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PURIFICATION OF α_1-PROTEINASE INHIBITOR BY TRIAZINE DYE AFFINITY CHROMATOGRAPHY, ION-EXCHANGE CHROMATOGRAPHY AND GEL FILTRATION ON FRACTOGEL TSK

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SUMMARY

The purification of plasma proteins by affinity chromatography on triazine dye matrices can be optimised with regard to the triazine dye used as a group-specific ligand. A comparison by electrophoretic and immunological means of the results of affinity chromatography with human plasma on Fractogel TSK-Blue and Fractogel TSK-Red demonstrated that the Procion Red HE-3B-containing gel was able to adsorb more plasma constituents than the Cibacron Blue F3-GA gel. The preparation of α_1-proteinase inhibitor obtained after chromatography on Fractogel TSK-Red showed a higher degree of purity and could easily be further purified by ion-exchange chromatography on Fractogel TSK DEAE-650 and gel filtration on Fractogel TSK HW 55, without significant loss of biological activity.

INTRODUCTION

Since Haeckel *et al.*[1] discovered interactions between pyruvate kinase and the dye Cibacron Blue F3-GA, triazine dyes have become well established as group-selective affinity ligands because of their high specificity and affinity for nucleotide-dependent enzymes.

Aside from these affinity interactions, other proteins are bound in a less specific manner by electrostatic and/or hydrophobic effects to these sulfonated polyaromatic ligands. Cibacron Blue F3-GA agarose matrices were reported to bind human plasma proteins, such as albumin[2] and α_1-antichymotrypsin[3]. Systematic studies of the chromatographic behaviour of serum and plasma components on Cibacron Blue F3-GA agarose gels have been reported by Angal and Dean[4] and recently by Gianazza and Arnaud[5]. A process for the isolation of α_1-proteinase inhibitor from human plasma was reported by Pannell *et al.*[6]. They removed albumin from plasma by affinity chromatography on immobilized Cibacron Blue F3-GA.

The present report compares the merits of Procion Red HE-3B and Cibacron Blue F3-GA, both immobilized to Fractogel TSK HW 65, in the isolation of α_1-proteinase inhibitor from human plasma. With two additional steps, ion-exchange chromatography on Fractogel TSK DEAE-650 and gel filtration on Fractogel TSK HW 55, we succeeded in purifying α_1-proteinase inhibitor while retaining high biological activity.

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EXPERIMENTAL

Reagents

All reagents were from E. Merck (Darmstadt, F.R.G.) unless stated otherwise. Cibacron Blue F3-GA was from Serva (Heidelberg, F.R.G.). Procion Red HE-3B was from Sigma (Taufkirchen, F.R.G.). As plastic support and cover film for 100-μm polyacrylamide gels we used Mylar D plastic sheets from Technoplast (Köln, F.R.G.). GelBond® PAG films were purchased from F.M.C. (Johnson & Co., Erkrath, F.R.G.). As carrier ampholytes we used Servalyt T 4-9 from Serva (Heidelberg, F.R.G.). Cellulose acetate gel, Cellogel was purchased from Chemetron (Biotec-Fischer, Reiskirchen, F.R.G.). Antisera were from Dakopatts (Boehringer Ingelheim, Garching, F.R.G.).

Chromatography

Human plasma was dialysed overnight at 2–8°C against 30 m*M* sodium phosphate, pH 7.0. The protein solution was cleared by centrifugation (20,000 *g*). Protein corresponding to 10 ml of plasma was applied to each affinity gel. Fractogel TSK-Blue (40 × 2.6 cm I.D.) and Fractogel TSK-Red (40 × 2.6 cm I.D.) columns were equilibrated with the above-mentioned buffer. The plasma sample was applied to the column and the nonretarded proteins were eluted with equilibration buffer. Adsorbed proteins were eluted by 30 m*M* sodium phosphate–2 *M* sodium chloride, pH 7.0. Final desorption of albumin and lipoproteins was carried out with 0.5 *M* sodium thiocyanate. α_1-Proteinase inhibitor appeared together with the unbound proteins. The fractions containing inhibitor activity were pooled and then concentrated in an ultrafiltration cell equipped with a PM-10 membrane, both from Amicon (Witten, F.R.G.). This protein sample was dialysed against 20 m*M* triethanolamine–hydrochloric acid (pH 7.5) and applied directly to a Fractogel TSK DEAE-650 column (30 × 1.6 cm I.D.), equilibrated with 20 m*M* triethanolamine–hydrochloric acid (pH 7.5). α_1-Proteinase inhibitor was eluted by a linear salt gradient 0–0.3 *M* sodium chloride (500 ml). Gel filtration on a Fractogel TSK HW 55 column (60 × 2.6 cm I.D.) was carried out in 50 m*M* Tris–HCl (pH 8.0).

Protein analysis

Polyacrylamide gel electrophoresis was performed as described by Ornstein[7] and Davis[8]. Two-dimensional electrophoresis was carried out in ultra-thin polyacrylamide gels[9]. We modified this technique by carrying out the isoelectric focusing in 100-μm polyacrylamide gels formed by the "flap technique"[10]. The gels, 5% T and 3% C[11], contained 3% (w/v) of carrier ampholytes. The gels were prefocused for 30 min at 400 V. After the sample application, focusing was continued for 30 min at 400 V, 30 min at 800 V, 60 min at 1200 V, and then raised to 2000 V for 10 min. As support films for the second dimension we used GelBond films. Two-dimensional immunoelectrophoresis[12] was performed on cellulose acetate films with 50 m*M* Tris–glycine, pH 8.9. For the first dimension 200 V was applied for 55 min and for the second dimension 120 V for 16 h. Samples were run against anti-whole human plasma. After the cellulose acetate films had been washed with phosphate buffered saline (PBS) for 1.5 h, precipitates were stained with 0.5% (w/v) Coomassie Brilliant Blue R-250 in destaining solution (methanol–water–acetic acid; 45:45:10, v/v/v).

For the inhibition assay, α_1-Proteinase inhibitor was quantified by its ability to inhibit bovine trypsin[13]. For the determination of total protein was measured by the method of Lowry *et al.*[14] was used.

RESULTS

Fractogel TSK HW 65 (25–40 μm particle size), a hydrophilic synthetic polymer, was used as matrix for affinity chromatography. Cibacron Blue F3-GA and Procion Red HE-3B were coupled to the gel by the method of Rinderknecht *et al.*[15]. The binding capacity was determined with bovine albumin in 50 m*M* sodium phosphate buffer, pH 7.0. For Fractogel TSK-Blue and Fractogel TSK-Red the capacity

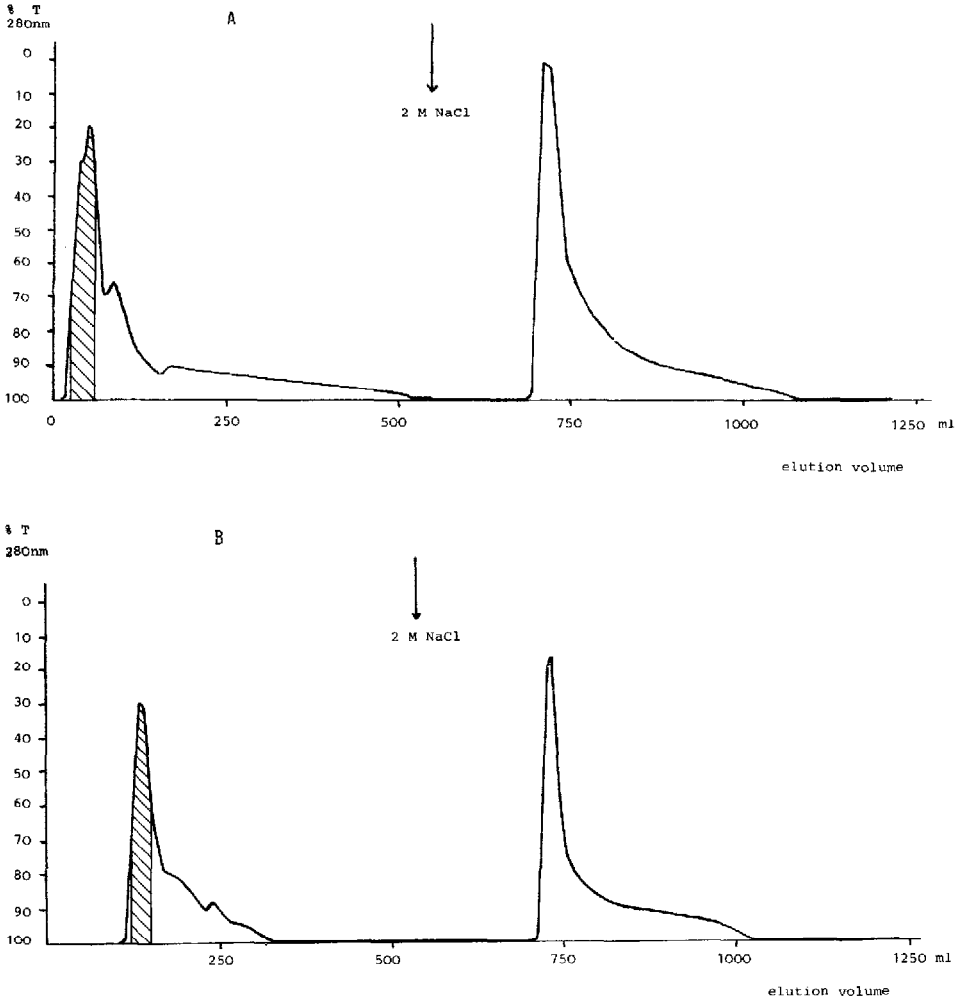

Fig. 1. Chromatography on Fractogel TSK-Blue (A) and Fractogel TSK-Red (B). Samples human plasma; column, 40 × 2.6 cm I.D.; equilibration buffer, 30 m*M* sodium phosphate, pH 7.0; eluent, 30 m*M* sodium phosphate–2 *M* NaCl, pH 7.0; flow-rate: 100 ml/h. Shaded area: α_1-proteinase inhibitor fractions.

was approximately 11 mg/ml and 6 mg/ml swollen gel, respectively. α_1-Proteinase inhibitor was always eluted in the early fractions of unbound or weakly retarded proteins (Fig. 1A and B). Polyacrylamide gel electrophoresis of the pooled α_1-proteinase inhibitor fractions after affinity chromatography revealed that the Fractogel TSK-Red eluate contained less contaminant proteins (Fig. 2 a and c). This was confirmed by two-dimensional immunoelectrophoresis (Fig. 3). The protein pattern of these preparations were examined by means of two-dimensional gel electrophoresis (Fig. 4). Both α_1-proteinase inhibitor pools contained albumin, prealbumin, and α_1-acid glycoprotein. Transferrin, IgG, and haptoglobin were also present in the Fractogel TSK-Blue pool. Prealbumin and most of the albumin could be separated from α_1-proteinase inhibitor on Fractogel TSK DEAE-650 (Fig. 5). The preparation of Fractogel TSK-Blue fractions caused two additional protein peaks (Fig. 5A). Most of the contaminant haptoglobin could be separated from α_1-proteinase inhibitor by

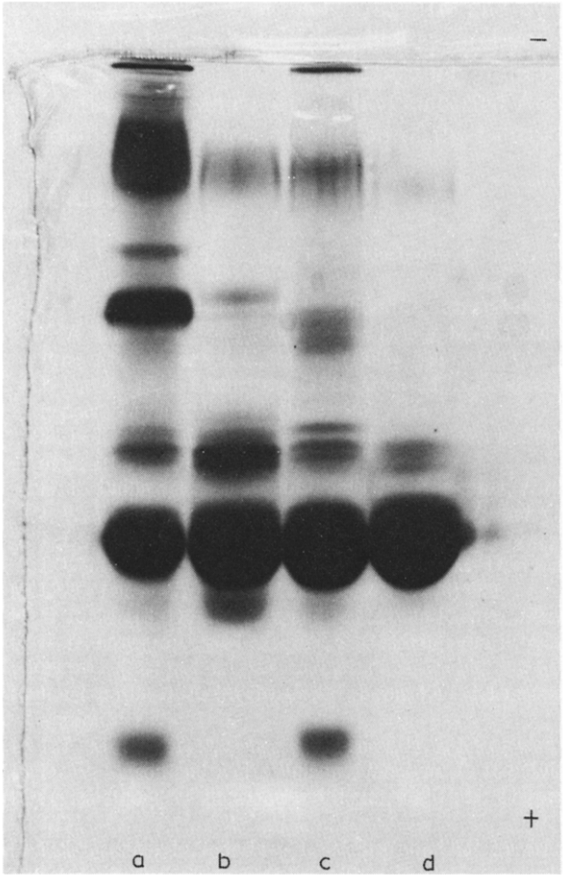

Fig. 2. Polyacrylamide gel electrophoresis with the anode at the bottom. Sample: 75 µg. (a) α_1-Proteinase inhibitor after chromatography on Fractogel TSK-Blue, (b) after additional chromatography on Fractogel TSK DEAE-650 and Fractogel TSK HW 55, (c) α_1-proteinase inhibitor after chromatography on Fractogel TSK-Red, and (d) after additional chromatography on Fractogel TSK DEAE-650 and Fractogel TSK HW 55.

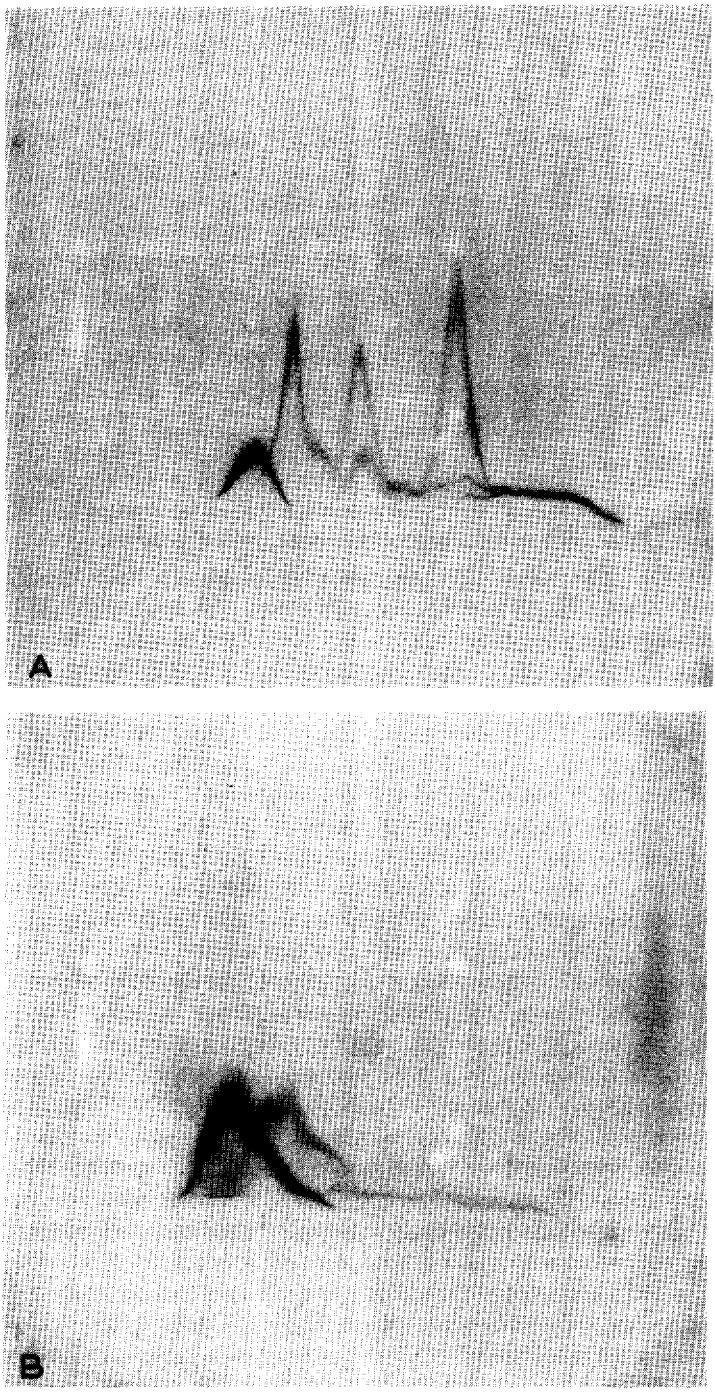

Fig. 3. Two-dimensional immunoelectrophoresis with α_1-proteinase inhibitor fraction on cellulose acetate gels. The anode of the first dimension was at the left and the anode of the second dimension was at the top. (A) Fractogel TSK-Blue fraction. (B) Fractogel TSK-Red fraction.

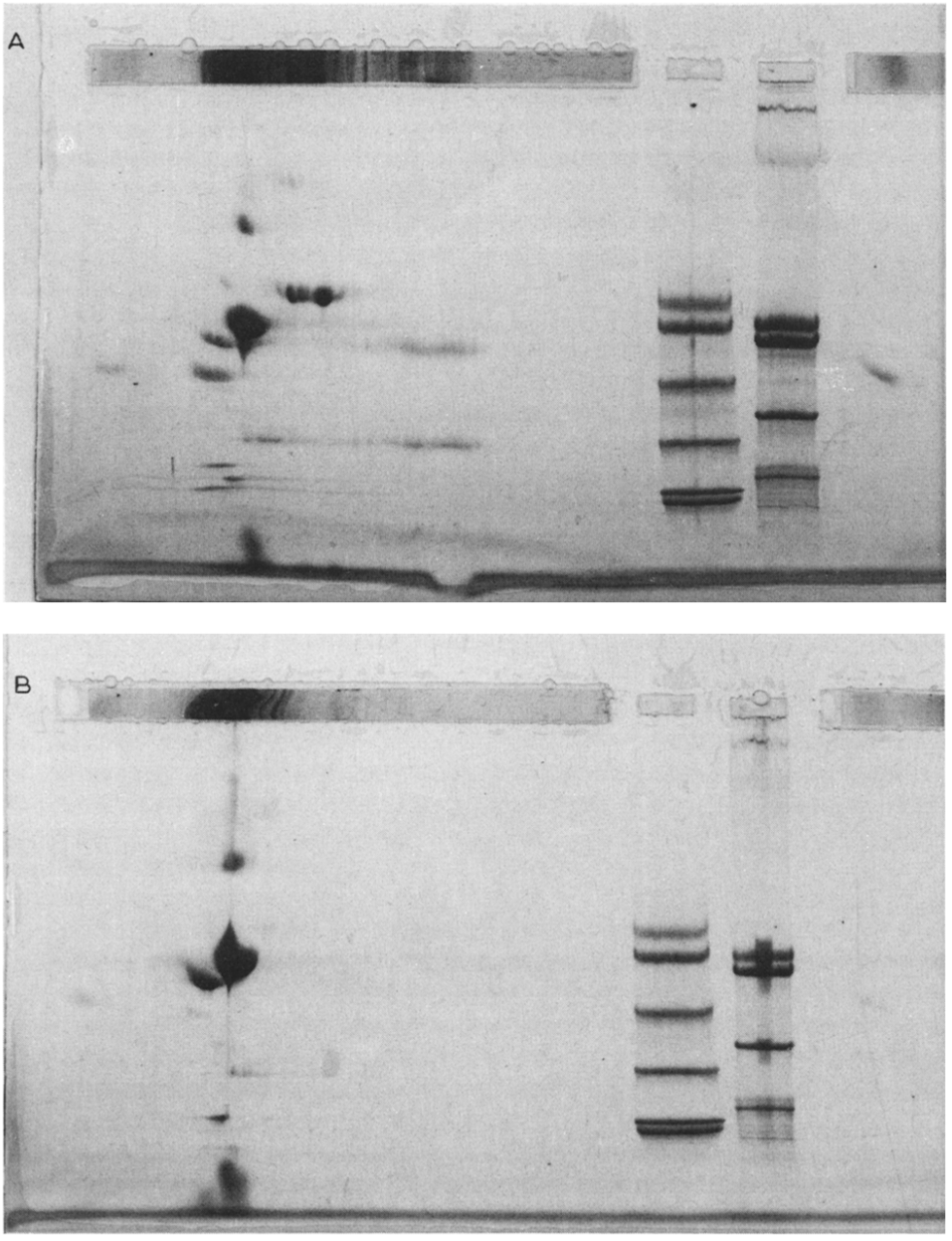

Fig. 4. Two-dimensional gel electrophoresis. The anode of the first dimension was at the left, the anode of the second dimension was at the bottom. α_1-Proteinase inhibitor preparations after chromatography on Fractogel TSK-Blue (A) and Fractogel TSK-Red (B). Calibration proteins (from bottom to top): right hand: ferritin (18,500), LDH (36,000), catalse (60,000), albumin (67,000), ferritin (220,000) and thyroglobulin (330,000); left hand: cytochrome *c* (12,300), myoglobin (17,200), chymotrypsinogen (25,000), ovalbumin (45,000), albumin (67,000) and ovotransferrin (76,000–78,000).

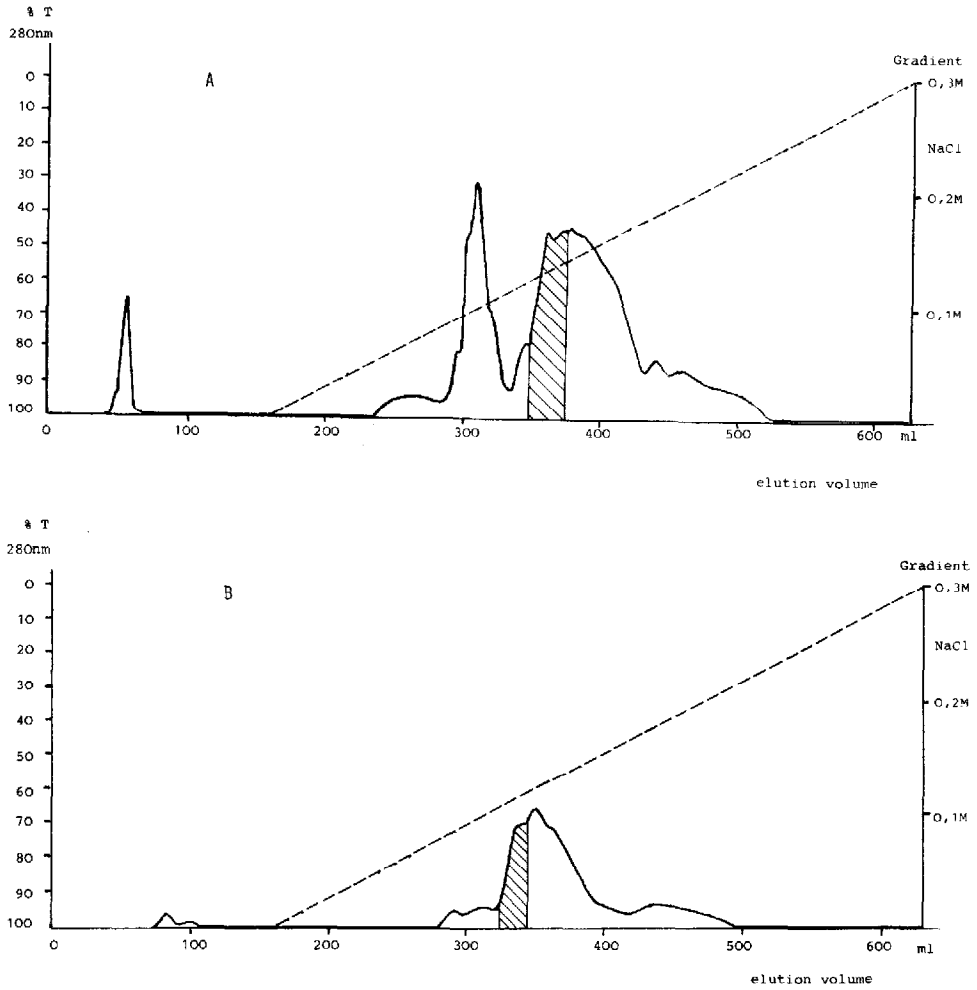

Fig. 5. Chromatography on Fractogel TSK DEAE-650. Samples: α_1-proteinase inhibitor fractions from Fractogel TSK-Blue (A) and from Fractogel TSK-Red (B). Column: 30 × 1.6 cm I.D.; equilibration buffer 20 m*M* triethanolamine–HCl, pH 7.5; gradient: linear 0–0.3 *M* NaCl in equilibration buffer (× 500 ml). Flow-rate: 40 ml/h. Shaded area: α_1-proteinase inhibitor fractions.

gel filtration on Fractogel TSK HW 55 (Fig. 6A). The elution diagram of the Fractogel TSK-Red preparation again stressed the effectiveness of this affinity chromatography step (Fig. 6B). Polyacrylamide gel electrophoresis of the final preparations demonstrated a higher degree of purity for the "Fractogel TSK-Red preparation" (Fig. 2b and d). Albumin present in both samples could be easily removed together with the trace amounts of Gc-globulin by chromatography on concanavalin A gels (not shown).

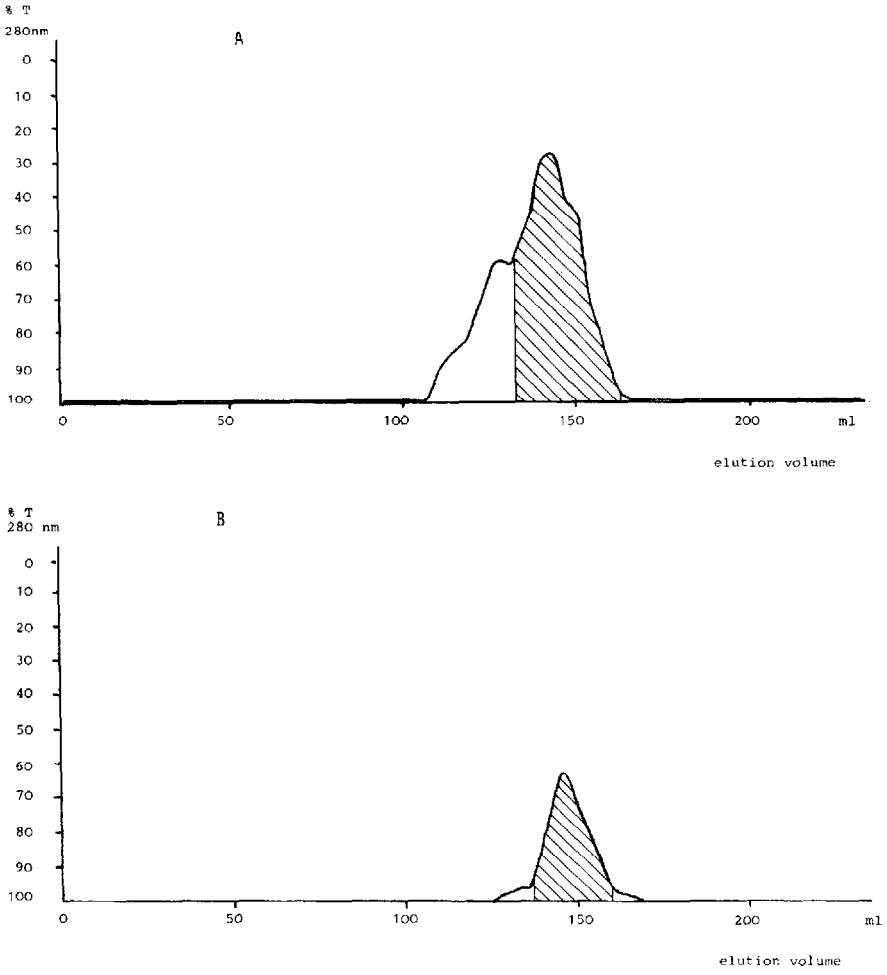

Fig. 6. Chromatography on Fracotgel TSK HW 55. Samples: α_1-proteinase inhibitor fractions from Fractogel TSK DEAE-650, (A) Fractogel TSK-Blue; (B) Fractogel TSK-Red). Column: 60 × 2.6 cm I.D. Equilibration buffer: 50 m*M* Tris–HCl, pH 8.0. Flow-rate: 100 ml/h. Shaded area: α_1-proteinase inhibitor fractions.

DISCUSSION

Unlike other investigators[4–6], we tried to improve the purification of α_1-proteinase inhibitor from human plasma with regard to the type of triazine dye used as an affinity ligand. The complexity of protein–dye interactions could be demonstrated by comparing the composition of α_1-proteinase inhibitor preparations purified with Fractogel TSK-Blue and Fractogel TSK-Red. The Fractogel TSK-Red matrix bound more contaminant plasma proteins (Figs. 2–4), facilitating the subsequent purification steps. With both affinity gels high recovery of biological inhibitor activity was found. By following ion-exchange chromatography on Fractogel TSK DEAE-650 with gel filtration on Fractogel TSK HW 55 highly purified α_1-proteinase inhibitor

preparations could be obtained. Gel electrophoresis with the inhibitor fractions from the final chromatography steps demonstrated the superiority of affinity chromatography on the Procion Red gel (Fig. 2b and d). Because of the high binding capacity of Fractogel TSK-Red and Fractogel TSK DEAE-650 and the possibility of speeding up gel filtration with Fractogel TSK HW 55, this method can easily be scaled up. The latest results have shown that the purification of α_1-proteinase inhibitor on Fractogel TSK-Red could be further optimised by lowering the pH value during chromatography. Under these conditions fractionation of α_1-proteinase inhibitor phenotypes could be achieved[16].

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