*Journal of Chromatography*, 397 (1987) 197–206
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1125

# FRACTIONATION OF PLASMA PROTEINS BY PSEUDO-LIGAND AFFINITY CHROMATOGRAPHY

## ELUTION FROM IMMOBILIZED BROWN FRACTOGEL TSK-AF

ANNE E. HANAHAN, LAURENT MIRIBEL* and PHILIPPE ARNAUD*,**
*Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina, Charleston, SC 29425 (U.S.A.)*

SUMMARY

The fractionation of human plasma by chromatography on immobilized Brown Fractogel TSK-AF was analyzed by following the elution profile of 25 different plasma proteins. A three-step procedure was used to elute proteins from the column. First, a low-molarity buffer (30 m*M* sodium phosphate, pH 7.0, $I = 0.053$) was applied; then a linear salt gradient (0–1.0 *M* sodium chloride in the above buffer) was followed by an additional wash with four bed volumes of 1.0 *M* sodium chloride. Tightly bound proteins were finally stripped with 0.5 *M* ammonium thiocyanate. The elution profile of the proteins obtained by this procedure appears to be very reproducible. Comparison with the profiles obtained by chromatography on Cibacron Blue 3-GA and on Green TSK-AF indicates significant differences between the binding properties of the three gels.

## INTRODUCTION

Affinity chromatography on immobilized dyes continues to be an important method for the purification of a wide variety of enzymes and proteins[1–5]. One area of application, the fractionation of plasma proteins, has received extensive attention, especially in efforts to isolate several minor components of plasma[6–8]. In view of the major advantages of the technique (high capacity, high flow-rate, versatile elution conditions, recovery of apparently non-denatured proteins in high yields), and considering that several dyes with different structures are commercially available, it seemed logical to explore the possibility of realizing tandem chromatography, in which a protein would be separated from its contaminants by their differential affinity

* Present address: Laboratory of Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.
** Present address: Laboratory of Biochemistry, National Cancer Institute, Bldg. 37, Rm. 4C09, National Institutes of Health, Bethesda, MD 20892, U.S.A.

0021-9673/87/$03.50 

for dyes. Indeed, some major and other minor (trace) plasma components have been purified according to this procedure.

In the present article, we report the elution profile of plasma proteins obtained by chromatography on immobilized Brown Fractogel TSK-AF, which, by comparison with that obtained from Cibacron Blue 3-GA and Green TSK-AF, allows the design of several procedures for the purification of specific plasma proteins.

## MATERIALS AND METHODS

### *Materials*

Fractogel TSK-AF Brown (TSK-AF) (Procion Brown, covalently bound to a hydrophilic synthetic polymer) was kindly provided by Dr. G. Gunzer (E. Merck, Darmstadt, F.R.G.). Gel bond PAG films, Seaplaque, and Seakem HGT agarose were purchased from FMC (Marine Colloids Div., Rockland, ME, U.S.A.). Acrylamide, bis-acrylamide, and molecular weight standards were obtained from BioRad Labs. (Richmond, CA, U.S.A.). As carrier ampholytes we used LKB 4–6 and 5–7 from LKB Instruments (Gaithersburg, MD, U.S.A.). Poly- and monospecific antisera against plasma proteins were purchased from Dako (distributed by Accurate Chemicals and Scientific Co., Westbury, NY, U.S.A.), Calbiochem-Behring (La Jolla, CA, U.S.A.), Hyland Labs. (Travenol Lab., Costa Mesa, CA, U.S.A.), Meloy (Springfield, CA, U.S.A.), and Atlantic Antibodies (Scarborough, MA, U.S.A.). Antisera against lipoproteins were kindly provided by Dr. Maria Lopes-Virella (Medical University of South Carolina, Charleston, SC, U.S.A.).

### *Methods*

*Chromatography.* Blood samples were obtained from individual healthy donors (with informed consent), collected in citrate–soybean trypsin inhibitor (SBTI) as described by Harpel[9], and centrifuged at 1500 *g* at 4°C for 15 min. The plasma was dialyzed overnight at 4°C against 0.03 *M* sodium phosphate buffer (pH 7.0). TSK-AF (25 ml) was packed in a column, 20 × 1.6 cm, equipped with plungers. A volume of 4 ml of plasma was applied to the gel, previously equilibrated with five bed volumes of the above-mentioned buffer. The chromatographic experiments were performed at room temperature, and the $A_{280}$ of the column effluents was monitored; the flow-rate was 10 ml/h, and 1.25-ml fractions were collected at 4°C. After loading the sample, the column was washed with 155 ml of the buffer, then a linear salt gradient (0–1.0 *M* sodium chloride in the buffer, total volume 120 ml) was applied, using a Pharmacia GM 10 gradient mixer. After an additional wash with 60 ml of 1.0 *M* sodium chloride in the buffer, the tightly bound proteins were stripped off with 110 ml of 0.5 *M* ammonium thiocyanate in the buffer. The column was then re-equilibrated with five bed volumes of the starting buffer.

*Protein analysis.* Fused-rocket immunoelectrophoresis was performed by the method of Svendsen[10]. Two-dimensional electrophoresis was carried out as described previously[11] with certain modifications (see the legend of Fig. 4). Total protein concentration was measured by the method of Bradford[12] with bovine serum albumin as a standard. The measurement of the concentration of individual plasma proteins was performed by electroimmunoassay[13] or single-radial immunodiffusion[14] and values were compared with standards obtained from Calbiochem-Behring.

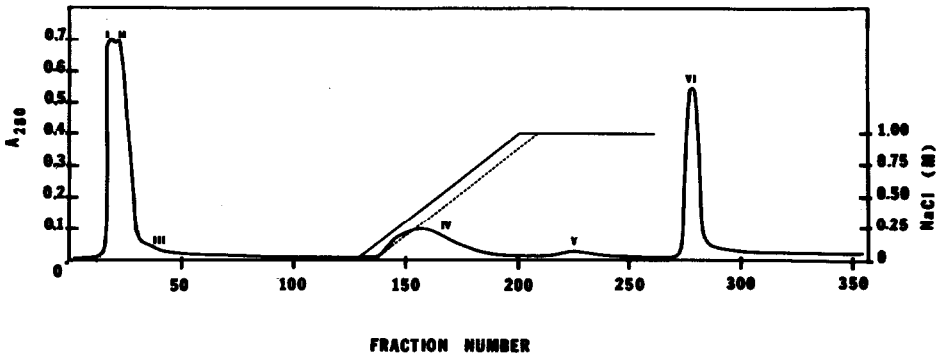


Fig. 1. Elution profile of plasma proteins from immobilized Brown TSK-AF. Plasma (4 ml) was chromatographed on a 25-ml column at room temperature. The equilibration buffer was 30 m*M* sodium phosphate (pH 7.0) and 1.25-ml fractions were collected. At fraction 126, a linear sodium chloride gradient (0–1.0 *M*, total volume 120 ml) was applied. The broken line indicates the development of the gradient, taking into account the void volume of the column. At fraction 203, the elution was continued with 1.0 *M* sodium chloride. At fraction 252, the column was stripped with 0.5 *M* ammonium thiocyanate.

## RESULTS*

Fig. 1 shows the elution profile of the plasma proteins, fractionated on TSK-AF, as measured by UV adsorbance. The location of 25 different proteins was determined by analysis of the eluted fractions by fused-rocket immunoelectrophoresis, using poly- and mono-specific antisera (Figs. 2 and 3; Table I), and by single-radial immunodiffusion for two of them (properdin and TBG).

Washing the column with the equilibration buffer led to an elution profile of three peaks (I, II, and III), the first two largely overlapping. Peak I contained mostly $A_1S$, $A_1AT$, Tf, TC, prealbumin, and Alb. Peak II contained Gc, together with 52% of the IgA and 67% of $A_2M$. The trailing part was represented by Peak III, containing mostly Hp (12%) and a part of the IgG, which were weakly retarded and overlapped with Peak II. Sixty-eight percent of the A1B and 72% of the HDL were detected in all the fractions, although their concentration was maximal in the first ones. Samples from each tube were pooled and analyzed by two-dimensional electrophoresis (Fig. 4B). To verify that this protein peak was not due, at least in part, to a saturation of the column, unbound proteins were pooled, concentrated and rechromatographed on the same column under the same conditions. Over 95% of the proteins appeared in the peak that was eluted with the equilibration buffer.

When a salt gradient was applied to the column, the elution of bound proteins began at a concentration of 0.04 *M* sodium chloride. First to be removed were a

* Abbreviations: Ig, immunoglobulin; $A_1S$, $\alpha_1$-acid glycoprotein; $A_1AT$, $\alpha_1$-antitrypsin; $A_1X$, $\alpha_1$-antichymotrypsin; $A_2M$, $\alpha_2$-macroglobulin; $B_2$ 1, $\beta_2$-glycoprotein 1; TBG, thyroxin-binding globulin; $A_1B$, $\alpha_1$-$\beta$-glycoprotein; TC, transcortin; Hp, haptoglobin; Alb, albumin; Hpx, hemopexin; Cp, ceruloplasmin; $AT_3$, antithrombin III; $C_3$ and $C_4$, third and fourth components of complement; RBP, retinol-binding protein; HDL, LDL and VLDL, high-density, low-density, and very-low-density lipoproteins; RID, single radial immunodiffusion; $M_r$, molecular-weight range; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis. The abbreviations for the protein names are according to Putnam[15].

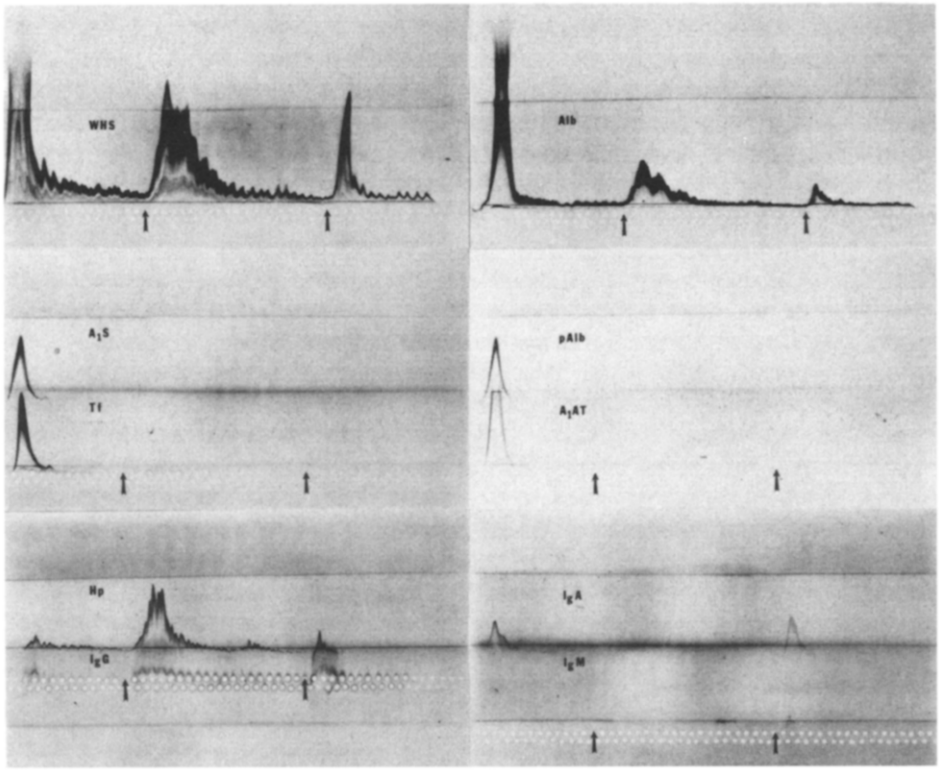


Fig. 2. Fused-rocket immunoelectrophoresis of fraction 8–83, 140–259, and 270–336, developed against poly- and mono-specific antisera. Gels were 1% agarose (2 mm thick) in 7.88 m*M* sodium barbital, 1.40 m*M* barbital, 93.67 m*M* glycine, 46.6 m*M* Tris (pH 8.6). Samples of 3 μl were applied to the wells. Antisera/agarose concentrations: serum protein (WHS), 18 μl/ml; $A_1S$, $A_1AT$, transferrin (Tf), pre-albumin (pAlb), 11 μl/ml; IgG and Hp, 10 μl/ml; IgA and IgM, 5 μl/ml; albumin (Alb), 20 μl/ml. The plates were electrophoresed for 3 h at 30 V/cm, then washed twice, dried, and stained with 0.5% Coomassie Blue R250 in ethanol–water–acetic acid (9:9:2, v/v/v). The anode is at the top. In each fused-rocket immunoelectrophoresis, the two arrows indicate the elution by the sodium chloride gradient (left) and by 0.5 *M* ammonium thiocyanate (right).

group of two high-molecular-weight ($M_r$) proteins, including Hp (83%) and IgG (Peak IV). Together with these two high-$M_r$ proteins, the majority of the Hpx (62%) was detected and the remaining Alb (24%), though eluted in all the salt fractions, presented an elution maximum corresponding to peak IV. Overlapping with peak IV, $A_1X$ was eluted next (starting at a sodium chloride molarity of 0.6 *M*). When the 1 *M* sodium chloride solution was applied to the column, peak V was obtained. The major components were small parts of the Hp, IgG, Hpx and $A_1X$. Properdin factor B (detected by RID) was found to be eluted in the salt gradient. Samples from each tube were pooled and analysed by two-dimensional electrophoresis (Fig. 4C).

The proteins still bound to the gel were removed by stripping the column with 0.5 *M* ammonium thiocyanate (peak VI). These included the remainder of Alb (8%), $A_2M$ (33%), HDL (28%), Hp (5%), Hpx (38%), IgA (48%), and the remaining part

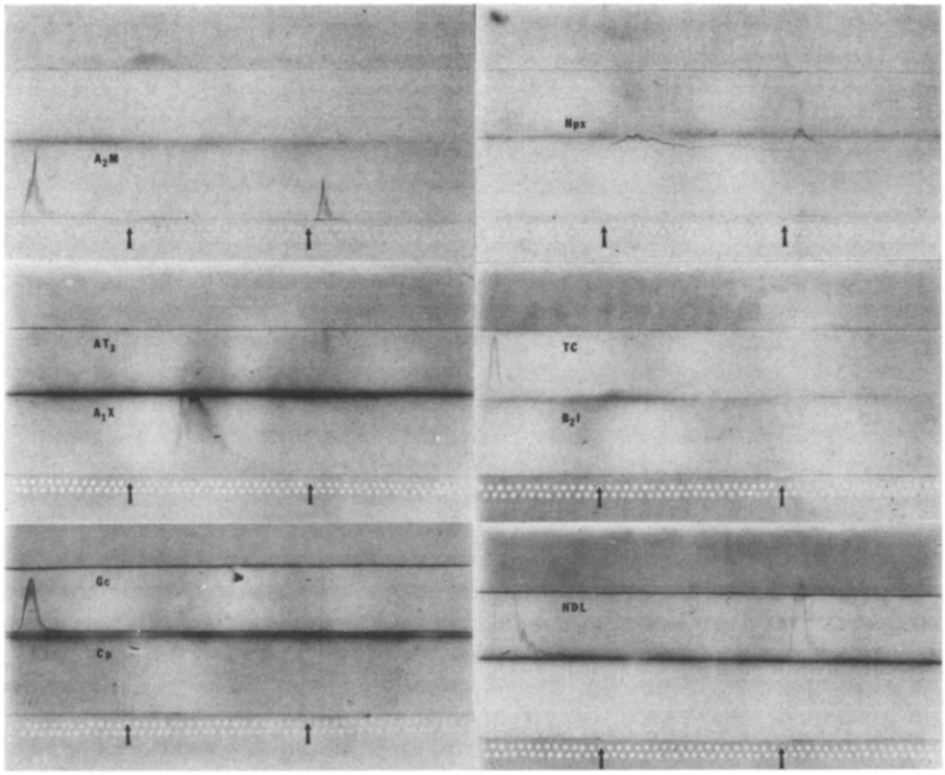


Fig. 3. Fused-rocket immunoelectrophoresis of fractions 8–83, 140–259, and 270–336 developed against mono-specific antisera. Experimental conditions were as in Fig. 2 (volume applied to the wells was 5 $\mu$l, except for $A_2M$, Gc and Cp: 3 $\mu$l). Antisera concentrations: group-specific component (Gc), ceruloplasmin (Cp) and $\alpha$-antichymotrypsin ($A_1X$), 10 $\mu$l/ml; antithrombin III ($AT_3$), 8 $\mu$l/ml; $\alpha_2$-macroglobulin ($A_2M$), 4 $\mu$l/ml; hemopexin (Hpx), 7 $\mu$l/ml; transcortin (TC), 6 $\mu$l/ml; $\beta_2$-glycoprotein 1 ($B_21$), 2 $\mu$l/ml; high density lipoproteins (HDL), 13 $\mu$l/ml.

of IgG. Cp, $AT_3$, RBP, $C_3$, $C_4$, IgM, LDL, and VLDL were also eluted in this peak. $B_2$l was found to be eluted later in the trailing part of peak VI. Samples from each tube were pooled and analysed by two-dimensional electrophoresis (Fig. 4D).

## DISCUSSION

Protein fractionation on immobilized TSK-AF presents several features of interest. These include easy regeneration of the gel, absence of obvious modification of its binding capacity with time, and apparent absence of protein alterations, as judged by the examination of the eluates by two-dimensional electrophoresis, which gave no evidence of modifications of the $M_r$ and/or p*I*. Also, since no saturation was observed, the approximate capacity of this gel can be evaluated between 0.10 and 0.16 ml of plasma per ml of gel.

In addition to these advantages, our results contribute to the empirical process of designing a suitable system for the purification of plasma proteins[1,7,16]. Indeed,

TABLE I

IDENTIFICATION OF 25 PLASMA PROTEINS FRACTIONATED ON IMMOBILIZED BROWN TSK-AF

All proteins were detected by fused-rocket immunoelectrophoresis[10] except for the last two, which were detected by single-radial immunodiffusion of the pools as indicated. When a protein was eluted in more than one peak, the percentage of each (shown in parenthesis) was calculated by electroimmunoassay (for albumin) or by integration of the areas of the immunoprecipitation peaks for the other proteins.

| *Protein* | *Fraction number* | |
|---|---|---|
| | *Range* | *Peak* |
| $\alpha_1$-Acid glycoprotein | 13– 35 | 18 |
| Transcortin | 14– 21 | 17 |
| $\alpha_1$-Antitrypsin | 15– 29 | 20 |
| Prealbumin | 14– 27 | 20 |
| Transferrin | 17– 37 | 20 |
| $\alpha_1$-$\beta$ Glycoprotein | 17– 62 | 20 |
| Group-specific component | 17– 32 | 22 |
| IgA | 17– 30 (52%) | 22 |
| | 276–286 (48%) | 279 |
| Albumin | 14– 59 (68%) | 23 |
| | 148–185 (24%) | |
| | 274–277 (8%) | 275 |
| Haptoglobin | 23– 62 (12%) | 27 |
| | 149–242 (83%) | 160 |
| | 274–285 (5%) | 276 |
| IgG | 20– 38 | 26 |
| | 146–242 | 161 |
| | 274–291 | 279 |
| HD lipoproteins | 15– 60 (72%) | 20 |
| | 273–294 (28%) | 279 |
| $\alpha_2$-Macroglobulin | 17– 32 (67%) | 23 |
| | 273–285 (33%) | 276 |
| Hemopexin | 146–242 (62%) | 164 |
| | 276–291 (38%) | 279 |
| $\alpha_1$-Antichymotrypsin | 170–218 | 189 |
| Ceruloplasmin | 276–288 | 279 |
| Antithrombin III | 276–280 | 278 |
| IgM | 276–280 | 277 |
| LD and VLD lipoproteins | 276–285 | 279 |
| Retinol-binding protein | 276–291 | 279 |
| Third component of complement | 276–291 | 279 |
| Fourth component of complement | 276–282 | 279 |
| $\beta_2$-Glycoprotein 1 | 297–309 | 303 |
| Properdin | Sodium chloride pool | |
| Thyroxine-binding protein | Unbound pool | |

comparison of the elution profiles of proteins between Cibacron Blue 3GA[7], Fractogel Green TSK-AF (results submitted elsewhere) and Fractogel Brown TSK-AF (Table II) can led to a "tandem chromatography" system. This system involves both "negative" and "positive" chromatography steps[17] and provides a useful separation method for certain proteins having different affinities for the three gels, for instance $AT_3$, TBG, $C_3$ and $C_4$. Another example is represented by Cp, which binds more or

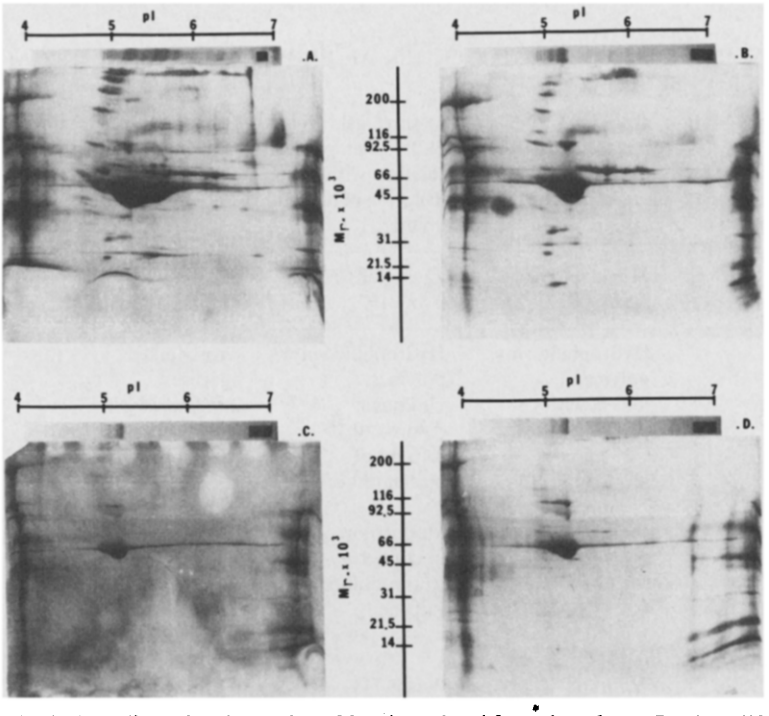


Fig. 4. Two-dimensional mapping of fractions eluted from the column. Portions (10 $\mu$l) were pooled from the following fractions: (B) fractions from the wash by the equilibrium buffer; (C) fractions from the salt gradient and plateau; (D) fractions from the ammonium thiocyanate peak. (A) represents the 2D-mapping of a dilution of the starting plasma sample. All the samples were previously dialyzed against a buffer containing 1% (w/v) glycine. Isoelectric focusing was performed on gel bond PAG sheets in 0.2-mm thick polyacrylamide gels ($T_{30}C_4$, 15% v/v), containing 6.5% (v/v) LKB Ampholines (pH range 4.0–7.0) and 6 *M* urea. Total protein amount deposited was for (A) 60 $\mu$g, for (B) 16 $\mu$g, for (C) 11 $\mu$g, for (D) 9 $\mu$g. A constant power of 3 W was applied for 3.5 h, with maximum voltage at 2000 V. After electrophoresis, the tracks were equilibrated for 5 min at room temperature with 0.025 *M* Tris–0.192 *M* glycine, containing 0.1% SDS (w/v), (pH 8.20) (electrode buffer) and then transferred to the top of the vertical SDS-polyacrylamide gel. The separating gel was a 5% (top) to 20% (bottom) of a $T_{40}C_{5.4}$ acrylamide stock solution in a 1.5 *M* Tris/0.4% SDS (w/v) (pH 8.8) buffer. The gel thickness was 0.75 mm. Two 10 × 5 mm rectangles of filter paper (LKB sample applicators) were impregnated with 3.5 $\mu$l of a 1:10 dilution of Bio-Rad $M_r$ standards. The IEF strips and $M_r$ standards were sealed in 1% low-gelling-temperature agarose (Seaplaque, FMC) in 0.5 *M* Tris–0.5% SDS (w/v) (pH 6.8) buffer. After solidification of the agarose, the gels were electrophoresed at 30 mA/gel. The electrophoresis was stopped 30 min after the dye (Phenol Red, diluted in the electrode buffer at the cathode — top of the gel) had reached the bottom of the gel. The gels were fixed in methanol–water (3:7, v/v) containing 11.4% (w/v) trichloroacetic acid and 3.4% (w/v) sulphosalicylic acid. IEF gels were stained with 0.2% Coomassie Blue and SDS-PAGE with silver. $M_r$ standards were myosin, $\beta$-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

less tightly to the Brown and Green gels, whereas its usual contaminant (Gc) does not bind to any of the three gels. $A_1B$ does not bind to the Brown gel, whereas it does to the Blue gel. Sixty-eight percent of the Alb on the Brown gel does not bind at all, whereas more than 90% binds tightly to the Cibacron Blue 3-GA. The slight changes in the structure which differentiate the gels could explain the difference in behavior.

TABLE II

MAJOR DIFFERENCES BETWEEN FRACTOGEL TSK-AF BROWN, FRACTOGEL TSK-AF GREEN AND CIBACRON BLUE 3-GA

The procedures of fractionation of plasma proteins on the three gels involved the same chromatographic parameters: temperature, ionic strength and pH of the buffers, sample loading. "Wash" corresponds to the elution with the equilibration buffer; "Salt" to the elution with a linear gradient 0–1.0 *M* sodium chloride; plateau wash of the column with 1.0 *M* sodium chloride in this buffer, and "Thiocyanate" to the elution with ammonium Thiocyanate 0.5 *M* in the same buffer.

| | *Fractogel Brown TSK-AF* | *Fractogel Green TSK-AF** | *Affi-gel Blue (Cibacron 3-GA)*** |
|---|---|---|---|
| Gel matrix | Hydrophilic vinyl polymer | Hydrophilic vinyl polymer | Cross-linked agarose |
| Ligand concentration | Unknown | Unknown | 2 mg/ml of gel |
| Gel capacity | Approx. 0.10–0.16 ml of plasma/ml of gel | Approx. 0.10–0.16 ml of plasma/ml of gel | 0.22–0.34 ml of plasma/ml of gel or 12–18 mg of albumin/ml of gel |
| Structure | Monochloro triazine dye | Monochloro triazine dye | Monochloro triazine dye |
| Chromophore class | Metal complexes (1 $Cr^{2+}$) | Anthraquinone-stilbene | Anthraquinone |
| Major differences in the protein elution profile: | | | |
| Albumin | Wash: 68%<br>Salt: 24%<br>Thiocyanate: 8% | Wash: 74%<br>Salt: 20%<br>Thiocyanate: 6% | Wash: traces<br>Salt: < 10%<br>Thiocyanate: > 90% |
| Ceruloplasmin | Thiocyanate | Salt | Wash |
| Hemopexin | Salt (62%) | Thiocyanate | Salt |
| Retinol-binding protein | Thiocyanate | Thiocyanate | Salt |
| $\alpha_1$-$\beta$ Glycoprotein | Wash | | Salt |
| Antithrombin III | Thiocyanate | Salt | Salt |
| Thyroxine-binding globulin | Wash | Salt | Salt |
| $\alpha_2$-Macroglobulin | Wash (67%) | Wash (70%) | Salt |
| Third component of complement | Thiocyanate | Salt | Salt |
| Fourth component of complement | Thiocyanate | Thiocyanate | Salt |
| IgM | Thiocyanate | Thiocyanate | Salt |

* Detailed results submitted elsewhere.
** From ref. 7.

The exact mechanism by which proteins and enzymes are fractionated by chromatography on Brown Fractogel TSK-AF is still unclear. Brown dyes are generally metal complexes of 0,0'-dihydroxyazo or 0-carboxy-0'-hydrazo dyes[2,5]. Unfortunately, the exact chemical structures of the majority of reactive dyes are secret due to existing patent rights of the main dye suppliers (ICI and CIBA). This represents a serious disadvantage, reducing the studies on dye protein interactions to the hypothesis level. Considering the purification systems described by Bruton and Atkinson[16] for the purification of a tryptophanyl-tRNA synthetase[18], Atkinson *et al.*[19] suggested that this brown dye could exhibit some structural resemblance to aromatic

amino acids. Since the dye contains a $Cr^{2+}$, four aromatic groups, one sulphonated group and one carboxyl groups[16], there are also possibilities for hydrophobic and electrostatic interactions with proteins[8,20]. Our results show that some proteins, like Hp, $A_2M$ and Hpx, are eluted in an apparently heterogeneous fashion. The possibility that partial saturation of the column could lead to an artefactual fractionation of the proteins was ruled out by a second run of the unbound proteins (see above). The ability of TSK-AF to generate multiple peaks from these proteins that had appeared as homogeneous peaks on Cibacron Blue 3-GA, is one of the most significant features of this gel. There is some controversy as to whether this heterogeneity is due to different variants of the protein, as already observed with Gc[21,22] or IgA[7] on Cibacron Blue 3-GA, or to the heterogeneity of the triazine dye itself[23,24], although this latter hypothesis is unlikely, according to Clonis *et al.*[25]. Our results also show that the elution of IgG and Hp tends to be identical qualitatively and quantitatively. Considering the dye–protein interactions, this similar behaviour could be in some way related to the apparent homology in the secondary structure between the two proteins, as reported by Putnam[26].

Another advantage of the studies performed here is that by comparing fractionation of plasma proteins by affinity chromatography on different immobilized dyes, with greater availability of the chemical structure of the dyes and with the beginnings of the elucidation of protein dye binding mechanisms, it will be possible to determine which structure(s) are responsible for the specific binding of a protein or a group of proteins. This information would consequently simplify the elution process. Up to now, the use of these dye-ligands is, and probably will continue to be, on a highly empirical basis.

## ACKNOWLEDGEMENTS

Publication No. 826 from the Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina. Supported in part by grants from the French Foreign Office, from the Philippe Foundation, Inc., from the American Medical Association, Education and Research Foundation, from S.C. General and Faculty Research Appropriations no. 22300-A-091 and CR-11, and from Labcatal Laboratories, Montrouge, France. L. Miribel was a postdoctoral fellow of the College of Graduate Studies and University Research, Medical University of South Carolina. We thank Dr. Maria Lopes-Virella, Department of Medicine, Medical University of South Carolina and Veterans Administration Medical Center, for supplying antisera to lipoproteins.

## REFERENCES

1 P. D. G. Dean and D. H. Watson, *J. Chromatogr.*, 165 (1979) 301.
2 C. R. Lowe, D. A. P. Small and A. Atkinson, *Int. J. Biochem.*, 13 (1981) 33.
3 S. Subramanian, in G. D. Fasman (Editor), *Critical Reviews in Biochemistry*, CRC Press, Boca Raton, U.S.A., Vol. 16, 1984, p. 169.
4 I. Lascu, H. Porumb, T. Porumb, I. Abrudan, C. Tarmure, I. Petrescu, E. Presecan, I. Proinov and M. Telia, *J. Chromatogr.*, 283 (1984) 199.
5 C. R. Lowe and J. C. Pearson, in W. B. Jakoby (Editor), *Methods in Enzymology: Enzyme Purification and Related Techniques*, Part C, Academic Press, New York, 1984, p. 97.

6 S. Angal and P. D. G. Dean, *FEBS Lett.*, 96(2) (1978) 346.
7 E. Gianazza and P. Arnaud, *Biochem. J.*, 201 (1982) 129.
8 E. Gianazza and P. Arnaud, *Biochem. J.*, 203 (1982) 637.
9 P. E. Harpel, *J. Exp. Med.*, 138 (1973) 508.
10 P. J. Svendsen, *Scand. J. Immunol.*, Suppl. 1 (1973) 69.
11 M. Petrini, R. M. Galbraith, D. L. Emerson, A. E. Nel and P. Arnaud, *J. Biol. Chem.*, 260(3) (1985) 1804.
12 M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
13 C. B. Laurell, *Anal. Biochem.*, 15 (1966) 45.
14 G. Mancini, A. O. Carbonara and J. F. Heremans, *Immunochemistry*, 2 (1965) 235.
15 F. W. Putnam, in F. W. Putnam (Editor), *The Plasma Proteins*, Vol. 4, Academic Press, New York, 2nd ed., 1984, p. 1.
16 C. J. Bruton and T. Atkinson, *Nucleic Acids Res.*, 7 (1979) 1579.
17 Y. Hey and P. D. G. Dean, *Chem. Ind.*, 20 (1981) 726.
18 A. R. Ashton and G. Polya, *Biochem. J.*, 175 (1978) 501.
19 T. Atkinson, P. M. Hammond, R. D. Hartwell, P. Hughes, M. D. Scawen, R. F. Sherwood, D. A. P. Small, C. J. Bruton, M. J. Harvey and C. R. Lowe, *Biochem. Soc. Trans.*, 9 (1981) 290.
20 J. R. Slemmon, P. M. Salvaterra, G. D. Crawford and E. Roberts, *J. Biol. Chem.*, 257(7) (1982) 3847.
21 C. Chapuis-Cellier, E. Gianazza and P. Arnaud, *Biochim. Biophys. Acta*, 709 (1982) 353.
22 E. Gianazza, D. L. Emerson, D. Dykes and P. Arnaud, *Biochem. J.*, 218 (1984) 969.
23 K. G. McFarthing, S. Angal and P. D. G. Dean, *Anal. Biochem.*, 122 (1982) 186.
24 D. Hanggi and P. Carr, *Anal. Biochem.*, 149 (1985) 91.
25 Y. D. Clonis, M. J. Goldfinch and C. R. Lowe, *Biochem. J.*, 197 (1981) 203.
26 F. W. Putnam, in F. W. Putnam (Editor), *The Plasma Proteins*, Vol. 4, Academic Press, New York, 2nd ed., 1984, p. 92.