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A Magnetizable Solid Phase for Enzyme Extraction

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ABSTRACT

A method for the convenient and reliable preparation of magnetizable agarose beads containing iron particles is described. The beads were treated with the triazine dye, Reactive Red 120, and the matrix was examined for the ability to extract proteins from crude preparations using lactate dehydrogenase from porcine muscle as a model. The recovery and specific activity values of enzyme obtained using this matrix and magnetic field separation were significantly greater than those for enzyme purified by centrifugation and conventional dye ligand chromatography.

Index Entries: Magnetizable solid phase; triazine dye; agarose; lactate dehydrogenase; enzyme purification.

INTRODUCTION

The potential for using magnetizable solid phases in protein purification has been recognized (*1–3*), but little appears to have been reported on the application of this approach.

Dunnill and Lilly (*4*) prepared a crude magnetizable solid phase by granulating a polyacrylamide gel containing magnetic iron oxide. Following the attachment of suitable ligands to the matrix, the solid phase was successfully used to isolate β-galactosidase and L-asparaginase from *Escherichia coli* homogenates. Hirschbein and Whitesides (*5*) rendered derivatized agarose beads magnetizable by treating them with a colloidal

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suspension of magnetite, and found their performance compared favorably with a more conventional technique using polyacrylamide immobilized on a glass-fiber cloth as solid phase. Postmagnetizing of Sepharose beads with ferro-fluids has been used to produce magnetically responsive solid phases (*6*). The biospecificity of the attached ligand was unchanged, but the binding capacity of the solid phase was reduced by about 50% compared to nontreated beads. Griffin et al. (*7*) postmagnetized 2′, 5′-ADP Sepharose in a similar way and subsequently used this to isolate glucose-6-phosphate dehydrogenase from yeast and red blood corpuscles, with a reported purification of 11,000-fold in only one step.

Our aim was to devise a method for the preparation of magnetizable beads for the biospecific adsorption of proteins from crude mixtures. Suitable beads were prepared by allowing agarose to gel in the presence of iron particles. We chose the triazine dye, Reactive Red 120, as a ligand and used it to evaluate the extraction and partial purification of the model enzyme, lactate dehydrogenase (LDH), from a porcine muscle homogenate.

MATERIALS AND METHODS

Preparation of Agarose Beads

Agarose beads containing iron particles were prepared using a method based on that of Pertoft and Hallen (*8*). Agarose, medium EEO (Sigma Chemical Co.) (3 g) was dissolved in about 700 mL of distilled water by heating in a water bath to 95°C. Polyethylene glycol (Sigma; mol wt 20,000) (45 g) and iron powder (Koch-Light) (1.75 g) were added to the hot agarose solution, the final volume adjusted to 750 mL with water, and the solution then allowed to cool. Stirring of the mixture throughout the whole process produced beads containing particles of iron. The Fe-agarose beads were collected by filtration on a Buchner funnel, and washed extensively with warm water to remove excess polyethylene glycol. Nonmagnetizable beads were prepared following the same procedure, with the exception that the iron was omitted. Both types of beads were stored in 20 m*M* sodium phosphate buffer, pH 7.2, containing 0.02% NaN_3 at room temperature.

Evaluation of the Agarose Beads

Beads in 20 m*M* sodium phosphate buffer, pH 7.2, containing 1*M* NaCl, were packed in columns (10×100–130 mm) and run at a rate of 4 cm/h. The porosities of the beads were determined by measuring the elution volumes (V_e) of the following markers: Blue Dextran 2000 (2000 kDa) (Pharmacia LKB) for the void volume (V_o); β-galactosidase (540 kDa); catalase (232 kDa); LDH (140 kDa); horseradish peroxidase (34 kDa); cyto-

chrome-*c* (12 kDa); and cytidine (250 Da) (all from Sigma) to estimate the total mobile phase volume, V_s. The distribution coefficient (Kd) for each of the marker proteins was calculated using the formula $Kd=(V_e-V_o)/V_s$. Fractions of 0.5 mL were collected; the enzymes were detected by standard assays, and the other markers by their characteristic absorbances. Sepharose CL6B beads (Pharmacia LKB) were examined under the same conditions.

The agarose content of the nonmagnetizable beads was estimated by drying triplicate samples of the beads, of known packed volume, to constant weight in a 37°C drying oven over a period of 14 d. The fraction of the packed volume actually occupied by the agarose beads (i.e., the packed bed volume less the void volume) was determined by size exclusion chromatography to be 66%, and this value was used to calculate the concentration of agarose in the solid phase.

Dye Ligand Immobilization

The triazine dye, Reactive Red 120 (Sigma), was immobilized on the magnetizable (Fe-agarose), the nonmagnetizable, and Sepharose CL6B beads using a method similar to that of Harris and Byfield (*9*). Dye powder was dissolved in distilled water at 2.5 mg/mL and 4 vol were added to 1 vol of the settled solid phase in a sealable glass bottle, and the suspension was mixed by rolling at 50 rpm for 30 min at room temperature. Sufficient NaCl was then added to the mixture to give a final concentration of 2%, and mixing continued for a further 30 min. The pH was adjusted to 11.8 with 1*M* NaOH and the mixture was incubated in a shaking water bath for 24 h at 28°C. The dyed beads were washed with distillled water, followed by 6*M* urea until no further dye was washed out. Beads were stored in 20 m*M* sodium phosphate buffer, pH 7.2, containing 0.02% NaN_3 at room temperature.

The amount of dye attached to the solid phases was estimated by determining the difference in the amount of dye applied in the immobilization reaction and the amount of unbound dye subsequently removed by washing. The absorbances of the dying solution and the washes were measured at 545 nm, after adjusting the pH to 7.

Enzyme Purification

Preparation of Homogenate

Fresh porcine muscle was obtained directly from an abbatoir, cut into pieces of about 30 g, and stored at −70°C until required. The pieces were thawed at room temperature and excess fat and connective tissue removed. The muscle was then finely chopped using a scalpel, and the weight of tissue determined. Homogenization was carried out (at 4°C) in 20 m*M*

sodium phosphate buffer, pH 7.2, containing 15 m*M* β-mercaptoethanol and 1 m*M* phenylmethyl sulfonyl fluoride (2 mL of buffer/g of tissue) using a VirTis Super 30 homogenizer at medium speed for 90 s.

Conventional Extraction

A sample (40 mL) of homogenate was centrifuged at 5000*g* for 30 min at 4°C. The supernatant was sieved through four layers of surgical gauze to remove remaining fat particles, and the volume of filtrate recorded. On reaching ambient temperature, supernatant was loaded onto a 15-mL column of the dyed agarose beads (equilibrated in 20 m*M* sodium phosphate buffer, pH 7.2) at a flow rate of 14 mL/h. The bed was washed with two bed volumes of the same buffer and bound proteins eluted with buffer containing 1*M* NaCl. Fractions containing more than 100 U of LDH were pooled so that maximum yield was obtained without undue dilution.

Magnetic Extraction

For the magnetic extraction process, 40 mL of homogenate were diluted with 40 mL of the homogenization buffer and filtered through four layers of surgical gauze. The dyed Fe–agarose beads (15-mL packed vol) were added and the suspension incubated at room temperature for 1 h with constant mixing by rolling at 50 rpm in a sealed glass vessel. The suspension was transferred to a 500-mL beaker and the magnetizable solid phase removed from the suspension using a 50×40×40 mm permanent magnet (Griffin) placed in a resealable waterproof bag. This was immersed in the suspension for 1 min and then carefully withdrawn with the beads attached. On removing the magnet from the bag, the solid phase was easily washed into a second vessel with buffer. During withdrawal of the magnet from the suspension, some of the outermost beads were retained by surface tension, necessitating five such extractions to remove all the beads. (The use of a stronger magnetic field would reduce this need.) To separate remaining particulate matter, the beads were extracted a second time and washed into a third vessel. The extracted beads were then packed in a 16-mm diameter column, and the bound proteins eluted and fractions collected, as in the conventional extraction. In one experiment, differential elution of bound proteins was carried out using a gradient of NaCl (0–1*M*) in 20 m*M* sodium phosphate buffer, pH 7.2. The LDH, malate dehydrogenase (MDH), and aspartate transaminase (AST) activities were assayed by standard methods.

Protein Determination

Protein concentrations were determined by the method of Lowry et al. (*10*) and by measuring the absorbance of chromatography fractions at 280 nm; bovine serum albumin was used as standard protein in both methods.

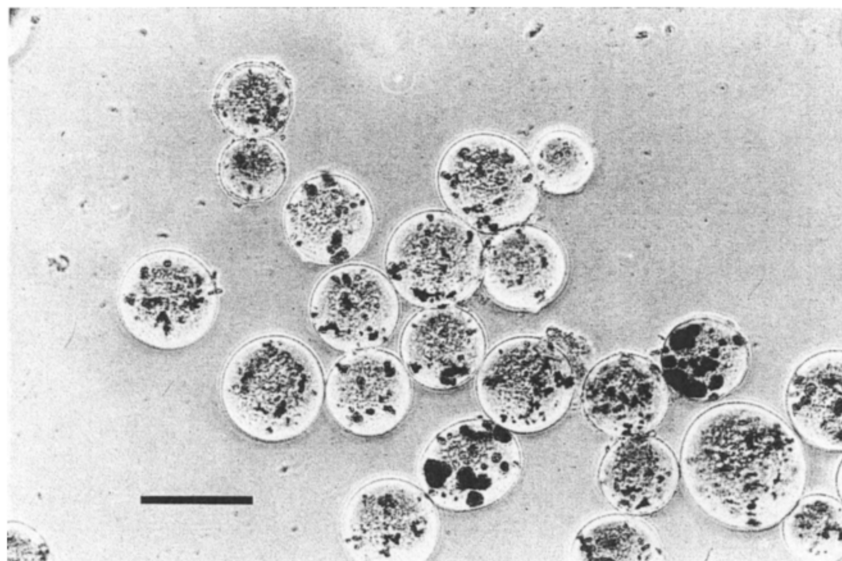

Fig. 1. Dyed Fe-agarose beads. The bar represents 100 μm.

Polyacrylamide Gel Electrophoresis

The method of Laemmli (*11*) was used with 5–15% gels. Molecular weight markers (BRL) were run with the LDH samples, and the gels were stained with Coomassie Brilliant Blue.

RESULTS AND DISCUSSION

Properties of Agarose Beads

For both the agarose and Fe-agarose beads, yields of 27 mL of packed vol were consistently obtained per batch. The beads were spherical, or very nearly so, with diameters ranging from 50 to 125 μm (Fig. 1). The agarose concentration of the beads was determined to be 9% (w/v). This resulted in a lower porosity of the solid phases compared to Sepharose CL6B, with an agarose concentration of 6%. The Kd for LDH (140 kDa) was 0.13 for the agarose and Fe-agarose beads and 0.45 for Sepharose CL6B (Fig. 2). However, the greater agarose content of the prepared solid phases allowed higher sustainable flow rates through packed columns.

Between 30 and 45% of the Reactive Red dye was routinely coupled to the beads after 24 h incubation. Extending the dyeing time to 4 d approximately doubled the amount of dye immobilized, but gave no significant increase in the enzyme binding capacity, 1000 U LDH/mL of beads (for both dyed agarose and dyed Fe-agarose beads). This capacity was almost

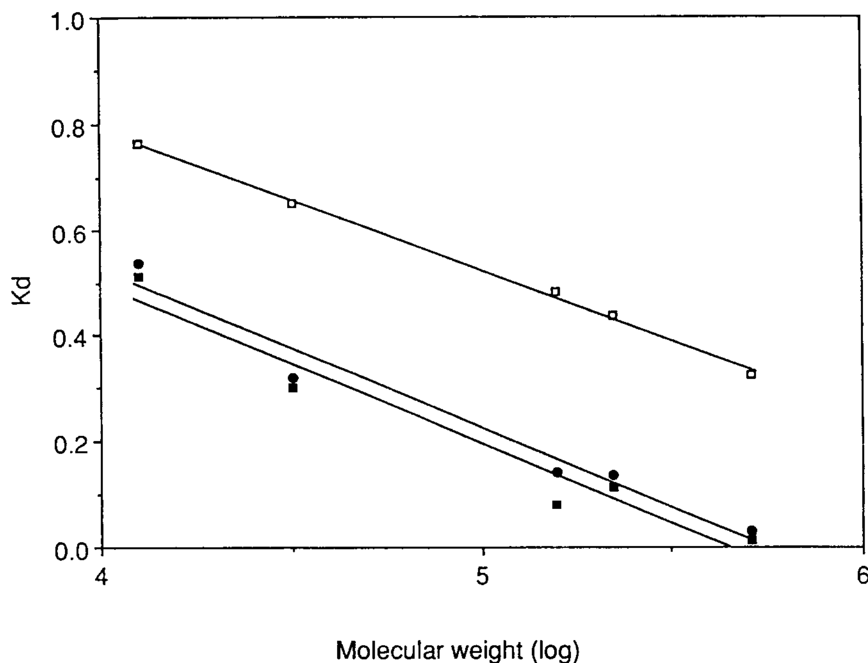

Fig. 2. Porosities of magnetizable Fe-agarose (■) and nonmagnetizable agarose beads (●) compared to Sepharose CL6B beads (□), using the mol wt markers listed in the text. The graph was plotted by computer, using a least squares fit method.

80% of that exhibited by dyed Sepharose CL6B (1275 U LDH/mL beads) and compares favorably with the 50% reduction in binding capacity of postmagnetized Sepharose reported by Mosbach and Andersson (*6*).

The presence of the Fe particles in the magnetizable Fe-agarose beads had little or no effect on the mechanical and enzyme-binding properties of the solid phase. Long-term stability of the dyed Fe-agarose beads was good, with no corrosion of the Fe or decrease in LDH binding observed after 12-mo storage in buffer at room temperature and several enzyme extractions.

Comparison of Conventional and Magnetic Extractions

The extraction of LDH from porcine muscle homogenate using a conventional process of centrifugation to remove particulate matter followed by affinity chromatography on the dyed agarose beads resulted in a yield of about 50% of the enzyme (Table 1). The greatest loss was incurred during centrifugation, where 40% of the activity remained in the pellet. Increasing the liquid:solids ratio by diluting the homogenate could reduce, but not completely eliminate, this loss at the expense of increasing the volume of material to be processed. Magnetic extraction, on the other hand, permitted a 100% recovery of enzyme activity in the pooled frac-

Table 1
Purification of LDH from Porcine Muscle Homogenate by Conventional Extraction (Centrifugation and Affinity Chromatography) and by Magnetic Extraction Using Reactive Red as a Ligand

	Volume (ml)	Protein Concentration (mg/ml)	LDH activity (U/ml)	Specific activity (U/mg)	Purification factor	Yield %
Homogenate	40	30	241	8	1	100
Conventional Extraction	24	5	200	40	5	50
Magnetic Extraction	27	3	357	119	15	100

tions. Direct addition of the magnetizable solid phase to the homogenate allows the entire liquid fraction to come into contact with the affinity ligand, reducing the losses encountered with centrifugation. In addition to the increased yield of enzyme, the magnetic extraction also resulted in a greater purification factor, 15, compared to only 5 obtained by the conventional method (Table 1).

Extensive washing of the conventional solid phase was required to remove the nonbound material from the column before elution of the LDH by the salt wash was carried out (Fig. 3). This preelution washing of the column was not necessary with the magnetizable beads (Fig. 3) as the removal of particulate matter by the buffer changes simultaneously removed nonbound protein. Elution of the magnetizable beads with a salt gradient (Fig. 4) indicated that bound proteins were eluted at different ionic strengths. This could provide a method of further increasing the specific activity of the extracted protein(s).

Analysis of the eluted LDH fractions by gel electrophoresis revealed the presence of a number of coextracted proteins (Fig. 5). This is not surprising since Reactive Red 120, like the other dye ligands, can bind many proteins (*12–15*).

CONCLUSION

Agarose beads containing Fe particles and the Reactive Red dye were easily prepared and their stability, porosity, and binding capacity made them suitable for protein purification. The direct application of the beads to the crude homogenate of porcine muscle allowed LDH to be extracted at maximum yield and at a higher specific activity than that obtained by

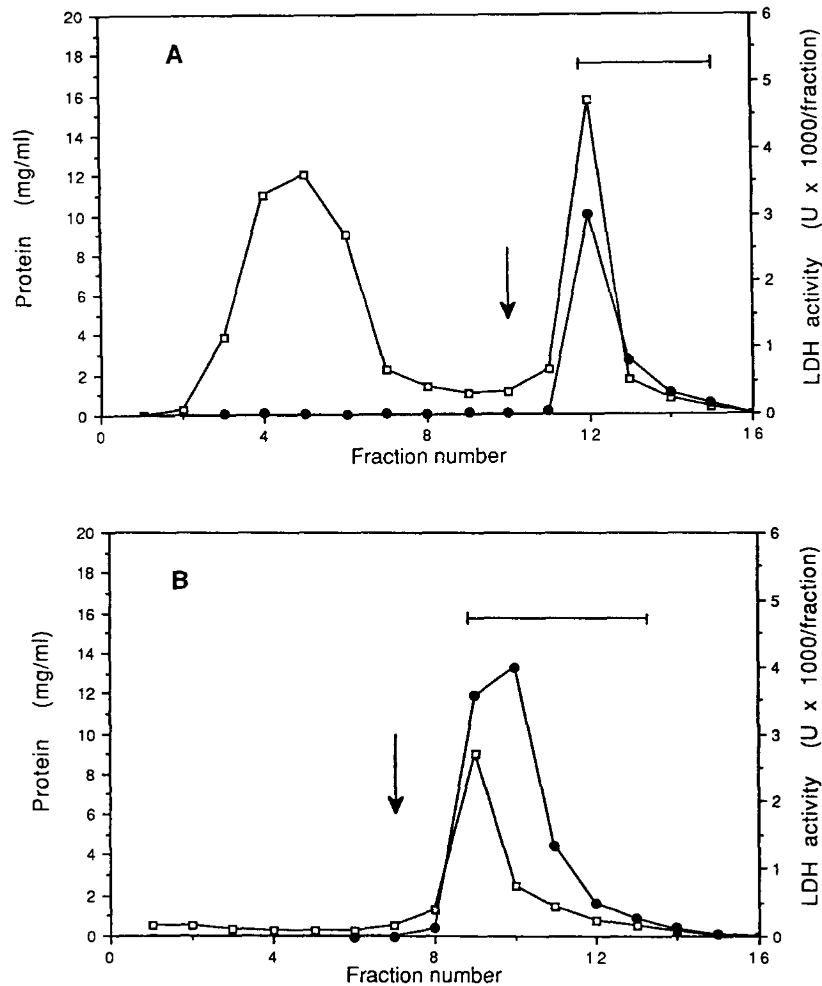

Fig. 3. Elution profiles of LDH from Reactive Red-dyed agarose beads (A) and dyed magnetizable Fe-beads (B). Sodium phosphate buffer, pH 7.2, containing 1*M* NaCl was applied where shown by the arrows, and fractions pooled as indicated by the bars. Fractions of 5.4 mL were collected and their LDH activity (●) and protein concentration (□) determined.

centrifugation and conventional dye ligand chromatography. This approach may be especially advantageous in preparing labile proteins. By substituting appropriate ligands for the Reactive Red 120 dye used in this case, the method should prove easily adaptable to many procedures employing an affinity purification step. The process should also lend itself to large-scale applications, in which the simplicity of the extractions and the elimination of centrifugation, the high enzyme yield, and the solid phase stability would all be major advantages.

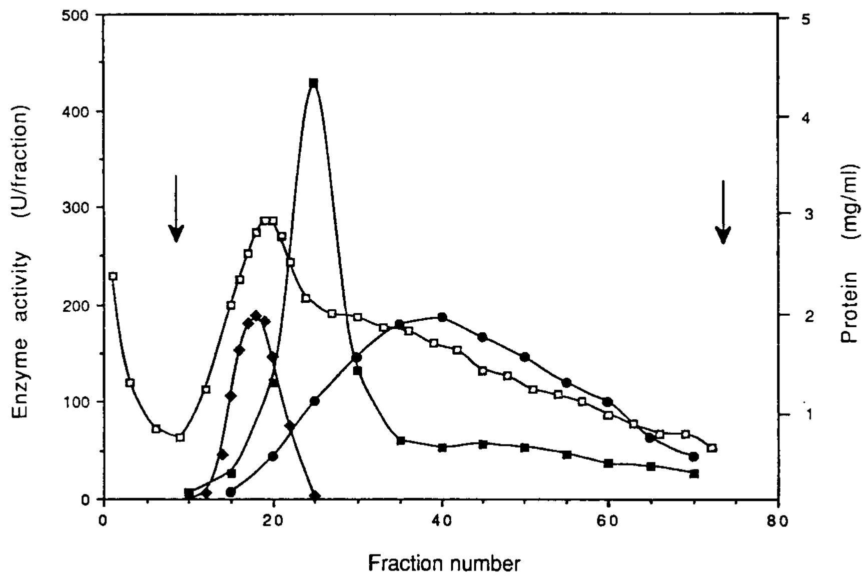

Fig. 4. Elution profile of Reactive Red-dyed magnetizable beads. A linear 0–1*M* NaCl gradient was applied between the arrows, and fractions of 4 mL were collected. Protein concentrations (□), LDH (●), MDH (■), and AST, x 10 (◆) activities of fractions are shown.

Fig. 5. Gel electrophoresis of LDH preparations. A, porcine muscle soluble fraction; B, conventionally extracted enzyme; C, magnetically extracted enzyme. The position of LDH is marked (subunit M_r 36,500).

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