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Journal of Chromatography A, 795 (1998) 211–217

JOURNAL OF CHROMATOGRAPHY A

Preparation of magnetic immobilized metal affinity separation media and its use in the isolation of proteins

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Received 18 June 1997; received in revised form 9 September 1997; accepted 11 September 1997

Abstract

A new method of pseudobiospecific protein isolation is developed and tested, which employs both metal affinity and magnetism as the basis for isolation. The chelating group iminodiacetic acid (IDA) has been coupled to the surface of magnetic agarose, and when charged with metal ions (Cu^{2+} or Zn^{2+}) is capable of binding model proteins which display metal affinity, and of separating protein mixtures. Magnetic properties of the medium facilitated the batch recovery of the adsorbent, as losses are minimized by concentrating and retaining the separation medium with the aid of a magnet. Model proteins were used to characterize protein adsorption, capacity, and stability of IDA magnetic agarose. Recovery from a cell lysate was demonstrated by protein isolation from extracts of *E. coli* containing a target protein. Overall, this study effectively illustrates the engineering of separation media which combine several desired properties for the development of a new branch of metal affinity-based bioseparation. © 1998 Elsevier Science B.V.

Keywords: Immobilized metal affinity chromatography; Magnetic media; Stationary phases, LC; Proteins

1. Introduction

Bioseparation techniques which utilize metal affinity as the basis for separation exploit differential interactions with immobilized metal atoms or ions [1]. Immobilized metal affinity has been central to many bioseparation techniques which include: immobilized metal affinity chromatography (IMAC), (metal affinity) precipitation, membrane-based separations (immobilized metal affinity membrane separation), and (metal enhanced) two-phase aqueous extraction [2–12]. Of the techniques which utilize metal affinity, IMAC has been widely used in the separation of biological materials on analytical, laboratory-scale, and pilot-scales [13–19]. Over the past 25 years since its first introduction, results with IMAC demonstrate the effectiveness of metal affinity as a basis for bioseparation, and several review articles document the potential of this and other metal affinity based bioseparation techniques [12,20–23].

Metal affinity-based separation methods utilize metal ions (Me^{2+}) as pseudobiospecific ligands for protein separation [1,24–26]. Advantages of pseudobiospecific versus conventional (e.g. cofactor, antibody) ligands include stability and low cost [27]. Chelate groups attached to either a stationary phase or mass-separating agent are used to retain the Me^{2+} ligand. Chelates most often encountered are iminodiacetic acid (IDA) and nitrilotriacetic acid, and the divalent metal ions commonly employed include divalent nickel, zinc, copper, and cobalt.

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0021-9673/98/$19.00
PII S0021-9673(97)00959-X

For a given protein, the location and frequency of electron donating groups on the exposed surface determines metal affinity [25,26,28,29]. Surface exposed histidine is the amino acid considered most responsible for interaction with chelated metal ions. This is largely attributed to the high association constant between the metal ion and imidazole side chain (relative to interactions with other amino acids). Solvent accessibility of binding residue(s), cooperation between neighboring amino acids, and microenvironment of the binding residue also affect metal affinity. Protein adsorption is further modulated by several factors such as the metal ion chosen, loading and column operating pH, addition of organic solvents or denaturants, and choice/molarity of antichaotropic salt used to promote adsorption [5,30,31].

The application of protein–metal interactions in recovery schemes is a rapid and expanding field, and metal affinity systems show great promise for the recovery of proteins with natural and enhanced metal affinity [32–36]. This paper describes a novel combination for protein separation, viz. metal affinity to achieve a high selectivity of protein adsorption, with magnetism to enhance the performance of the medium. Separation systems which employ magnetism have been recently investigated, and their ability to recover enzymes, antibodies or nucleic acid fragments have been documented [37–46]. In batch recovery systems, once adsorption of the target biological molecule occurs the application of a magnetic field causes a physical separation to occur through the enhancement of settling rates, or through concentration of the medium against the vessel side. Alternatively, chromatography in fluidized beds with magnetic media has been touted as a method to recover protein from minimally clarified cellular lysates. In these systems, the application of a field reduces bubbling and mixing within the system, and media for use in magnetically stabilized fluidized beds have been prepared and include ion-exchange, and cyanuric dye [43,47].

This article reports on the preparation and use of magnetic immobilized metal affinity separation media. Magnetic agarose has previously found several niches in biotechnology which include: recovery of polymerase-chain reaction products through complementary nucleic acid binding; and cell sorting, or the isolation of proteins through antibody–antigen interactions. In this study a new separation medium is prepared by covalent coupling of the chelating group IDA to magnetic agarose. When charged with Cu^{2+}, or with Zn^{2+} the medium is used to adsorb model proteins via surface exposed histidine(s), or to recover a target protein from crude lysate.

2. Experimental

Reagents were obtained from Aldrich (Milwaukee, WI, USA). Model protein samples were from Sigma (St. Louis, MO, USA). Magnetic agarose beads were obtained from Advanced Magnetics (Boston, MA, USA). AngioI-TEM-β-lactmamse was from resolubilized, refolded inclusion body protein or cell extracts obtained from *E. coli* DH5α:pBAM105 [32].

2.1. Preparation of IDA magnetic agarose

The procedure used to prepare iminodiacetic acid-containing magnetic agarose beads is based on methods developed by Porath and Sunberg wherein diepoxides are used to couple the chelating group to the stationary phase (Fig. 1) [1]. Suction dried magnetic agarose (1 ml) and 1,4-butanediol diglycidyl ether (1 ml) are mixed in a solution of 0.6 *M* sodium hydroxide containing 2 mg/ml sodium

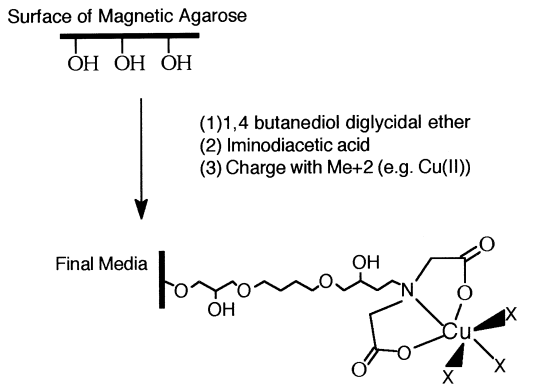

Fig. 1. Structure of IDA magnetic agarose charged with Me^{2+}. By reaction of surface hydroxyls, magnetic agarose (crosslinked agarose containing iron oxide) is modified so as to contain IDA. In the first step, a diepoxide is used to couple a pendant epoxide ring, which then reacts with IDA. After synthesis is complete, any divalent metal ion may be chelated (e.g. copper(II)).

borohydride. The reaction is carried out for 8 h at 25°C with gentle agitation, and is stopped by washing the product with approximately 500 ml water. At this point, the bisoxirane is coupled via addition to a free hydroxyl present on the agarose. The product of the first step is then mixed with a 0.2 g of IDA, and 5.0 mg of sodium borohydride in a solution of 2.0 *M* sodium carbonate. After an overnight incubation with gentle agitation, the final product is washed thoroughly with water, dilute acetic acid, and again with water until the washings are neutral. IDA magnetic agarose is stored at 4°C until needed.

To charge the media with metal ions, a salt of the appropriate cation (e.g. $CuSO_4$) is dissolved in water (50 mg/ml). IDA magnetic agarose is mixed with this solution, and allowed to stand for >15 min. Metal charged media is rinsed thoroughly with water and equilibrated in 0.05 *M* $NaPO_4$ buffer+1.0 *M* NaCl (pH 7.35) prior to use in equilibrium binding studies or protein isolation experiments.

2.2. Batch protein uptake

Equilibrium experiments to examine the batch uptake of model proteins are done in the following manner. A known mass of media previously charged with Me^{2+} and equilibrated in buffer (pH 7.35) is placed in 5-ml PTFE vials (Cole-Parmer, Niles, IL, USA). Media samples are incubated with varying concentrations of protein, or crude cell lysate predissolved in 1–3 ml buffer (pH 7.35) and are gently shaken at 4°C. To elute bound protein, washes with buffers of varying pH, or ethylenediaminetetraacetic acid (EDTA) content are used (EDTA will displace chelated Me^{2+} and concurrently release proteins). Fluid is decanted from the sample vials by placing a magnet against the vessel to collect media against the sidewall, or against the bottom of the vial. Results are obtained from data of multiple vials or samples ($n=5$) when possible.

2.3. Protein determination

Concentration of protein in solution (C_s) is determined by one of three methods: absorbance at 280 nm using a DU-640 spectrophotometer (Beckman, Fullerton, CA, USA), enzymatic activity, or HPLC size-exclusion chromatography. A simple mass balance between the total concentration and concentration remaining in solution is used to calculate bound protein. Calibration curves are prepared for a given protein by measuring samples (either adsorbance, or peak area) of known concentration, and used to calculate C_s. For the case of angioI-TEM-β-lactamase, penicillin-G (5 mg/ml) hydrolysis in a solution of 0.1 *M* $NaPO_4$ is followed spectrophotometrically at 240 nm [48].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is used to visualize protein samples. Samples diluted by 4× cracking buffer [0.125 *M* Tris–HCl, bromophenol blue, 3% (v/v) SDS, 3% (v/v) β-mercaptoethanol, 30% (v/v) glycerol] are boiled for 5 min, then loaded onto a 3% stacking+12% resolving gel. Electrophoresis employing a Bio-Rad Mini Protean II apparatus for 45 min at 75 V is carried out in buffer (25 m*M* Tris, 200 m*M* glycine, 0.1% SDS). Gels were stained using comassie blue to visualize protein samples.

3. Results

3.1. IDA-magnetic agarose preparation and metal uptake

Metal ion retention was qualitatively established by washing Cu^{2+} charged media with excess 1 *M* sodium chloride, followed by several washes with 0.05 *M* phosphate+1 *M* sodium chloride. After decanting the final, colorless wash, 1 ml of 0.05 *M* EDTA+1 *M* sodium chloride was added. The EDTA solution turned light blue, indicating that Cu^{2+} was readily chelated, and bound Me^{2+} can be stripped from the media.

3.2. Model protein retention and adsorption isotherm

Cytochrome *c* adsorption and desorption were examined by mixing a slurry of 0.125 ml of Cu^{2+} charged media with 200 μg of either tuna or horse heart cytochrome *c* solution for 12 h at 4°C. After decanting the solution, the beads were then washed with 10 mg of buffer (pH 7.35) followed by 1 ml buffer (pH 5). HPLC analysis (Table 1) indicated

Table 1
Cytochrome *c* adsorption with Cu^{2+} charged media

Protein	pH 7.35 wash		pH 5.0 wash		Capacity, q_{max} (mg/g)	Dissociation constant, K_d (mg/ml)
	Solution	Bound	Solution	Bound		
Tuna heart	300±7	0	0	0	0	∞
Horse heart	0	300±8	290±10	0	42	0.34

complete uptake of horse heart cytochrome *c*, whereas the protein of tuna origin was not adsorbed.

An adsorption isotherm was prepared for both variants of cytochrome *c* with media charged with $Cu^{+2.}$ (Fig. 2). For the case of tuna, a flat profile indicating zero protein adsorption was obtained, whereas a saturation-type isotherm was observed with horse heart cytochrome *c*. The curve of Fig. 2 is based on a Langmuir isotherm, defined by

$$q = \frac{K_d q_{max} C_s}{1 + K_d C_s} \quad (1)$$

where q is the amount of protein bound, q_{max} is the amount of protein bound at capacity and K_d is the equilibrium constant. Values from a Lineweaver–Burke (double-reciprocal) plot of the isotherm data were used as initial estimates for a least-squares solution of Eq. (1), which yielded $q_{max} = 5.6$ mg and $K_d = 0.34$ mg/ml (basis = 0.125 g of media).

3.3. History and time dependent properties

Loss of capacity and rate of protein uptake were also examined using horse heart cytochrome *c* as a probe molecule. Protein was adsorbed by samples of Cu^{2+} charged media in buffer (pH 7.35). Once a week over an 8-week period, both the bound protein and chelated Cu^{2+} was stripped from the support by the addition of EDTA. Metal ion and protein were then reloaded, and allowed to equilibrate again for 7 days. Overall, when 2.5 mg cytochrome *c* is mixed with media, the protein partitions between the adsorbant (1.6±0.1 mg) and liquid phases (0.9±0.1 mg) in a consistent fashion.

In a separate experiment, the rate of protein uptake was determined by incubating identically prepared samples (Cu^{2+} charged media + protein) for various times, quickly decanting the solution, and analyzing the supernatant for protein content. Samples demonstrated rapid uptake of protein (Fig. 3), with >90% of the protein adsorbed within the first 5 min.

3.4. Recovery of protein from crude lysate

Media charged with Zn^{2+} was exposed to *E. coli*

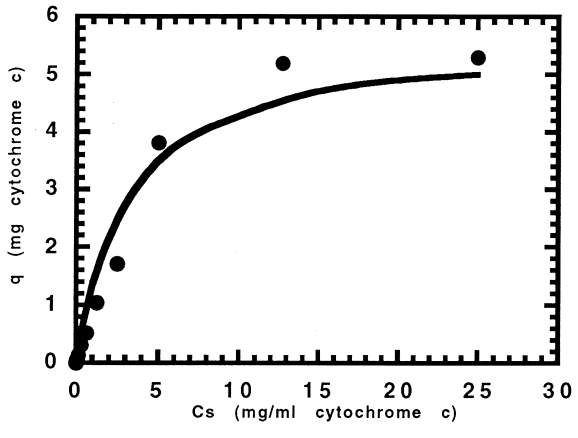

Fig. 2. Adsorption isotherm for horse heart cytochrome *c*. Horse heart cytochrome *c* displays a single surface exposed histidine, which may readily interact with Cu^{2+} chelates.

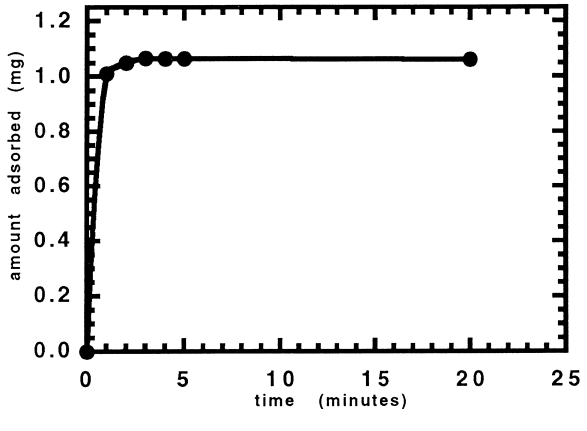

Fig. 3. Binding kinetics for horse heart cytochrome *c* employing Cu^{2+} as the affinity ligand. As indicated in the figure, the bulk of protein adsorption occurs quite fast, with over 90% of protein adsorbed in the first few minutes.

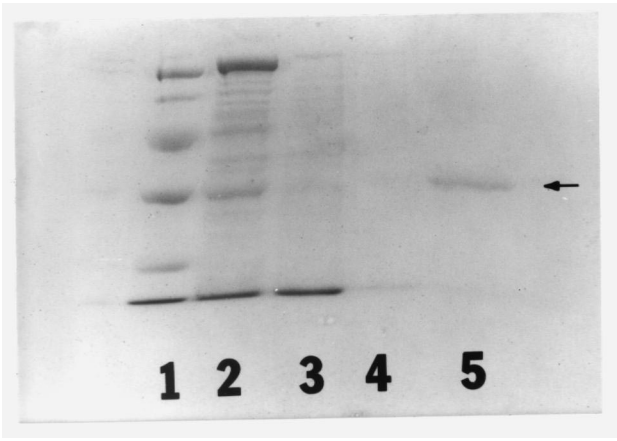

Fig. 4. SDS-PAGE of model protein recovery. As indicated by the presence of a single band (lane 5), the model protein (angioI-TEM-β-lactamase) is efficiently isolated by Zn^{2+} charged IDA magnetic agarose. Lanes 1–4 contain molecular mass standards, crude cell lysate, and pH washes, respectively. See text for details.

cell extracts containing angioI-TEM-β-lactamase, a protein with enhanced metal affinity. After gentle mixing at 4°C, the lysate is decanted, and washes of varying pH values are then used to desorb any non-desired proteins, and to desorb angioI-TEM-β-lactamase.

SDS-PAGE of the cell extract, supernatant, and washes is shown in Fig. 4. Lane 1 contains molecular mass standards (phosphorylase B at 97 400, bovine serum albumin at 66 200, ovalbumin at 45 000, carbonic anhydrase at 31 000, trypsin inhibitor at 21 500, lysozyme at 14 400), whereas lanes 2–5 contain cell lysate, or decanted washes of pH values 7.35, 6.8, and 4.0 respectively. Both SDS-PAGE and enzymatic activity indicate that angioI-TEM-β-lactamase is recovered and highly purified, as evident by a single, strong band similar in mobility to carbonic anhydrase (M_r of angioI-TEM-β-lactamase$\approx$29 000) concurrent with penicillinase activity in the active fraction (lane 5 of Fig. 4).

4. Discussion

The functionalization of agar derivatives or other materials to yield separation media with group specific adsorbants has been the focus of many past efforts. Central to this work is the preparation of IDA containing agarose particles which function in a similar manner to conventional IMAC media, and contain a ferromagnetic core. By combining these characteristics, both metal affinity and magnetism may be exploited in a fashion which facilitates recovery. IDA magnetic agarose displays the requisite properties of a metal affinity adsorbant. These properties include reversible binding of Me^{2+}, selectivity towards surface exposed histidine, Langmurian-style adsorption of proteins, and long term stability of the chelate group coupled to the support. Metal ions may be easily loaded, stripped, and changed by the addition of EDTA or other chelating agent. The ability to regenerate/reuse media employing the same or entirely different Me^{2+} provides an additional degree of flexibility, since the choice of ion dictates the strength of interaction for this system, a common feature for all metal affinity based techniques. Thus, if metal affinity is a viable method of recovery for a particular target protein, separation may be tailored by the appropriate choice of ion.

Adsorption of cytochrome *c* (horse and tuna heart) as model indicates that retention is dependent on the number of solvent exposed histidine residues. Historically, isozymes that differ in exposed histidine content have been used in IMAC experiments to positively correlate this particular amino acid with protein retention. The two cytochromes employed in these experiments differ primarily by the presence, or absence of a single histidine displayed on the protein surface. Lack of adsorption of tuna heart cytochrome *c* adsorption (zero surface histidine), and

its presence in experiments with horse heart cytochrome *c* (single surface histidine) demonstrate the selectivity of IDA magnetic agarose.

In the case of horse heart cytochrome *c*, the assumed Langmuir isotherm agrees well with the data obtained. The dissociation constant obtained in this study agrees with previous work which similarly employed IDA chelated Cu^{2+} as the affinity ligand ($K_d = 0.34$ versus 0.435, 0.455 mg/ml) [9]. Capacity of the media (~40 mg/ml media) determined from equilibrium binding experiments is of sufficient magnitude to attractively compete with commercially available IMAC media on a per milliliter basis. As a batch adsorbant, IMAC media has been previously used in this operational mode both at bench (recovery from a few milliliters) and pilot scales (recovery from 40 l or more) to recover a target protein. IDA magnetic agarose can effectively be employed to recover a protein from cell lysates, as illustrated by the recovery of angioI-TEM-β-lactamase from *E. coli* DH5α:pBAM105 extracts. After protein adsorption, concentration of the media with the aid of magnet has the added advantages of no centrifugation, and the ability to stabilize the media against a side wall or bottom during the decanting of washes. This strategy may be applied to lysates which are of high viscosity and/or particulate count, and are not easily centrifuged, filtered, or amenable to packed bed operation. Containment in this fashion also helps to minimize loss during recovery. This caveat may be of particular importance when the scale of the system is small, due either to a low concentration of target protein or a desire to use as little media as possible in order to provide a concentrated sample.

5. Conclusions

Separation media which combines the selectivity of metal-affinity with the use of magnetic properties during recovery has been formulated. Media illustrated in Fig. 1 mimics both the structure of commercial IMAC media, and displays a characteristic selectivity toward histidine-containing proteins over those without this amino acid. When charged with a divalent metal ion (e.g. Cu^{2+} or Zn^{2+}), media efficiently binds model proteins, and may be used to isolate a target protein from a cell lysate.

6. Nomenclature

C_s	concentration of protein in solution (mg/ml)
q	amount of protein bound per gram media (mg)
q_{max}	maximum amount of protein bound per gram media (mg)
K_d	equilibrium constant (mg/ml)
Me^{2+}	divalent metal ion

Acknowledgements

The authors wish to acknowledge the University of Arkansas for financial support, Sanjay Chandru, Drs. I. Fritsch and M. Coleman for their input during the work. The helpful comments of Professor M.M. Ataai (University of Pittsburgh) during the preparation of the manuscript are greatly appreciated.

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