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Separation of immunoglobulin G by high-performance pseudo-bioaffinity chromatography with immobilized histidine

I. Preliminary report on the influence of the silica support and the coupling mode

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ABSTRACT

High-performance liquid affinity chromatography with immobilized histidine as a preudo-biospecific ligand has been used for the fractionation of human immunoglobulin G (IgG). Histidine was immobilized onto silica in two different modes: directly onto silica after epoxy activation or using an intermediate amino derivatization of silica and then coupling histidine using water-soluble carbodiimide. The behaviours and capacities of the obtained affinity supports as well as the influence of pH, silica type, pore diameter and coupling mode have been studied. IgG was effectively separated from human plasma and high maximal binding capacities were obtained.

INTRODUCTION

Affinity chromatography is a very selective method for the purification and separation of proteins and other biomolecules. It is based on a specific interaction between an immobilized ligand and the molecule to be purified. As most biospecific ligands are expensive or unstable in chromatographic systems, they are often replaced by pseudo-biospecific ligands, which normally are simpler molecules [1]. Thus, amino acids such as arginine, tryptophan and lysine have been used to purify fibronectin, cellulase and plasminogen, respectively [2–4]. Previous work in our laboratory has shown that histidine can also be used as a ligand for the purification of proteins such as chymosin, acid protease of *Aspergillus niger* and yeast carboxypeptidase [5], myxaline (bacterial human blood anticoagulant) [6], phycocyanin chromopeptides [7] and IgG [8,9]. Now we hope to combine the specificity of histidine ligand affinity chromatography with HPLC technology to obtain a fast, selective and effective method for the separation of IgG subclasses. In this first part of our work we have studied the influence of silica type and pore size as well as the coupling mode of histidine.

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EXPERIMENTAL

Materials

Human placental IgG was from Institut Mérieux Recherches (Lyon, France). 3-Glycidoxypropyltrimethoxysilane was from Serva (Heidelberg, Germany), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and histidine were from Sigma (St. Louis, MO, USA).

LiChrosorb SI 100 (amorphous silica, 10 μm, 100 Å) was purchased from Merck, Nucleosil 300-10 (spherical silica, 10 μm, 300 Å) was from Macherey–Nagel (Düren, Germany); Chromatorex (spherical aminopropyl silica, 10 μm, 100, 320 and 540 Å) was supplied by Chromato Group, Fuji Davison Chemical (Nagoya, Japan).

Immobilization of histidine on silica

Histidyl-epoxy silica. Silica was activated with 3-glycidoxypropyltrimethoxysilane as described by Chang *et al.* [10]. Then the activated silica was added to a 20% histidine in a dimethylformamide–water (1:1, v/v) solution and stirred for 48 h at room temperature.

Histidyl-aminopropyl silica. A 3-g aliquot of Chromatorex aminopropyl silica was added to 40 ml of a 10% histidine solution at pH 5.0, then 120 mg of EDC were added and the solution stirred for 30 h at room temperature.

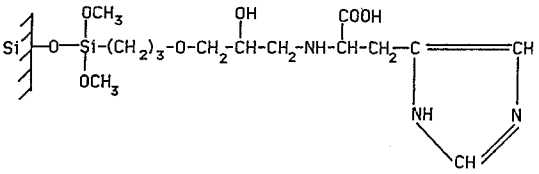
The structures of the synthesized gels are shown in Fig. 1.

High-performance liquid chromatography

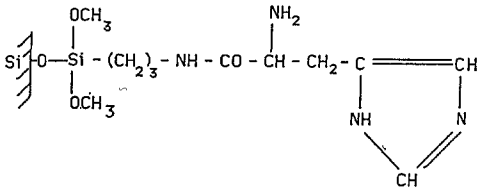
Histidyl silica materials in 25 m*M* Tris–HCl buffer, pH 7.4, were slurry packed into columns of 50 × 4.7 mm I.D., at a constant pressure of 250–300 bar of nitrogen, with a Touzart et Matignon unit (Vitry, France). All chromatographic procedures were performed with an HPLC system consisting of a Waters F6000 A pump (Milford, MA, USA), an LKB 2138 UVICORD UV detector (Bromma, Sweden), a 7161 Rheodyne injector (Cotati, CA, USA) with 500-μl filling loop and a Servotrace recorder (Paris, France).

Protein determination

IgG was determined spectrophotometrically at



Histidyl-epoxy silica



Histidyl-amino silica

Fig. 1. Structure of histidyl silica.

280 nm using a molar absorptivity of 14 for a 1% solution with a Beckman (Fullerton, CA, USA) DU-70 spectrophotometer.

Electrophoresis

The chromatographic fractions were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [11] using a 10% polyacrylamide gel. Samples diluted with water were applied under reducing conditions. The gel was stained with Coomassie brilliant blue.

Determination of capacities and apparent dissociation constants of the affinity matrices

The capacity and dissociation constant (K_D) were determined in a batch mode: 100 mg of different silica gels were added to IgG solutions at different protein concentrations in Tris–HCl buffer 25 m*M*, pH 7.4, to a final volume of 3 ml and stirred for 30 min. Then the gels were allowed to settle and the supernatants were discharged. The gels were washed three times with the same buffer, then the bound protein was elut-

ed with Tris–HCl buffer containing 1 *M* sodium chloride and the quantity of adsorbed protein was determined.

RESULTS AND DISCUSSION

For the immobilization of histidine to the silica matrix, two different coupling methods were used. In the first method, histidine was immobilized to an epoxy-activated silica by its amino group and the carboxyl groups and imidazole ring remained free. In the second method, histidine was coupled through its carboxyl group to an amino silica and the amino group of the histidine as well as the imidazole ring were free (Fig. 1).

Early work in our laboratory [8,9] using Sepharose as support matrix showed that IgG can be adsorbed onto immobilized histidine at pH 7.4 and elution is achieved with sodium chloride-containing buffers. We obtained the same adsorption pH optimum for the gels studied in this work. A typical result is shown in Fig. 2; maximal adsorption was achieved at pH 7.4; at pH values lower than 6 and higher than 8, the adsorption was only 50% of that at pH 7.4.

Fig. 3 shows the elution profiles of two columns containing histidyl-epoxy silica (a) and histidyl-amino silica (b). For the first gel, even at

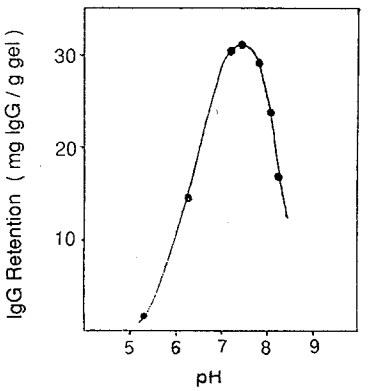


Fig. 2. Influence of pH on IgG retention on a histidyl silica column. Column: Histidyl-epoxy silica (Nucleosil, 10 μm, 300 Å), 50 × 4.7 mm I.D. Injected IgG: 140–150 mg. Equilibration buffer (EB): 25 m*M* Tris–HCl, pH 7.4. Elution buffer: EB + 1.0 *M* sodium chloride, pH 7.4. Detection: UV 277 nm, 0.5 a.u.f.s. Flow-rate: 1.0 ml/min.

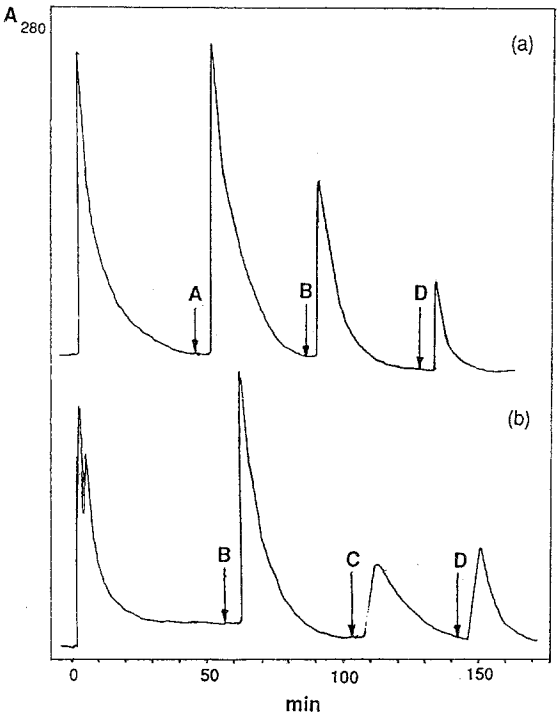


Fig. 3. Comparison of separation of human placental IgG on histidyl-epoxy silica and histidyl-amino silica. (a) Histidyl-amino silica (Nucleosil, 10 μm, 300 Å), 50 × 4.7 mm I.D. (b) Histidyl-epoxy silica (Chromatorex, 10 μm, 320 Å), 50 × 4.7 mm I.D. Injected IgG: 33 mg. Equilibration buffer (EB): 25 m*M* Tris–HCl, pH 7.4. (A) EB + 0.05 *M* sodium chloride, pH 7.4; (B) EB + 0.10 *M* sodium chloride, pH 7.4; (C) EB + 0.20 *M* sodium chloride, pH 7.4; (D) EB + 1.0 *M* sodium chloride, pH 7.4. For other conditions, see Fig. 2.

low salt concentrations (0.05 *M* sodium chloride) some IgG fractions (subsets) were eluted, while 0.1 *M* sodium chloride was needed in the case of histidyl-amino silica for the first appearance of the IgG-containing fraction. The differences in the net charge pattern of the two adsorbents studied might cause the retention of some IgG subsets with different binding strengths. This is borne out by the fact that in one of our previous papers we have in fact shown a difference in the isoelectric point distribution of fractions eluted from affinity adsorbents with immobilized histidine with different sodium chloride concentrations [9]. However, based on the optimal adsorption parameters, we suppose that adsorption is mainly due to the imidazole ring. The amino group or the carboxyl group further helps in de-

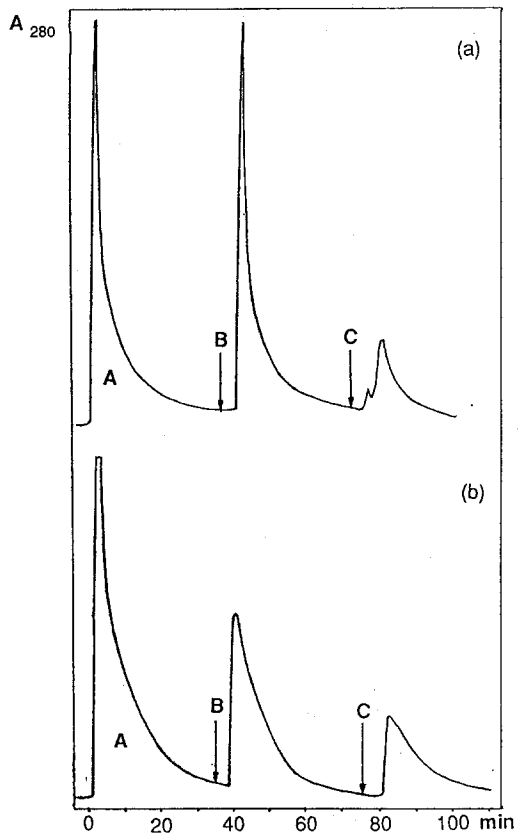


Fig. 4. Comparison of separation of human placental IgG on histidyl silica matrices with different pore diameters. (a) Histidyl-amino silica (Chromatorex, 10 μm, 320 Å), 50 × 4.7 mm, I.D. (b) Histidyl-amino silica (Chromatorex, 10 μm, 540 Å), 50 × 4.7 mm, I.D. (A) 25 m*M* Tris–HCl, pH 7.4; (B) A + 0.2 *M* sodium chloride, pH 7.4; (C) A + 1.0 *M* sodium chloride, pH 7.4. For other conditions, see Fig. 2.

termining the differential binding strength of the IgG subsets.

The elution pattern of IgG was also influenced by the pore size of the silica matrix. Fig. 4 shows that with a pore diameter of 320 Å sharp peaks are obtained, whereas with a pore diameter of 540 Å the peaks are broadened. This effect can be explained by the diffusion of the IgG molecules into the larger pores, which causes retardartion; in contrast, diffusion into the smaller pores is limited and the protein is mostly excluded.

The specificity for IgG binding on histidyl silica obtained with pure IgG was verified by comparing the results obtained with prepurified human placental IgG (Fig. 5a) and with microfiltered human plasma (Fig. 5b) as starting material. The samples were injected in Tris–HCl buffer pH 7.4. After elution with different sodium chloride concentrations in the Tris–HCl buffer, we obtained for both starting materials three peaks at 0.1, 0.2 and 1 *M* sodium chloride. As shown in an SDS-PAGE (Fig. 6), the first peak in the chromatography of human plasma, which represents the unbound protein, contains albumin ($M_r \approx$ 68 000) and some other proteins (lane 6). The main bands in the three elution peaks of the two starting materials correspond to IgG (heavy chain, M_r 50 000; light chain, 25 000; lanes 2–5 and 7–9). The purest fractions were eluted at 0.2 *M* sodium chloride (lanes 4 and 8). IgG is also

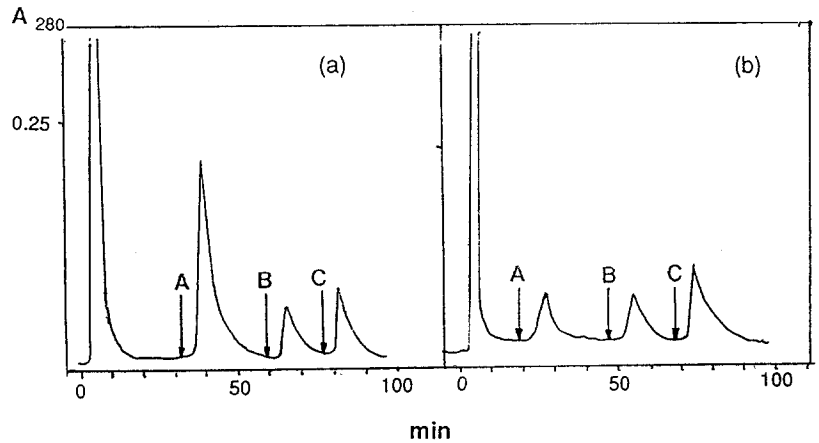


Fig. 5. Separation of human placental IgG from human plasma on histidyl silica (Chromatorex, 10 μm, 320 Å), 50 × 4.7 mm I.D. (A) EB + 0.1 *M* sodium chloride, pH 7.4; (B) EB + 0.2 *M* sodium chloride, pH 7.4; (C) EB + 1.0 *M* sodium chloride, pH 7.4. (a) Injected IgG: 26 mg; (b) Injected human plasma: 0.4 ml. For other conditions, see Fig. 2.

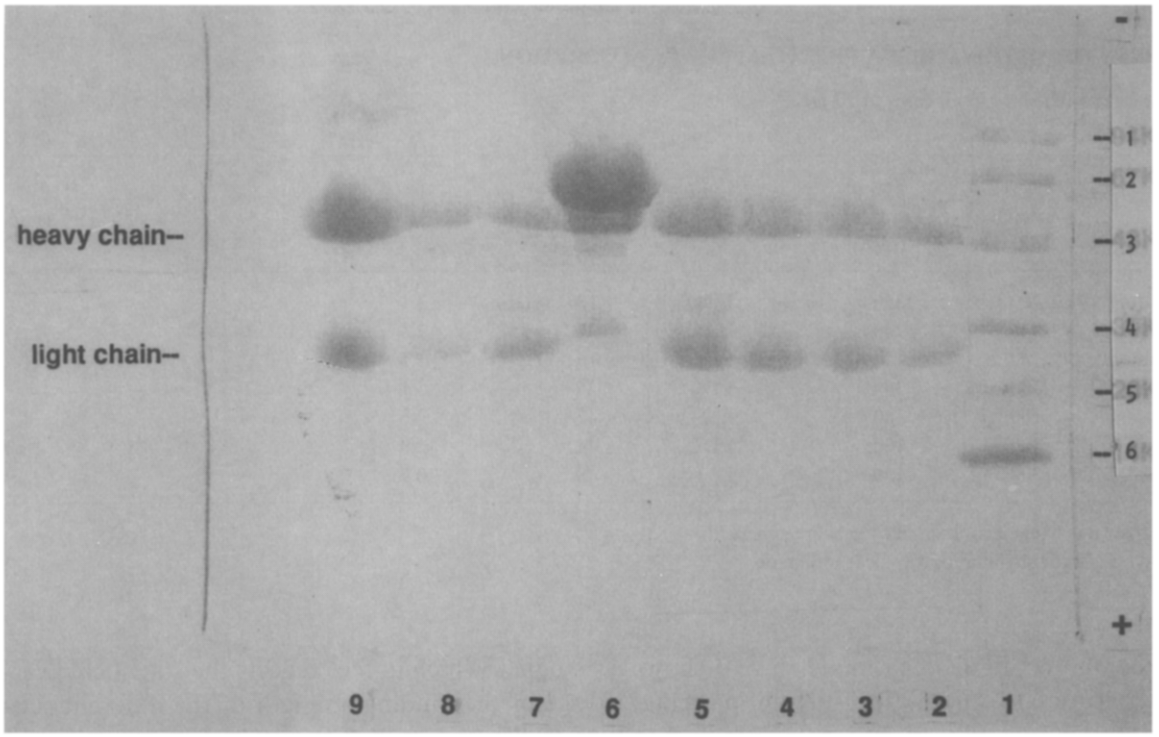


Fig. 6. SDS-PAGE (10% T, Laemmli discontinuous buffer system) of the eluted fractions in Fig. 5. Lanes 2, 3, 4 and 5 represent peaks 1, 2, 3 and 4 of human placental IgG in Fig. 5a; lanes 6, 7, 8 and 9 represent peaks 1, 2, 3 and 4 of microfiltered human plasma in Fig. 5b. Lane 1 contains molecular mass markers (1 = phosphorylase *b*, 94 000; 2 = bovine serum albumin, 67 000; 3 = ovalbumin, 43 000; 4 = carbonic anhydrase, 30 000; 5 = soybean trypsin inhibitor, 20 100; 6 = lactalbumin, 14 400).

contained in the non-retained fractions (lanes 2 and 6).

The elution of IgG at different sodium chloride concentrations indicates that there are different subsets of IgG with different affinities for histidine. In addition, previous work in our laboratory using agarose gels as well as silica gels has shown that the affinity of the four IgG subclasses for immobilized histidine was different. IgG_1 is retained and preferentially eluted with 0.2 *M* sodium chloride added to the starting buffer. The separation and quantitative determination of the different IgG subclasses and subsets will be the subject of the following paper.

An important factor in the scale-up separation of IgG by affinity chromatography is the capacity of the affinity matrix. In our work we have determined the capacities of different silica supports with immobilized histidine as well as the dissociation constants of the corresponding protein–li-

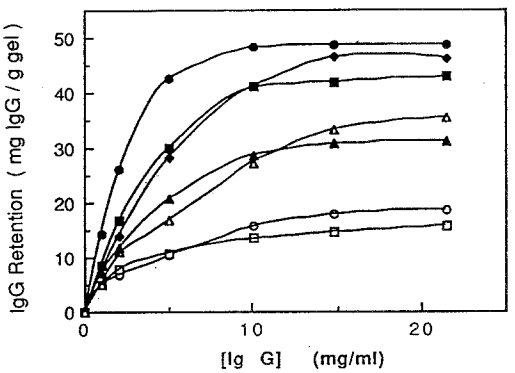


Fig. 7. Adsorption isotherm of human placental IgG on different histidyl silica gels in a batch mode. Equilibration and washing with 25 m*M* Tris–HCl buffer, pH 7.4 (A). Elution: A + 1.0 *M* sodium chloride, pH 7.4. ● = Histidyl-epoxy silica (300 Å, Nucleosil); ◆ = histidyl-amino silica (300 Å, Nucleosil); ■ = histidyl-epoxy silica (100 Å, LiChrosorb); △ = histidyl-amino silica (100 Å, LiChrosorb); ▲ = histidyl-amino silica (320 Å, Chromatorex); ○ = histidyl-amino silica (100 Å, Chromatorex); □ = histidyl-amino silica (540 Å, Chromatorex).

TABLE I

CAPACITY OF HISTIDYL SILICA FOR IgG AFFINITY ADSORPTION

Particle size of the silica in all cases in 10 μm.

Trade name	Pore diameter (Å)	Specific surface area (m^2/g)	Coupling method	Capacity in batch (mg IgG/g gel)	K_D (10^{-5} M)
Chromatorex	100	350	EDC[b]	18	3.1
Chromatorex	320	100	EDC	31	2.4
Chromatorex	540	70	EDC	16	1.5
LiChrosorb[a]	100	300	EDC	35	5.1
LiChrosorb	100	300	Epoxy	42	2.6
Nucleosil	300	100	EDC	45	2.9
Nucleosil	300	100	Epoxy	48	0.97

[a] LiChrosorb is irregular; other silica gels are spherical.

[b] 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide.

gand complexes in a batch mode. Fig. 7 shows the adsorption isotherm for human placental IgG, taken as a whole, on different histidyl silica gels. The apparent K_D and the capacity values, determined with a non-linear regression based on the Langmuir adsorption isotherm, are shown in Table I. In fact, Nucleosil with 300 Å pore size gives the highest capacity, but all the tested gels showed a high capacity for IgG which was between two and four times higher than that obtained with soft agarose gel (result not shown). For both spherical (Nucleosil) and amorphous (LiChrosorb) silica, epoxy silica shows slightly higher capacity than amino silica, whereas amino silica gels from Chromatorex showed comparatively lower capacities. (However, within this group the 320 Å pore size silica had clearly higher capacities than the gels of 100 Å or 540 Å pore size.)

The dissociation constants determined in batch mode are in apparent contradiction to the results in Fig. 3 indicating that the adsorption is always stronger for the epoxy-derivatized gels. This can be at least partially explained by the fact that in dynamic chromatographic mode the differences in binding strength of different IgG subsets are clearly reflected in a step gradient elution mode. In contrast, in the batch mode, only adsorption is studied, which in fact reflects the statistical average of the binding strength of the different subsets. This has to be further investigated using the chromatographically separated subset fractions for these studies.

The higher capacities obtained for the histidyl-epoxy silica gels are certainly due to the higher ligand concentration, which is about 100 μmol per gram of gel for the epoxy-activated gels but only 58 μmol/g for the amino silica (Nucleosil).

The binding capacity is influenced by the pore size of the silica particles as shown for histidyl-amino silica (Chromatorex). The highest capacity was obtained with a pore diameter of 320 Å. For the gel with 540-Å pores, the capacity decreased to 50%, which is probably because of the smaller specific surface area, whereas in the case of the gel with 100 Å pore diameter the lower capacity (60%) may be the result of mass transfer limited by diffusion of the IgG molecules (M_r 150 000) into these small pores. The relatively high capacity of the amorphous gel LiChrosorb in comparison with that of spherical Chromatorex with the same pore size is related to the different number of active hydroxyl groups which remain on the surface after the production of the silica beads and which are in fact the potential anchoring points for histidine coupling.

On the basis of these results we can formulate the following rules: (i) Gels with pore sizes of 300 Å are the most suitable for good working capacity. This is in accordance with the observations in ref. 12 for proteins in general. (ii) Histidine coupled to silica through an oxirane group shows a higher capacity. but the binding strength of the different IgG subsets depends on the functional group remaining free after immobilization of the ligand. (iii) Independent of the coupling chemistry and the pore size, the silica itself, *i.e.* its chemistry, has a significant role to play in determining the capacity, even when the specificity is the same in all cases.

CONCLUSIONS

High-performance pseudo-bioaffinity chromatography with immobilized histidine is a selective, simple and rapid method for the purification and separation of IgG from microfiltered human plasma and for the separation of IgG subsets. It is an attractive alternative to protein A or G affinity chromatography because of its very low cost and because the low molecular mass of histidine makes it free of any eventual denaturation and hence confers good working stability.

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