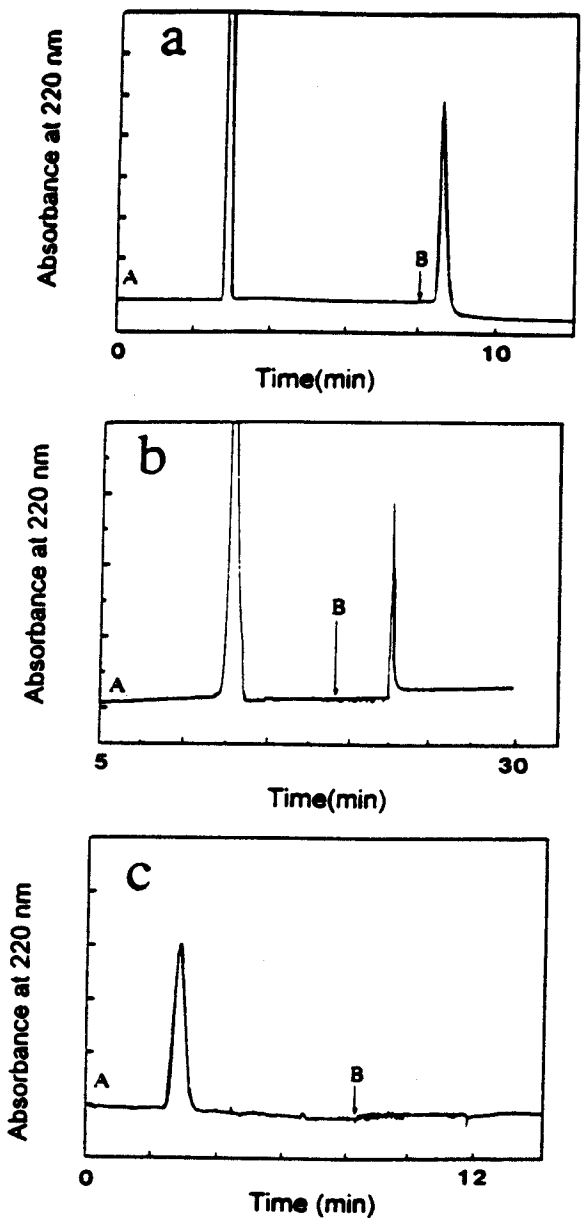

Figure 2. CAC using an etched capillary with peptidoglycan heparin-bound. (a) SLPI concentration, 10 mg/mL. CAC conditions: etched capillary, 75 μm ID × 55 cm (47 cm from injection to detection window), heparin bound *via* silane spacer. Injection mode: gravity, height 55 cm, time 15 s. Washing and elution mode: pressure injection, 2 psi, 300 s. Buffer A, 25 mM sodium phosphate, pH 7.4; buffer B, buffer A + 1.0 M NaCl. Detection wavelength, 220 nm. (b) ATIII concentration, 4.5 mg/mL; other conditions as in (a). (c) Bovine serum albumin, 0.3 mg/mL; other conditions as in (a). The flow rate is controlled by applying N_2 at 2 psi. Such a low pressure is difficult to control, resulting in variable retention times for interacting protein (10–22 min).

presence of LMW heparin, a slight change in migration time was observed (Fig. 1). This increase in protein migration time was not observed if the capillary was washed extensively with 0.1 N NaOH after every run, suggesting it was an artifact of the separation method. The use of additives such as ethylene glycol ($OHCH_2CH_2OH$) was also examined to reduce the protein absorption on the capillary wall (not shown). However, these failed to afford an improvement in the ACE separation of ATIII or SLPI with the LMW heparin.

3.2 Capillary affinity chromatography

A new approach, CAC, was undertaken in which an affinit ligand was bound on the inner wall of an etched capillar Using standard capillary etching methods, the inner surfac area can be enhanced 1000-fold, compared to a ba capillary [22, 23]. A bare unetched capillary, measurin 40 cm × 50 μm ID, has an inner surface area of approx mately 6.3×10^{-5} m^2. This surface area is apparentl insufficient to immobilize the ligand for affinity-base fractionation. By increasing the rough inner surface of a etched capillary to 0.06 m^2, sufficient bound ligand migh be immobilized to affinity-fractionate detectable levels o proteins. If a bare unetched capillary of the same size wa filled with 5 μm silica spheres (pore diameter 30 nm specific area 50 m^2/g, density 1 g/mL), the total specifi area would be approximately the same, 0.05 m^2. Thus, i became clear that either etched or filled capillaries, wit similar specific internal surface areas, might be useful fo CAC.

A silica bead-filled capillary was first prepared for testing A 4 cm capillary closed with a silica frit at one end wa slurry-packed with 5 μm silica spheres [29–32]. The pressure of this packed capillary using a flow rate o 0.1 mL/min reached 200 kg/cm^2. This pressure was clearly too high to be compatible with commercially available CE equipment with low pressure injection and rinse systems. Next, an etched capillary was prepared using ammonium hydrogen fluoride etching at 250°C for 5 h as previously described [23]. The hydroxide groups on the inner surface of the etched capillary were highly exposed, permitting their efficient reaction with silane reagent. The epoxide is opened under acid catalysis and activated using tresyl-chloride/pyridine. Semipurified (raw) heparin contains approximately 10% peptidoglycan heparin consisting of a reactive peptide with a terminal amino group. This peptidoglycan heparin is easily reacted with the tresyl activated diol on the inner surface of the capillary. Peptidoglycan heparin has a molecular weight of 12 000 and is highly negatively charged (~ −100). Once bound through its short peptide to a surface, it adopts an extended

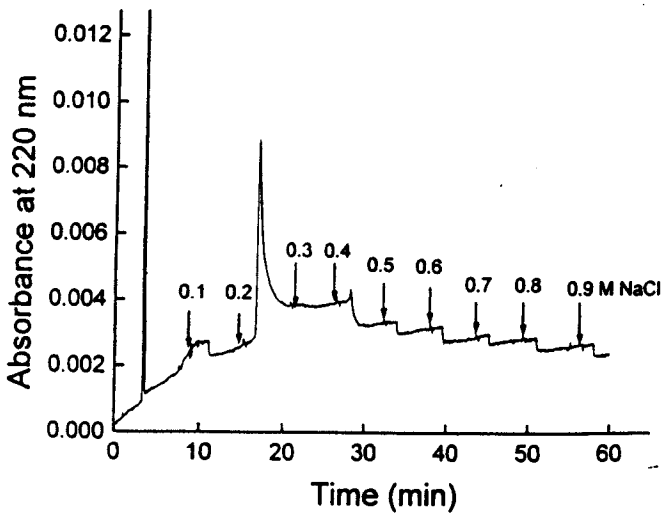

Figure 3. CAC step elution of SLPI by various concentrations of NaCl containing buffer from peptidoglycan heparin-bound etched capillary. NaCl concentration in elution buffer: see Fig. Other conditions as in Fig. 2.

rod conformation placing it away from the capillary surface, promoting its affinity interaction with the protein [18, 19].

Heparin is a polydisperse, highly sulfated polysaccharide consisting of a repeating 1→4 linked uronic acid and glucosamine sugar residues that binds to a large number of proteins [33]. The separation of SLPI was examined by CAC using a gravity injection mode. The injected amount of SLPI calculated at approximately 135 ng (capillary length 55 cm, 75 μm ID, SLPI concentration 10 mg/mL). Noninteracting protein eluted first in the 25 mM sodium phosphate buffer. The heparinized, etched capillary retained SLPI (~ 50 ng) which was then eluted using 1 M NaCl in sodium phosphate buffer (Fig. 2a). On release, the SLPI peak observed was both symmetric and sharp. The baseline was slightly altered due to the large increase in salt concentration. A similar result was obtained when ATIII was applied to the same capillary (Fig. 2b). Bovine serum albumin had no affinity to the heparinized capillary under identical experimental conditions (Fig. 2c), suggesting that both the SLPI and ATIII interactions with heparin ligand were specific. Injection of buffer alone resulted in no peaks in the electropherogram (not shown) and the application of decreasing amounts of bovine serum albumin resulted in a reduced area of the peak eluting with 25 mM sodium phosphate, confirming the identity of this peak as the noninteracting protein.

A stepwise elution, from 0.1 to 0.9 M NaCl, was next used to examine the affinity of SLPI for the heparinized capillary (Fig. 3). SLPI could be eluted from the capillary with 0.2 M NaCl, a concentration similar to the 0.20–0.27 M NaCl required to release it from a heparin-Sepharose matrix in traditional affinity chromatography [17]. However, the linear velocity (122 cm/min at 2 psi, with a length of 55 cm) of the separation on the capillary column washed by pressure injections was 200-fold greater than in traditional affinity chromatography (0.64 cm/min, 1 cm diameter column). The capacity of this column (~ 50 ng) decreased slightly following each day of use, suggesting some inherent stability problems with the immobilized heparin. After washing the columns with water, the capillaries were filled with Tris-buffer, pH 7.5, containing 0.01% sodium azide, the ends were sealed, and the capillaries were stored at 4°C. Under these conditions the capillaries could be stored for one month without substantial loss in performance characteristics.

4 Concluding remarks

New instrumentation with lower pressure of injection permitting operation at lower linear velocities should improve ligand-protein interactions, permitting CAC analysis of protein with very low affinity or slow on-rates. In addition, the development of more sensitive detection methods, such as mass detectors, might enhance the sensitivity of CAC sufficiently to allow lower ligand loading on the capillary, facilitating the analysis of proteins that bind very tightly to heparin. Further studies are also required to examine alternative capillary derivatization strategies.

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