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Immunochromatographic analysis of proteins

Identification, characterization and purity determination

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ABSTRACT

Antibodies specific to a protein and its structural variants were immobilized on a high-performance Protein G column. This column recognized and selectively subtracted specific molecules from a sample. When a size-exclusion column was coupled with this high-performance affinity column, a comparison between the elution profile before and after the antibody immobilization was used to study antigen components present in the sample. Various human growth hormone structural variants and aggregates were studied using this approach. The technique is simple, fast and does not involve the usage of radioactive material.

INTRODUCTION

Immunoaffinity chromatography has been used widely both in the purification of macromolecules [1–3] and as an analytical tool for studying molecular interactions and protein structure [4–6]. Affinity chromatographic (AC) determinations of equilibrium and rate constants of solute-ligand interactants [8–10] through both frontal and zonal elution are some examples of analytical applications of the technique. Other examples are quantitative analysis of specific proteins [11–14] and the estimation of reaction rate constants [15,161.

Unfortunately, most proteins are so strongly retained by an immunoaffinity column that they must be denatured to effect their elution. Under these severe elution conditions, antibodies can lose specificity and many proteins are irreversibly denatured. This paper introduces the approach of monitoring antigen components that are *not* retained by the affinity column. When antibodies specific to a protein and its structural variants are immobilized on an affinity column, this affinity column should recognize and selectively subtract specific molecules from the sample. Comparing the elution profiles before and after the antibody immobilization through a subtraction process may reveal antigen components present in the sample. Quantification of these components is achieved by measuring differences in peak areas.

Because of its great flexiblity and ease of use, a short protein G column (30 × 2.1 mm I.D.) was used to immobilize antibodies. Affinity columns were prepared by immobilizing specific antibodies onto the protein G column as reported previously [13]. Although a size-exclusion column was used as the separation column in these studies, reversed-phase or ion-exchange columns can also be used.

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The experiments reported in this paper were performed with a tandem column system consisting of an immunosorbent column directed against human growth hormone (hGH) and a size-exclusion column.

EXPERIMENTAL

Materials

Antibodies. Monoclonal antibodies to hGH (GHC101 and GHC072) were the same material described previously [12]. Rabbit antiserum to hGH (lot No. 184-15) was prepared by immunization techniques as previously described [17]. The serum was taken ten days after the third monthly booster injection.

Growth hormone and derivatives. Monomeric hGH (somatropin, 22.1 kilodalton) of recombinat DNA origin was obtained from Lilly Research Labs. (Indianapolis, IN USA) and determined to be greater than 99% pure by size-exclusion chromatography (SEC) at Lilly [18]. The purity determined by reversed-phased HPLC was greater than 95%. Non-covalent hGH dimer was recovered from a pool of high-molecular-mass materials generated during the production of hGH and purified by SEC [19]. N-Terminal methionyl hGH (met-hGH) was prepared as described previously [20]. Methionyl- 14 sulfoxide hGH was obtained by treating hGH with 3% hydrogen peroxide [21]. Desamido hGH, with the major deamination site at Asn-149 and the minor site at Asn-152, was produced by storage of hGH in ammonium bicarbonate (pH 9) for 72 h at 37°C [22]. An N-terminal methionyl, hGH variant. molecular mass 20 kilodalton (20K met-hGH) was prepared by rDNA methods. Except for the N-terminal methionine residue, this material is identical to the natural 20 kilodalton hGH variant in which the 32 -45 peptide fragment is omitted and the 1 -31 segment is connected directly to the 46– 191 segment. All the hGH derivatives described above were obtained from Lilly Research Labs.

Synthetic. peptides. Homologous peptides of hGH, synthesized on a Beckman Model 990B automated synthesizer according to the Merrifield solid-phase method [23], were obtained from Lilly Research Labs. Peptide identities were confirmed by N-terminal sequence and total amino acid analysis. These peptides are 15 to 28 residues in length and are numbered according to the primary sequence of hGH. They are referred to as peptide 1–28, peptide 25–45, peptide 126–151, and peptide 171-191.

Other materials. Bovine serum albumin (BSA). Blue Dextran 2000, ferritin. aldolase, ovalbumin, chymotrypsinogen A and ribonuclease A are from the gel filtration calibration kits for protein molecular mass determination supplied by Pharmacia (Piscataway, NJ, USA). All other chemicals were analytical-reagent grade unless otherwise indicated. Reagent water was obtained from a Millipore Milli-Q water-purification system.

Apparatus

The chromatographic system used for this experiment is shown in Fig. 1. Valves V-l and V-2 are two-position, six-port switching valves (Rheodyne, Model 7000, with pneumatic actuator). V-l was coupled to the system as shown in the diagram so

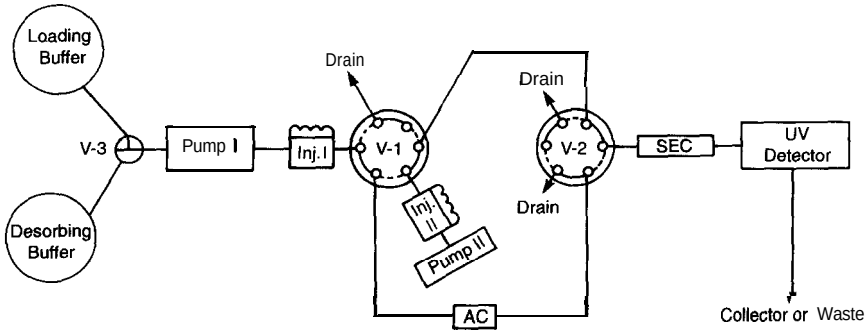


Fig. 1. Apparatus for Protein G AC-SEC tandem column immunochromatographic analysis. See text

that either pump I or pump II can deliver mobile phase to the protein G affinity column (pore size 500 A; 30 × 2.1 mm I.D., custom packed by Chromatochem, Missoula, MT, USA). V-2 was installed so that the protein G affinity column and an SEC column could be operated either in "column-tandem" mode (Fig. 1, connection of V-2 ports shown by broken line) or "column-independent" mode (Fig. 1, connection of V-2 ports shown by solid line). Either a GF-250 (DuPont, Zorbax Bioseries, 25 cm × 9.4 mm I.D.) or Ultraspherogel SEC3000 (Beckman, 30 cm × 7.5 mm I.D.) column was used in the SEC separations. Pump I (Beckman, Altex Model 110A) was used for antibody immobilization or regeneration of the protein G column. A simple switching "T" valve was installed at the solvent inlet of the pump to allow rapid switching between loading and desorbing buffers. Two Rheodyne (Model 7125) injection valves, equipped with a 20-μl sample loop, were used. One was installed between pump I and V-l for immobilization of antibodies. Another one was installed between pump II and V1 for sample introduction. Pump II (Spectra-Physics, SP8700XR, extended-range LC pump) was used for delivering the mobile phase to achieve the SEC separation.

The column eluate was monitored by a UV detector (Spectra-Physics SP8440XR). The detector output was interfaced with an HP1000 minicomputer where all data were collected, stored and processed using an in-house (Lilly Research Labs.) chromatographic software package.

Immobilization of antibodies onto the Protein G column

To immobilize antibodies onto the protein G column, V-l was set at the pump I mode, and V-2 was set at the "column independent" mode. Tris–acetate buffer (50 mM, pH 7.4) was used as loading buffer. Antibody solutions were diluted to approximately 10 μg/ml, and an aliquot of 20 μl (about 200 μg antibody) was injected via injector I onto the protein G column at a flow-rate of 0.5 ml/min for 5 min. Then the flow-rate was increased to 2 ml/min for 10 min to elute the unbound materials.

Regeneration of the Protein G column

To regenerate the protein G column, V-l remained at the pump I mode, V-2 remained at the "column independent" mode, and the column was eluted with 20% acetic acid at a flow-rate of 3 ml/min for 10 min to desorb any bound immunoglobulin G. Following acid-initiated desorption, the column was equilibrated with Tris-acetate buffer (50 mM, pH 7.4) for 15 min at 2 ml/min.

Protein G AC-SEC tandem column immunochromatographic procedure

The protein G column was regenerated using the procedure described above. Then, V-2 was switched to the "column-tandem" mode and V-l was switched to allow pump II to deliver mobile phase onto the columns. After equilibrating the columns with Tris-acetate or ammonium hydrogencarbonate-acetate, 50 mM, pH 7.5 ± 0.1, 20 μl of sample were injected onto the protein G column via injector II, using a flow-rate of either 1.0 or 0.6 ml/min, depending on the experiment. The column eluent was monitored at 214 nm when ammonium hydrogencarbonate buffer was the mobile phase or at 280 nm when Tris-acetate buffer was the mobile phase. Antibody was then immobilized on the protein G column using the procedure described earlier. During antibody immobilization, the SEC column was equilibrated with appropriate mobile phase using pump II. After antibody was immobilized onto the protein G column, V-2 was again switched to the column-tandem mode and V-l was switched to allow pump II to deliver mobile phase onto both columns. Another 20-μl sample was injected onto the immunosorbent column and unretained solutes were eluted through the tandem column system. The chromatograms acquired before and after antibody immobilization were compared, and the difference obtained by subtracting each data point of the second chromatogram from the first were used to plot a difference chromatogram.

Antibody recognition of hGH

In an initial study of hGH recognition by anti-hGH mono- or polyclonal antibodies, samples were chromatographed on a Beckman Ultraspherogel SEC3000 SEC column (30 cm × 7.5 mm I.D.) and eluted with Tris-acetate buffer (50 mM, pH 7.4) at a flow-rate of 1 ml/min. Sample solution A was prepared by dissolving 1 mg each of BSA, hGH and ribonuclease A in mobile phase and adjusting the final volume to 1 ml. Sample solution B was pre-

pared by dissolving 1 mg each of BSA, hGH and chymotrypsinogen A in the mobile phase and adjusting the final volume to 1 ml. Sample solution A was analyzed by the immunochromatographic procedure as described above with 200 μg of immobilized GHC072 monoclonal antibody. Sample solution B was chromatographed on an immunoaffinity column containing approximately 200 μg of immobilized anti-hGH from a rabbit anti-serum (lot No. 184-15).

Binding study of hGH-related compounds

Stock solutions of hGH, met-hGH, desamido hGH, met-14-sulfoxide hGH, 20K met-hGH and hGH dimer were prepared by dissolving 1 mg of each protein in 1 ml Tris-acetate buffer (50 m*M*, pH 7.4) and stored refrigerated (5°C). Stock solutions of ferritin and cytidine were also prepared by dissolving 1 mg each in 1 ml of the above Trissacetate buffer and stored in dark at 5°C. The synthetic peptide solutions were used directly.

Sample solutions of hGH, met-hGH, desamido hGH, met-14-sulfoxide hGH and hGH dimer were prepared by mixing 100 μl of each protein stock solution with 200 μl of ferritin and 20 μl of cytidine (used as high- and low-molecular-mass internal standards), and the final volume was adjusted to 1 ml with NH_4HCO_3 (50 m*M*, pH 7.6). A sample solution of 20K met-hGH was prepared similarly except cytidine was not added. Sample solutions of the peptides were prepared by mixing 20 μl of each stock solution with 20 μl of ferritin solution, and the final volume was adjusted to 100 μl with ammonium hydrogencarbonate buffer.

For the binding study of hGH-related compounds, a GF-250 column was selected for the SEC separation. Ammonium hydrogencarbonate–acetate buffer (50 m*M*, pH 7.6) was used as the mobile phase at flow-rates of both 0.6 ml/min and 1.0 ml!' min.

The binding of hGH to both GHC072 and GHClOl monoclonal antibodies was examined. The immunoaffinity column was prepared by immobilizing 200 μg of either antibody onto the protein G column using the procedure described above.

Binding of hGH monomer and dimer

A test solution containing both hGH monomer and dimer was prepared by mixing 100 μl of both the hGH monomer and dimer stock solutions with 200 μl of ferritin and 20 μl of the cytidine stock solutions and adjusting the final volume to 1 ml with the ammonium hydrogencarbonate buffer (50 m*M*, pH 7.6). This test solution was analyzed using the AC-SEC immunochromatographic procedure as previously described. The immunoaffinity column was prepared by the immobilization of approximately 300 μg of GHC072 or GHClOl antibody onto the protein G column.

A sample solution for further investigation of the competition of hGH monomer and dimer for binding to GHC072 antibody was prepared by adding the appropriate amount of hGH monomer and dimer stock solutions to ammonium hydrogencarbonate buffer (50 m*M*, pH 7.6) to a final concentration of 400 μg/ml of each component. This mixture was analyzed using the ACSEC immunochromatographic method at a flow-rate of 1 ml/min and a GHC072 immunoaffinity column with 100 μg of antibody. This experiment was then repeated. with less antigen and a lower flow-rate. The mixture of hGH monomer and dimer solution was diluted 1:2 with the mobile phase so that the final solution contained 200 μg/ml each of hGH monomer and dimer. The AC–SEC immunochromatographic procedure was repeated with this diluted solution at a mobile phase flow-rate of 0.6 ml/min.

Band-broadening investigation

To investigate the possible band-broadening effect of the protein G column on the SEC separation, a test solution was injected via injector II onto the protein G column. V-2 was then switched to the "column independent" mode and V-l was switched so that pump II was connected directly to the SEC column instead of the protein G column. Another 20-μl aliquot of the test solution was again injected onto the SEC column. The chromatograms obtained using the SEC column alone and the tandem protein G-SEC column were compared.

A test solution for the GF-250 column was prepared by mixing 100 μl each of the hGH monomer and dimer stock solutions, 200 μl of ferritin, and 20 μl of cytidine stock solutions; the final volume was adjusted to 1 ml with the ammonium hydrogencarbonate (50 m*M*, pH 7.6) mobile phase. The flow-rate was set at 0.6 ml/min and column eluent was monitored at 214 nm.

A test solution for the Ultraspherogel SEC3000 column was prepared by dissolving 1 mg of ovalbumin and ribonuclease A, 4 mg of aldolase, 0.4 mg of Blue Dextran 2000 and 0.5 mg of cytidine in 1 ml of Tris-acetate (50 m*M*, pH 7.4) mobile phase. The flow-rate was set at 1 ml/min and column eluent was monitored at 280 nm.

RESULTS AND DISCUSSION

Recognition of hGH by both mono- and polyclonal anti-hGH antibodies is demonstrated in Fig. 2. The hGH peak in Fig. 2A(a) disappeared [Fig. 2A(b)] when 200 μg of GHC072 anti-hGH monoclonal antibody was immobilized on the protein G column. In contrast, the peak areas of BSA and ribonuclease A were not affected. The difference chromatogram (c) obtained by subtracting each data point of (b) from (a) re-created the hGH peak, which was retained by the affinity column. The hGH peak area of(c) was 100.2% that of (a). Similar results are seen in Fig. 2B where the area of the regenerated hGH peak in (c) was 99.6% of that in (a). Fig. 2B also demonstrated that in a sample matrix containing hGH, BSA and chymotrypsinogen A, the anti-hGH in the rabbit anti-serum can selectively bind to hGH and allow non-specific components (BSA and chymotrypsinogen A) to elute unretained, as shown in trace (b). Coelution of antigen with impurities, with the peaks of interest, or a slight shift in retention times between chromatograms (a) and (c) could potentially generate false peaks in the difference chromatogram. Such an artifact peak with a retention time of 600 s is seen in trace (c). However, the hGH peak was correctly regenerated.

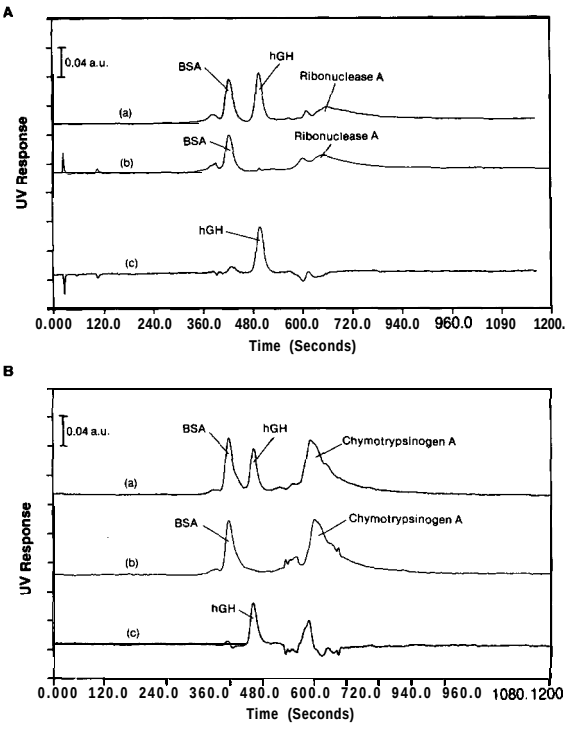


Fig. 2. Recognition of hGH by (A) GHC072 monoclonal antibody and (B) anti-hGH in rabbit anti serum, (a) before and (b) after immobilization of 200 μg antibodies onto the Protein G column. The difference chromatogram (c) was obtained by subtracting each data point of(b) from (a). Experimental conditions are described in the text.

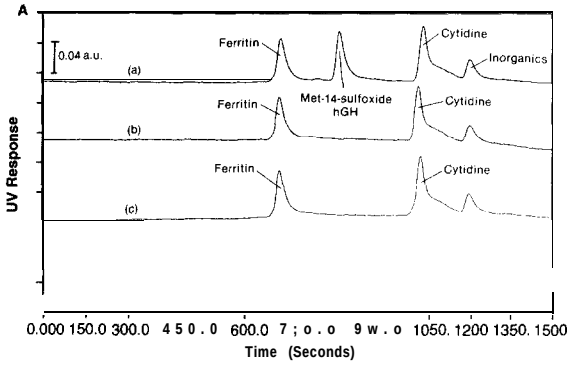
Results from a study on reactivity of hGH related compounds with anti-hGH monoclonal antibodies GHC072 and GHClOl are listed in Table I. hGH, met-hGH, desamido hGH, and met-14-sulfoxide hGH all bind to both GHClOl and GHC072 anti hGH monoclonal antibodies with equal affinity, except for 20K met-hGH and hGH dimer. Protein binding to the antibody was indicated by the disappearance of the protein peak in the chromatogram after antibody immobilization on the protein G col-

TABLE I

BINDING OF hGH, HOMOLOGOUS PEPTIDES OF hGH AND hGH DERIVATIVES TO MONOCLONAL ANTIBODIES TESTED BY AC–SEC IMMUNOCHROMATOGRAPHIC ANALYSIS

+ = Antibody binding; − = no significant binding; ± = partial binding.

Proteins/peptides	Monoclonal antibodies	
	GHC101	GHC072
hGH	+	+
hGH dimer	+	±
Desamide hGH	+	+
Met-hGH	+	+
20K met-hGH	−	+
Met-14-sulfoxide hGH	+	+
Peptide 1-28	−	−
25–45	−	−
126–131	−	−
171–191	−	−



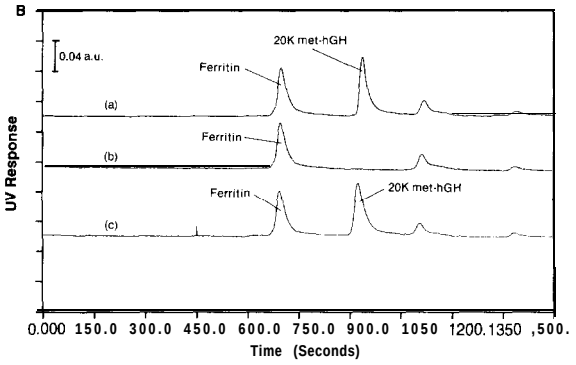


Fig. 3. Antibody bmding of (A) met 14-sulfoxide hGH and (B) 20K met-hGH. AC-SEC chromatogram obtained (a) with no antibody immobilization on Protein G column. (b) with 200-μg of GHC072, (c) with 200-μg of GHC101 immobilized on the Protein G column. Experimental conditions are described in the text.

umn (Fig. 3A). Ferritin and cytidine were used as high and low molecular weight internal standards. 20K met-hGH binds to GHC072 antibody but does not bind to GHClOl, as seen in Fig. 3B. The peak height ratio of 20K met-hGH to ferritin in trace (b) did not show significant change when the mobile phase flow-rate was changed from 1 ml/min to 0.6 ml/min. This indicates that the affinity of binding is sufficiently low that small changes in the flow-rate do not influence the binding of 20K methGH to GHClOl. It is likely that the 32-45 peptide fragment that is omitted in this variant is important for the binding of GHClOl antibody. This is not too surprising because the residues 29-41 are thought to be solvent-exposed [24]. This conclusion is consistent with other studies [25–27].

It was found that 2 μg each of hGH monomer

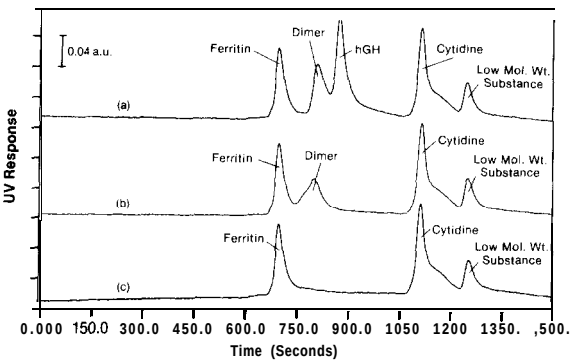


Fig. 4. Binding of 2 μg each of hGH monomer and dimer to anti-hGH monoclonal antibodies. (a) Without antibody immobilization, (b) with 300 μg GHC072 immobilized. (c) with 300 μg GHC101 immobilized. Experimental conditions as described in the text.

and dimer bind very differently to GHClOl and GHC072 on the tandem AC -SEC immunochromatographic system. Fig. 4 demonstrates that both hGH dimer and monomer were retained (trace b) when 300 μg of GHClOl was immobilized onto the protein G column. However, when 300 μg of GHC072 was immobilized onto the protein G column, all the hGH monomer and only a portion of the dimer were retained. Trace (c) still exhibits a

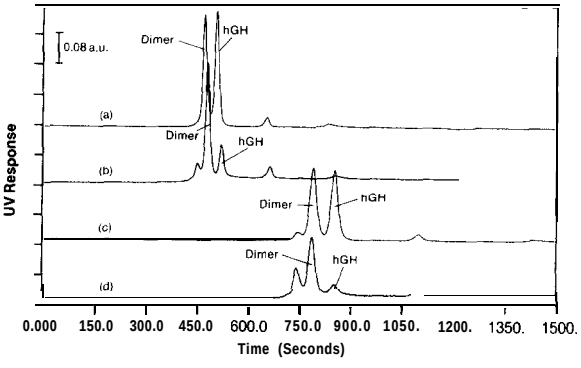


Fig. 5. Binding of hGH monomer and dtmer to GHC072 monoclonal antibody. (a), (b): Sample containing 8 μg each of hGH monomer and dimer, elution flow-rate is 1.0 ml/min; (a) without antibody, (b) with 100 μg of GHC072 immobilized on Protein G column. (c), (d): Sample containing 4 μg each of hGH, elution flow-rate is 0.6 ml/min; (c) without antibody, (d) with 200 μg of GHC072 immobilized on Protein G column. Other experimental conditions as described in the text.

small peak of hGH dimer. Based on these AC-SEC immunochromatographic analysis, hGH dimer binds well to the GHClOl antibody but binds poorly to GHC072.

The realtive binding of hGH monomer and dimer was further investigated by chromatographing a sample containing 8 μg each of hGH monomer and dimer on a protein G column with 100 μg of immobilized GHC072. Unbound material was chromatographed on an SEC column operated at a flow-rate of 1 ml/min. As shown in Fig. 5, only a portion of hGH dimer and monomer was retained by the affinity column (trace b). When the amount of hGH dimer and monomer was reduced to 4 μg each and the elution flow-rate was also reduced to 0.6 ml/min, there was still a difference in binding (trace d). Because the lower percentage of binding between GHC072 antibody and hGH dimer does not seem to be influenced by either the amount of antigen or the small change of the flow-rate, it is possible that the hGH dimer has more than one conformation and these conformations may have different affinities for GHC072.

None of the four synthetic peptides (l-28, 25–45, 126131 or 171-191) tested showed any binding to either GHC072 or GHC101. In view of the fact that amino acids in the 25–45 region of hGH are involved in binding to GHClOl, it is surprising that this peptide did not bind to the antibody. It is probable that the epitope for this antibody is either discontinuous or the binding of the antibody to hGH is sensitive to conformation [27].

The possiblity of band-broadening introduced by the protein G column was investigated by comparing the chromatograms of the test solutions obtained using the SEC column alone to those obtained using protein G and the SEC column in tandem. Results of the investigation are shown in Fig. 6. As shown, the protein G column used in our experiments does exhibit a slight band-broadening effect but is should not affect the utility of the AC–SEC system. This effect can be further reduced if a shorter protein G column of a smaller diameter is used.

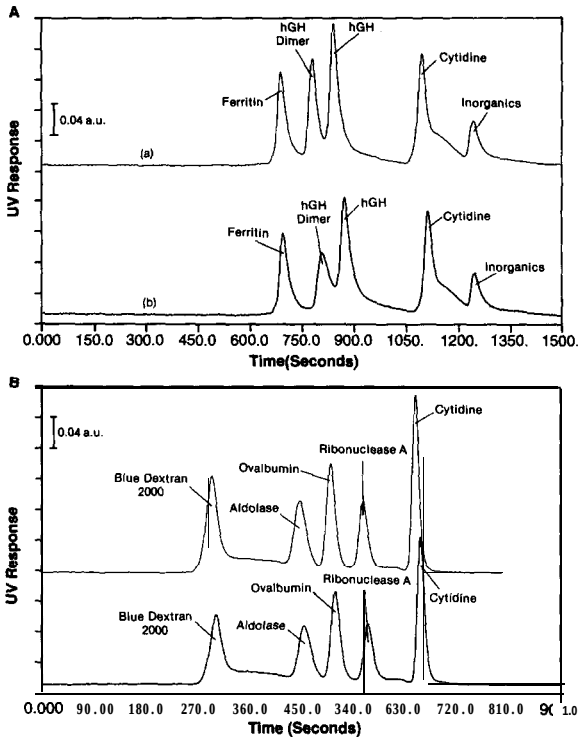


Fig. 6. Slight band-broadening introduced by Protein G column. (A) Protein G–GF250 system, at 0.6 ml/mm flow-rate, (B) Protein G-Ultraspherogel 3000 system, at l .O ml/min. (a) SEC column only, (b) Protein G-SEC tandem column.

CONCLUSIONS

The objective of this research was to design a simple, fast, and economical approach for the study of molecular recognition in affinity chromatography. It is demonstrated in this paper that the tandem AC-SEC immunochromatographic technique can be used to rapidly characterize the interactions between antigen and antibodies. The work presented here also shows that protein G can be used to rapidly prepare an immunosorbent column that wil interact specifically with antigens. The AC-SEC immunochromatographic system may be used as a powerful tool to differentiate between cross-reacting variants and the reactivity of antibodies for these variants.

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