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IMMUNOLOGICAL-CHROMATOGRAPHIC ANALYSIS

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SUMMARY

Tandem immunoaffinity and conventional high-performance liquid chromatography columns, coupled with a switching valve, were used for the analysis of single and multicomponent antigen samples. Immunoaffinity columns were prepared by hydrophobic adsorption or covalent immobilization of poly- or monoclonal antibodies on macroporous poly(styrene–divinylbenzene) packing materials. Capacities of these columns were constant through at least 77 cycles. Macromolecular antigens were analyzed at submicrogram levels. Antigens bound to the immunoaffinity column were desorbed and concentrated on a conventional analytical column. Gradient elution on the analytical column separated the desorbed antigen(s) from interfering species and permitted the analysis of all species which bound to the immunoaffinity column. Immunological-chromatographic analysis was useful for purification and discrimination of polypeptides of similar three dimensional structures, such as several lysozyme variants.

INTRODUCTION

The emergence of genetic engineering has been one of the most exciting practical advances in the life sciences during the past decade. Accompanying the large-scale production of human and animal proteins in cell culture are a series of separation challenges. Typically, the recovery of an expressed protein from tissue culture media or fermentation broths is difficult because host cell contaminants and artifacts of the recombinant product must be removed. Artifacts arising from translation errors, improper refolding, incomplete or incorrect post-translational modification, and chemical or proteolytic degradation during purification all contribute to the production of polypeptide species with structures very similar to the desired native polypeptide. Monitoring biosynthetic fidelity and protein purity are major analytical endeavors in the production of recombinant proteins.

Protein identification and discrimination between similar polypeptide species is often achieved by surface-mediated separation techniques. For example, small changes in protein conformation or amino acid composition can change chromatographic retention. This makes it possible to separate six out of seven lysozyme variants by hydrophobic interaction chromatography[1]. Retention mechanism studies show that many proteins have a specific chromatographic contact area in ion-exchange

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(IEC), hydrophobic interaction (HIC) and reversed-phase chromatography (RPC), which controls their chromatographic behavior[1–3]. The size of this area varies with each type of separation mode. It may be on one side of the protein, or cover the entire surface[4]. Structural changes within this contact region have a high probability of altering chromatographic behavior.

In comparison to conventional modes of chromatographic analysis, immunological methods examine a much smaller region of the protein called the epitope. Antibodies recognize unique spatial arrangements of amino acids on the surface of an antigen and bind to this region with great affinity. Immunosorbent assays are often unable to distinguish between mutant, variant, chemically modified, fragmented, and multimeric forms of a protein[5–8] when the changes do not influence either the composition or spatial arrangement of amino acids in the epitope. Thus, immunoaffinity chromatography is very powerful in the isolation and purification of proteins of similar three-dimensional structure, but is of limited utility in discriminating between species with random structural differences. In contrast, other, less specific chromatographic methods, which are not as effective in the initial purification of proteins, are able to discriminate between similar structural forms.

A combination of these, immunological–chromatographic analysis (ICA), in which the immunoaffinity and conventional (*e.g.* reversed-phase) columns are coupled directly, should be superior to either method alone. All polypeptide species bound to the antibody are eluted from the immunoaffinity column, and concentrated onto a conventional analytical column, where they are separated by a different retention mechanism. The transfer of polypeptides from an immunoaffinity to a RPC column, coupled with a switching valve, has been described by Rybacek *et al.*[8] and Roy *et al.*[9]. A modification of this system was used in these studies. The objective of this research was to examine the utility of an ICA system in discriminating between similar structural forms of proteins. A general scheme for the design of such a system is presented.

MATERIALS AND METHODS

Reagents

Carbonyl diimidazole (CDI) was purchased from Sigma (St. Louis, MO, U.S.A.). Tween-20 [polyoxyethylene (20) sorbitan monolaurate] was a gift from ICI Americas (Wilmington, DE, U.S.A.). Cyanogen bromide was purchased from Pierce (Rockford, IL, U.S.A.). Inorganic reagents were of analytical-reagent grade or comparable quality. HPLC-grade trifluoroacetic acid (TFA) (Pierce), acetonitrile and water (American Burdick & Jackson, Muskegon, MI, U.S.A.) were used in the chromatographic analyses.

Proteins

All polyclonal antibodies were obtained from ICN ImmunoBiologicals (Lisle, IL, U.S.A.). Rabbit anti-human hemoglobin (anti-HHb), rabbit anti-bovine serum albumin (anti-BSA) and goat anti-rabbit serum were antisera in the form of a lyophilized powder and sheep anti-human lysozyme (anti-HLZM) was obtained as an immunoglobulin (IgG) fraction dissolved in phosphate-buffered saline (0.30 *M* phosphate, 0.14 *M* sodium chloride, pH 7.4). Mouse ascites fluid with monoclonal

antibodies to hen egg-white lysozyme (anti-HEWLZM) was a generous gift from Dr. S. Smith-Gill (National Institutes of Health, Bethesda, MD, U.S.A.)[5,6]. Human milk lysozyme (HLZM) and L-1-tosylamide-α-phenylethylchloromethyl ketone (TPCK) treated trypsin were purchased from U.S. Biochemical (Cleveland, OH, U.S.A.). Avian lysozyme variants were purified as described by Fausnaugh and Regnier[1]. Bovine serum albumin, HEWLZM, HHb, conalbumin, rabbit IgG, ovalbumin, and β-lactoglobulin were obtained from Sigma. All biochemicals were of the purest grade available.

Preparation of immunoaffinity columns by hydrophobic adsorption

Conventional methods[10,11] for immobilizing antisera, by hydrophobic adsorption on polystyrene, for immunoassays were adapted for *in situ* immobilization of antisera on microparticulate, macroporous poly(styrene–divinylbenzene) (PLRP-S, 8 μm, 300 Å; a generous gift from Polymer Labs., Shropshire, U.K.). The polystyrene resin was slurry-packed from 2-propanol into a 5 × 0.46 cm I.D. stainless-steel column at 3300 p.s.i., using an HPLC packing pump (Shandon Southern Instruments, Sewickley, PA, U.S.A.). Polyclonal antisera were reconstituted to 2 ml with 0.50 *M* carbonate buffer (pH 9.6) and then diluted to 25 ml (final concentration, *ca.* 5 mg/ml). The column was equilibrated in the carbonate buffer. The antisera were repeatedly injected (4.6-ml volumes) into the polystyrene column, maintaining a flow-rate of 0.3 ml/min, until a breakthrough peak appeared. One more injection was made to ensure that the column was saturated. An amount of 35–40 mg of protein was immobilized per 5-cm column (42–48 mg/ml column volume). Either the detergent, Tween-20, or proteins, were used to saturate any non-specific binding sites that were not covered by the antisera as follows. A 0.05% solution of Tween-20 in phosphate buffered saline was pumped through the column at 0.5 ml/min for 1 h. The proteins, BSA or conalbumin (20 μl, 10 mg/ml), were injected into the column until a constant peak height was achieved.

Preparation of immunoaffinity columns by covalent immobilization

The hydrophilic, macroporous poly(styrene–divinylbenzene)-based packing material (300 Å, 20 μm) used in these studies was prepared by Thévenon and Regnier at this laboratory[12]. The surface of this resin contained hydroxyl groups which could be derivatized with CDI. Approximately 400 mg of the packing material was activated with 400 mg of CDI in 3 ml of dry dioxane for 5 h. The CDI-activated resin was washed with water and 0.05 *M* carbonate buffer (pH 6.0), then suspended in 20 ml of a 5 mg/ml solution of the antibodies to be immobilized. (Polyclonal antisera were used as purchased; monoclonal antibodies to HEWLZM were purified from mouse ascites fluid by precipitation in 42% ammonium sulfate.) The reaction mixture was slowly agitated at 4°C for 3–4 days. After washing with 0.10 *M* phosphate buffer (pH 7.0), the packing material was slurry-packed from this buffer into a stainless-steel column (5 × 0.46 cm I.D.) at 1300 p.s.i. using an Constametric I and III G pumping system (Laboratory Data Control, Riviera Beach, FL, U.S.A.).

Determination of antigen-load capacities

The antigen-load capacities of the immunoaffinity columns, prepared by hydrophobic antibody adsorption, were determined by frontal analysis[13]. A volume of 2 ml of 0.1–0.3 mg/ml solution of antigen (depending on the column capacity) in 0.10

M phosphate buffer (pH 7.0) was loaded onto the affinity column at 0.5 ml/min until a breakthrough curve appeared. Duplicate injections of 2 ml of 0.10 *M* glycine buffer (pH 2.2) were used to desorb the antigen and regenerate the column.

The dual column technique described in *ICA procedure* below was used to estimate antigen-load capacities of the covalently prepared immunoaffinity columns. Antigens, saturating the affinity column, were desorbed and analyzed on the RPC column. Reversed-phase peak heights of the desorbed antigens were compared to the peak heights of antigen samples of known concentration generated using the RPC column alone.

Digestion of lysozyme

Cleavage at methionine residues was achieved by dissolving *ca.* 5 mg of human lysozyme in 2 ml of 77% formic acid, followed by the addition of a 100-fold molar excess (over methionine) of cyanogen bromide. The solution was stirred for 24 h at room temperature in the dark, then lyophilized.

The cyanogen bromide-treated lysozyme was first dissolved in 1 ml of 0.1% TFA and adjusted to pH 7.8 with 0.10 *M* ammonium bicarbonate (pH 8.0). TPCK-treated trypsin (2% by weight of lysozyme) was added to this mixture. After incubation at 37°C for 4 h, the solution was lyophilized.

Apparatus

The immunoaffinity and analytical chromatography columns were coupled by an automatic switching valve (Model 7010, Rheodyne, Cotati, CA, U.S.A.) which was controlled by a binary-gradient liquid chromatograph (Model 1090L, Hewlett-Packard, Waldbronn, F.R.G.) fitted with a manual injector (Model 7125, Rheodyne). The switching valve contained the analytical column in place of a sample loop. This allowed the analytical column to be used either in series after the affinity column, or alone with the gradient pump. A second pump (Model 110, Altex Scientific, Berkeley, CA, U.S.A.) and injection valve (Model C6U, Valco Instruments, Houston, TX, U.S.A.), equipped with a 2-ml injection loop (for samples and desorbing agent) was connected to the immunoaffinity column. Duplicate pumps and injection valves permitted the simultaneous loading, washing, elution, and re-equilibration of both columns. The two types of analytical columns used were (a) reversed-phase columns (Synchropak RP-8; 25 × 0.46 cm I.D., and 5 × 0.46 cm I.D., Synchrom, Lafayette, IN, U.S.A.) and (b) a strong cation-exchange column (Synchropak S-300, 5 × 0.46 cm I.D. Synchrom). Absorbance was monitored with a variable-wavelength detector (Spectroflow 757, equipped with a 0.5-μl flow cell; kindly lent by Kratos Division, Applied Biosystems, Ramsey, NJ, U.S.A.).

ICA procedure

The position on the switching valve determined whether the immunoaffinity and analytical columns would be used in series or separately. The samples were loaded onto the affinity column in 0.10 *M* phosphate buffer (pH 7.0) at a flow-rate of 0.5 ml/min. Non-retained proteins could either be concentrated on the analytical column for subsequent analysis, or sent directly to waste. Before the analysis of antigens bound to the affinity column, the column was washed with at least 50 column volumes of buffer. Then the valve was switched to place both columns in-line. A 2-ml plug of desorbing

agent (either 0.10 *M* glycine, pH 2.2, or 0.1% TFA) was used to desorb bound antigens from the immunoaffinity column into the analytical column, at a flow-rate of 1 ml/min. [The plug is illustrated in the ICA chromatograms (Figs. 1–4) as a darkened rectangle on the baseline prior to the start of the gradient.] After 3 or 6 min, the valve was switched, placing the immunoaffinity column off-line, to permit the analysis of the antigens by gradient elution on the analytical column. All analytical RPC separations were performed with acetonitrile gradients in 0.1% TFA at a flow-rate of 1 ml/min. Analytical cation-exchange chromatography was performed with a 20-min linear gradient from 0 to 1.0 *M* sodium chloride in 0.10 *M* phosphate (pH 8.0) at 1 ml/min. The affinity column was then treated with another 2 ml of desorbing agent to ensure total removal of antigen, and equilibrated and reloaded.

RESULTS AND DISCUSSION

Evaluation of immunoaffinity columns

Hydrophobically adsorbed immunoaffinity columns. Antigen-load capacities for the immunoaffinity columns prepared by hydrophobic adsorption are shown in Table I. All columns were conditioned for several cycles to achieve a steady-state capacity of 40–45% of the initial value. Capacities varied with the length of equilibration time, as reflected by the small variations in the capacities listed in Table I. These columns can be used for at least 77 cycles.

TABLE I

ANTIGEN-LOAD CAPACITIES FOR IMMUNOAFFINITY COLUMNS PREPARED BY ANTIBODY ADSORPTION ON POLY(STYRENE–DIVINYLBENZENE) PACKING MATERIALS

Capacity determined by frontal analysis (see text).

Cycle No.	*Capacity (μg/ml column volume)*		
	*Anti-human hemoglobin**	*Anti-BSA*	
		*I**	*II***
1	220	217	635
2	118	128	597
3	111	117	420
4	95	108	386
5	91		338
6	102		283
7	103		
20			272
32	103		
40	97		270
56	90		
77	100		
78***	42		

* Non-specific binding sites, saturated with Tween-20.

** Non-specific binding sites, saturated with conalbumin.

*** Pumped 60 ml of 0.05% Tween-20 solution through the column before measuring capacity.

The anti-HHb and anti-BSA I columns showed a 40–50% decrease in capacity after the first cycle. This initial decrease in capacity of immunoaffinity columns has also been observed by other workers using covalently immobilized polyclonal antisera. Eveleigh and Levy[14] attributed the decrease to be due to irreversible adsorption of antigen. One possible explanation for this phenomenon may be that antigen remained bound to high-affinity subclasses of the polyclonal antiserum and was not desorbed by the pH 2.2 glycine buffer.

The steady-state capacity of the anti-BSA II column was three times larger than that of the anti-BSA I column. The anti-BSA II column was prepared by using conalbumin rather than Tween-20 to saturate nonspecific binding sites. The hydrophobic tail of the Tween-20 detergent may enable it to displace adsorbed proteins from the hydrophobic polystyrene surface. After 77 cycles, 60 ml of Tween-20 solution was pumped through the column, decreasing the antigen-load capacity dramatically from 100 to 42 μg/ml column volume.

Elution of the adsorbed antiserum from the column by Tween-20 was verified using the ICA column-switching system in which a hydrophobically adsorbed goat anti-rabbit serum affinity column was placed before the RPC column. The proteins eluted from the affinity column by a 0.05% Tween-20 PBS solution were collected on the RPC column and analyzed. The chromatogram showed that both albumin and IgG were eluted. Throughout all other ICA studies no other significant desorption of the adsorbed antisera from immunoaffinity columns was noted on the reversed-phase chromatograms. Proteins are a better choice than Tween-20 for saturating the non-specific bonding sites of a hydrophobically adsorbed immunoaffinity column.

Non-specific binding to the immunoaffinity matrix, prepared by hydrophobic adsorption, was investigated with a number of protein and peptide samples. Triplicate injections of BSA, rabbit IgG, ovalbumin, conalbumin, and β-lactoglobulin (20 μl, 10 mg/ml) were made on the anti-HHb column. All were eluted in the void volume with small differences due to size-exclusion effects. Constant peak heights, following successive injections, indicated that the proteins were not retained. (Non-specific binding would have resulted in peak heights slowly increasing with each injection until a maximum was reached.) Only the antigen, HHb, was bound to the immunoaffinity column, indicating that the anti-HHb column was specific. The anti-BSA column was also specific. Fig. 1A illustrates a chromatogram of BSA in undiluted bovine serum from the ICA. BSA was the major peak eluting from the immunoaffinity column onto the RPC column. Interferences were also present in the blank (Fig. 1B).

Although proteins were not retained on these immunoaffinity columns, smaller molecules, *i.e.* peptides, were bound. Injections of HLZM tryptic digest (50 μg) into the adsorbed anti-rabbit serum affinity column indicated no recovery of the peptides. Apparently, small molecules were able to penetrate the adsorbed antibody layer while large protein molecules were excluded. This suggests that immunoaffinity columns with adsorbed antibodies are most useful for the analysis of large polypeptides.

Covalently immobilized immunoaffinity columns. The antigen-load capacities of the covalently prepared immunoaffinity columns were estimated using the column-switching ICA system to be 1 μg/ml column volume or less. These low capacities may be due to low activation of the matrix with CDI. Work on improving the capacities of these columns is in progress. Non-specific binding of protein and peptide samples on a covalently-immobilized affinity column was investigated using a non-immune rabbit

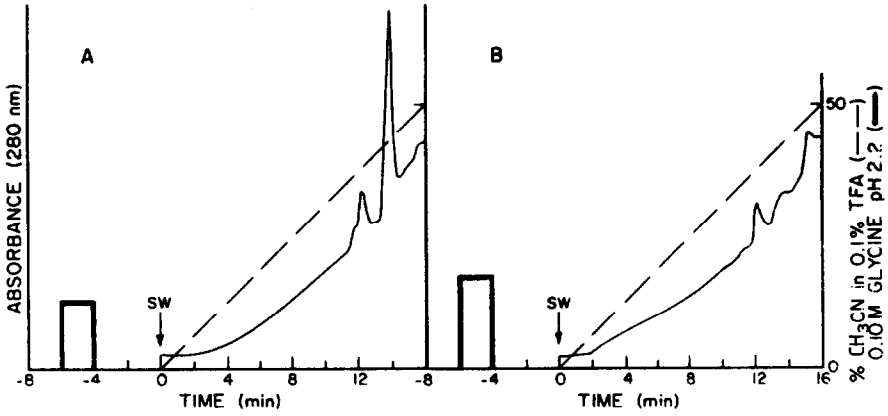

Fig. 1. ICA of BSA. (A) BSA from 20 μl undiluted bovine serum; (B) blank. Affinity column: rabbit anti-BSA, hydrophobically adsorbed. RPC column: Synchropak RP-8 (5 × 0.46 cm I.D.). Event sequence: −6.0 min, affinity and RPC column in-line, inject 2-ml plug of 0.10 *M* glycine (pH 2.2) (rectangular box); 0.0 min, affinity column switched (SW) off-line, RPC analysis started with a 16-min linear gradient from 0.1% TFA to 50% acetonitrile in 0.1% TFA at a flow-rate of 1 ml/min.

IgG column. As was observed for the hydrophobically adsorbed affinity column, constant peak heights of (triplicate injections) of BSA, HEWLZM and HLZM (20 μl, 1 mg/ml) indicated no significant non-specific binding of proteins. The ICA system using tandem immunoaffinity and RPC columns was used to monitor non-specific binding of peptide samples. Peptides not retained on the non-immune rabbit IgG column were routed into the RPC column for analysis. Differences between this chromatogram of the non-retained peptides and the chromatogram of the entire HLZM tryptic digest, generated on the RPC column alone, indicated that a significant amount of the peptides remained bound to the immunoaffinity column. When the rabbit IgG column had been saturated with Tween-20, chromatograms of the entire digest and of the peptides, which were not bound to the immunoaffinity column, were nearly identical. This indicates that treatment with Tween-20 decreased non-specific binding of peptides. These data imply that most non-specific binding of peptides is due to interactions with the matrix, not with the immobilized antibodies. As previously stated, Tween-20 cannot be used on adsorbed immunoaffinity columns because it slowly displaces hydrophobically adsorbed antiserum from the matrix. Thus, a covalently immobilized antibody column (treated with Tween-20) is a better choice for the analysis of small peptides.

Immunological–chromatographic analysis

Affinity chromatography is based on the selective adsorption–desorption of a single species and generally results in only two peaks —non-retained and bound–desorbed species. Commonly, the kinetics of desorption are slow, so that a broad second peak with severe tailing is observed[15]. Concentrating the desorbed species on a second more efficient column, as is done in ICA, can significantly improve the detection limits of an affinity analysis. Often the large differences in absorbance between the loading buffer and the desorbing agent result in a baseline disturbance (noise) when a step gradient is used for antigen desorption. This limits the amount of

antigen (signal) which can be quantitated. Analyses are restricted to high wavelengths (280 nm) and low detector gains.

Other baseline interferences, caused by non-specifically bound species eluting simultaneously with the antigen, are common in affinity chromatography. The use of a second column to separate the antigen from these interferences, as is done in ICA, enables much lower concentrations of antigen to be quantitated. Detection limits can also be increased by concentrating dilute samples on the immunoaffinity column using larger sample volumes. Although this may lead to higher non-specific binding, chromatographic conditions can be designed to separate the analyte from interfering species. In cases where information on non-specific binding to affinity supports is needed, the ICA system can be used to monitor this as discussed previously. Compared to fixed-volume immunoassays in non-flow-through systems, a much broader range of antigen concentrations may be quantitated by ICA.

The ICA system first was investigated using standard samples containing only one protein which was able to bind to the immobilized antibodies. Next, its utility in the analysis of samples containing several immunologically reactive proteins was examined. Chromatograms from the ICA of BSA, HLZM and HHb are illustrated in Figs. 1–3. The analyses of HLZM (Fig. 2) and HHb (Fig. 3) were recorded at 225 nm. These chromatograms show that the protein peaks are separated from the desorption buffer peaks at the beginning of the analyses, as well as from any interferences present in the blank (Fig. 2B). Without the use of the second RPC column, HLZM and HHb could not have been detected at these low levels. Since 400 ng of HLZM was analyzed at 0.1 a.u.f.s. it is estimated that detection limits could be further decreased at least ten-fold, to 40 ng, by exploiting the most sensitive range (0.005 a.u.f.s.) of the detector.

The ICA system may also be used for structural analysis. For example the chromatogram from the ICA of HHb in Fig. 3 illustrates that the alpha and beta chains of HHb are resolved. Hemoglobin mutations occurring in both chains have been identified by RPC[16,17]. Thus, ICA may be useful for the rapid screening of blood samples for Hb mutations.

The ability of ICA to discriminate between similar structural forms of a protein was investigated with several lysozyme variants. Although human and avian

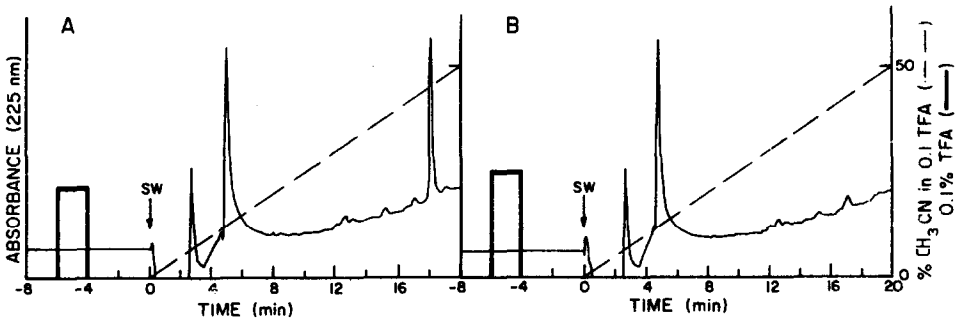

Fig. 2. ICA of HLZM. (A) HLZM, 400 ng; (B) blank. Affinity column: sheep anti-HLZM, covalently immobilized. RPC column: Synchropak RP-8 (25 × 0.46 cm I.D.). Event sequence: −6.0 min, affinity and RPC column in-line, inject 2 ml plug of 0.1% TFA (rectangular box); 0 min, affinity column switched (SW) off-line, RPC analysis started with a 20-min linear gradient from 0.1% TFA to 50% acetonitrile in 0.1% TFA at a flow-rate of 1 ml/min.

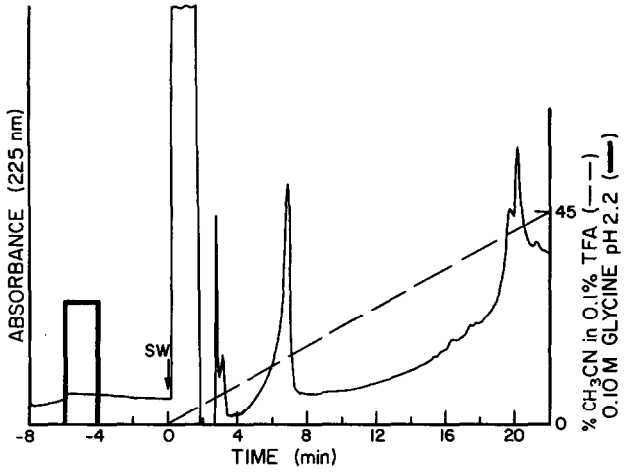

Fig. 3. ICA of HHb. Both α and β chains were resolved. Affinity column: rabbit anti-HHb, covalently immobilized. RPC column: Synchropak RP-8 (25 × 0.46 cm I.D.). Event sequence same as for Fig. 1. RPC was performed with a 22-min linear gradient from 0.1% TFA to 45% acetonitrile in 0.1% TFA at a flow-rate of 1 ml/min.

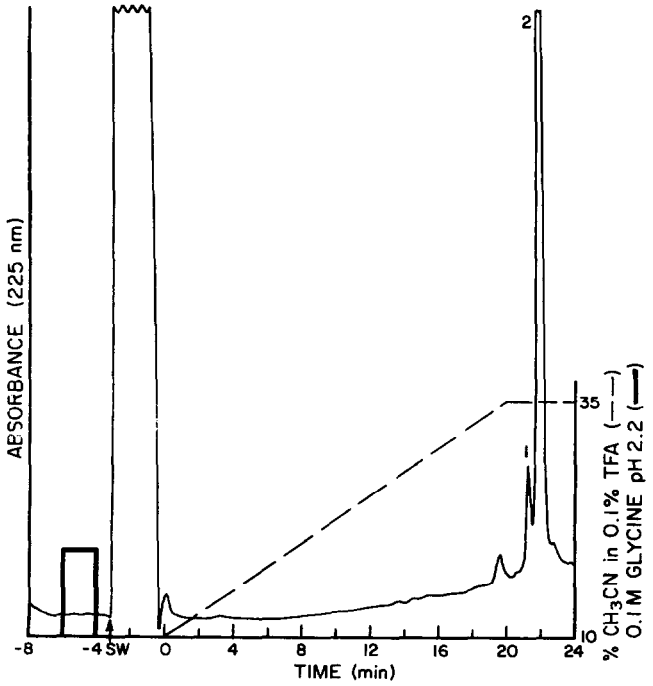

Fig. 4. ICA of lysozyme variants (1 = hen egg-white; 2 = human). Affinity column: sheep anti-HLZM, hydrophobically adsorbed. RPC column: Synchropak RP-8 (25 × 0.46 cm I.D.). Event sequence: −6.0 min, affinity and RPC column in-line, inject 2-ml plug of 0.10 *M* glycine (pH 2.2) (rectangular box); −3.0 min, affinity column switched (SW) off-line; 0.0 min RPC analysis started with a 20-min linear gradient from 10 to 50% acetonitrile in 0.1% TFA at a flow-rate of 1 ml/min.

lysozymes differ by more than 40% of their amino acids[18], Miller *et al.*[19] found that polyclonal rabbit anti-HLZM antibodies cross-reacted with several avian lysozymes. The immobilized polyclonal sheep anti-HLZM antibodies used in these studies bound both HLZM and HEWLZM. A chromatogram of the two variants desorbed from the immunoaffinity column and resolved by RPC is shown in Fig. 4. An ICA system using a covalently-immobilized monoclonal anti-HEWLZM antibody affinity column coupled to a strong-cation-exchange column, was able to discriminate between the avian lysozyme variants, ring-necked pheasant and duck A, B and C lysozymes (data not shown). The variants could be distinguished[3], but resolution was diminished due to denaturation of the lysozymes by the pH 2.2 glycine desorption buffer.

The utility of ICA in evaluating species of similar three-dimensional structure may be expanded to the simultaneous analysis of multiple antigens. Several antibodies immobilized on the same support should remove corresponding antigens from a sample for subsequent desorption and chromatographic separation on the second column.

CONCLUSIONS

The combination of immunological and chromatographic methods provides a sensitive technique for analysis of single and multicomponent polypeptide samples. We have shown that ICA is capable of discriminating between similar structural forms of a protein. This makes it potentially useful for the analysis of proteins produced by recombinant DNA technology, where contaminants arising from genetic aberrations and faulty processing are very similar in structure to the desired protein product.

An important attribute of an ICA system is its ease of implementation. Providing the antiserum is available, an immunoaffinity column can be prepared by hydrophobic adsorption *in situ* in less than 1 h. Modification of existing high-performance liquid chromatography instrumentation requires only a switching valve and an additional pump. The entire operation could be fully automated by the incorporation of an autosampler and data system. It is anticipated that the use of small columns and non-porous packing materials would reduce the chromatographic analysis time at least ten-fold[20,21].

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