Journal of Chromatography, 323 (1985) 247–254
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 491

DEVELOPMENT OF IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY

I. COMPARISON OF TWO IMINODIACETATE GELS

ESMAT SAYEDA HEMDAN and JERKER PORATH*
Institute of Biochemistry, Uppsala Biomedical Centre, Box 576, S-751 23 Uppsala (Sweden)
(First received October 12th, 1984; revised manuscript received December 20th, 1984)

SUMMARY

Two iminodiacetate (IDA) gels were prepared by different synthetic routes: (a) direct coupling of IDA to epoxy-activated Sephadex G-25; and (b) a two-step synthesis involving initial introduction of amino groups into epoxy-activated Sephadex G-25 and their subsequent carboxymethylation. The efficiency of coupling of IDA to the epoxy-activated matrix was studied in detail as a function of pH in the range from 8.0 to 12.5. The IDA gel produced by the direct coupling procedure at the optimum pH of 10.5 was found to retain *ca.* 450 μmol of Cu^{2+} per g dry weight. Different α-haloacids were explored as carboxymethylating agents for the second step of the two-step preparation. IDA gels produced with the use of bromoacetic acid were found to retain *ca.* 370 μmol of Cu^{2+} per g dry weight. A novel chelating gel having a "hybrid" carboxyl/sulphonic ligand was also synthesized and its chelating properties were compared to the standard IDA gels. This gel retained only 180 μmol of Cu^{2+} per g dry weight. The retention of Cu^{2+} in the presence of the competing ligand, glycine, was inferior to that obtained with the IDA ligand.

INTRODUCTION

Carboxymethylated amines (chelones) are excellent complexing agents for di-, tri- and tetravalent metal ions. Therefore, the immobilization of such groups on carriers should convert the latter into useful metal-chelating sorbents. These can be used for selective removal of heavy metal ions from aqueous solutions and, in particular, for immobilized metal affinity chromatography (IMAC). The usefulness of IMAC for the isolation of several proteins has amply been demonstrated[1–3]. Since IMAC is relatively new, there is a need to improve the already accepted iminodiacetate (IDA)-type gels and to search for new metal ligands as well. The present report is addressed to both of these goals.

In the past, the IDA function has been used exclusively among the chelones to prepare metal-chelating adsorbents. However, recently we have found that other metal chelone adsorbents have selective affinities for proteins, differing from those

0021-9673/85/$03.30

of the IDA gels, and that these differences may be used to advantage for group fractionation of complex protein mixtures[1,4].

The chelone gels may be synthesized most conveniently from amino gels, but it is necessary to work out conditions for satisfactory conversion without concomitant introduction of carboxymethyl ether groups into the matrix. The IDA gels can easily be obtained either by coupling iminodiacetate to an epoxy-activated gel or according to the suggested general route, *i.e.* carboxymethylating a precursor gel, in this case amino gel.

We report now a detailed evaluation of the conditions (pH) for coupling of the IDA function to an epoxy-activated matrix (direct coupling procedure). Synthesis of the IDA function on a matrix via preparation of an amino gel and its subsequent carboxymethylation (two-step procedure) has also been accomplished. In addition, a novel mixed carboxyl/sulphonic acid "hybrid" ligand has been prepared and its metal-chelating properties studied.

EXPERIMENTAL

Materials

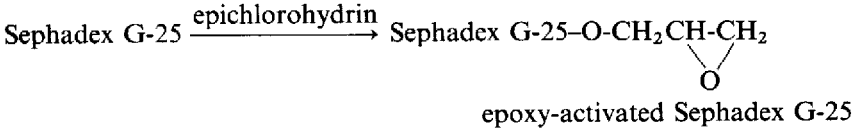
Sephadex G-25 and Sepharose 6B were used exclusively as the solid matrices (Pharmacia, Uppsala, Sweden). Bromoacetic acid, bromosuccinic acid and dibromosuccinic acid were all purchased from Aldrich (Milwaukee, WI, U.S.A.). Disodium iminodiacetate was from Fluka (Buchs, Switzerland). Copper chloride was obtained from Merck (Darmstadt, F.R.G.).

Analytical procedures

In order to measure the chelating capacity of the prepared gels, 8.5-g samples were washed thoroughly with water on a fritted-glass funnel, treated with 300 ml of copper (II) chloride solution (20 m*M*) and washed again with water to remove the unbound copper ions. Finally, after a wash with acetone, the gel samples were freeze-dried and analyzed for copper, nitrogen and sulphur according to established procedures[5]. The capacity of the gels for Cu^{2+} was expressed in μmol of Cu^{2+} per gram of dry weight.

Activation of Sephadex G-25 and Sepharose 6B

The activation of both matrices was performed with epichlorohydrin according to an established procedure[4]:

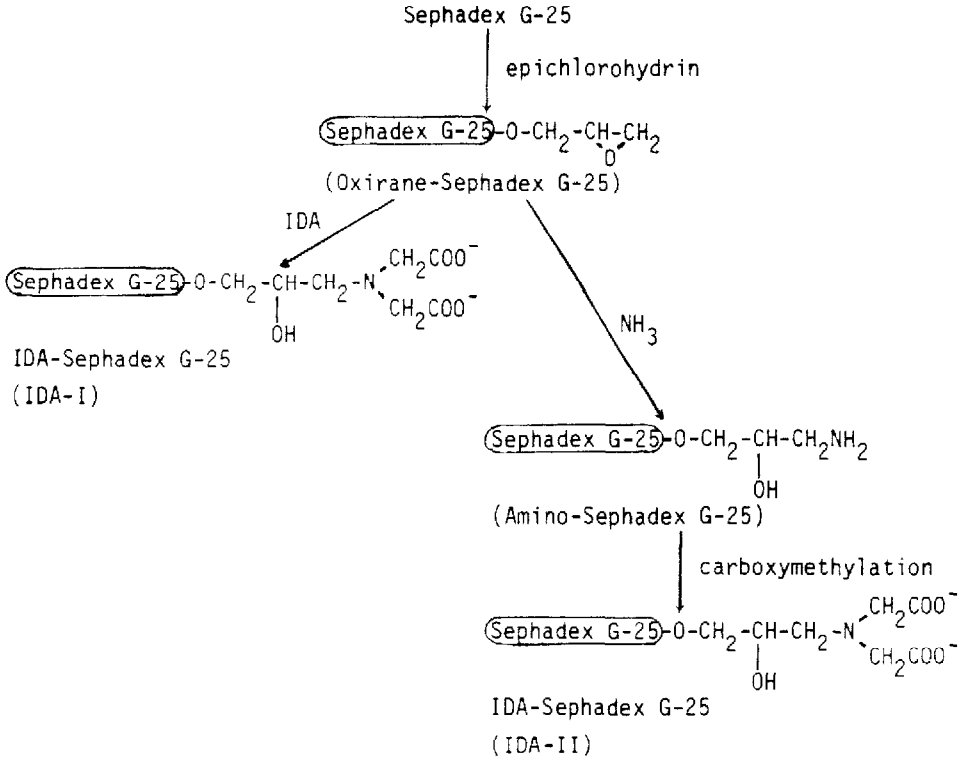


Briefly, a 200-g sample of suction-dried Sephadex G-25, was added to a 2-l round-bottom flask containing 100 ml of sodium hydroxide (2 *M*), 10 ml of epichlorohydrin and 375 mg of sodium borohydride ($NaBH_4$). The reaction mixture was stirred at room temperature for 2 h and during that time an additional 100 ml of sodium

hydroxide (2 *M*) and 50 ml of epichlorohydrin were added. The reaction was allowed to continue overnight.

Direct coupling of iminodiacetate (IDA-I)

This was accomplished as in Scheme 1[6]. A 2-g sample of disodium iminodiacetate was dissolved in 50 ml of $Na_2CO_3/NaHCO_3$ (2 *M*) buffer, pH 8–12.5. A 10-g sample of suction-dried epoxy-activated Sephadex G-25 was transferred into a reaction flask in 20 ml of Na_2CO_3–$NaHCO_3$ (2 *M*) buffer containing 3 g of IDA and adjusted to the appropriate pH. The flask was placed in a water-bath (60–65°C) and the contents were stirred for 24 h. The reaction product, *i.e.*, IDA-Sephadex G-25 gel, was washed with water (2 l), 10% acetic acid (0.5 l) and finally with water (2 l).



Scheme 1.

Synthesis of iminodiacetate function on epoxy-activated gels — "indirect coupling" (IDA-II)

Introduction of the iminodiacetate function onto an epoxy-activated gel was accomplished by a two-step procedure as shown in Scheme 1. First, an amino group was introduced by coupling via the epoxy function and then the amino group was carboxymethylated with α-haloacids. The preparation of the amino derivative from the epoxy-activated matrix was accomplished by using several previous reports[7–9] as broad guidelines. A 100-g sample of suction-dried epoxy-activated Sephadex G-25

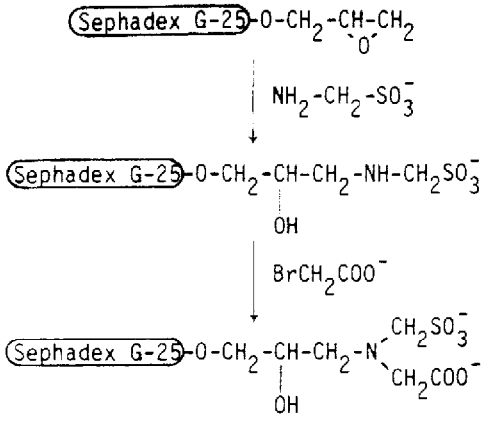
was washed with water (1 l) and then with 0.2 *M* sodium carbonate (0.5 l) on a büchner funnel. The washed gel was transferred into a 2-l round-bottom flask which was placed in a water-bath at 60–65°C. Concentrated ammonia, diluted two-fold (100 ml), was added and the suspension was stirred continuously. Further ammonia solution (50 ml) was added dropwise during the initial 3-h period of the reaction. The reaction was continued up to 24 h. The resulting amino gel was rinsed with water (2 l), 10% acetic acid (0.5 l) and water (2 l). The washed gel was kept in the cold room when not used immediately.

The carboxymethylation reaction is usually accomplished with α-haloacids. In order to N,N-biscarboxymethylate the prepared amino gel, the bromoacetic acid procedure was employed using as a general guideline the information given by Gurd[10]. The carboxymethylation procedure, as described below, was identical for all the α-bromoacids tested (bromoacetic, bromosuccinic and dibromosuccinic acids).

Routinely, a 2-g sample of a bromoacetic acid was placed into a 250-ml round-bottom flask in an ice-bath. After 15 min a cold solution of sodium carbonate (1 *M*, 80 ml) was added dropwise and the mixture was stirred slowly. The desired pH, within the range 8–12.5, was adjusted by addition of sodium hydroxide (2 *M*). A 10-g sample, of suction-dried amino-Sephadex G-25 (previously washed with 200 ml of 0.2 *M* sodium bicarbonate) was then added to the flask. The pH of the reaction mixture was again adjusted to the desired value with sodium hydroxide (2 *M*). The flask was rotated at a slow speed at 25°C for 24 h. The reaction product, *i.e.*, bis-carboxymethylamino-Sephadex G-25 (IDA-II) was washed on a fritted-glass funnel with water (1 l), 10% acetic acid (200 ml) and water (1 l).

Preparation of mixed carboxyl/sulphonic chelating gel

This was done as shown in Scheme 2. A 30-g sample of suction-dried oxirane-Sephadex G-25 was washed with carbonate/bicarbonate buffer (2 *M*), pH 10.5. The washed gel was transferred into a round-bottom flask. A 3-g sample of aminomethane–sulphonic acid was dissolved in 50 ml carbonate/bicarbonate buffer (2 *M*) and the pH was adjusted to 10.5 with sodium hydroxide. This solution was added to the gel in the flask. A further portion of aminomethane–sulphonic acid, 20



(Carboxymethyl-aminomethane sulphonic acid-Sephadex G-25)

Scheme 2.

ml, adjusted to pH 10.5, was added to the reaction mixture dropwise during the first hour of the reaction, while the mixture was stirred slowly at room temperature. The reaction vessel was then transferred into a water-bath (60–65°C) and the mixture was stirred slowly overnight. The product was washed on a büchner funnel with water, 10% acetic acid and finally water. The carboxymethylation of the gel was performed as described above for the preparation of IDA-II gel.

Evaluation of the stability of copper(II) chelation

The stability of the association between a chelating gel and the metal ion was tested as follows: the gels were packed into columns (10 × 1 cm), washed with water and charged with Cu^{2+} (20 m*M*) until saturated. The excess, *i.e.*, unbound copper ion, was washed out with water (two bed volumes). The bound copper was measured as described in Analytical procedures. In order to test the stability of the metal chelates all sorbents were then developed with glycine (1 *M*, pH 9.0) as a competing ligand.

RESULTS

Table I gives the copper contents of IDA-I, IDA-II and samples of Sephadex G-25 and Sepharose 6B that had been carboxymethylated without prior activation with epichlorohydrin. A graphic representation of the data is given in Fig. 1. The coupling efficiency of IDA to epoxy-activated Sephadex G-25 increases rapidly with the pH of the reaction, starting at pH 9.5 and going through a maximum at pH 10.5; there is an abrupt drop in the coupling above pH 11.5. Therefore, the most effective coupling range is between pH 10 and 11, which gives ample leeway for ready control of the coupling reaction.

TABLE I

CHELATING CAPACITY OF THE GEL (μmol Cu^{2+} PER g DRY WEIGHT)

IDA-I = Iminodiacetate-Sephadex G-25; IDA-II = amino-Sephadex G-25, carboxymethylated. Control gels: A = non-activated but carboxymethylated Sephadex G-25; B = non-activated but carboxymethylated Sepharose 6B.

pH	*IDA-I*	*IDA-II*	*Control gels*	
			A	*B*
8.0	195	121	14	0
9.0	228	152	15	2
9.2	231	296	15	2
9.5	258	320	18	2
9.6	270	342	18	2
10.0	393	367	20	2
10.5	459	349	17	2
10.6	452	347	14	2
11.0	397	337	14	2
11.5	382	330	64	2
12.0	336	321	114	16
12.5	80	132	150	30

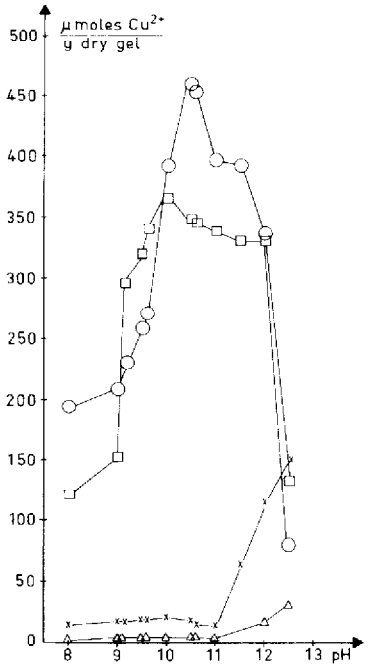


Fig. 1. The influence of pH on the extent of coupling of the IDA function to epoxy-activated Sephadex G-25 and on the extent of carboxymethylation of amino-Sephadex G-25. ○—○, IDA-I; □—□, IDA-II; × — ×, carboxymethylated Sephadex G-25, non-activated by epichlorohydrin; △—△, carboxymethylated Sepharose 6B, non-activated by epichlorohydrin.

It is clear (Table I, Fig. 1) that the control gels (A and B) contain only traces of tightly bound Cu^{2+} when they are carboxymethylated at pH < 11. The retention of Cu^{2+} by Sephadex G-25 carboxymethylated above pH 11 increases with increasing pH of the reaction. This occurs in the conspicuous absence of an IDA group. Apparently, some carboxyl functions are introduced at this high pH and these may account for the retention of Cu^{2+}: when the gel prepared at pH 12.5 is washed with glycine buffer, all retained Cu^{2+} is readily displaced. By contrast, IDA gel prepared at the same pH and having a similar copper content cannot readily be washed free of copper by glycine buffer. The carboxymethylation reaction of amino-Sephadex (IDA-II) displays the same general pH dependence as does coupling of IDA to epoxy-activated Sephadex G-25. The coupling efficiency range is wider, spanning pH 10–12. However, the extent of substitution is lower.

The molar ratio of Cu^{2+}/N in IDA-I is *ca.* 1, as expected if all IDA functions on the gel are occupied by the chelated Cu^{2+}. This indicates (a) the accessibility of all IDA functions for metal chelation and (b) the spatial distribution of IDA functions within the three-dimensional network of the gel matrix is such as to prohibit sharing of a Cu^{2+}. In the case of IDA-II, the ratio of immobilized Cu^{2+} to nitrogen (IDA function) is 0.8, somewhat lower than that for IDA-I. Plausibly, this discrepancy can be explained by assuming that IDA groups are less homogeneously distributed in the IDA-II than in the IDA-I gel. The existence of a small percentage of local clusters of IDA groups which could share the Cu^{2+} might account for the experi-

TABLE II

COPPER, NITROGEN AND SULPHUR CONTENTS OF THE GELS (μmol per g dry weight)

Amino C = amino-Sephadex G-25; Sulph. D = aminomethane-sulphonic acid Sephadex G-25.

Gel	*Cu*	*N*	*S*
IDA-I	459	443	–
IDA-II	367	451	–
Amino C	29	451	–
Sulph. D	183	156	148

mentally observed lower ratio of Cu^{2+} to N (IDA ligand).

The nitrogen content of the amino-Sephadex G-25 gel was similar, within experimental error, to those of IDA-I and IDA-II. Aminomethane-sulphonic acid Sephadex G-25 displayed the anticipated equimolar ratio of copper/nitrogen/sulphur (Table II). Its capacity for copper was only *ca.* 39% of the capacity of IDA-I. Moreover, the chelated copper was readily removed by competing upon elution of column with glycine buffer. In view of its relatively low capacity for Cu^{2+} and the labile complexation with Cu^{2+}, further study of this sorbent was put in abeyance for the present time.

The efficiency of carboxymethylation of the amino groups of the gel, as reflected by the chelating capacity of the resulting IDA function, was found to increase in the following order: α-bromoacetic acid > α-bromosuccinic acid > dibromosuccinic acid (Table III).

DISCUSSION

Two procedures for the preparation of IDA gels have been described. The coupling of the IDA function to epoxy-activated Sephadex G-25 ("direct coupling") yielded a chelating sorbent with a high capacity for a metal ion. Taking into accounts the swelling of the dry gel in an aqueous solvent (factor of *ca.* 5), one obtains a value of *ca.* 90 μmol of the ligand per ml bed volume of the swollen gel. Both products thus compare favourably with some commercially available gels.

An attempt to prepare a novel chelating gel having a hybrid carboxyl/sulphonic acid function met with only qualified success in view of the considerably weaker retention of the metal ion (Cu^{2+}). This is consistent with the observation that the phosphonic acid analogue was found to be inferior to IDA in its chelating properties[11]. At present, we see no advantage in using this mixed type of metal adsorbent.

TABLE III

CARBOXYMETHYLATION EFFICIENCY OF VARIOUS α-BROMOACIDS

Compound	*μmol Cu^{2+}/g dry gel*
Bromoacetic acid	367
Bromosuccinic acid	101
Dibromosuccinic acid	21

However, the preparation of other hybrid ligands, by the same synthetic strategy as employed for the carboxyl/sulphonic acid ligand, may be possible.

ACKNOWLEDGEMENTS

We thank Drs. E. Sulkowski and D. Eaker for invaluable suggestions and help with linguistic revision of the manuscript. This work was financially supported by The Swedish Natural Science Research Council, The Swedish Board for Technical Development, Erna and Victor Hasselblad Foundation, Alice and Knut Wallenberg Foundation and LKB Products.

REFERENCES

1 J. Porath and B. Olin, *Biochemistry*, 22 (1983) 1621–1630.
2 B. Lönnerdal and C. L. Keen, *J. Appl. Biochem.*, 4 (1982) 203–208.
3 E. Sulkowski, K. Vastola, D. Oleszek and W. von Muenchhausen, *Anal. Chem. Symp. Ser.*, 9 (1982) 313–322.
4 L. Andersson, *J. Chromatogr.*, 315 (1984) 167–174.
5 L. Gustavsson, *Talanta*, 4 (1960) 227–243.
6 J. Porath and L. Sundberg, *Protides Biol. Fluids*, 18 (1970) 401–407.
7 L. Sundberg and J. Porath, *J. Chromatogr.*, 90 (1974) 87–98.
8 P. Hubert and J. Porath, *J. Chromatogr.*, 198 (1980) 247–255.
9 J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature (London)*, 258 (1975) 598–599.
10 F. R. N. Gurd, *Methods Enzymol.*, 11 (1967) 532–541.
11 M. Lederer, personal communication.