Journal of Chromatography, 376 (1986) 259—267
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2950

NEW SUPPORT FOR THE LARGE-SCALE PURIFICATION OF PROTEINS

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SUMMARY

We propose a new affinity sorbent, matrix-linked histidine, for the large-scale purification of proteins. A variety of proteins and certain peptides, each distinct from the other, were purified. Immunoglobulin G from human placenta was chosen for a detailed study concerning the effects of coupling, spacer-arm and other parameters. Multiple interactions such as charge-transfer and other ionic reactions have been suggested to be responsible for the interactions between proteins and ligand.

INTRODUCTION

Recently, the term affinity chromatography has achieved a very wide sense of meaning, unlike in the beginning when it was applied only to cases where pure biospecificity was the force behind the ligand—biomolecule interactions (by biospecificity is meant the interactions between antibody and antigen, enzyme and inhibitor/substrate analogue, hormone and receptor, etc). Today, dye—ligand chromatography, immobilized-metal affinity chromatography (IMAC) and amino acid ligand affinity (AALA) chromatography, though not actually biospecific techniques, are considered as forms of affinity chromatography. The ligands employed in these techniques exhibit multiple interactions with various proteins, depending on their nature and behaviour under different conditions. These ligands have been termed general ligands and they include dyes, metals, amino acids and various molecules possessing potential sites for these interactions.

Amino acids such as lysine, arginine and tryptophan have been coupled to Sepharose for the purification of proteins [1–3]. The use of histidine as a coupled ligand for the purification of chymosin and certain chromopeptides

0378-4347/86/$03.50

has been reported [4, 5]. Davankov et al. [6] and Boue et al. [7] have used N-decylhistidine and proline coupled to silica for the resolution of amino acids.

In this paper, we demonstrate the versatile nature of histidine as a general ligand for the pseudo-specific purification of many biological molecules (i.e. proteins and peptides), each different from one another.

EXPERIMENTAL

Histidine was coupled to Sepharose 4B, preferentially through the α-NH_2, after epoxy activation of the Sepharose either with epichlorhydrin (short chain length) or with 1,4-butanediol diglycidyl ether (long chain length). According to Vijayalakshmi and Porath [3] and Sundberg and Porath [8], oxirane silica was prepared by the method of Chang et al. [9], then histidine was coupled as described by Amourache and Vijayalakshmi [4].

Protein concentration was determined by measuring the absorbance at 280 nm. The acid protease was assayed using the method described by Ichishima [10], with casein as substrate. Chymosin was assayed by testing for its milk-clotting activity as described by Amourache and Vijayalakshmi [4]. Immunoglobulin G (IgG) was assayed by radioimmunodiffusion as outlined by Glasner and Wenig [11]. Carboxypeptidase Y activity was measured using benzoyl-L-tyrosine-*p*-nitroaniline (BTPNA) as substrate, based on the method suggested by Aibara et al. [12]. Chromatography was carried out at 4 and 20°C. Flow-rates as high as 3 ml/min were used, with satisfactory results in the case of silica columns.

RESULTS

Characteristics of the adsorbents

The coupling method employed and the support matrices used are important parameters. The use of longer oxirans to link L-histidine to the matrix plays an important role in determining the purity of the protein to be purified. This will be clearly demonstrated in one of the examples cited: purification of IgG from placenta.

A detailed study was carried out for IgG, to determine the effects of the different supports silica and Sepharose, and of the length of the chain between ligand and support. While the method of fixation of histidine to epoxy-Sepharose and oxirane-silica are known, it was necessary to establish the procedure of fixation of histidine to bis(oxirane)-Sepharose. The ideal conditions for coupling were found to be: (i) an incubation time of 12—16 h under agitation, (ii) an incubation temperature of 75°C and (iii) a histidine concentration of 20% in carbonate–bicarbonate buffer (0.5 *M*, pH 11). Using these parameters, a maximal concentration of 250 μM His per g of dry gel was found to be fixed.

Chromatography was carried out using histidyloxirane-Sepharose 4B, histidyloxirane-silica and histidylbis(oxirane)-Sepharose 4B. Studies using histidine coupled to silica using a spacer-arm are under way. Table I compares the purities and the yield of IgG obtained using the three types of affinity sorbents.

TABLE I

COMPARATIVE EFFICIENCIES OF DIFFERENT GELS TESTED FOR IgG PURIFICATION

Affinity sorbent	Purification factor	Yield (%)
Histidyloxirane-Sepharose	53.5	17
Histityloxirane-silica	22	26
Histidylbis(oxirane)-Sepharose	157	7

TABLE II

CAPACITIES OF THE DIFFERENT GELS TESTED

Gel	Capacity of IgG per ml of gel (mg)
Histidyloxirane-Sepharose	0.05
Histityloxirane-silica	0.15
Histidylbis(oxirane)-Sepharose	0.235

The capacities of the different columns were also studied. Tables I and II show the capacities of the different gels used. The differences in the capacities of the three types of gels studied show that steric hindrances could be the cause of the low capacity of histidyloxirane-Sepharose.

L-histidine is apparently bound to the matrix through the α-NH_2 group, as the free imidazole group plays an important role in the binding mechanism. In the presence of imidazole buffer, the retention was totally suppressed.

Purification of different proteins on the histidine-coupled matrix

Aspergillus niger acid protease. A 60–65% saturated ammonium sulphate precipitate of an *Aspergillus niger* culture medium containing the acid protease was chromatographed at +4°C on a histidyloxirane-Sepharose column. The purification profile is shown in Fig. 1. A ten-fold purification with a yield of 65% was obtained. The purified enzyme showed a single band on polyacrylamide gel electrophoresis (PAGE).

Carboxypeptidase from Saccharomyces cerevisiae. Histidyloxirane-Sepharose 4B was also employed for the purification of carboxypeptidase Y. A filtered and centrifuged extract was injected into a column at 4°C (carboxypeptidase Y being intracellular, it was necessary to disrupt the yeast cells prior to filtration and centrifugation). The enzyme was eluted using 0.5 *M* sodium chloride added to a Tris—HCl buffer at pH 7.2. The eluted enzyme was 25-fold pure and the yield in activity was 77% (Fig. 2). A similar run at 20°C gave all carboxypeptidase activity in the flow-through peak.

Chymosin from different sources. Crude dialysed extracts from calf and kid abomassa were chromatographed on a histidyloxirane-Sepharose column, without any prior treatment. Increased yield, purification and specificity towards the milk-clotting activity was obtained at +4°C rather than +20°C, as seen in Table III. The retained chymosin could be eluted by adding 0.5 *M*

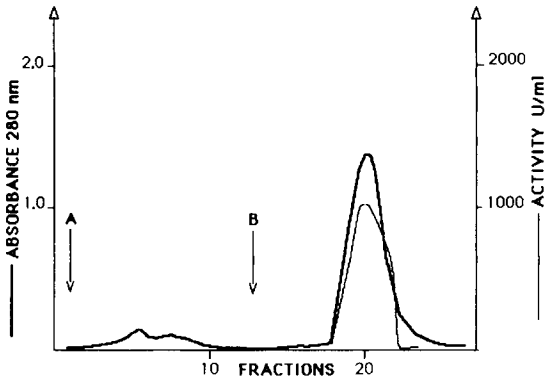


Fig. 1. Purification of acid protease on histidyl-Sepharose: (A) 0.05 *M* sodium acetate buffer (pH 3.5); (B) 0.05 *M* acetate buffer (pH 5.5) + 0.5 *M* sodium chloride. Flow-rate 1.2 ml/min; temperature 4° C; volume of fractions 5 ml.

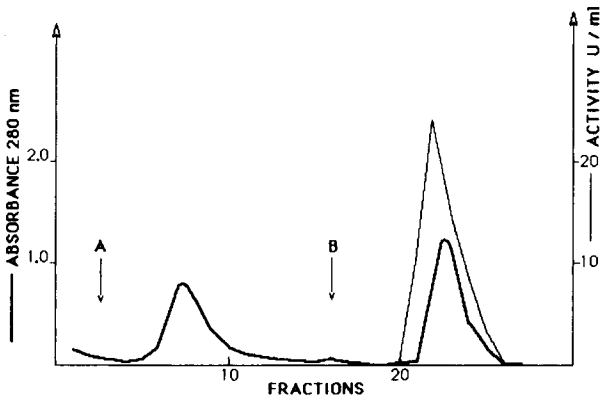


Fig. 2. Purification of carboxypeptidase Y on histidyl-Sepharose: (A) 0.025 *M* Tris—HCl (pH 3.5); (B) 0.025 *M* Tris—HCl (pH 7.2) + 0.5 *M* sodium chloride. Flow-rate 1.6 ml/cm²/min; temperature 4° C; volume of fractions 6 ml.

TABLE III

COMPARISON OF PROTEOLYTIC AND MILK-CLOTTING ACTIVITIES OF THE ENZYME PURIFIED AT 4 AND 20° C

Conditions (° C)	*n*-Fold purification	Yield (%)	Ratio milk clotting/proteolytic
4	26	331	1.692
20	18	108	711

sodium chloride to the equilibrating buffer. The purity of kid chymosin was greater than that of calf chymosin; however, the proteases of microbial origin having chymosin-like activity could also be successfully purified on this column. Fig. 3 shows the elution profile obtained at +4° C on the histidyl-Sepharose 4B column.

Another interesting feature is that a silica-based histidine-coupled column could be successfully used, with a Waters high-performance liquid chromato-

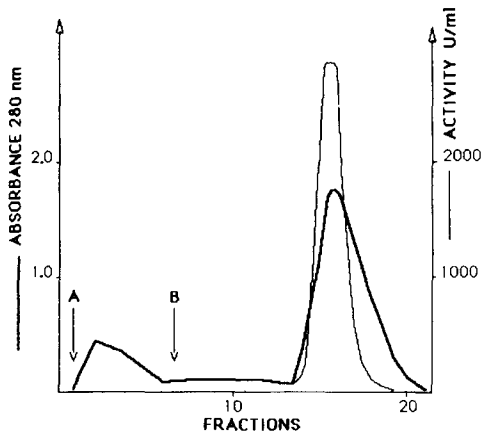


Fig. 3. Purification of chymosin on histidyl-Sepharose: (A) 0.01 *M* sodium acetate (pH 5.5); (B) 0.01 *M* sodium acetate (pH 5.5) + 0.5 *M* sodium chloride. Flow-rate 1.5 ml/cm²/min; temperature 4° C; volume of fractions 7 ml.

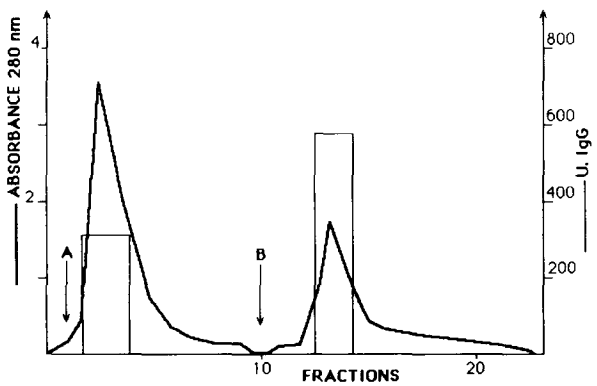


Fig. 4. Purification of IgG on histidyl-Sepharose: (A) 0.025 *M* Tris—HCl (pH 7.4); (B) 0.025 *M* Tris—HCl (pH 7.4) + 0.2 *M* sodium chloride. Flow-rate 2.0 ml/cm²/min; temperature 4° C; volume of fractions 8 ml.

graphic (HPLC) analytical apparatus, for the purification of chymosin from extracts or for the recovery of residual activity from milk sera.

Purification of IgG from human placenta. A delipidated ammonium sulphate extract of pulverized human placenta was chromatographed under different conditions on either histidyl-Sepharose 4B or histidyl-silica. Fig. 4 shows the elution profile obtained at +4° C on the histidyl-Sepharose 4B column.

As in the case of protease purification, a better purification is obtained at +4°C when Sepharose is used as the support matrix. Thus, a maximum purification of 157-fold could be obtained (Table IV).

The histidine silica, however, shows a certain amount of non-specific interaction at +4° C, which results in a decrease in purification (Table I).

Further characterization of the purified fractions on cellulose acetate, immunoelectrophoresis, etc. revealed only one major band (Figs. 5 and 6), and the non-retained peak is rich in albumin while the eluted peak contains only traces of albumin.

TABLE IV

PURIFICATION OF IgG FROM HUMAN PLACENTA ON HISTIDYL-SEPHAROSE 4B AT 4°C

Fraction	Protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	123 610	591.72	$4.8 \cdot 10^{-3}$	100	1
After precipitation with 8% chloroform	10 980	544.4	$49.6 \cdot 10^{-3}$	92	10.4
After precipitation with $(NH_4)_2SO_4$ (0—55%) and dialysis	2597	544.0	$209.5 \cdot 10^{-3}$	92	43.8
Peak I after chromatography	1856.27	398.0	$214.5 \cdot 10^{-3}$	67	44.8
Peak II after chromatography	50.1	37.6	$750.1 \cdot 10^{-3}$	6.3	156.7

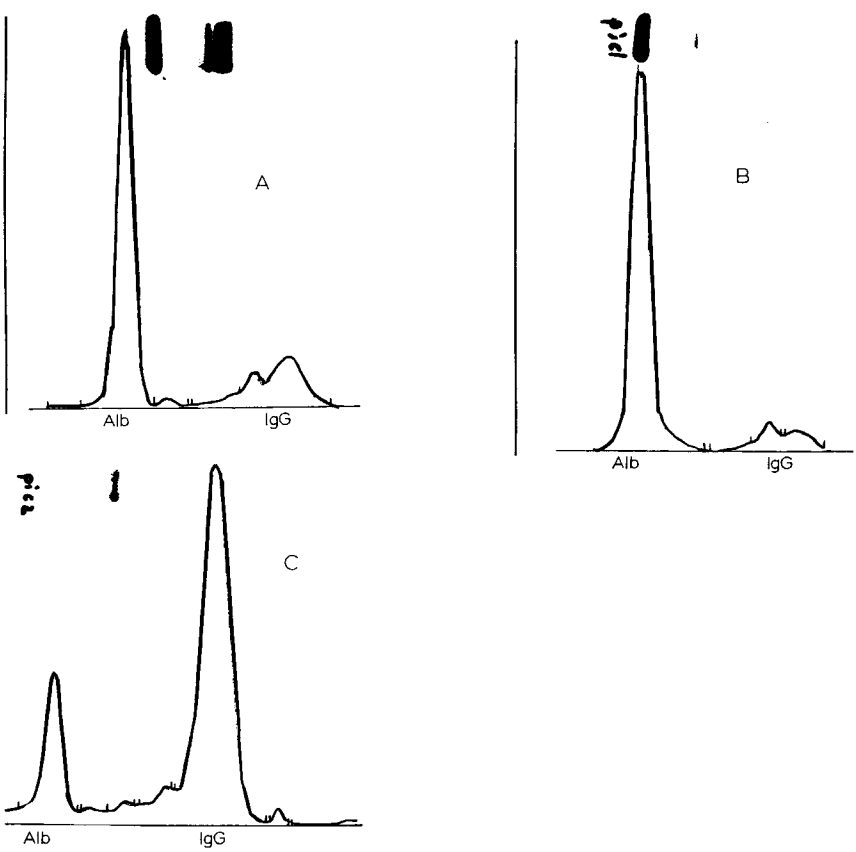
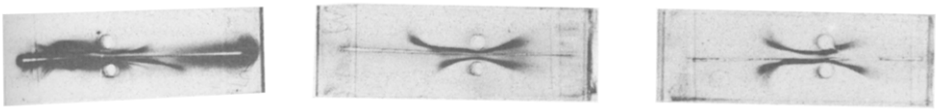


Fig. 5. Electrophoresis on cellulose of the initial and the purified IgG fractions: (A) initial extract; (B) peak 1; (C) peak 2.



$(NH_4)_2SO_4$ Extract Peak 1 Peak 2

Fig. 6. Immunoelectrophoresis of IgG purified on a histidine-silica-Sepharose column.

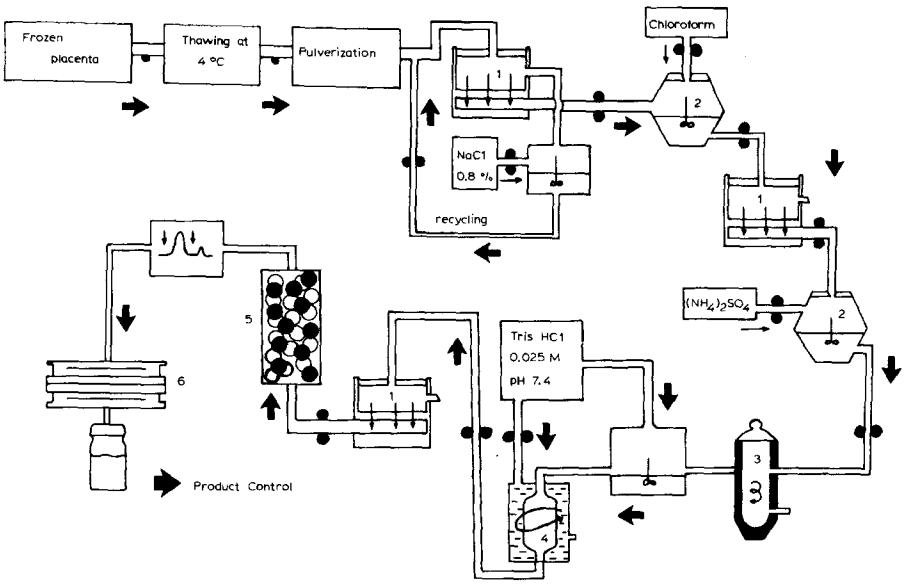


Fig. 7. Schematic diagram of the semi-pilot-scale plant: (1) filtration; (2) precipitation; (3) centrifugation; (4) dialysis; (5) chromatography; (6) concentration.

Further investigation of the retained peak showed that it is the IgG_1 that is more specifically bound and the non-retained peak contains the IgG_2, IgG_3 and IgG_4 along with albumin and only traces of other serum proteins.

Based on these results, a pilot-scale purification of placental IgG was undertaken. The flow-diagram shown in Fig. 7 indicates the various steps used in this purification scheme. Thus, a semi-pilot-scale plant was set up in our laboratory. The adsorbent is quite stable and could be used for up to ten cycles. It could be regenerated by a thorough washing with 3 *M* potassium chloride followed by 0.1 *M* sodium hydroxide.

Other molecules of interest. Apart from these proteins, many peptides of biological interest could be recovered in a pure form from complex mixtures using these novel histidine-coupled gels. To cite an example: a glycopeptide of high biological interest, which is a secondary metabolite of a *Myxococcus xanthus* strain, is recovered in its active form. A 50-l culture medium was run on the column and the peptide of interest could be recovered as a single retarded peak.

DISCUSSION

Immobilized histidine has permitted the purification of several proteins and peptides at high flow-rates. The use of model peptides and the work carried out by Rabier et al. [5] allow us to draw several conclusions concerning the possible solute—ligand interactions that could occur.

The presence of the imidazole ring and the carboxyl group makes it an excellent candidate for charge-transfer (CT) and ionic interactions. Histidine has also been attributed with the capacity for hydrophobic interactions with proteins [5, 13]. Pommerening et al. [13] have already indicated that the imidazole group is a good hydrophobic ligand. Optimal retention of all four purified proteins occurred only at pH values near their respective isoelectric points (pI). This was also the case with chromopeptides. Acid protease and carboxypeptidase Y with p$I \approx 3.6$ were retained only if the pH of the starting buffer was between 3 and 4. Chymosin with pI near 6 had an optimal retention only at ca. pH 5.5. IgG, which has a pI between 6.8 and 7.2, was optimally retained on histidyl-silica only at pH 7.4. Optimal retention of all these proteins occurred only at low temperature (4° C), and for effective desorption the presence of sodium chloride in the eluting buffer was necessary. However, the ionic interactions cannot be solely responsible for the retention, as our efforts to purify these proteins and peptides on DEAE-Sepharose did not give comparable and satisfactory results. The zwitterionic nature of the histidine residues suggests more of a dipole-induced interaction than an electrostatic one.

Detailed kinetic studies are yet to be undertaken; however, in the case of chymosin, competitive inhibition by L-histidine has been observed [4], with an inhibition constant (K_i) of $17.5 \cdot 10^{-3}$ M. Carboxypeptidase Y is inhibited by acetylhistidine [14] and Ichishima [10] has observed inhibition of a protease from *Aspergillus saitoi* by acetylimidazole. These data suggest a sort of specificity in the interaction, especially in the case of carboxypeptidase Y, which possesses an essential histidine in its active site [14]. Acid proteases, however, are not known to possess histidine in their active sites. Charge-transfer, ionic and maybe hydrophobic interactions could all participate in the case of the acid protease since the enzyme was also purified using DEAE-Sephadex followed by phenyl-Sepharose. The same could apply to chymosin, which possesses an enormous quantity of hydrophobic amino acid residues on its surfaces. We are, however, quite persuaded that charge-transfer (induced by a dipole) and ionic interactions are predominant in the case of the protein-histidine–ligand interactions, since the proteins injected on histidyl-silica in the presence of 0.5 M sodium chloride were not retained. Rabier et al. [5] have nevertheless noticed hydrophobic interactions between chromopeptides and the histidine ligand, and also in the case of model peptides such as Trp-Tyr and His-Tyr.

It seems extremely difficult to propose, conclusively, the type of interaction that occurs between the histidyl ligand and the proteins studied. Hydrophobicity seems to predominate in the case of the model peptides, while charge-transfer and ionic interactions seem to be the chief cause of retention of IgG, chymosin, acid protease and carboxypeptidase Y. Also, there seems to be a kind of specificity in the reaction of the ligand with chymosin and the chromopeptides.

However, it is evident that our approach is different from those of Davankov et al. [6] and Grushka and co-workers [16, 17], where they used a silica matrix coated with either N-decylhistidine or di- and tripeptides for the fractionation of amino acids and their phenylthiohydantoin derivatives. In these cases, the chiral and hydrophobic properties of the ligands are predominant.

The adsorbents reported here with covalently coupled histidine are very valuable for the simple and efficient purification of biologically active proteins and peptides.

CONCLUSION

Thus, from these few examples, it is very clearly evidenced that the histidine-coupled support matrices have high-potential, versatile applications in the purification of biological molecules.

ACKNOWLEDGEMENTS

The fruitful collaborations with Mr. Ali Akoum and Dr. J. Guespin from the Microbial Genetics group of our University, and Drs. Rabier and Lambert from the University of Marseille, are thankfully acknowledged.

REFERENCES

1 L. Suamri, F. Spitz and L. Arzadon, J. Biol. Chem., 251 (1976) 3693.
2 M. Vuento and A. Vaheri, Biochem. J., 183 (1979) 331.
3 M.A. Vijayalakshmi and J. Porath, J. Chromatogr., 177 (1979) 201.
4 L. Amourache and M.A. Vijayalakshmi, J. Chromatogr., 303 (1984) 285.
5 J. Rabier, M.A. Vijayalakshmi and W. Rudiger, Z. Naturforsch., 38 (1983) 230.
6 V.A. Davankov, A.S. Bochkov and Yu. P. Belov, J. Chromatogr., 218 (1981) 547.
7 J. Boue, R. Audebert and C. Quivoron, J. Chromatogr., 204 (1981) 185.
8 L. Sundberg and J. Porath, J. Chromatogr., 90 (1974) 87.
9 S.H. Chang, K.M. Gooding and F.E. Regnier, J. Chromatogr., 120 (1976) 321.
10 E. Ichishima, Methods Enzymol., 19 (1979) 397.
11 H. Glasner and C. Wenig, Klin. Wochenschr., 51 (1973) 806.
12 S. Aibara, R. Hayashi and T. Hata, Agric. Biol. Chem., 35 (1971) 658.
13 K. Pommerening, M. Kuhn, H. Jung, K. Buttergereit, P. Mohr, J. Stamberg and M. Benes, Int. J. Biol. Macromol., 1 (1979) 79.
14 R. Hayashi, Y. Bai and T. Hata, J. Biol. Chem., 250 (1975) 5221.
15 M.A. Vijayalakshmi, unpublished results.
16 E. Grushka and R.P.W. Scott, Anal. Chem., 45 (1973) 1626.
17 G.W.K. Fong and E. Grushka, J. Chromatogr., 142 (1977) 299.