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HIGH-PERFORMANCE ADSORPTION CHROMATOGRAPHY OF PROTEINS ON DEFORMED NON-POROUS AGAROSE BEADS COATED WITH INSOLUBLE METAL COMPOUNDS

I. COATING: FERRIC OXYHYDROXIDE WITH STOICHIOMETRICALLY BOUND PHOSPHATE

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SUMMARY

A new support for adsorption chromatography of proteins based on cross-linked agarose gels containing ferric (hydr)oxide is described. Two methods have been used for immobilization of the ferric (hydr)oxide on agarose beads; namely,entrapment of preformed ferric oxide particles in connection with the preparation of macroporous agarose beads and precipitation of ferric oxyhydroxide on and in preformed non-porous agarose beads. In the former method many of the adsorption sites of the ferric oxide may become sterically blocked, resulting in chromatographic properties which are similar to those of cation exchangers. Therefore, attention was focused on the second method. Upon using compressed beds of non-porous agarose beads the resolution became independent of the flow-rate, even when the bead diameter was relatively large. When ferric oxyhydroxide agarose is equilibrated with phosphate buffer, some phosphate becomes stoichiometrically and irreversibly bound to the matrix. The separation mechanism of this ferric oxyhydroxide phosphate agarose is discussed. It differs from those of ion-exchange, hydrophobic-interaction, and molecular-sieve chromatography. One characteristic is that phosphate buffers are the most efficient eluting agents. Chromatography on ferric oxyhydroxide phosphate–agarose is accordingly a good complement to these three conventional methods. Some chromatographic properties of the adsorbents are described (resolution as a function of gradient time, flow-rate and protein load; and the pH dependence of the adsorption). A successful separation of a model mixture of six proteins was achieved. Purification of a commercial preparation of β-glucosidase is also presented.

INTRODUCTION

Macromolecules, such as proteins, bind to many insoluble metal salts, oxides and hydroxides, which are therefore potential chromatographic adsorbents. These metal compounds often appear in the form of gelatinous precipitates or as very fine,

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mechanically unstable particles. For this reason they give high resistance against hydrodynamic flow, which in practice prevents their direct use as chromatographic bed materials for column operation. This problem can be overcome by entrapping the adsorbing particles in a macroporous gel, for instance agarose[1–3]. However, this latter method may have the drawback that many of the adsorption sites become sterically blocked by the gel polymer chains. To avoid this, we have developed a technique based on direct precipitation of the metal compound in and on the surface of preformed gel beads. As a matrix we have used non-porous agarose beads because, following compression of the bed, they have the important property of giving a high resolution which is independent of the flow-rate even when their diameter is large[4–6].

In this paper we report the application of ferric oxide, Fe_2O_3, and ferric oxyhydroxide, FeO(OH), immobilized on beads of non-porous cross-linked agarose to the separation of proteins. Some characteristic features of those chromatographic materials are described. Chromatographic supports based on aluminium and zirconium (hydr)oxides will be presented in a forthcoming paper[7].

EXPERIMENTAL AND RESULTS

Chemicals and equipment

Ferric oxide particles (Fe_2O_3) were Bayferrox Red special grade 1120 Z of size 0.1 × 0.8 μm from Bayer (Leverkusen, F.R.G.). Agarose was a kind gift from Dr. R. Armisén, Hispanagar S.A., Spain. All salts were of analytical grade from Merck (Darmstadt, F.R.G.) Ovalbumin (molecular weight, MW = 43 000, p*I* 4.7), bovine serum albumin (MW 69 000, p*I* 4.9), carbonic anhydrase (MW 30 000, p*I* 5.9), α-chymotrypsinogen A (MW 25 000, p*I* 8.8), ribonuclease (MW 13 700, p*I* 8.9), aldolase (MW 158 000, p*I* 9.1), cytochrome *c* (MW 12 200, p*I* 9.2) and lysozyme (MW 14 600, p*I* 10) were from Sigma (St. Louis, MO, U.S.A.). β-Glucosidase from sweet almonds and 3-nitrophenyl-β-glucopyranoside were obtained from Serva (Heidelberg, F.R.G.). Thyroglobulin (MW 670 000, p*I* 4.5) was obtained from Pharmacia (Uppsala, Sweden). Human transferrin (p*I* 5.5) and human serum albumin (p*I* 4.9) were from Kabi (Stockholm, Sweden). Haemoglobin A_{1c} (p*I* 6.9) was from Bio-Rad (Richmond, CA, U.S.A.). Before use all buffer solutions were filtered through a 0.22-μm Millipore filter and degassed.

The chromatographic system, including a Model 2150 HPLC pump, a Model 2152 gradient controller, a Model 2151 variable-wavelength UV monitor and a Model 2220 reporting integrator, was obtained from LKB (Bromma, Sweden) and the HPLC sample injector from Rheodyne (Berkeley, CA, U.S.A.).

Free zone electrophoresis was performed in a 400 mm × 3 mm I.D. quartz tube which was rotated around its long axis at a speed of 40 rpm for stabilization against convection[8].

Preparation of Fe_2O_3–agarose by entrapment of ferric oxide particles into macroporous agarose beads

Agarose beads were prepared by an emulsion-gelation method[9] with some modificatons: 1 g of ferric oxide (Fe_2O_3) powder was added to the hot 12% aqueous agarose solution before the addition of the heated organic phase containing the emulsifier and the suspension was agitated extensively for 10 min before cooling. The

beads containing Fe_2O_3 were sized by elutriation in water[10]. Beads with diameters in the range 15–20 μm were collected and cross-linked twice with γ-glycidoxypropyltrimethoxysilane by a procedure described previously[4]. This material will be referred to as Fe_2O_3–agarose. The Fe_2O_3–agarose beads were packed in water at a constant flow-rate of 2 ml/min into a Plexiglas column with an inner diameter of 6 mm.

Preparation of non-porous agarose beads

Beads of 12% agarose were prepared by the emulsion-gelation method described earlier[9] and were sized by elutriation in water[10]. Beads with diameters of 15–20 μm were collected and shrunk in an organic solvent according to a previous method[5] and then cross-linked twice with γ-glycidoxypropyltrimethoxysilane[4] or butanediol diglycidyl ether[5]. Upon shrinkage, the diameters of the beads decreased to 10–15 μm. A microscopic investigation of the shrunken and cross-linked agarose beads removed from the column immediately after packing at 2 ml/min revealed that the beads had become deformed upon compression, leading to tight contact between their surfaces and to a decrease in the void volume of the bed.

To investigate indirectly the change in pore size of the agarose beads as a result of the shrinkage, a column (30 mm × 6 mm I.D.) was packed with the shrunken agarose beads cross-linked with γ-glycidoxypropyltrimethoxysilane. The following substances were applied separately on each column: potassium chromate (MW 194), ribonuclease (MW 13 700), chymotrypsinogen A (MW 25 000), bovine serum albumin (MW 69 000), aldolase (MW 158 000) and thyroglubilin (MW 670 000); 0.3 *M* potassium phosphate buffer, pH 7.0, was used for elution.

The retention times were plotted against the molecular weights (Fig. 1). It is evident that the shrunken beads were impermeable to proteins, at least to those with molecular weights higher than about 10 000, since the retention times of the proteins were independent of their molecular weights. Similar results were obtained when the experiment was repeated with shrunken agarose beads cross-linked with butanediol diglycidyl ether (Fig. 1). The shrunken, cross-linked beads will be referred to as non-porous agarose beads. Agarose beads cross-linked with γ-glycidoxypropyltrimethoxysilane were used in all the experiments described below, unless otherwise stated.

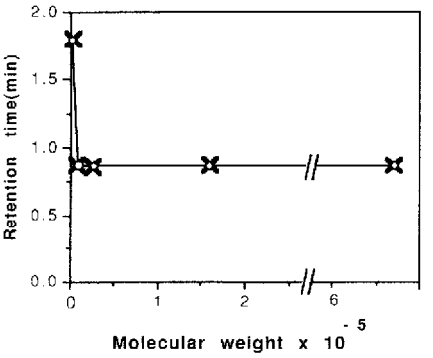

Fig. 1. Retention times of proteins with different molecular weights on a column of deformed non-porous agarose beads. ○ = cross-linked with γ-glycidoxypropyltrimethoxysilane; × = cross-linked with butanediol diglycidyl ether.

Precipitation of ferric oxyhydroxide onto preformed non-porous agarose beads

A 5-g amount of sedimented non-porous agarose beads was incubated for 15 min in 10 ml of water in which 1 g of $FeCl_3$ had been dissolved. The beads were then collected by centrifugation and a 10-ml volume of 2 *M* NH_4OH was added. The suspension was mixed thoroughly and shaken for 5 min. The beads became dark brown, indicating the formation of ferric oxyhydroxide, FeO(OH). To eliminate ferric oxyhydroxide not bound to agarose, the suspension was washed six times with water by centrifugation at 800 *g* until the supernatant became clear or until no free oxyhydroxide clusters were observed by light microscopy.

The beads were packed in distilled water into a Plexiglas column, 6 mm in diameter, to a height of 30–35 mm under such a high pressure (corresponding to a flow-rate of 2 ml/min) that the beads became deformed. The upper piston of the column tube was then pressed down to make contact with the surface of the agarose bed. Otherwise, the bed will expand when the experiments are performed at a lower flow-rate (= lower pressure).

The mechanical properties of FeO(OH)–agarose beads

Two columns with the same total volume were packed: one containing non-porous cross-linked agarose beads, the other containing identical beads coated with ferric oxyhydroxide. Both columns were equilibrated with 0.3 *M* potassium phosphate buffer, pH 7.0, at flow-rates ranging from 0.7 to 5 ml/min. The relationship between the flow-rate and pressure was the same for the two columns. The continuous use of flow-rates above 3 ml/min resulted in some leakage of the ferric oxyhydroxide coating.

Free zone electrophoresis of ferric oxyhydroxide and ferric oxide

Ferric oxyhydroxide was prepared as described under *Precipitation of ferric oxyhydroxide onto preformed non-porous agarose beads* but in the absence of agarose beads. Electrophoresis was performed in both 0.01 *M* potassium phosphate, pH 6.0, and 0.01 *M* sodium cacodylate, pH 6.0, using 1500 V and 3 mA for 15 min. The experiment was repeated with particles of ferric oxide suspended in the same buffers. In both buffers, ferric oxyhydroxide and ferric oxide migrated toward the positive electrode.

Elemental analysis of ferric oxyhydroxide-coated agarose beads

A column of ferric oxyhydroxide agarose was equilibrated with phosphate buffer (phosphate buffers were used for desorption), washed overnight with water and then analyzed for iron and phosphorus. The elemental analysis showed that phosphate had become irreversibly bound to the adsorbent at a molar metal-to-phosphate ratio of 2:1. Since some of the ligands of the ferric ions accordingly are replaced by the phosphate ions and these ions are used for equilibration and elution, it is appropriate to designate the adsorbent ferric oxyhydroxide phosphate by the *simplified* notation $FeO(OH)(PO_4)$.

Chromatographic characterization of Fe_2O_3– and $FeO(OH)(PO_4)$–agarose

Two columns were packed with Fe_2O_3–agarose and $FeO(OH)(PO_4)$–agarose, respectively, to a total volume of 2.5 ml each and equilibrated with 0.005 *M* potassium

phosphate buffer, pH 7.0. These columns were used to investigate the influence of different ions on the elution of proteins. A sample containing 75 μg each of the proteins myoglobin, chymotrypsinogen A and cytochrome *c* dissolved in the equilibration buffer was applied onto each column. In another set of experiments a sample containing 75 μg each of the proteins ovalbumin, human serum albumin and human transferrin was applied to each column. The columns were washed with the equilibration buffer and then eluted with the same buffer using a gradient in salt concentration from 0 to 0.2 *M* over 30 min. The following salts were used: sodium, potassium and ammonium chloride, ammonium hydrogencarbonate, sodium EDTA and ammonium acetate. An experiment was also performed with only a gradient in potassium phosphate concentration from 0.005 to 0.2 *M* over 30 min (without adding any salt). The same experiments were then performed on both columns equilibrated with 0.01 *M* sodium acetate buffer, pH 5.5, or 0.01 *M* sodium borate buffer, pH 9.1, instead of 0.005 *M* potassium phosphate buffer, pH 7.0. In brief, the above experiments gave the following results.

Proteins with p*I* values below the pH of the equilibration buffer did not adsorb to the ferric oxide–agarose column. The adsorbed proteins were eluted in the order of increasing p*I* values (for p*I* values, see the *Chemicals and equipment* section). There was no significant difference between the elution patterns of the proteins eluted with gradients formed by the different salts.

Equilibration of the column with acetate buffer at pH 5.5 instead of phosphate buffer, pH 7.0, resulted in stronger adsorption of the proteins. Upon equilibration with borate buffer at pH 9.1, cytochrome *c* was the only protein bound. The ferric oxide–agarose column thus had cation-exchange properties.

Similarly, proteins were eluted from ferric oxyhydroxide phosphate agarose roughly in the order of increasing p*I* values, but only with a gradient in potassium phosphate concentration and not by the other salts. Of the proteins tested, only ovalbumin did not adsorb to the column. Successful separations were not obtained when the column was equilibrated with 0.01 *M* sodium acetate buffer at pH 5.5, owing to very strong adsorption of the proteins. After equilibration with sodium borate buffer at pH 9.1, the resolution of more acidic proteins was unsatisfactory, due to weak adsorption. Some further experiments indicated that equilibration with a cacodylate buffer, pH 6.0, gave a moderate adsorption and this buffer was therefore used in all subsequent experiments.

The above experiments indicated that Fe_2O_3–agarose had chromatographically less interesting properties than $FeO(OH)(PO_4)$–agarose. Therefore, no further experiments with the former material were designed.

Fig. 2a shows the chromatography of a model protein mixture on the column of $FeO(OH)(PO_4)$–agarose equilibrated with 0.002 *M* potassium phosphate buffer, pH 6.0, and eluted with a 30-min linear gradient in potassium phosphate concentration from 0.002 to 0.2 *M* (pH 6.0). Since dihydrogenphosphate has a pK_a value of 7.2 and therefore a relatively low buffering capacity at pH 6.0 (the pH to be preferred, see above), we repeated the experiment in the presence of a "background buffer" of sodium cacodylate (pK_a 6.27). The column was equilibrated with 0.01 *M* sodium cacodylate buffer, pH 6.0, and eluted with a linear gradient in potassium phosphate concentration from 0 to 0.2 *M* over 30 min in the presence of the equilibration buffer (Fig. 2b). A comparison of Fig. 2a and b shows that somewhat more narrow peaks were obtained in the presence of cacodylate.

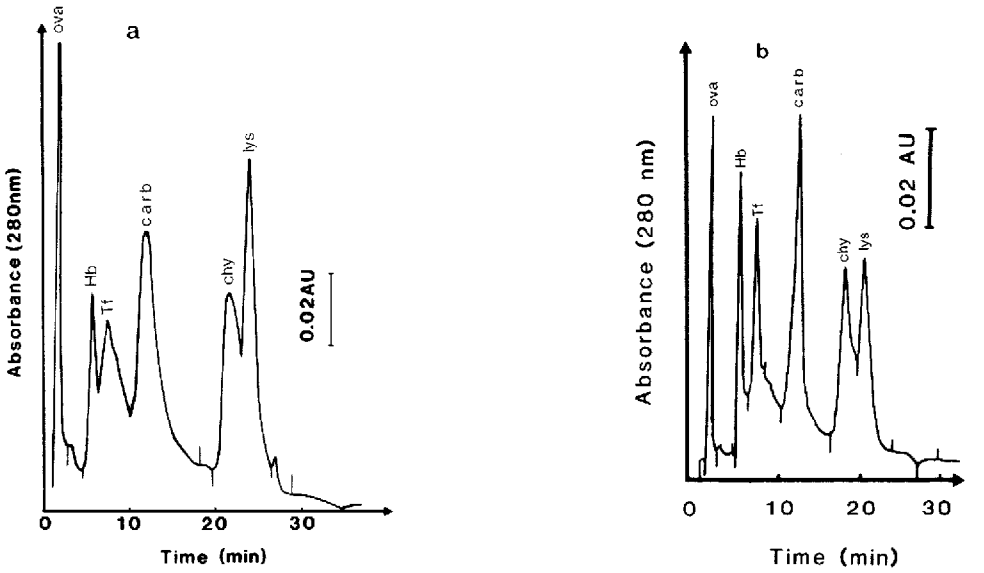

Fig. 2. Chromatography of a model protein mixture on a $FeO(OH)(PO_4)$–agarose column. Column dimensions: 60 mm × 6 mm I.D. Flow-rate: 1 ml/min. Sample: 75 μg of each of the following proteins in a total volume of 60 μl: ovalbumin (ova), haemoglobin A_{1c} (Hb), human transferrin (Tf), carbonic anhydrase (carb), chymotrypsinogen A (chy) and lysozyme (lys). Elution: (a) a linear gradient in potassium phosphate from 0.002 to 0.2 *M* over 30 min, pH 6.0; (b) a linear gradient in potassium phosphate from 0 to 0.2 *M* over 30 min in 0.01 *M* sodium cacodylate, pH 6.0. Sharper peaks were obtained in the presence of cacodylate.

In all experiments to be discussed we used cacodylate as a buffering ion (and phosphate as a desorbing ion) on ferric oxyhydroxide phosphate–agarose columns (*i.e.*, $FeO(OH)$–agarose equilibrated with phosphate). The cacodylate ions alone did not desorb the proteins.

Resolution as a function of some different chromatographic parameters

The influence of different experimental conditions on the resolution of two proteins on a $FeO(OH)(PO_4)$–agarose column was investigated. Eqn. 1 was used for the calculation of the resolution, R_s, between two components (α-chymotrypsinogen A and carbonic anhydrase),

$$R_s = \frac{t_2 - t_1}{0.5\ (t_{w2} + t_{w1})} \tag{1}$$

where t_2 and t_1 are the retention times and t_{w2} and t_{w1} are the peak widths at half the height of the peaks (in time units) of α-chymotrypsinogen A and carbonic anhydrase, respectively.

Gradient time. Aliquots containing 50 μg each of α-chymotrypsinogen A and carbonic anhydrase dissolved in 100 μl of the equilibration buffer (0.01 *M* sodium cacodylate, pH 6.0), were applied onto the $FeO(OH)(PO_4)$–agarose column and eluted with a gradient in potassium phosphate concentration from 0 to 0.15 *M* in the presence

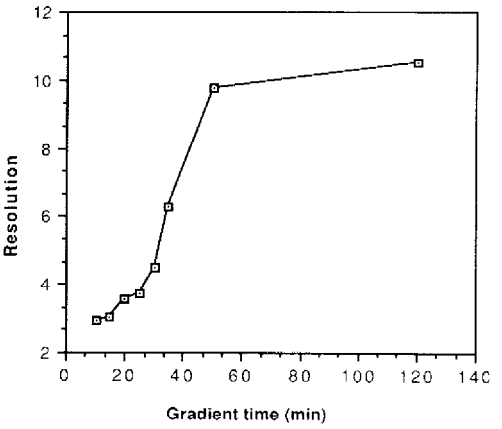

Fig. 3. Resolution of chymotrypsinogen A and carbonic anhydrase *versus* gradient time on a column of $FeO(OH)(PO_4)$–agarose. For details see the Experimental and results section.

of the cacodylate buffer to get a higher buffering capacity. Gradient times were varied between 10 and 120 min. The flow-rate was kept constant at 1 ml/min. Fig. 3 shows that the resolution increased with the gradient time up to about 50 min.

Flow-rate. The same sample as described under *Gradient time* was applied on the column and eluted with the same gradient at flow-rates ranging from 0.2 to 3 ml/min. The total volume of the gradient was kept constant at 25 ml and the gradient times were changed in proportion to the flow-rates to keep dc/dv constant, *i.e.*, the concentration gradient with respect to volume[5]. Fig. 4 shows that there was little, if any, dependence of the resolution on the flow-rate. The recorder chart speed was chosen proportional to the gradient time to give all of the chromatograms the same width, thereby facilitating visual comparison of the separation patterns.

Protein load. Samples containing different amounts of α-chymotrypsinogen A and carbonic anhydrase, ranging from 50 to 1250 μg of each protein dissolved in the equilibration buffer, were applied onto a $FeO(OH)(PO_4)$–agarose column with a total volume of 1 ml equilibrated with 0.01 *M* sodium cacodylate, pH 6.0. Proteins were eluted with the same gradient as described under *Gradient time* over 10 min. The

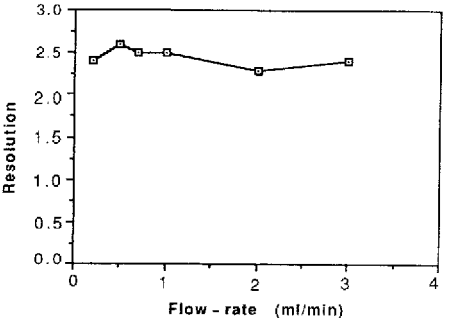

Fig. 4. Resolution of chymotrypsinogen A and carbonic anhydrase *versus* flow-rate on a column of $FeO(OH)(PO_4)$–agarose. For details see the Experimental and results section.

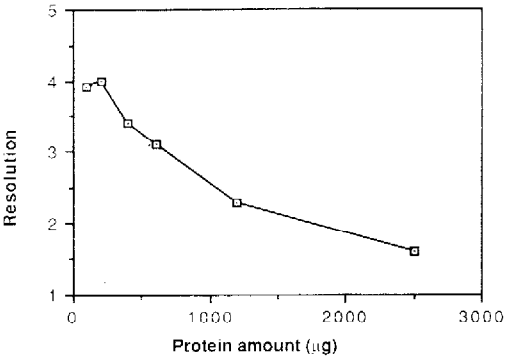

Fig. 5. Resolution of chymotrypsinogen A and carbonic anhydrase *versus* total amount of protein in the sample on a column of $FeO(OH)(PO_4)$–agarose. For details see the Experimental and Results section.

flow-rate was kept constant at 1 ml/min. Fig. 5 shows the decrease in resolution observed when the load of protein was increased.

Chromatography of a commercial preparation of β-glucosidase on $FeO(OH)(PO_4)$–agarose

β-Glucosidase (50 μg) dissolved in 50 μl of the equilibration buffer was applied onto the ferric oxyhydroxide phosphate–agarose column equilibrated with 0.01 *M* sodium cacodylate buffer, pH 6.0. The column was eluted with a gradient in concentration of potassium phosphate from 0 to 0.3 *M* over 30 min in the presence of 0.01 *M* sodium cacodylate, pH 6.0, at a flow-rate of 1 ml/min. Fractions of 1 ml were collected. The activity of β-glucosidase in each fraction was measured with 3-nitrophenyl-β-glucopyranoside as a substrate as described previously[11]. Fig. 6 shows several components without β-glucosidase activity and one peak containing 97% of the initial enzyme activity, indicating an extensive purification of the commercial preparation of β-glucosidase.

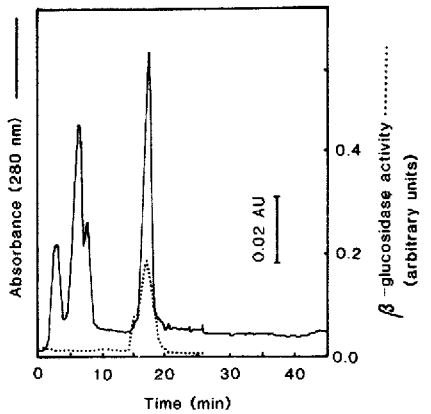

Fig. 6. Chromatography of a commercial preparation of β-glucosidase on a column of $FeO(OH)(PO_4)$–agarose. Elution: a gradient in potassium phosphate from 0 to 0.3 *M* over 30 min in 0.01 *M* sodium cacodylate, pH 6.0. Column dimensions: 60 mm × 6 mm I.D. Flow-rate: 1 ml/min. Sample: 50 μg of β-glucosidase dissolved in 50 μl of the equilibration buffer; substrate: 3-nitrophenyl-β-glucopyranoside.

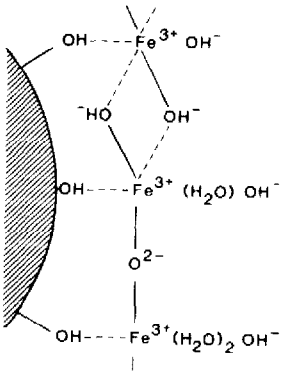

Fig. 7. Proposed possible structure of the ferric oxyhydroxide coating on agarose beads. The coordination number of ferric ions is six. One or more of the coordination sites is thought to be occupied by hydroxyl groups of the agarose. The remaining sites are coordinating water molecules or hydroxide ions, the ratio between these depending on the pH of the surrounding liquid. The hydroxide ions can be shared by other ferric ions forming double hydroxy bridges or, upon rearrangement of water molecules, oxy bridges. In this way a network of ferric oxyhydroxide is formed on and in the agarose bead. After equilibration with phosphate buffer and washing with distilled water overnight, some of the phosphate ions cannot be removed, but are irreversibly bound in a molar ratio between iron and phosphate close to 2.0. How these are incorporated into the ferric oxyhydroxide agarose is not known.

DISCUSSION

The phenomenon that proteins adsorb to ferric (hydr)oxides can be utilized in different ways. For instance, particles of ferric oxide with antibodies or antigens adsorbed on their surfaces have been used for batch affinity separation of proteins[12] and in immunoagglutination assays for the detection of the corresponding antigen or antibody[13]. The separation mechanism of ferric oxyhydroxide phosphate is unknown. We know, however, that it differs from those of ion-exchange, hydrophobic-interaction and molecular-sieve chromatography, since it is not in a simple way related to the charge, hydrophobicity or size of the proteins. Chromatography on ferric oxyhydroxide phosphate–agarose columns in combination with one of these other methods can therefore be expected to give a high purification.

When iron trichloride is dissolved in water, the ferric ion coordinates six aquo groups[a]. One or more of these can probably be displaced by hydroxyl groups on and in the agarose beads in the same way as hydrated titanium oxide is bound by polysaccharides[14]. A layer of ferric ions coordinating OH^- or aquo groups may thus form on the surface and other accessible parts of agarose beads (Fig. 7). This formation may also be stabilized by hydrogen bonding between the coordinated water and the hydroxyl groups of the agarose. To a certain extent, some of the adsorbed water molecules spontaneously lose a proton to the bulk solution, thereby forming hydroxide ions. This deprotonation is strongly facilitated when the pH is raised by the addition of ammonium hydroxide to the solution. The deprotonated water molcules form double hydroxy bridges between ferric ions[15]. This process continues in three

[a] Chloride ions are also coordinated to the ferric ions in solution, but they are omitted in the text and the figure.

dimensions until the agarose beads become covered by a network of ferric ions coordinating water molecules and binding to each other by shared hydroxyl groups.

Upon aging, the hydroxy bridges lose water molecules to form a ferric polyoxy complex on and in the bead[15]. Depending on the pH of the surrounding solution, the remaining coordinated water molcules can lose more protons, thus giving the ferric oxyhydroxide layer a negatively charged surface.

Elemental analysis of phosphate-treated FeO(OH)–agarose shows that some phosphate is irreversibly bound in a molar metal-to-phosphate ratio of 2:1. This is further evidence that the outer layer of the bead should have negative charges.

In free zone electrophoresis in cacodylate and phosphate buffers of pH 6.0, ferric oxyhydroxide migrated towards the positive electrode, which showed that the surface of the oxyhydroxide is negatively charged. It should therefore not be surprising that this support shows pronounced ion-exchange properties. However, the binding of acidic proteins to ferric oxyhydroxide phosphate agarose shows that mechanisms other than ion exchange also take part in the adsorption of proteins to the matrix. The bed material differs from ion exchangers also in that, among the ions tested, only phosphate ions desorb proteins. On the other hand, the elution order of proteins is roughly in accord with their p*I* values.

Certain groups on the protein molecule might have the ability to displace aquo groups coordinated to the ferric ions (Fig. 7). These groups are the carboxyl groups of the C-terminus, aspartyl and glutamyl residues, the phenoxy groups of tyrosyl residues, the imidazole groups of histidyl residues, the guanidinium groups of arginyl residues, the thiol groups of cysteinyl residues and the amino groups of the N-terminus and lysyl residues[14]. Another mode of adsorption might involve an ionic interaction between the negatively charged hydroxide and phosphate ions of ferric oxyhydroxide and positively charged amino acid residues in the protein. If all of the above interactions occur, ferric oxyhydroxide may display both anionic and cationic properties.

Such a combination of different modes of adsorption might explain the differences between chromatography on $FeO(OH)(PO_4)$–agarose and the more conventional methods mentioned above.

In Fe_2O_3–agarose, protein groups may be prevented from displacing water coordinated to ferric ions by steric hindrance arising from the tight contact between the surface of the ferric oxide particles and the carbohydrate chains of the agarose which is created during the preparation of the beads. However, it is likely that the negatively charged surface of the metal oxide can still attract positive groups in the protein, thus allowing Fe_2O_3–agarose to funtion as a cation exchanger. Although the application of this bed material is limited, it was used successfully for the separation of γ-globulin from human serum[2] and for the enrichment of gp70, an envelope protein from the cell culture fluid of feline leukemia virus[3].

Comparison of Fig. 2a and b shows that sharper peaks were obtained when the elution was performed in the presence of cacodylate ions to increase the buffering capacity. A stable pH is essential, since the adsorption is pH dependent (see *Chromatographic characterization of Fe_2O_3- and $FeO(OH)(PO_4)$–agarose*). However, we cannot exclude the possibility that cacodylate ions have some specific desorbing properties analogous to those of "modifiers" used in reversed-phase chromatography.

Chromatography of a model protein mixture on non-shrunken macroporous cross-linked agarose beads coated with ferric oxyhydroxide phosphate gave broader peaks as compared to chromatography on $FeO(OH)(PO_4)$-coated shrunken non-porous cross-linked agarose beads (results not shown). This is not surprising, since the use of non-porous agarose beads prevents zone broadening caused by diffusion of proteins in and out of the beads. We have increased the resolution further by compressing the beds so that the diffusion paths between them are reduced[4-6,16]. The favourable effects of shrinkage, non-porosity and compression of the beads are discussed briefly in refs. 4–6.

The resolution on columns packed with compressed non-porous agarose beads is independent of the flow-rate (Fig. 4 and refs. 4–6), which permits short analysis times without loss of resolution.

Probably the ferric oxyhydroxide on the surface of a bead extends into the cavities of the bead. This anchorage of the ferric oxyhydroxide, together with the proposed mechanism for the binding of ferric oxyhydroxide to agarose, may explain why no leakage from the column was observed at moderate flow-rates. However, continuous use of flow-rates above 3 ml/min on ferric oxyhydroxide–agarose should be avoided owing to mechanical loss of ferric oxyhydroxide.

We have shown herein that ferric oxyhydroxide phosphate agarose under suitable conditions can yield a good separation of proteins. The mechanism of this separation, although not completely understood, is obviously different from that of conventional chromatographic media. It therefore seems appropriate to describe this new adsorbent even at this early stage of development. However, more data should be collected concerning the specificity of ferric oxyhydroxide phosphate for different proteins and other groups of biological substances and the applicability of this new chromatographic material for their separation.

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REFERENCES

1 S. Hjertén, I. Zelikman, I. Kourteva and J. Lindeberg, presented at *6th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Baden-Baden, October 20–22, 1986*, Abstract No. 113.
2 S. Hjertén and I. Zelikman, *J. Biochem. Biophys. Methods*, 17 (1988) 67–74.
3 I. Zelikman, L. Åkerblom, S. Hjertén and B. Morein, *Biotech. Appl. Biochem.*, 11 (1989) 209–216.
4 S. Hjertén, Y. Kunquan and J.-l. Liao, *Makromol. Chem.*, 17 (1988) 349–357.
5 S. Hjertén and J.-l. Liao, *J. Chromatogr.*, 457 (1988) 165–174.
6 J.-l. Liao and S. Hjertén, *J. Chromatogr.*, 457 (1988) 175–182.
7 S. Hjertén, I. Zelikman, J. Lindeberg and M. Lederer, *J. Chromatogr.*, 481 (1989) 187–199.
8 S. Hjertén, *Chromatogr. Rev.*, 9 (1967) 122–219.
9 S. Hjertén, *Biochim. Biophys. Acta*, 79 (1964) 393–398.
10 S. Hjertén, in M. T. W. Hearn (Editor), *HPLC of Proteins, Peptides and Polynucleotides*, VCH, Weinheim, in press.
11 W. Hössel and A. Nahrstedt, *Hoppe-Seyler's Z. Physiol. Chem.*, 356 (1975) 1265–1275.
12 I. Giaever, *U.S. Pat.*, 4 018 886 (1977).

13 I. Zelikman and S. Hjertén, *J. Immunol. Meth.*, 114 (1988) 267–273.
14 J. F. Kennedy, *Chemical Soc. Rev.*, 8 (1979) 221–257.
15 A. Müller, *Arzneim.-Forsch.*, 17 (1967) 920–931.
16 S. Hjertén, Z.-q. Liu and D. Yang, *J. Chromatogr.*, 296 (1984) 115–120.