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APPLICATION OF DYE–LIGAND CHROMATOGRAPHY TO THE ISOLATION OF α-1-PROTEINASE INHIBITOR AND α-1-ACID GLYCOPROTEIN

G. BIRKENMEIER and G. KOPPERSCHLÄGER*

Institute of Physiological Chemistry, Karl-Marx-University Leipzig. DDR-7010 Leipzig (G.D.R.)

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SUMMARY

Various Cibacron blue F3G-A substituted Sephadex G-100 gels which differ in the density of the bound dye were investigated for their applicability in the affinity chromatography of human serum proteins. Protein adsorption was found to depend strongly on the density of the covalently attached dye and on the pH of the applied buffer.

A high degree of dye substitution of the gel caused binding of most of the serum proteins. Only a small number of proteins were found to appear in the breakthrough fraction. On this basis a simple and relatively mild procedure for the isolation of homogeneous α-1-proteinase inhibitor and α-1-acid glycoprotein from human serum was developed. Isolation of α-1-acid glycoprotein succeeded in only a single step, whereas that of α-1-proteinase inhibitor required two additional steps: a prior ammonium sulphate precipitation and a chromatographic step on DEAE-cellulose at pH 6.5.

INTRODUCTION

Immobilized Cibacron blue F3G-A is frequently used as a powerful tool for the affinity chromatography of dehydrogenases and kinases[1–3]. The binding of these enzymes to dye matrix conjugates is regarded as a specific interaction of the dye with a typical structural element in these enzymes, designated as "dinucleotide fold"[4]. In addition to these groups of enzymes, other proteins apparently not having this fold in their molecules[5,6] have also been shown to bind to immobilized Cibacron blue F3G-A.

Travis and Pannell[7] originally reported the interaction of human serum albumin with this dye and developed an efficient procedure for the removal of albumin from other serum proteins. Since then, dye-ligand chromatography has been applied to the purification of various serum proteins[8–10].

The strong interaction of human albumin with Cibacron blue F3G-A is apparently due to the binding of the dye to the bilirubin binding site of albumin[11]. Other plasma proteins have also been found to interact with Cibacron blue F3G-A. These

interactions depend strongly on the pH[12], on the chemical properties of the matrix[13] and on the mode of covalent attachment of the dye to the insoluble support[14].

This paper deals with the influence of the loading density of the gel and of pH on the binding of human serum proteins to immobilized Cibacron blue. By taking advantage of these interactions simple procedures for the isolation of pure α-1-proteinase inhibitor and of α-1-acid glycoprotein are presented.

EXPERIMENTAL

Chemicals

Cyanogum 41, agarose, Coomassie Brilliant Blue G-250 and DEAE-cellulose were purchased from Serva (Heidelberg, G.F.R.). Cibacron blue F3G-A was obtained from Ciba AG (Basle, Switzerland) and the chemicals for the immunological methods from Behringwerke (Marburg, G.F.R.). Bovine trypsin and cytochrome c were purchased from Boehringer (Mannheim, G.F.R.). Benzoylarginine-p-nitroanilide (BAPNA) was obtained from Ferak (Berlin, G.F.R.) and Sephadex G-100 from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical-reagent grade. Human serum was a gift from the blood bank of the Medical Centre of the Karl-Marx-University (Leipzig, G.D.R.).

Coupling of Cibacron blue F3G-A to Sephadex G-100

Covalent coupling of Cibacron blue F3G-A to Sephadex G-100 was effected by an ether link between the triazine ring and the polysaccharide matrix[3]. For preparation of gels with different degrees of substitution, different amounts of Cibacron blue F3G-A were applied in the range between 0.006 and 0.85 g per gram of dried Sephadex G-100. For maximal dye substitution the reaction temperature was kept constant at 80°C.

Determination of Cibacron blue substitution

The determination of the amount of dye covalently bound to the matrix was carried out according to Chambers[15]. The gel was dried by washing with methanol and kept in a vacuum desiccator until the weight became constant.

Preparative column chromatography with Blue Sephadex

The dye-Sephadex conjugate (Blue Sephadex) was equilibrated with 10 mM sodium phosphate solution (pH 5.8 or 6.8) in a column (50 × 5 cm) at 4°C. In the case of α-1-proteinase inhibitor, solid ammonium sulphate was added to fresh human serum until 50 % saturation was reached. The supernatant of the resulting precipitate was exhaustively dialysed against equilibration buffer. Then a small amount of precipitate was removed by centrifugation and the supernatant was applied to the column. For preparation of α-1-acid glycoprotein the salt precipitation could be omitted. The flow-rate through the column was adjusted to 50 ml/h and fractions of 15 ml were collected. Unless mentioned otherwise, the pooled fractions were concentrated by employing an Amicon UM-10 membrane filter and dialysed against phosphate buffer (50 mM, pH 7.0) or against the equilibration buffer as applied in the subsequent chromatographic step.

For regeneration, the column containing the Cibacron blue–Sephadex con-

jugate was washed with 10 mM sodium hydroxide solution and finally with distilled water. The gel was stored at 4°C in 3 M ammonium sulphate solution containing 0.01% of sodium azide.

DEAE-cellulose chromatography

After chromatography on Blue Sephadex the fractions containing the α-1-proteinase inhibitor were applied to a column (20 × 1.5 cm) of DEAE-cellulose equilibrated with 5 mM sodium phosphate buffer (pH 6.5) containing 50 mM sodium chloride. The column was washed with two volumes of this buffer and then a linear gradient from 50 to 200 mM sodium chloride in 5 mM sodium phosphate (pH 6.5) was employed. The protein content of the effluent was monitored at 280 nm and the antitryptic activity was measured. The active fractions were pooled and concentrated. The preparation was either subjected to further tests or kept precipitated at 4°C.

Electrophoresis

Polyacrylamide gel electrophoresis was carried out in 8 × 0.5 cm tubes by using a linear gradient of acrylamide (3–15%)[16]. The electrophoretic runs were performed at 3 mA per tube and room temperature. For staining, the method of Diezel et al.[17] was applied.

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis was carried out in 7.0% gels by application of the technique of Weber and Osborn[18]. For molecular weight determinations, cross-linked cytochrome c polymers served as a standard. The proteins were denatured in 1% SDS solution containing 1% of 2-mercaptoethanol at 100°C for 5 min and then loaded onto the gels. The runs were carried out for 4 h at 8 mA per tube.

Assay of antitryptic activity

The inhibitory power of the individual fractions on trypsin was measured according to Eriksson[19] by using crystallized, salt-free trypsin in 100 mM Tris–hydrochloric acid buffer (pH 7.8) containing 5 mM calcium chloride. After pre-incubation of 25 µg of trypsin with the samples for 15 min at 25°C, the residual activity was measured by addition of BAPNA dissolved in dimethylformamide. The release of p-nitroaniline at 405 nm was monitored.

Immunological methods

Immunodiffusion was carried out according to Ouchterlony[20] in 100 mM sodium phosphate buffer (pH 7.5) and 1.5% agarose. The purity of the antigen preparation was checked by immunoelectrophoresis[21]. The concentration of α-1-acid glycoprotein was determined by the method of Mancini et al.[22] on M-Partigen plates using standard serum.

Ultracentrifugation

Sedimentation velocity experiments were performed with a Phywe Model U 60 L analytical ultracentrifuge by following the Schlieren pattern. The resulting apparent s-values obtained with five different protein concentrations were corrected to water and infinite dilution at 20°C.

The determination of protein was performed according to the method of Janatova et al.[23] using bovine serum albumin as standard.

RESULTS

Variation of the degree of dye substitution

The influence of the degree of dye substitution of the matrix on the binding of serum proteins was investigated at pH 7.0 by employing three types of Blue Sephadex: low-substituted, medium-substituted and high-substituted gels.

With low-substituted gels the serum proteins are separated into two fractions: the breakthrough fraction and a fraction of retarded proteins which can be eluted by washing the column with 1 M sodium chloride solution (Fig. 1A).

Medium-substituted gel (Fig. 1B) shows a shift in protein distribution toward the retarded fractions, whereas high-substituted gels are capable of adsorbing most of the serum proteins (Fig. 1C). The electrophoretic analysis of the eluted fractions
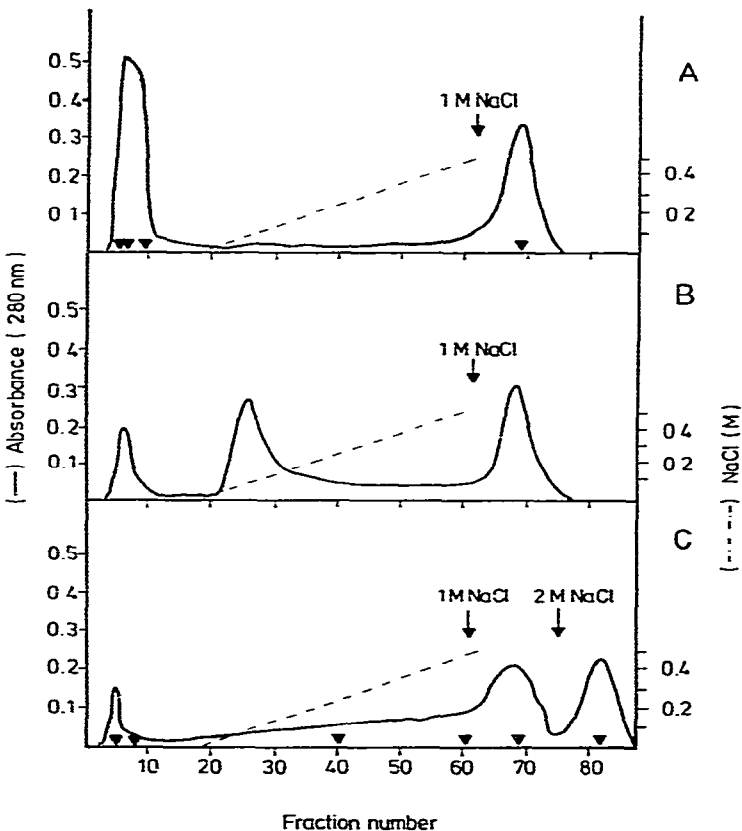

Fig. 1. Elution diagrams for human serum proteins chromatographed at pH 7.0 on Cibacron blue F3G-A–Sephadex G-100 gels with different degrees of dye substitution. (A) 4 μg of dye per mg of Sephadex; (B) 73 μg of dye per mg of Sephadex; (C) 214 μg of dye per mg of Sephadex. A 2-ml volume of fresh human serum was dialysed against 10 mM sodium phosphate buffer (pH 7.0) and then applied to a column (40 × 1.5 cm) previously equilibrated with the same buffer. After removing the unbound protein fraction, elution was started by applying a gradient of 0–0.5 M sodium chloride in 10 mM sodium phosphate buffer (pH 7.0). Finally, the column was washed with 1 and 2 M sodium chloride solution. The flow-rate was 12 ml/h and 2-ml fractions were collected. The black triangles indicate the fractions subjected to polyacrylamide gel electrophoresis presented in Fig. 2.

showed that at low dye substitution only albumin is bound, whereas in the breakthrough fraction most of the other serum proteins appear. An asymmetric protein distribution in this fraction indicates weak interactions occurring between some protein components and the gel (Fig. 2A). With the high-substituted gels the breakthrough fraction contains only four proteins, which could be identified immunologically as α-2-macroglobulin, α-1-proteinase inhibitor, α-1-acid glycoprotein and prealbumin (Figs. 1C and 2B).

Apparently, α-1-acid glycoprotein does not exert any interaction with the immobilized Cibacron blue F3G-A. This protein is found in the leading edge of the wash-out fraction and so can be partially separated from the other serum proteins.
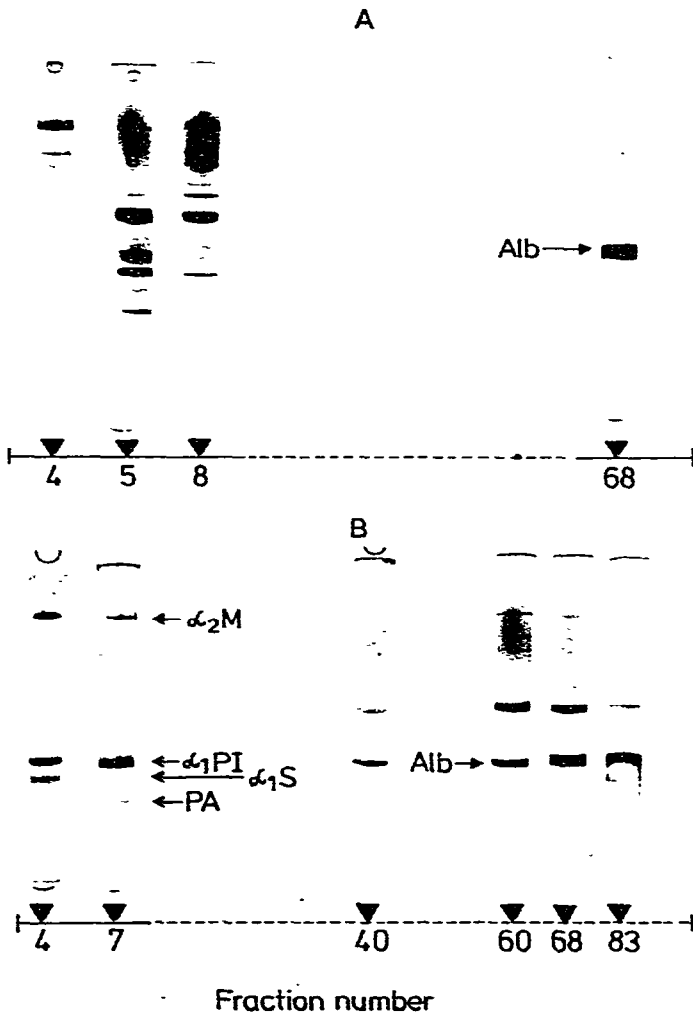

Fraction number

Fig. 2. Polyacrylamide gel electrophoresis of selected fractions from (A) Fig. 1A and (B) Fig. 1C. Each gel was loaded with 50–100 μl of the respective eluate. The numbers correspond to the fraction numbers in Fig. 1A and C. Symbols: Alb = albumin; α_2M = α-2-macroglobulin; α_1PI = α-1-proteinase inhibitor; α_1S = α-1-acid glycoprotein; PA = prealbumin.

The subsequently eluted protein component was identified as α-1-proteinase inhibitor. Because the electrophoretic mobility of this protein in alkaline polyacrylamide gel electrophoresis is similar to that of albumin, immunodiffusion tests using monospecific antisera against α-1-proteinase inhibitor and albumin had to clarify whether this fraction is contaminated by albumin or not. Clear evidence of the absence of albumin from this fraction was obtained.

Attempts were made to fractionate the bound proteins, using a sodium chloride gradient from 0 to 0.5 M. However, all of the experiments were unsuccessful.

At pH 6.0 the affinity of serum proteins for the substituted gels increases considerably. With high-substituted gels, only α-1-acid glycoprotein and a very small amount of α-2-macroglobulin constitute the breakthrough fraction, whereas all of the other proteins are adsorbed, including α-1-proteinase inhibitor and prealbumin (not shown). On the basis of these results simple methods for the purification of α-1-proteinase inhibitor and α-1-acid glycoprotein were developed.

Isolation of α-1-proteinase inhibitor

As mentioned above, high-substituted Sephadex G-100 binds at pH 7.0 all of the serum proteins except α-1-proteinase inhibitor, α-1-acid glycoprotein, α-2-macroglobulin and prealbumin. When a Blue Sephadex column (loading density 170 μg dye per milligram of dried gel) at pH 6.8 is charged with the supernatant resulting from ammonium sulphate precipitation of serum at 50 % saturation, the breakthrough fraction differs from that of the whole serum. A typical example is shown in Fig. 3.
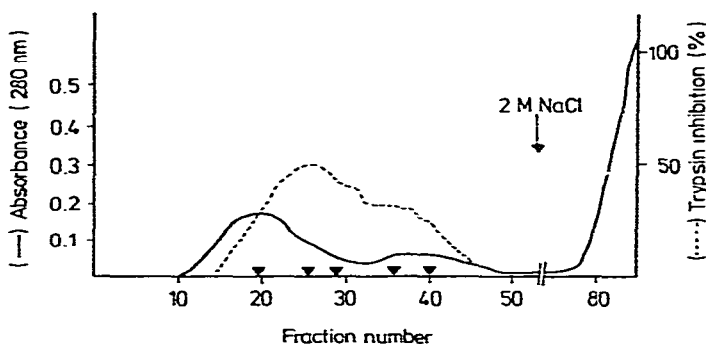

Fig. 3. Chromatography of human serum on Cibacron blue F3G-A–Sephadex G-100 at pH 6.8. A 30-ml volume of human serum was treated with ammonium sulphate at 50 % saturation and then centrifuged. The supernatant was dialysed against 10 mM sodium phosphate buffer (pH 6.8) and charged on to a gel column (50 × 5 cm) previously equilibrated with the same buffer. The unbound protein fraction was eluted with equilibration buffer, then the bound proteins were removed by applying 2 M sodium chloride solution. Samples 10–24 (I) and 25–50 (II) were pooled. The black triangles indicate the fractions subjected to polyacrylamide gel electrophoresis as presented in Fig. 4.

Some asymmetry in the diagram is observable, caused by slight retardation of the α-1-proteinase inhibitor and prealbumin in comparison with the α-1-acid glycoprotein. α-2-Macroglobulin is lacking in the breakthrough fraction. The pattern of trypsin inhibition coincides with the protein bands of α-1-proteinase inhibitor, as demonstrated in gel electrophoresis (Fig. 4).
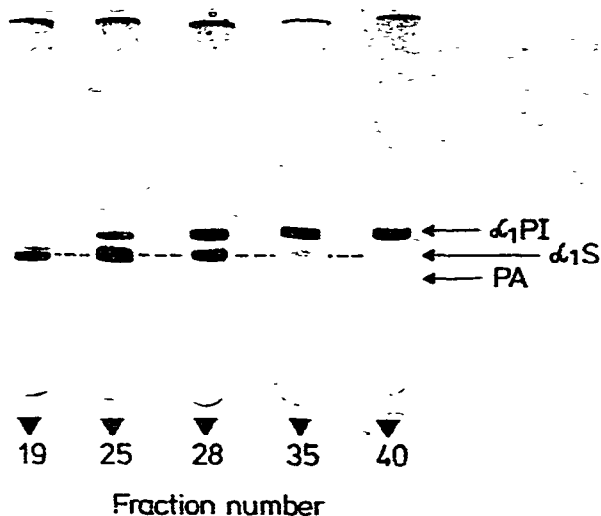
Fig. 4. Polyacrylamide gel electrophoresis of selected fractions from Fig. 3. Volumes of 50 μl (fraction 19) and 100 μl (fractions 25, 28, 35 and 40) of the samples were subjected to electrophoresis.

The partial separation of α-1-proteinase inhibitor from α-1-acid glycoprotein permits partition of the breakthrough fraction into two pools (I and II), one containing the major part of α-1-acid glycoprotein (pool I) and the other that of α-1-proteinase inhibitor (pool II).

The fractions collected in pool II were subjected to DEAE-cellulose chromatography at pH 6.5 according to Pannell et al.[24] (see Experimental). By this procedure a complete separation of α-1-proteinase inhibitor from α-1-acid glycoprotein and pre-albumin could be achieved. Polyacrylamide gel electrophoresis of the isolated α-1-proteinase inhibitor yields in general a single band. Sometimes two bands moving closely together can be observed, and both were identified as α-1-proteinase inhibitor (Fig. 5A). Apparently, this is due to the polymorphism of this protein, which can more distinctly be shown by isoelectric focusing. The homogeneity of the isolated α-1-proteinase inhibitor can unequivocally be demonstrated by means of SDS–polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol (Fig. 5B) and immunoelectrophoresis (Fig. 6A). By SDS electrophoresis a single band is obtained. The comparison with the electrophoretic mobilities of cross-linked cytochrome c polymers permitted the molecular weight of the inhibitor to be calculated to be 52,500.

Immunoelectrophoresis yielded a single precipitation arc when tested against anti-whole human serum and monospecific anti-human α-1-proteinase inhibitor. No precipitation was detected with antisera against human α-1-acid glycoprotein, human serum albumin, human α-1-lipoprotein and human Gc-globulin.

In the analytical ultracentrifugation in 50 mM sodium phosphate buffer (pH 7.0), α-1-proteinase inhibitor showed a single symmetrical peak with a sedimentation coefficient of $s_{20,w}^0 = 3.5$ S. This value is in close agreement with the sedimentation coefficient of 3.6 S published by Pannell et al.[24].
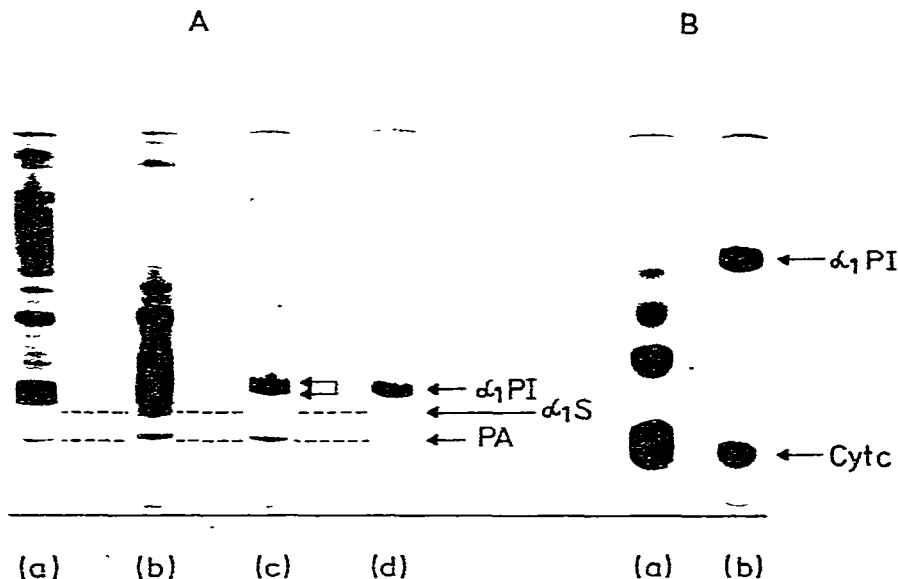
Fig. 5. (A) Polyacrylamide gel electrophoresis of samples containing α-1-proteinase inhibitor. (a) Whole human serum (400 μg): (b) supernatant after ammonium sulphate precipitation (300 μg): (c) α-1-proteinase inhibitor after ammonium sulphate precipitation and Blue Sephadex chromatography (40 μg): (d) α-1-proteinase inhibitor after ammonium sulphate precipitation, Blue Sephadex chromatography and DEAE-cellulose chromatography (30 μg). The black arrows indicate the two bands of α-1-proteinase inhibitor (for explanation see text). (B) SDS-electrophoresis of purified α-1-proteinase inhibitor. (a) Cytochrome c (Cyt c) oligomers (40 μg): (b) α-1-proteinase inhibitor (30 μg).

The yield of α-1-proteinase inhibitor obtained by ammonium sulphate precipitation at 50 % (first step), dye-ligand chromatography (second step) and DEAE-cellulose chromatography (third step) is about 30 % relative to the total antitryptic activity of the serum. From 30 ml of serum 8.3 mg of purified human α-1-proteinase inhibitor could be obtained.

Isolation of α-1-acid glycoprotein

When dialysed serum without prior ammonium sulphate precipitation is applied to a Cibacronblue Sephadex column (loading density 170 μg of dye per milligram of dried gel) at pH 5.8 the only constituent of the breakthrough fraction is α-1-acid glycoprotein (Fig. 7). After concentrating this fraction on an Amicon UM-10 membrane filter and dialysis against 50 mM sodium phosphate buffer of pH 7.0, 75–80 % of the total serum α-1-acid glycoprotein is recovered. Hence, 100 ml of human serum yield 40–45 mg of α-1-acid glycoprotein from a total concentration of 55 mg per 100 ml.

In alkaline polyacrylamide gel electrophoresis (Fig. 8A) and SDS-polyacrylamide gel electrophoresis (Fig. 8B) the preparation shows a single protein band. By SDS electrophoresis a molecular weight of 42,500 was calculated.

Analytical ultracentrifugation of the α-1-acid glycoprotein in 50 mM sodium phosphate buffer at pH 6.0 showed a single symmetrical peak having a sedimentation coefficient of $s_{20,w}^{0} = 3.27$ S.
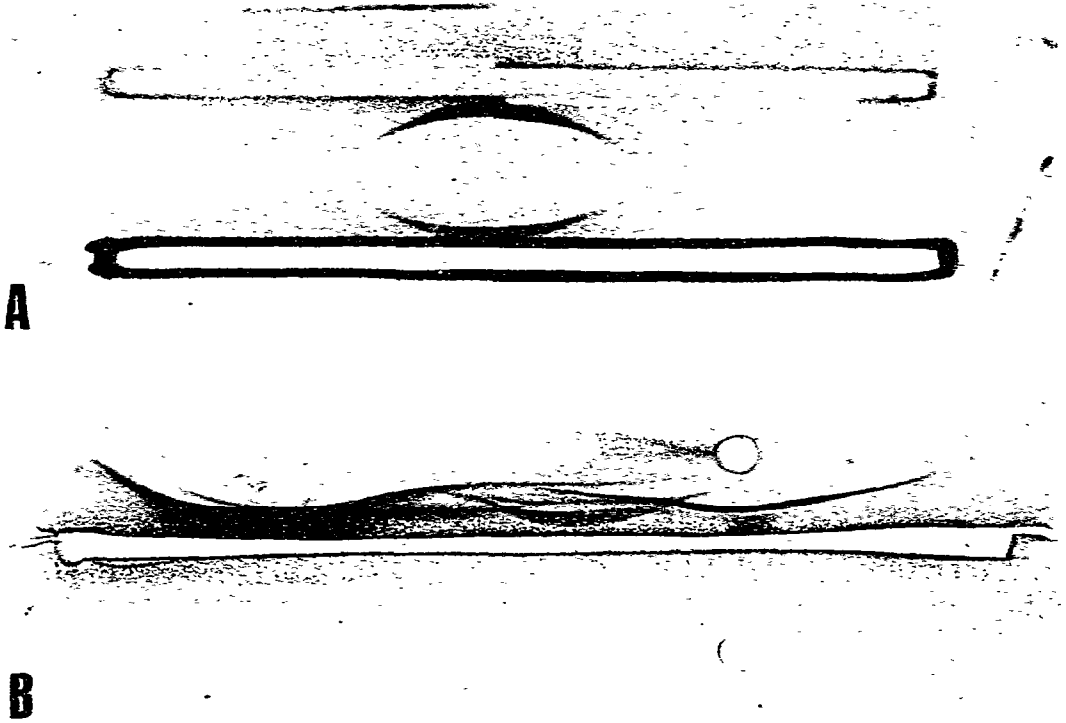
Fig. 6. Immunoelectrophoresis of (A) α-1-proteinase inhibitor and (B) α-1-acid glycoprotein. (A) Upper trough, monospecific rabbit anti-human α-1-proteinase inhibitor; bottom trough, rabbit anti-human serum; well, purified α-1-proteinase inhibitor. (B) Top well, normal human serum; bottom well, purified α-1-acid glycoprotein; trough, anti-human serum fortified with monospecific anti-human α-1-acid glycoprotein from rabbit.

Against anti-whole human serum fortified with monospecific anti-human α-1-acid glycoprotein and monospecific anti-human α-1-acid glycoprotein a single precipitation line was obtained (Fig. 6B).
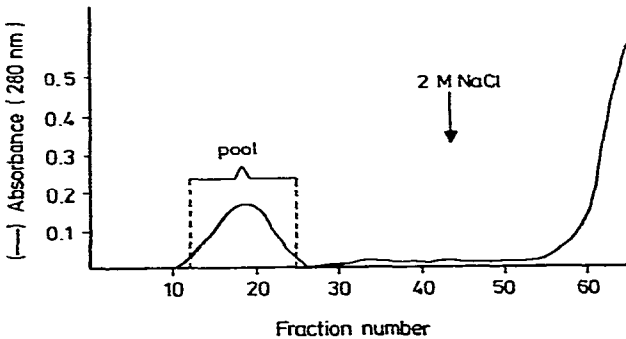

Fig. 7. Chromatography of human serum on Cibacron blue F3G-A–Sephadex G-100 at pH 5.8. A 50-ml volume of human serum was dialysed against 10 mM sodium phosphate buffer (pH 5.8) and applied to a gel column (50 × 5 cm) equilibrated with the same buffer. After eluting the unbound protein fraction with 10 mM sodium phosphate buffer (pH 5.8), the bound proteins were removed with 2 M sodium chloride solution. The fractions indicated were pooled and concentrated.
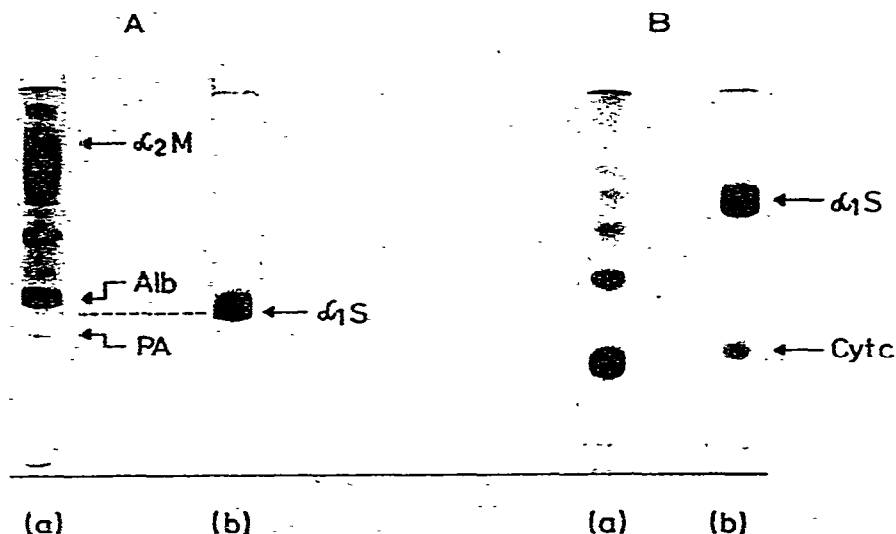
Fig. 8. (A) Polyacrylamide gel electrophoresis of purified α-1-acid glycoprotein. (a) Human serum (400 μg); (b) purified α-1-acid glycoprotein (60 μg). (B) SDS electrophoresis of purified α-1-acid glycoprotein. (a) Cytochrome c oligomers (40 μg); (b) α-1-acid glycoprotein (30 μg).

DISCUSSION

Immobilized Cibacron blue F3G-A is capable of adsorbing a great number of serum proteins. In addition to albumin, lipoproteins[11], blood coagulation factors[25], α-1-antichymotrypsin[9], complement factors[10], interferon[14] and other proteins have also been reported to bind to the immobilized dye. As shown in this paper, highly dye-substituted gels at appropriate ionic strength and pH of the buffer are capable of adsorbing almost all serum proteins.

In order to prepare gels, the covalent attachment of Cibacron blue F3G-A to the polysaccharide chain of Sephadex G-100 via an ether bridge was found to be superior to other coupling procedures[26]. The degree of substitution can easily be altered by changing the reaction temperature and the amount of the reacting dye per gram of Sephadex. The modified Sephadex G-100 is sufficiently stable also at high ligand concentrations. It exhibits good flow-rates in a column and can be used for large-scale chromatography.

There are many reports of the use of Sepharose gels for dye immobilization[8,12,14]. However, in contrast to the Sephadex used in our laboratory, the polysaccharide matrix of the Sepharose itself has a considerable non-specific binding capacity for proteins, especially at pH values below 7.0[27].

Our results show that the nature and amount of the adsorbed serum proteins depend directly on the loading density of the Sephadex G-100 and are not seriously influenced by matrix–protein interactions.

The adsorption of the great variety of proteins to immobilized Cibacron blue F3G-A can hardly be explained on the basis of specific interactions as they have been postulated for several dehydrogenases[4] and kinases[3] or for albumin[11]. According to

theoretical considerations by Kopperschläger *et al.*[28], the dye binding site is apparently an apolar region in the surface of the protein molecule surrounded by hydrophilic amino acid residues. All data indicate the involvement of hydrophobic and electrostatic forces in the dye–protein interaction. Their specificity depends primarily on hydrophobic forces, whereas their stability is governed by electrostatic forces. The nucleotide fold as present in dehydrogenases and in some of the kinases is only a special case in a dye binding site of a protein. Further, in a number of instances a second type of protein interaction can be recognized. This is based on the ability of the dye to act as weak cationic exchanger owing to its sulphonic acid groups. Consequently, below the isoelectric point almost all proteins should be able to bind to the dye.

These considerations are in line with the findings reported in this paper. Both lowering the pH and increasing the dye ligand concentration cause an increase in the binding strength and the binding capacity of the immobilized dye.

The significance of electrostatic interactions is stressed by the fact that α-1-acid glycoprotein, having an isoelectric point of 2.8, does not show any affinity for the gel under the applied conditions. By taking advantage of these properties a rapid and efficient one-step purification procedure is possible. With respect to the yield and operating time the described method is superior to other techniques[29]. This procedure yields an immunochemically pure α-1-glycoprotein preparation and can be applied to the isolation of this protein from small volumes of serum as obtained from individual humans. The high degree of purity was confirmed by several methods. The sedimentation coefficient of $s_{20,w}^0 = 3.27$ S agrees well with the value of 3.19 S previously reported for human α-1-acid glycoprotein[29]. The estimated molecular weight of 42,500 is close to the published value of 40,800[29].

Various methods have been reported for the isolation of α-1-proteinase inhibitor from plasma or serum. Albumin as the main contaminant of such preparations was removed by either concanavalin A[30], zinc chelate[31] or Sepharose Blue Dextran[24]. In contrast to other reports in which immobilized Cibacron blue was also used for the purification of the α-1proteinase inhibitor, Blue Sephadex as applied in the work is able to retain not only albumin but also most other serum proteins. This contributes significantly to the high degree of purification.

In order to achieve an efficient purification on the α-1-proteinase inhibitor, the ligand concentration on the gel should be between 170 and 250 μg of dye per milligram of dry gel. The recovery of approximately 30% of the original inhibitory capacity by our procedure is comparable to that reported by Kurecki *et al.*[31], but less than the best preparation (60%) described by Pannell *et al.*[24]. However, these two procedures take longer as they involve at least three chromatography steps.

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