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FRACTIONATION OF HUMAN SERUM PROTEINS BY IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY

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SUMMARY

Human serum was fractionated on zinc and cadmium iminodiacetate and tris-(carboxymethyl)ethylenediamine chelates. Zinc iminodiacetate was found to be useful in the isolation of hemopexin and α_2-macroglobulin. The chromatographic behaviour of serum proteins on zinc iminodiacetate could be modulated by the nature of the eluent; a sulphate-containing solvent increased the binding of serum proteins to immobilized metal ions.

INTRODUCTION

Protein chromatography on gels containing immobilized metal ions was introduced in 1975 by Porath *et al.*[1]. Several subsequent papers reported the isolation of various proteins[2–4], but not until 1983 was a more detailed account of the method, now termed IMAC (immobilized metal affinity chromatography), published[5]. However, there is still a need to investigate many aspects of the method, particularly the effects of different salts, pH, temperature, the nature of the metal, and so on. A detailed investigation of these parameters in the resolution of serum proteins is the subject of this report.

MATERIALS AND METHODS

Iminodiacetate-Sepharose 6B (IDA-Sepharose) and tris(carboxymethyl)ethylenediamine-Sepharose 6B (TED-Sepharose) were synthesized as described earlier[5]. The capacity for Cu^{2+} in water was 37 μmol per ml of IDA-Sepharose and 45 μmol per ml of TED-Sepharose. Gels were packed into columns of 1-cm bore. After initial washing with distilled water, 20 m*M* zinc sulphate or 20 m*M* cadmium sulphate solutions were applied until metal appeared in the eluate. The loosely bound metal was washed off with water and then the columns were equilibrated with two or three volumes of appropriate buffers. The columns were routinely regenerated after each run with 0.05 *M* EDTA solution. All chromatographic experiments were performed at 20–22°C, unless otherwise indicated. Flow-rates were maintained at 15 ml cm^{-2} h^{-1}.

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Protein concentrations were estimated from the absorbance at 280 nm. Fractions containing protein were concentrated in Minicon concentrators and analysed by electrophoresis in polyacrylamide gradient gels (4/30) according to the manufacturer's instructions (Pharmacia). The purity of some protein fractions was ascertained by crossed immunoelectrophoresis in agarose gels containing antibodies against human serum. Immunoelectrophoresis was also used for the identification purposes.

Human serum was routinely dialysed overnight against the buffer to be used in the chromatographic experiment.

RESULTS

Affinity of serum proteins for IDA-Zn^{2+}, IDA-Cd^{2+}, TED-Zn^{2+} and TED-Cd^{2+}

A 3-ml sample of human serum was applied on an appropriate metal chelate column (10 × 1 cm I.D.) in 0.02 *M* Tris–0.5 *M* potassium sulphate–sulphuric acid, pH 8.0. The column was washed with the equilibrating buffer to yield unbound (breakthrough) proteins and then developed with 0.05 *M* histidine–0.02 *M* Tris–sul-

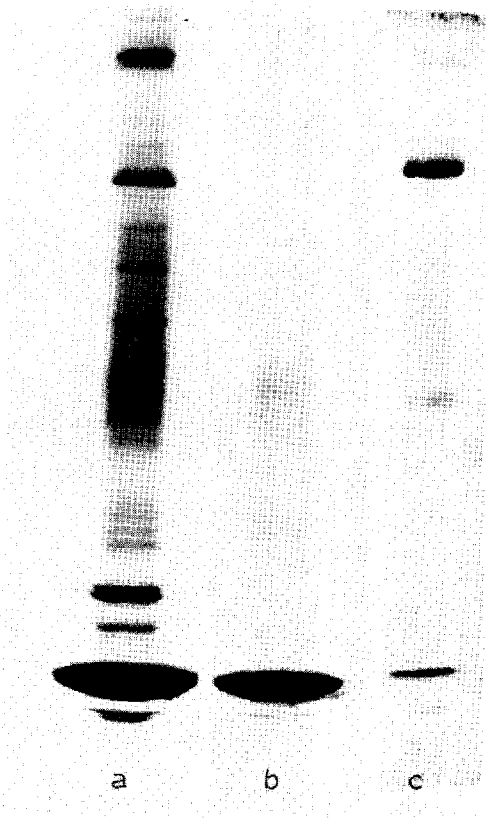

Fig. 1. Polyacrylamide gradient gel electrophoresis of serum and serum protein fractions from IMA chromatography in 0.5 *M* potassium sulphate–0.02 *M* Tris–sulphuric acid, pH 8.0. (a) Human serum; (b) serum proteins unadsorbed on IDA-Zn^{2+} gel; (c) serum proteins adsorbed on IDA-Cd^{2+} gel at 4°C.

phuric acid, pH 8.0 to yield proteins that were retained under the application conditions employed. The pooled fractions of unbound and bound proteins were analysed by electrophoresis. Fig. 1 illustrates the outcome of the experiment on IDA-Zn^{2+}. A comparison of lane a (whole serum) with lane b (breakthrough fractions) clearly shows that serum albumin is the major "breakthrough" constituent. A minor modification of the chromatographic conditions can yield the serum albumin monomer in greater than 99% purity (Fig. 2).

When Zn^{2+} was immobilized on TED, the resulting chelating gel displayed only a marginal capacity for serum proteins: only 4% of the applied sample was retained and no individual protein was selectively retained. TED-Zn^{2+} is, therefore, of no particular advantage in the resolution of serum proteins.

The affinity of serum proteins for Cd^{2+} was also studied. It was found that neither IDA-Cd^{2+} or TED-Cd^{2+} retained serum proteins in the presence of potassium sulphate or acetate (0.5 *M*) as the equilibrating solvent. However, at 4°C there was rather selective retention of α_2-macroglobulin (Fig. 1, lane c). Thus, IDA-Zn^{2+} could be useful for the isolation of serum albumin ("negative" adsorption) and

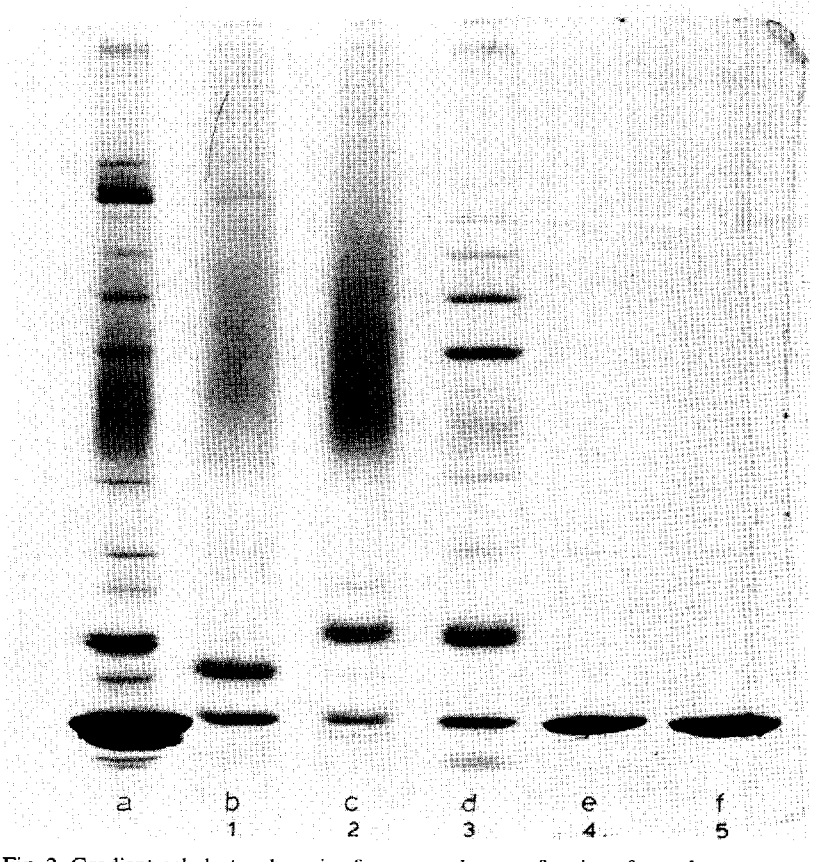

Fig. 2. Gradient gel electrophoresis of serum and serum fractions from chromatography on a sectioned column of IDA-Zn^{2+}-Sepharose in 0.5 *M* potassium sulphate–0.02 *M* Tris–sulphuric acid, pH 8.0; wash volume, 25 ml. (a) Human serum; (b–f) fractions from column sections 1–5, respectively, eluted with 0.5 *M* ammonium sulphate–0.02 *M* Tris–sulphuric acid, pH 8.0.

IDA-Cd^{2+} for the isolation of α_2-macroglobulin ("positive" adsorption), under appropriate experimental conditions.

Tandem IDA-Zn^{2+} column chromatography of serum proteins

In order to appreciate the relative affinity of serum proteins for IDA-Zn^{2+} the following chromatographic strategy was employed. Seven columns of different sizes (two 1 × 1 cm I.D., four 2 × 1 cm I.D. and one 6 × 1 cm I.D.) were connected to give a "train" tandem of columns. A 4-ml sample of human serum was applied to the first column in the "train" and a pulse of 25 ml of wash solvent (0.5 *M* potassium sulphate–0.02 *M* Tris–sulphuric acid, pH 8.0) was applied. The columns were then disconnected and developed individually to evaluate the distribution of serum proteins within the chromatographic tandem. The eluates from individual columns were analysed by electrophoresis. The results are illustrated in Fig. 2. Lane a depicts the whole serum, and lanes 1–5 indicate the protein composition of the corresponding column sections in the chromatographic "train". It is clear from Fig. 2 that serum albumin was the fastest moving serum protein, in agreement with the results illustrated in Fig. 1. Some albumin was also appeared in all the other eluates. Lane b shows a prominent protein band in the vicinity of albumin; this protein, which was almost totally retained by the first column in the series, was identified as hemopexin. A different protein band in lane b in the higher molecular weight region was identified as immunoglobulin. An attempt was made to improve the resolution of hemopexin from the remaining immunoglobulin. To this end the wash volume of the column "train" was increased from 25 ml to 50 ml. As a result, the immunoglobulin was transferred downstream whereas the hemopexin was retained on the first column section.

Fig. 2 also reveals the presence of several distinct protein bands in the eluate of column section 3. All in all, this experiment demonstrates the various affinities of many serum proteins for IDA-Zn^{2+} under the conditions employed. This observation clearly points to the potential of IDA-Zn^{2+} for the resolution of serum proteins.

TABLE I

DISTRIBUTION OF PROTEINS IN HUMAN SERUM AMONG THE IDA-Zn^{2+}-SEPHAROSE COLUMN SECTIONS IN TWO DIFFERENT CHROMATOGRAPAHIC RUNS

Serum volume, 4 ml; total elution volume, 50 ml.

Column section	*Length (cm)*	*Amounts of applied material adsorbed (%)*	
		In potassium acetate	*In potassium sulphate*
1	1	1.5	10.5
2	1	6.5	10
3	2	17	16
4	2	12	8.5
5	2	7.5	5
6	2	2	2
7	6	1.5	1.5
Total adsorbed material (%)		48	53.5

Table I summarizes the data on the chromatography of human serum on IDA-Zn^{2+} in potassium acetate and potassium sulphate. It is evident that the migration rates of proteins are lower in sulphate than in acetate, indicating the influence of structure-forming ions (sulphates) on the stability of IDA-Zn^{2+}-protein ternary complex. In acetate-containing solvent, α_2-macroglobulin is found on column section 1 (Fig. 3, lane 1). In sulphate-containing solvent, α_2-macroglobulin is strongly bound to column section 1 and is not eluted by the desorption solvent (0.5 *M* ammonium sulphate–0.02 *M* Tris–sulphuric acid, pH 8.0).

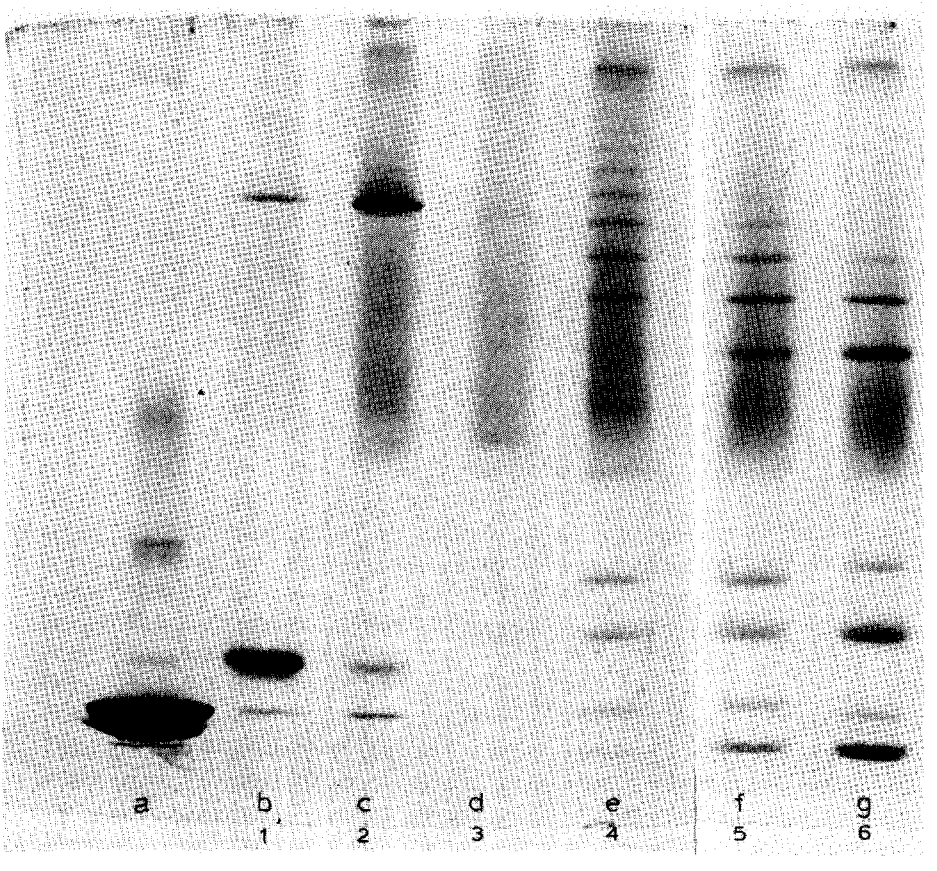

Fig. 3. Gradient gel electrophoresis of serum fractions from chromatography on a sectioned column of IDA-Zn^{2+}-Sepharose in 0.5 *M* potassium acetate–0.02 *M* Tris–acetic acid, pH 8.0; wash volume, 50 ml. (a) Breakthrough fraction; (b–g) fractions from column sections 1–6, respectively, desorbed with 0.5 *M* ammonium sulphate–0.02 *M* Tris–sulphuric acid, pH 8.0.

The outcome of the experiments illustrated in Figs. 2 and 3 prompted an attempt to isolate hemopexin and α_2-macroglobulin in a single step and also to measure the capacity of the IDA-Zn^{2+} sorbent for those proteins. To this end, 40 ml of human serum was applied on the tandem of columns in 0.5 *M* potassium acetate–0.02 *M* Tris–acetic acid, pH 8.0. As can be seen in Fig. 4, both proteins were separated from

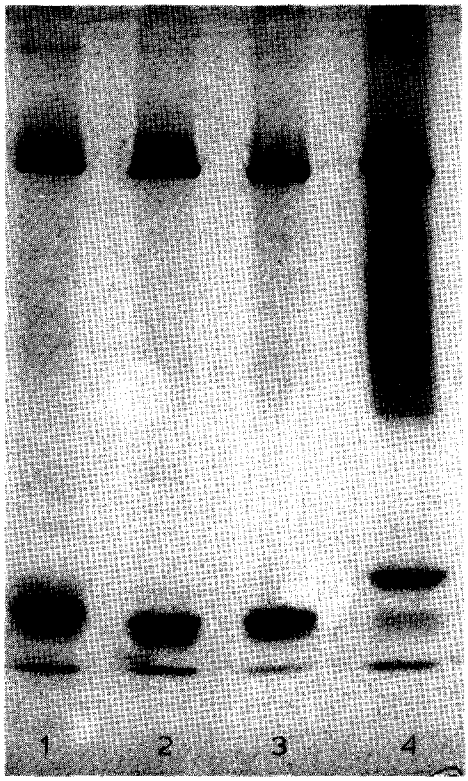

Fig. 4. Gradient gel electrophoresis of serum proteins obtained from chromatography of a large amount (40 ml) of human serum on the tandem column in 0.5 *M* potassium acetate–0.02 *M* Tris–acetic acid, pH 8.0; column sections 1–4, respectively.

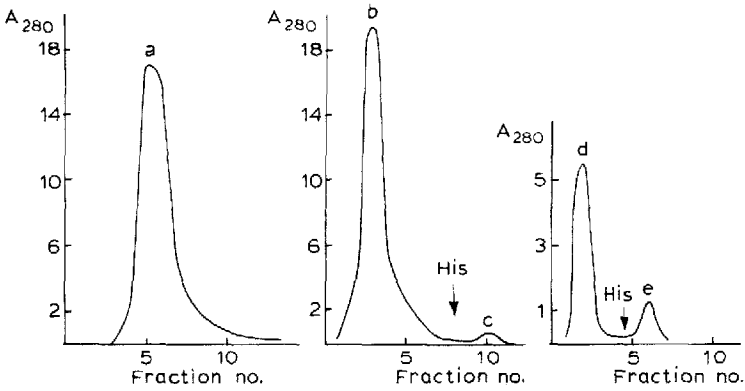

Fig. 5. Elution patterns of human serum obtained by chromatography on two columns of IDA-Zn^{2+}-Sepharose (1 × 1 cm I.D. and 12 × 1 cm I.D.) in 0.5 *M* potassium sulphate–0.02 *M* Tris–sulphuric acid, pH 8.0; fraction volumes, 3.2 ml. (a) Unadsorbed material; (b) material desorbed from the 12 × 1 cm I.D. column with 0.5 *M* ammonium sulphate–0.02 *M* Tris–sulphuric acid, pH 8.0; (c) material desorbed from the 12 × 1 cm I.D. column with 0.05 *M* histidine–0.02 *M* Tris–acetic acid, pH 8.0; (d) material desorbed from the 1 × 1 cm I.D. column with 0.5 *M* ammonium sulphate–0.02 *M* Tris–sulphuric acid, pH 8.0; (e) material desorbed from the 1 × 1 cm I.D. column with 0.05 *M* histidine–0.02 *M* Tris–acetic acid, pH 8.0.

other serum proteins with only minor cross-contamination with other serum proteins. Hemopexin and α_2-macroglobulin can be thus readily concentrated and significantly purified on IDA-Zn^{2+} under these chromatographic conditions. It can be readily calculated that 1 ml of the IDA-Zn^{2+} gel adsorbed the hemopexin from 12 ml of human serum. The complete adsorption of all the α_2-macroglobulin would require a larger column. A follow-up experiment was therefore performed in which a 1 × 1 cm I.D. and a 12 × 1 cm I.D. column were coupled. Human serum (4 ml) was applied and the column was washed with 46 ml of equilibration solvent. After disassembly, the columns were individually eluted with 0.5 *M* ammonium sulphate–0.02 *M* Tris–sulphuric acid, pH 8.0 and with 0.05 *M* histidine–0.02 *M* Tris–acetic acid, pH 8.0. The elution profiles of the individual columns are shown in Fig. 5. In both columns the only protein eluted by histidine is α_2-macroglobulin. As the short column adsorbed 65% of the α_2-macroglobulin present in 4 ml of human serum, this means that 1 ml of the gel can adsorb this protein from *ca.* 3 ml of human serum.

Analysis of ammonium sulphate eluate from the short column by electrophoresis indicated fairly pure protein (Fig. 6), although crossed immunoelectrophoresis demonstrated the presence of minor compounds. The α_2-macroglobulin recovered from both columns was more than 95% pure, as judged by crossed immunoelectrophoresis.

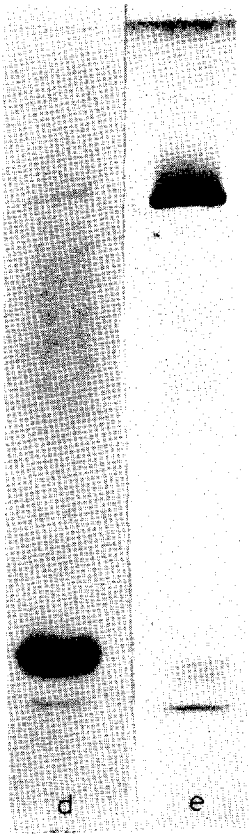

Fig. 6. Gradient gel electrophoresis of fractions (d) and (e) from the experiment in Fig. 5.

CONCLUSIONS

This report demonstrates the usefulness of IDA-Zn^{2+} for both group separation and purification of specific proteins in human serum. It also stresses that proper choice of the solvent is beneficial for the purification.

IDA-Cd^{2+} is useful at low temperatures, *e.g.* 4°C, for the isolation of α_2-macroglobulin in human serum. This study showed that TED gels have low capacities for serum proteins, but further investigation of the usefulness of these sorbents is required.

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