Journal of Chromatography, 550 (1991) 751–764
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2077

Cascade-mode multiaffinity chromatography

Fractionation of human serum proteins

JERKER PORATH* and PER HANSEN
Biochemical Separation Centre, Biomedical Center, Uppsala University, Box 577, S-751 23 Uppsala (Sweden)

ABSTRACT

The group-resolving power of cascade-mode multiaffinity column chromatography (CASMAC), was demonstrated with human serum as a model mixture. More than 99% of the serum proteins were adsorbed in the same high salt-containing buffer on a tandem column consisting of (1) immobilized Zn^{2+} on triscarboxymethyl diamine gel followed by (2) thiophilic (T) gel, (3) Zn^{2+} bound to the new tridentate chelating adsorbent dipicolylamine (DPA) agarose, (4) hexyl–thioether C_6-S agarose and (5) Ni^{2+}–DPA agarose. After the adsorption step the immobilized metal ion affinity gels were attached to the top of tandem columns of other adsorbents (T gel, Sephadex G-25 for desalting and Mono-Q) and the elution conditions were selected such that further group separation was achieved. High resolution, high recovery, easy manipulation and high capacity are characteristic features of the cascade process with these adsorbents. The advantage of CASMAC is particularly striking when, with a given number of adsorbents, the overall number of operations involving adsorption, desorption, washing, buffer change and substance concentration can be effectively minimized.

INTRODUCTION

Two main principles of separation have been developed and have become the most powerful tools for the isolation of biopolymers: chromatography and electrophoresis. Dr. Lloyd R. Snyder is one of the leading scientists who have made the most significant contributions in the field of liquid chromatography. In this paper, in his honor, we would like to pay him our homage by describing some developmental steps in another direction of liquid chromatography suited to the purification and isolation of biological substances. We stress the use of chemical affinities as important factors for separation.

The importance of separation methods, chromatography in particular, for progress in biochemical sciences can hardly be overestimated. Chromatographic materials are flooding the market, but most adsobents consist of variations on a few themes, many of them being ion exchangers with the same kind of simple ligands. Assessment of their relative merits is not an easy matter. The published methods have usually been selected by trial and error or accidently applied; only a few are based on a rational approach to the solution of a particular separation or isolation problem.

0021-9673/91/$03.50

There are two strategies for increasing the efficiency of chromatographic fractionation of a complex mixture: (1) to maximize the separation power in a bed of a given adsorbent or (2) to separate the components according to their affinities for a number of selected adsorbents, operating ideally according to different separation principles such as metal ion affinity or charge, supplemented, if necessary, by molecular size-descriminating methods. By use of rapid batch methods, Scopes and Porath [1] have recently shown how it is possible to screen a large number of different adsorbents in a sequential manner to produce a rational scheme for the fractionation of a number of enzymes in bacterial and yeast extracts.

To give this multiple affinity technique a high resolving power and ease of application, we suggest the use of sequential adsorption of material in tandem-coupled beds, each bed containing a different adsorbent. To increase further the resolution it is possible (1) to use selective elution or displacement from each bed separately or (2) to transfer the eluent to a new bed or a tandem column with a supplementary kind of affinity characteristics. The latter technique results in a cascade scheme of fractionation of the original mixture. This technique has been described previously by Porath [2] and Scopes [3]. In this paper such a cascade mode of fractionation will be exemplified by use of immobilized metal ion affinity (IMAC), hydrophobic, thiophilic interaction and ion-exchange chromatography as applied to human serum proteins.

EXPERIMENTAL

All chemicals were of analytical-reagent grade, obtained from commercial sources, and were used as purchased. Normal human serum was obtained from the blood bank at the University Hospital in Uppsala (Sweden). LC- and M-Partigen Immunodiffusion plates were purchased from Beringwerke (Marburg, Germany).

The triscarboxymethyldiamine (TED) gel, the hydrophobic C_6-S agarose and the thiophilic (T) agarose were synthesized as described previously by Porath and co-workers [4–6]. Sepharose 6B was activated with 1,4-butanediol diglycidyl ether, then treated with ethylene diamine followed by carboxymethylation with bromoacetic acid under alkaline conditions to produce the TED gel, with a nitrogen content of 920 μmol/g dry weight. The Zn^{2+}-loaded TED gel was found to contain 990 μmol/g dry weight. The hydrophobic C_6-S gel was synthesized from the 1,4-butanediol diglycidyl ether-activated Sepharose 6B by coupling hexanethiol in alkaline suspension under reducing condition. The T gel was obtained by treatment of Sepharose 6B with divinyl sulphone in carbonate buffer (pH 11.0) followed by coupling mercaptoethanol in sodium carbonate buffer (pH 9.0). The sulphur contents of the C_6-S agarose and T gel were 100 μmol/g and 8.1%, respectively, calculated on the dry adsorbents.

Dipicolylamine Sepharose 6B (DPA agarose)[a]

This new adsorbent was prepared as follows. Sepharose 6B (600 g) was washed thoroughly with deionized water and the excess water was removed on a glass filter under gentle suction. The moist gel was suspended in 120 ml of deionized water and 200 ml of 4 *M* NaOH containing 38 ml of epichlorohydrin and 1.72 g of $NaBH_4$. The suspension was stirred for 2 h. A 200-ml volume of 4 *M* NaOH and 200 ml of ep-

[a] Patent pending.

ichlorohydrin were added in portions of about 10 ml each for 3 h with adequate stirring. The stirring was continued overnight and the suspension was then washed thoroughly with deionized water. The moist activated gel was suspended in 600 ml of 1 *M* Na_2CO_3 containing 16.8 ml of picolylamine. The suspension was stirred for 48 h and then washed thoroughly with water and 1 *M* Na_2CO_3.

The picolylamine-coupled gel was suspended in 1.5 l of 1 *M* Na_2CO_3 (pH 12) and 1 l of ethylene glycol containing 40 g of picolyl chloride hydrochloride was slowly added. The suspension was coupled at 80°C in a water-bath for 6 h and was then cooled to room temperature overnight. The final dipicolylamine gel, DPA agarose, was thoroughly washed with water, 10% acetic acid and water again until neutral. The DPA agarose used had a nitrogen content of 590 μmol/g dry weight. The metal ion content of the Zn^{2+}- and Ni^{2+}-loaded gels was found to be 290 and 480 μmol/g dry weight, respectively.

Analytical procedures

The absorbance of the chromatographic fractions was measured at 280 nm with an Ultraspec II spectrophotometer (LKB, Bromma, Sweden). The analysis of the chromatographic fractions was performed by electrophoresis in slabs of 4–30% polyacrylamide gradient gel (PAA 4/30; Pharmacia, Uppsala, Sweden) according to the manufacturer's manual using a GE-2/4 gel electrophoresis apparatus with an EPS 500/400 electrophoresis power supply (Pharmacia). The electrophoresis buffer was 0.09 *M* Tris–0.08 *M* boric acid–0.0025 *M* Na_2EDTA (pH 8.4) and the running conditions were 150 V for 16 h. Fixing was performed with 10% sulphosalicylic acid solution for 30 min, staining with 0.02% Coomassie Brilliant Blue R-250–7% acetic acid in water overnight and destaining with 7% acetic acid overnight. Discontinuous sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (T = 12.2%, C = 1%)[a] was performed by using a gel electrophoresis apparatus from the BMC workshop (Biomedical Centre, Uppsala, Sweden) and the same power supply as above. The upper buffer was 0.040 *M* boric acid–0.41 *M* Tris–0.1% SDS (pH 8.64) and the lower buffer 0.42 *M* Tris (pH 9.18). The running conditions were 200 V for 5 h, the fixing conditions 50% methanol–7% acetic acid in water overnight, the staining conditions 0.05% Coomassie Brilliant Blue R-250 in the above fixing solution for 2–3 h and the destaining conditions were 10% methanol–10% acetic acid in water overnight. Identification and determination of protein concentration were performed by immunodiffusion using LC- and M-partigen immunodiffusion plates according to the manufacturer's manual.

Chromatography

The experiments were performed at room temperature using the Pharmacia FPLC system consisting of two P-500 pumps, an LCC-500 liquid chromatography controller, a Frac-100 fraction collector, a UV-1 single-path monitor and a Rec-482 two-channel recorder. Unless stated otherwise, the following buffers were used as eluents and will be referred to in abbreviated form throughout. Each buffer was de-aerated prior to use.

---

[a] C = g N,N'-methylenebisacrylamide (Bis)/%T; T = g acrylamide + g Bis per 100 ml of solution.

Buffer I (equilibration buffer): 50 m*M* sodium phosphate, 0.5 *M* in NaCl, 0.5 *M* in K_2SO_4 (pH 7.6).

Buffer II: buffer I, 0.1 *M* in imidazole.

Buffer III: 50 m*M* sodium phosphate (pH 7.6).

Buffer IV: 40% (v/v) ethylene glycol in buffer III.

Buffer V: 30% (v/v) 2-propanol in buffer III.

Buffer VI: 20 m*M* sodium phosphate (pH 8.0).

Buffer VII (final gradient buffer): buffer VI, 0–0.5 *M* in NaCl.

Immobilization of metal ions (Ni^{2+} and Zn^{2+}) was done as follows. A 4–5-g amount of the appropriate gel was thoroughly washed with deionized water over a glass filter. The gel was then transferred to a beaker containing 50 ml of a 20 m*M* metal ion solution. After *ca.* 15 min the gel was thoroughly washed with water over a glass filter to remove excess of and loosely bound metal ions. All gels (except Mono-Q, which is a prepacked column purchased from Pharmacia) were degassed prior to packing and packed at a constant flow-rate of 1 ml/min. The total gel volume was *ca.* 6 ml, except for the T and C_6-S gels, which were *ca.* ten and five times larger, respectively, to ensure the capacity needed for adsorption of immunoglobulins and albumin, respectively. The effect of human serum on the new adsorbent was then determined.

A column containing Zn^{2+}-DPA agarose was equilibrated with 50 m*M* sodium phosphate (pH 7.6), 0.5 *M* in NaCl, at a flow-rate of 0.5 ml/min. Human serum (1 ml) was applied and the chromatogram was developed with 0.5 *M* sodium acetate (pH 5.5), 0.5 *M* in NaCl, followed by 50 m*M* sodium phosphate (pH 7.6), 0.5 *M* in NaCl, 50 m*M* in imidazole. The fractions were read manually, pooled and further analysed by electrophoresis in polyacrylamide gradient gels. This procedure was repeated with Ni^{2+}-DPA agarose.

The beds were connected in tandem to produce a main-line tandem column consisting of the following beds in series: Zn^{2+}-TED agarose, T gel, Zn^{2+}-DPA agarose, C_6-S agarose and Ni^{2+}-DPA agarose (see Fig. 1 left). The tandem was equilibrated with five volumes of buffer I. The flow-rate was kept constant at 0.5 ml/min throughout the development of the chromatogram. The sample of human serum was equilibrated to buffer I by using a PD-10 column (1 ml of equilibrated human serum was found to have an absorbance $A_{280} \approx 42$). A 10-ml volume of human serum was applied to the column and the elution proceeded isocratically until the effluent adsorbance reached the baseline. The material passing through the train of beds without adsorption is denoted fraction A. The beds were disconnected and the adsorbed material was displaced from each bed separately, in some instances being transferred to the top of new "side-line" columns consisting in one instance of the T gel and in others of Sephadex G-25 followed by Mono Q. The following fractions were thus obtained (see Fig. 1).

Fraction B: the Zn^{2+}-TED agarose was connected in tandem to a T gel bed (6 ml). The material adsorbed on the Zn^{2+}-TED agarose was displaced with buffer II and allowed to pass into the T gel. These two columns were disconnected and the T gel bed was eluted as described previously [4], *i.e.*, using buffers III, IV and V in sequence.

Fraction C: the column containing T gel was developed as described previously [4].

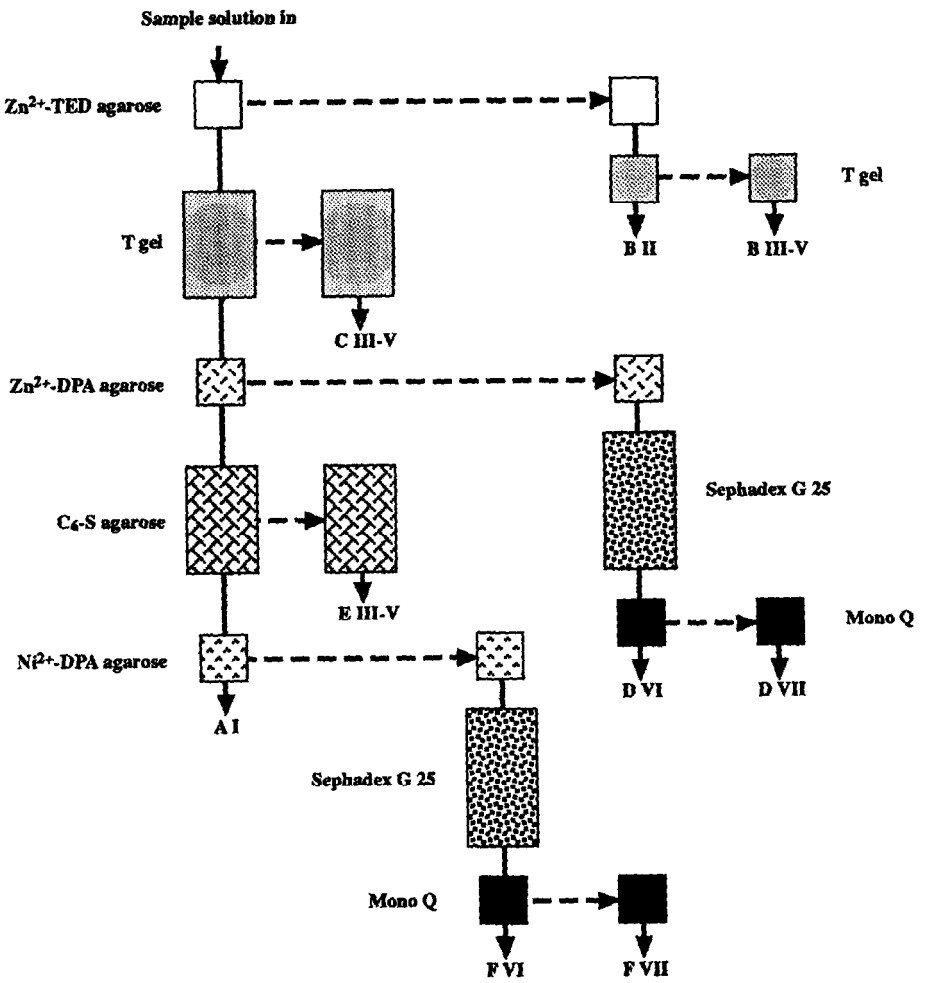


Fig. 1. Schematic diagram of the set-up of the tandem columns. The five columns to the left were connected in series at the start of the experiment. The columns were equilibrated with 50 m*M* sodium phosphate, 0.5 *M* in NaCl and 0.5 *M* in K_2SO_4 (pH 7.6) and 10 ml of human serum (equilibrated in the same buffer) were applied. The beds were washed until the A_{280} of the effluent reached the baseline. The beds were then disconnected and developed separately (see text). Solid arrows indicate liquid flow and dashed arrows indicate disconnection of a bed followed either by elution of adsorbed material directly or elution into a new tandem column. The letters and numbers refer to the fractions collected and the buffers used, respectively.

Fraction D: the adsorbed material on the Zn^{2+}-DPA agarose was displaced with buffer II into a Sephadex G-25 column (120 ml) equilibrated with buffer VI. The effluent was monitored continuously with a recording photometer and conductivity meter. The washing was stopped when the conductivity was increasing and A_{280} had decreased to the baseline. The Sephadex bed was disconnected. The desorbed material from the Zn^{2+}-DPA agarose gel, now in buffer VI, was transferred to a Mono-Q column, equilibrated with the same buffer. Isocratic elution proceeded until baseline

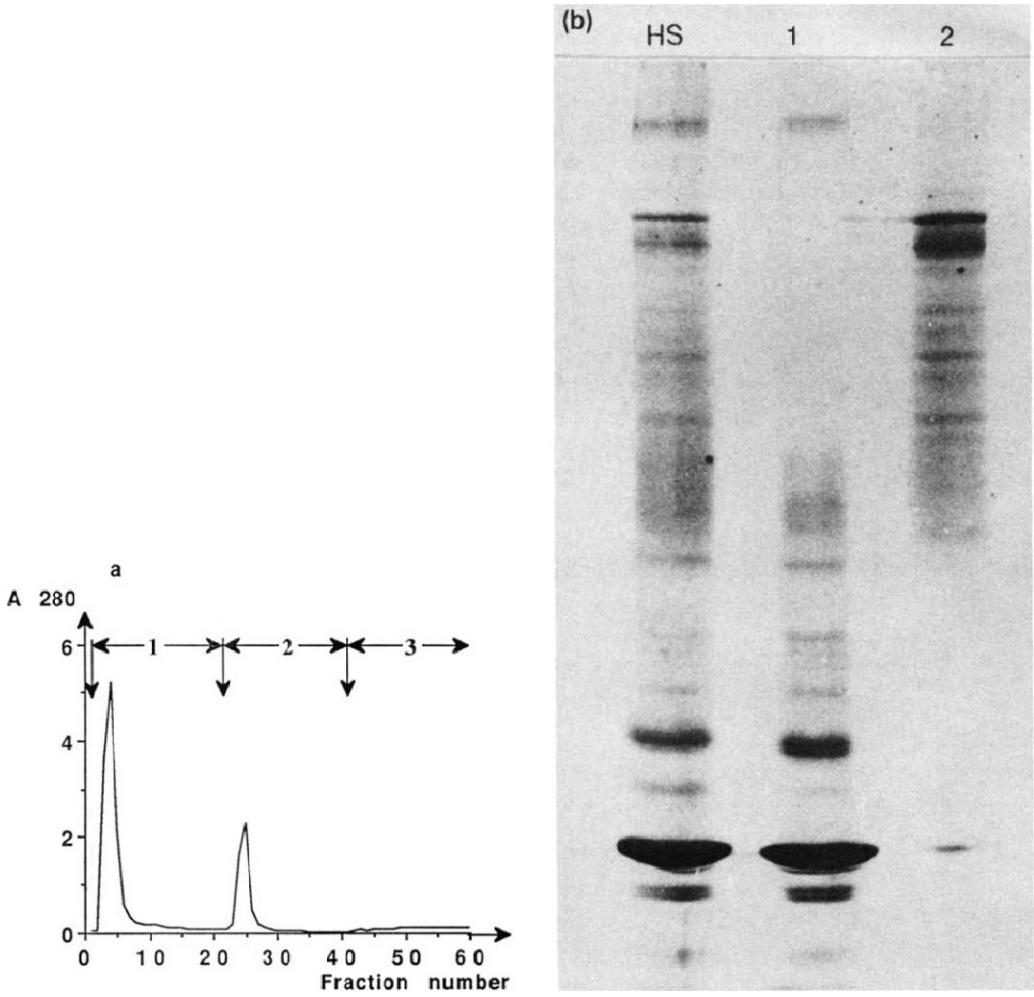


Fig. 2. (a) Chromatogram of Zn^{2+}-DPA agarose. A 1-ml volume of human serum was applied and the column was developed as follows: (1) 50 m*M* sodium phosphate, 0.5 *M* in NaCl (pH 7.6) (equilibration buffer); (2) 0.5 *M* sodium acetate, 0.5 *M* in NaCl (pH 5.5); (3) 0.5 *M* sodium acetate, 0.5 *M* in NaCl and 50 m*M* in imidazole (pH 5.5). (b) Electrophoresis of the peaks collected from the Zn^{2+}-DPA agarose. HS refers to human serum and 1 and 2 to peaks 1 and 2 in the chromatogram in (a).

adsorbance was reached. The Mono-Q bed in turn was disconnected and the adsorbed material in the bed separately eluted with a rising NaCl gradient using buffer VII.

Fraction E: the bed containing C_6-S agarose was developed as described previously [4], *i.e.*, using buffers III, IV and V.

Fraction F: the bed containing Ni^{2+}-DPA agarose was treated in the same way as the bed containing Zn^{2+}-DPA agarose.

RESULTS

Extensive experience in our laboratory has given us the necessary understanding of the properties of the adsorbents for the design of this kind of cascade chromatography. This includes careful matching of the metal ion, adsorbent and the mixture to be fractionated.

Effects of metal ion on human serum

The choice of metal ion to be loaded on the column is important. In our example, Zn^{2+} and Ni^{2+} show different adsorption properties. Thus, a Zn^{2+}-loaded DPA agarose adsorbs about 33% of applied serum proteins whereas a Ni^{2+}-loaded gel adsorbs about 96% (Figs. 2a and 3a). PAGE of the fractions from each chromatogram also reveals different adsorption properties. For example, the Ni^{2+}-DPA agarose adsorbs most of the albumin in human serum whereas Zn^{2+}-DPA agarose does not (Figs. 2b and 3b). α_2-Macroglobulin adsorbs very strongly to Ni^{2+}-DPA agarose (material in the strong band in the upper half of HS in Fig. 3b is not displaced by 50 m*M* imidazole), whereas Zn^{2+}-DPA agarose adsorbs α_2-macroglobulin more weakly. Material in the same band is displaced by buffer not containing imidazole (Fig. 2b).

Adsorption behaviour towards serum proteins of the tandem-coupled beds

For group separation of human serum proteins, we can now design an efficient strategy consisting of sequential adsorption in tandem-coupled beds as follows. Knowing that a Zn^{2+}-TED agarose adsorbs very little material from human serum, we place this gel first in the train of beds. The T gel adsorbs the majority of immunoglobulins and α_2-macroglobulins. It is placed second in the train ahead of the beds of DPA gel. The C_6-S gel adsorbs most of the albumin in human serum. The Ni^{2+}-DPA agarose adsorbs among other proteins albumin, which is not adsorbed by the corresponding Zn^{2+} gel. We therefore insert a bed of C_6-S gel between Zn^{2+} and Ni^{2+} beds of DPA gel to increase the separation efficiency of the last bed of the train (as indicated in the left part of Fig. 1). The arrangement of the side-line beds is also shown schematically in Fig. 1. The protein distribution in each fraction is shown in Table I. Fraction A, consisting of material passing the tandem column, contains less than 1% of the proteins in the sample.

Fig. 4 shows the composite chromatogram and Figs. 5 and 6 the diagrams from gel electrophoresis, gradient PAGE and discontinuous SDS-PAGE, respectively. The lower molecular weight limit for efficient analysis is M_r = 50 000 in Fig. 5 and 10 000 in Fig. 6. Two bands in Fig. 6 cannot be seen in Fig. 5 (the two lower bands in lane Da VII and E V). One of them corresponds to α_1-antitrypsin according to molecular weight and immunodiffusion, whereas the other component cannot be detected by immunodiffusion. It is probably κ- or λ-light chain of immunoglobulin. Some of the other bands in Figs. 5 and 6 can be correlated with the proteins listed in Table I.

DISCUSSION

Successful application of cascade chromatography requires a prior knowledge of the selectivity and capacity of the adsorbents and the relative proportions of the

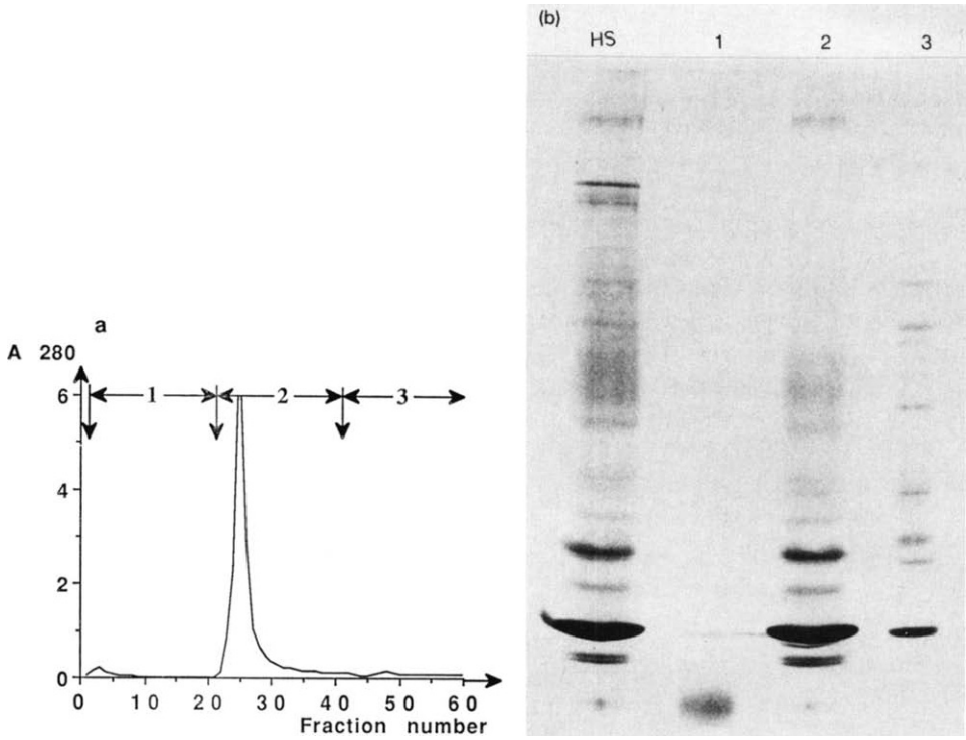


Fig. 3. (a) Chromatogram of Ni^{2+}-DPA agarose. A 1-ml volume of human serum was applied and the column was developed as follows: (1) 50 m*M* sodium phosphate, 0.5 *M* in NaCl (pH 7.6) (equilibration buffer); (2) 0.5 *M* sodium acetate, 0.5 *M* in NaCl (pH 5.5); (3) 0.5 *M* sodium acetate, 0.5 *M* in NaCl and 50 m*M* in imidazole (pH 5.5). (b) Electrophoresis of the peaks collected from the Ni^{2+}-DPA agarose. HS refers to human serum and 1, 2 and 3 to peaks 1, 2 and 3 in the chromatogram in (a).

various species in the mixture to be fractionated. Obviously, therefore, preliminary studies must be undertaken to optimize the efficiency of cascade chromatography whether the intention is the isolation of one, two or many of the components in the mixture. Once all the parameters are known, fractionation can be scaled up or down with retention of the resolving power.

One aim with the cascade technique is to minimize the number of operations. Adsorbents operating according to different affinity parameters should be used. In this study we selected (1) hydrophobic (C_6-S gel), (2) thiophilic (T gel) interactions, (3) size exclusion (Sephadex G-25 for buffer exchange), (4) electrostatic interaction (Mono-Q) and (5–7) three kinds of immobilized metal ion affinity (IMA). The use of IMA adsorbents is exploratory as we do not know *a priori* how the operations of the selected IMA gels complement or overlap each other.

IMA gels in conjunction with the other adsorbents permit a suitable selection of elution conditions and avoid extra buffer changes and separate concentration steps. For example (see Figs. 1 and 5 and Table I), fraction B adsorbed on the T gel is displaced by including imidazole in the buffer. By keeping the ionic strength high,

immunoglobulin M (IgM) is captured in the T bed but α_2-macroglobulin is allowed to pass.

The proteins adsorbed on the DPA gels can be further fractionated on an ion exchanger such as Mono-Q (Fig. 1), but this requires a buffer change. This can be accomplished by transferring the IMA gel to a penultimate Sephadex bed preceding an end bed of Mono-Q. The latter is disconnected after the proteins have just passed the Sephadex.

Albumin and immunoglobulin G (IgG) are the major components in serum and require larger beds for nearly complete adsorption than do the other proteins. If the amounts of gel are insufficient the proteins in question will, to a lesser or greater extent, pass the beds and contaminate fractions further down in the scheme. The T gel captures all IgG.

The major portion of the albumin (*ca.* 97%) is obtained in the C_6-S-adsorbed fraction (E) in almost pure form. The distribution in the other fractions may reflect

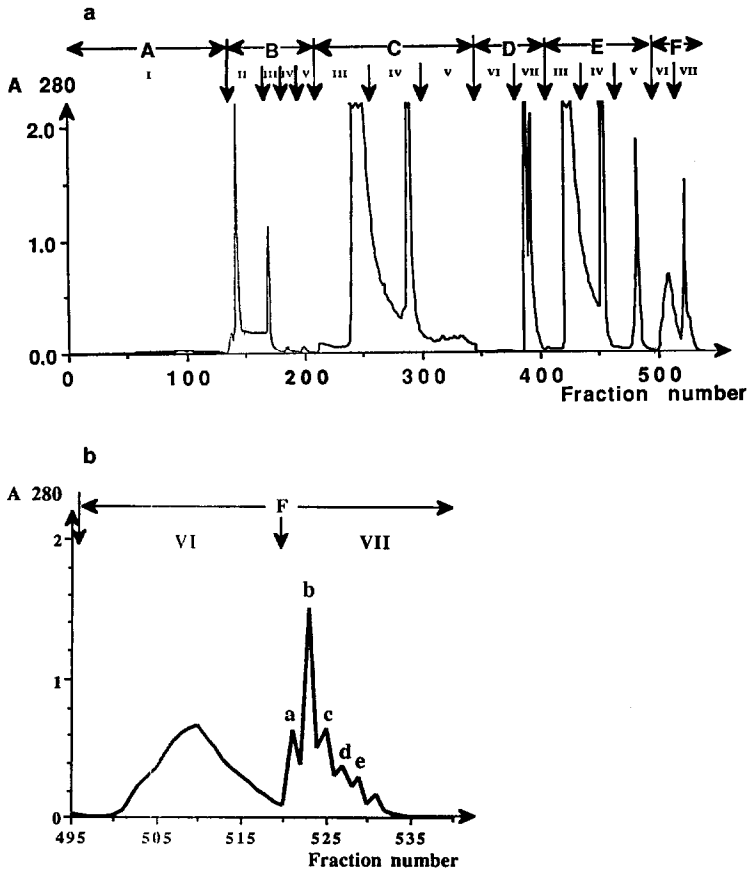


Fig. 4. (a) CASMAC chromatogram: A is the material passing the tandem columns, B is the material from the Zn^{2+}-TED gel, C the T gel, D the Zn^{2+}-DPA gel, E the C_6-S gel and F is the material from the development of the Ni^{2+}-DPA agarose. (b) Expanded part of (a).

TABLE I

AMOUNTS AND RECOVERIES OF SOME SERUM PROTEINS IN THE DIFFERENT FRACTIONS FROM THE DEVELOPMENT OF THE TANDEM COLUMNS

The components were determined using LC- or M-partigen immunodiffusion plates.

Subfraction	Serum protein (mg/l)													
	Albumin	α_2-Macroglobulin	α_1-Microglobulin	α_1-Antitrypsin	Transferrin	Ceruloplasmin	κ light chain	λ light chain	IgG	IgM	IgA	IgD	IgE	Recovery (%)
A I														0.6
B II		64												
B III	14	670							< 1					
B IV		13							0.02	5.7				6.6
B V		2.2								7.4				
C III	< 30	344				< 3	140	126	980		310			
C IV		< 14					56	77	780		64			40.1
C V							< 1.9	< 1.9	12		< 0.2			
Da VII	470			3.5			< 1.8	< 1.8			> 220			
Db VII	385			< 1.8		440	< 1.8	< 1.8			124			7.0
E III	16 000													
E IV	14 000													38.3
E V	49													
F VI					220									
Fa VII					65									
Fb VII			< 0.4	210	48						< 0.4			
Fc VII				55	< 1.0									5.6
Fd VII	< 5			105	≪ 2.6									
Fe VII	< 1.8			< 0.2										
Total														98.2

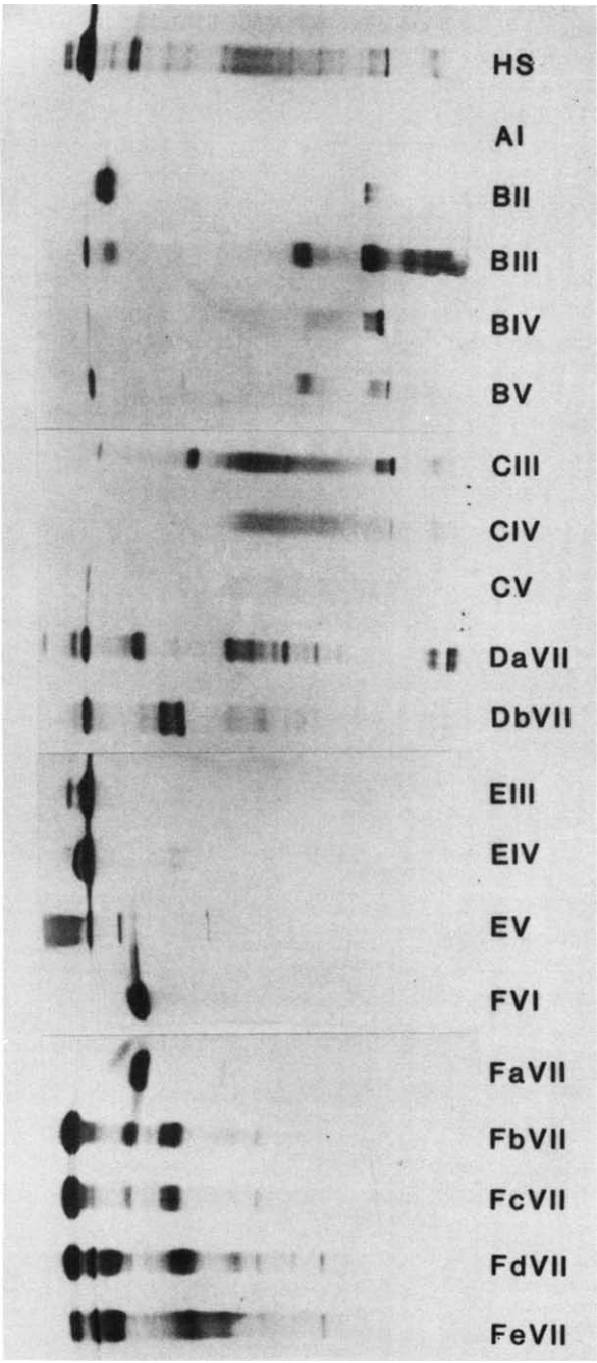


Fig. 5. Electrophoresis of the materials in the peaks collected from the chromatogram in Fig. 4a. Pharmacia PAA 4/30 polyacrylamide gradient gels were used.

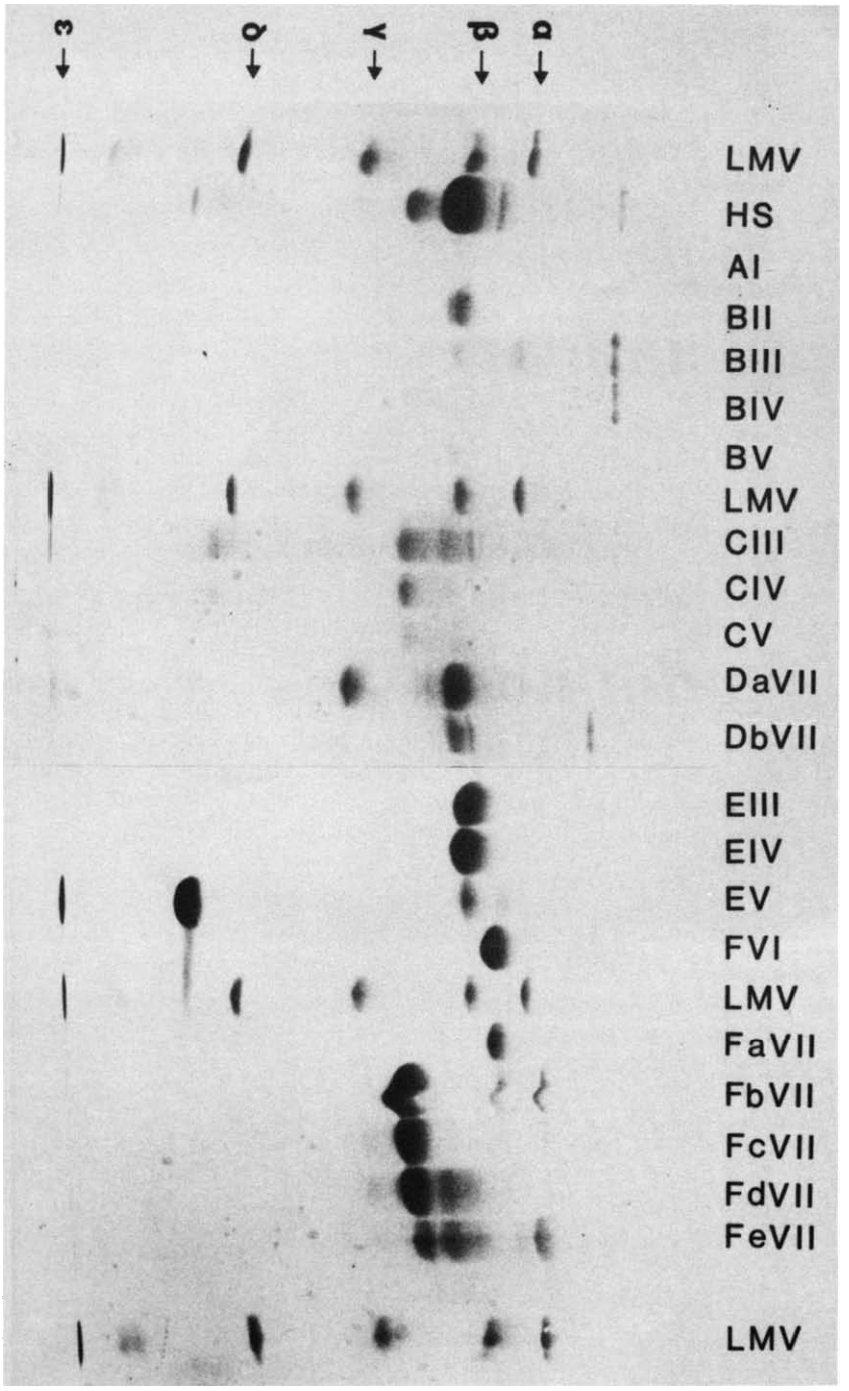


Fig. 6. Discontinuous SDS-PAGE of the materials in the peaks collected from the chromatogram in Fig. 4a. α = Phosphorylase *b* (M_r 94 000); β = bovine serum albumin (M_r 67 000); γ = ovalbumin (M_r 43 000); δ = carbonic anhydrase (M_r 29 000); ε = α-lactalbumin (M_r 14 400).

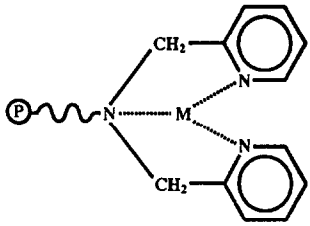
albumin heterogeneity. No albumin is found in fraction F, which proves that the C_6-S bed has sufficient capacity. The importance of proper bed order should be emphasized. If Ni^{2+}-DPA agarose had preceded the C_6-S gel, the albumin would have been distributed in both beds.

IgA has less affinity for the T gel than has IgG. Whereas the adsorption capacity is sufficient for the IgG (Fig. 1), it is not for IgA. The IgA that passes the T gel is captured in the Zn^{2+}-DPA bed where also all ceruloplasmin present in the serum is recovered together with some albumin. Transferrin and α_1-antitrypsin pass all the beds except the Ni^{2+}-DPA and they can subsequently be separated on Mono-Q.

CASMAC is a technique of general potential utility but its future success depends on the availability of a large number of selective adsorbents that can be applied generally to bioseparations. The number of possible combinations of chelate moieties and metal ions is enormous. IDA, TED and DPA are just some variants of the ligand part and Zn^{2+} and Ni^{2+} of the possible metal ions. However, a limited number of ligands with simple structures may serve most needs. In this paper some additional comments on the properties and use of DPA agarose may be justified.

Dipicolylamine (DPA), is a tridentate ligand like iminodiacetate (IDA) [7]. On binding a metal ion, an adsorption site is formed from DPA which may differ from IDA in its affinity for proteins. DPA possesses three nitrogen atoms for coordination whereas IDA has only one. Owing to the two carboxyls in IDA, the net charge of the adsorption site is lowered in the pH range 5–8 to a greater extent than for DPA. However, at the high ionic strength used in the experiments reported here, ionic attraction should be effectively suppressed. The π-electrons, the heteroaromatic nitrogen and the bulkiness of the pyridyl groups are all factors that should affect the affinity character of the DPA gel.

A DPA gel presumably has the structure



where P is the polymer matrix and M the metal ion. If, as is likely, Zn^{2+} and Ni^{2+} form hexacoordinate adsorption complexes, there should be three coordination sites per metal ion available for binding water or solutes. For steric reasons only one site on a protein will be bound, *e.g.*, an imidazole group from a histidine residue or, possibly, two oxygens from a phosphate group on a phosphoprotein.

Systematic stability studies of the immobilized metal ion complexes with the DPA ligand have not yet been undertaken. Therefore, in this study we deliberately avoided prewashing with weak chelators such as glycine. No overt signs of metal ion transfer have been discovered. Under the conditions used the metal ions are not bound in stoichiometric proportions to the fixed ligand; what that means remains to be established.

A comparison of the results shown in Figs. 2 and 3 with those obtained under

similar conditions for Zn^{2+}- and Ni^{2+}-IDA revealed both similarities and differences. The DPA gels seem to have broader selectivity and relatively higher capacity for human serum proteins. The structure–adsorption properties for tridentate liganded gels have to be studied in more detail.

The strength of protein interaction appears to be stronger with DPA than IDA. The protein adsorption capacity is of the same order (10–50 mg/ml gel bed). The metal ions can be easily removed from the adsorbents with a strong soluble chelator such as EDTA or by washing with acid.

The cascade adsorption procedures can be further extended and the separation power much improved by the introduction of gradient and affinity elution methods, *e.g.*, CASMAC can be combined with high-performance liquid chromatography by using the individual beds from the cascade as precolumns in the latter technique. It should also be possible to automate CASMAC. For large-scale, especially industrial, applications, batch operations in tanks is likely to be a preferred technique. This presumably requires the use of cheaper matrix materials such as cellulose, polyacrylates and hydrophilized silica.

ACKNOWLEDGEMENTS

We thank Professor John Law for valuable suggestions regarding the preparation of the manuscript and Ms. Karin Hellström and Mrs. Birgitta Hansson for technical assistance. This work was financially supported by the Swedish Board for Technical Development (STUF), an early grant from the Swedish Natural Science Research Council and the Erna and Victor Hasselblad Foundation.

REFERENCES

1 R. K. Scopes and J. Porath, *Bioseparation*, 1 (1990) 3.
2 J. Porath, *J. Chromatogr.*, 376 (1986) 331.
3 R. K. Scopes, *J. Chromatogr.*, 376 (1986) 131.
4 J. Porath and B. Olin, *Biochemistry*, 22 (1983) 1621.
5 F. Maisano, M. Belew and J. Porath, *J. Chromatogr.*, 321 (1985) 305.
6 J. Porath, F. Maisano and M. Belew, *FEBS Lett.*, 185 (1985) 306.
7 J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature (London)*, 258 (1975) 598.