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AFFINITY CHROMATOGRAPHY OF LACTATE DEHYDROGENASE AND WHEAT GERM LECTIN ON NEW GELS BEARING CARBOXYLIC FUNCTIONS

E. BROWN and R. JOYEAU

Laboratoire de Synthèse Totale de Produits Naturels, ERA No.394, Faculté des Sciences, Route de Laval, B P. 535, 72017 Le Mans Cedex (France)

and

E. BOSCHETTI and Y. MOROUX

L'Industrie Biologique Française, 92231 Gennevilliers (France)

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SUMMARY

The suitability of two new functionalized copolymer gels for use in affinity chromatography has been examined. Both gels were substituted by two ligands, one being specific for lactate dehydrogenase and the other for wheat germ lectin. The derivatives thus obtained were used successfully for the purification of two proteins with different biological activities.

INTRODUCTION

In a previous paper[1], we described the preparation and the characteristics of new copolymers for affinity chromatography. They consist of gels in the form of hydrophilic beads, obtained by copolymerization, in the presence of 4% agarose, of acrylamide, N,N'-methylenebisacrylamide and an acrylic monomer such as acrylamido-6-hexanoic acid or methacryloylglycylglycine. This paper reports on the suitability of these carriers for use in affinity chromatography.

The separation or purification of lactate dehydrogenase (LDH) and wheat germ lectin (WGA) were studied. The ligands used were *p*-aminophenyllactic acid (APL) as a specific effector of LDH, and *p*-aminobenzyl-1-thio-2-acetamido-2-deoxy-β-D-glucopyranoside (ABTAG) as a specific sugar of WGA[2]. The results were compared with those from a standard carrier, *i.e.* Sepharose 4B activated by cyanogen bromide (CNBr).

EXPERIMENTAL

Chemicals

Crystallized rabbit muscle LDH titrating 34 units per mg was obtained from Koch-Light (Colnbrook, Great Britain), and WGA from the reagent department of

L'Industrie Biologique Française (Gennevilliers, France), which also supplied the copolymers AB–MGG (a copolymer of N,N'-methylenebisacrylamide, methacryloylglycylglycine and agarose), and AB–A6NH (a copolymer of N,N'-methylenebisacrylamide, acrylamido-6-hexanoic acid and agarose) in bead form.

The various reagents used, such as CNBr, N-acetyl-glucosamine, L-phenylalanine, N-cyclohexyl-N-[β-(N-methylmorpholino)ethyl]carbodiimide toluene-*p*-sulphonate (CMC) and the other chemicals were supplied by Aldrich (Milwaukee, Wisc., U.S.A.). Sepharose 4B was provided by Pharmacia France (Parly, France).

Preparation and immobilization of p-aminophenyllactic acid (APL)

Nitration of L-phenylalanine, followed by nitrous acid deamination, yielded *p*-nitrophenyllactic acid, m.p. 121–126°. Catalytic reduction of the latter provided APL. A detailed description of this synthesis is to be published separately[3]. APL has a m.p. 178–185° and a specific rotation $[\alpha]_D^{20°} = -45°$ in a 10 mg/ml solution in $NaHCO_3$ (0.2 *M*). APL was immobilized on three different carriers: Sepharose 4B, AB–MGG and AB–A6NH.

Sepharose 4B was first activated by CNBr and substituted by 6-aminohexanoic acid, using the method of Porath *et al.*[4], which produced a gel which also carries, like the two others, side-chains ending with carboxylic acid groups. APL was fixed on these three gels by using a carbodiimide as condensing agent[5]. Thus, to 5 ml of an aqueous suspension of each gel (previously washed with water) are added 36 mg of APL dissolved in 2 ml of demineralized water. The pH of these three suspensions was adjusted to 4.7 before adding 150 mg of CMC, and maintained at this value by the addition of hydrochloric acid (0.1 *M*) for 1 h; the suspension was stirred slowly overnight. The gels were washed successsively with demineralized water, 1 *M* NaCl solution and 0.1 *M* phosphate buffer, pH 7.5.

Measurement of the specific rotations of the filtrates revealed that the amounts of APL fixed were *ca.* 9–10 μmole/ml on derivatized Sepharose 4B, and *ca.* 7 μmole/ml on AB–MGG and AB–A6NH.

Preparation and immobilization of p-aminobenzyl-1-thio-2-acetamido-2-deoxy-β-D-glucopyranoside

ABTAG, a specific ligand for WGA, was obtained by catalytic reduction[2] of *p*-nitrobenzyl-1-thio-2-acetamido-2-deoxy-β-D-glucopyranoside, prepared according to the method of Rafestin *et al.*[6]. This compound was immobilized on the AB–MGG copolymer and on Sepharose 4B, the latter being previously substituted by hexamethylenediamine followed by succinoylation. Then 25 ml of each gel were brought into contact with 0.25 μmole of ABTAG, at pH 4.7, and 250 mg of CMC were added. The suspension was stirred for 24 h at 4°, and the gel washed with a 0.05 *M* Tris–HCl, 0.15 *M* NaCl buffer, pH 8.6. The amounts of ABTAG immobilized on the gels, determined immediately after hydrolysis of a given amount of gel at 100° in 4 *N* HCl for 4 h, according to the method of Bouchard *et al.*[2], were 0.5 μmole/ml on the derivatized Sepharose 4B and 1.1 μmole/ml on the AB–MGG copolymer.

Affinity chromatography of LDH

The three gels, carrying immobilized APL, were packed into chromatography columns (1 cm I.D.) to a height of 6.5 cm. On each column were placed 34 LDH units

(*ca.* 1 mg) and 2 mg of bovine serum albumin (BSA), dissolved in 1 ml of 0.1 *M* phosphate buffer, pH 7.5.

The columns were successively washed with 0.1 *M* phosphate buffer, pH 7.5, the same buffer containing 0.15 m*M* NADH, and the initial buffer, in each case until the eluate was transparent at 280 nm. The elution rate was 15 ml/h. Fractions of 8.2 ml were collected. For each fraction, the protein concentration was determined by absorption at 280 nm, and the enzymatic activity of LDH was determined by kinetic measurements at 340 nm, in the presence of pyruvate[7].

Affinity chromatography of WGA

Aliquots of the gels (16 ml) carrying the ligand ABTAG (Sepharose 4B and AB–MGG) are put into columns 8 cm × 1.6 cm I.D. The columns were washed successively with a 0.05 *M* Tris–HCl, 0.15 *M* NaCl buffer, pH 8.6, by 1 *M* NaCl, by a 0.05 *M* acetate buffer, pH 4.4, by 0.05 *M* HCl and finally re-equilibrated in the first buffer at pH 8.6. At this stage, two different experiments were performed: first, the purification of 10 mg of commercial WGA, and secondly the separation of WGA, starting from 250 ml of a crude extract obtained by the method of Bouchard *et al.*[2].

The gels were washed until no absorbance was detectable at 282 nm. They were rinsed with 1 *M* NaCl and then with 0.05 *M* acetate buffer, pH 4.4. The immobilized WGA was then eluted with 0.05 *M* HCl, pH 1.6. The protein content of each isolated peak was determined by the method of Lowry *et al.*[8], and the agglutinating activity by the method of Salk[8].

RESULTS

Purification of LDH

We first ascertained that the unsubstituted AB–MGG and AB–A6NH carriers interact neither with BSA nor with LDH under the experimental conditions. Two columns made with these two carriers, loaded with 34 units of LDH (1 mg) and 2 mg of BSA, presented the same features as observed following chromatography on gel. In each case, we collected over 95% of protein, and enzymatic activity in the first fraction of eluate. We also made sure that the carrier derived from Sepharose 4B did not interact with LDH, following treatment by CMC in the absence of APL.

The results obtained during the affinity chromatography experiments are summarized in Fig. 1 and Table I.

Albumin and the other inert proteins appear to be totally eluted by the phosphate buffer used; no LDH enzymatic activity was detectable in the corresponding eluates. On the contrary, on adding 0.15 m*M* NADH to the buffer, a high enzymatic activity appeared in the corresponding eluate.

The results obtained with all three carriers indicate a real biospecific affinity of LDH. The overall yield in enzymatic activity was more than 80% in every case.

Purification of WGA

We followed Bouchard *et al.*[2] in using as a blank a column of ABTAG immobilized on Sepharose 4B. Likewise, we tested the suitability for affinity chromatography of the same ligand immobilized on AB–MGG. Although the quantities of ligand fixed on the two gels are very different (0.5 μmole/ml for derivatized Sepharose

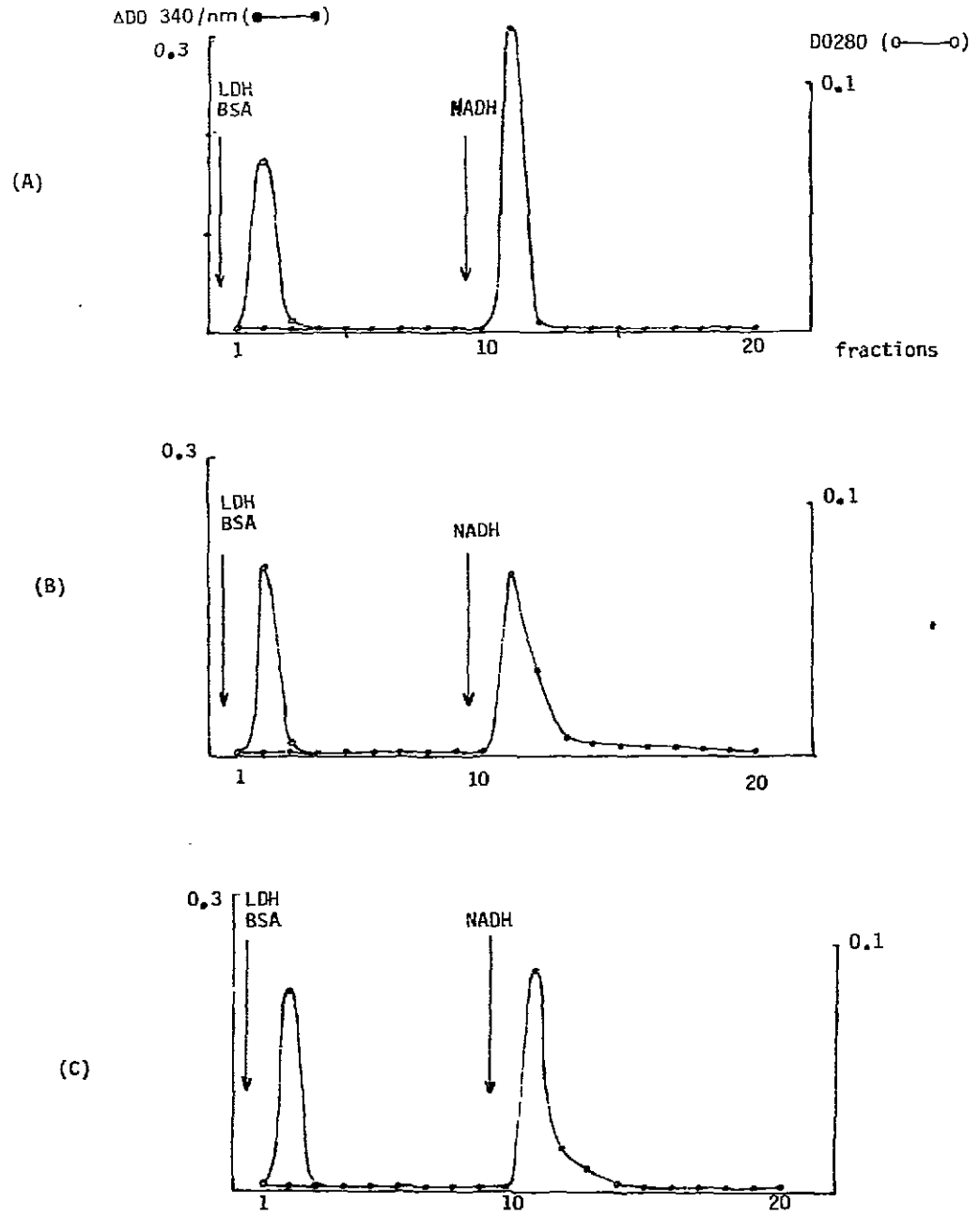

Fig. 1. Affinity chromatography of LDH on immobilized APL. The three diagrams correspond to the use of Sepharose 4B (A), AB-MGG (B) and AB-A6NH (C).

TABLE I

COMPARATIVE AFFINITY CHROMATOGRAPHIES OF LDH ON IMMOBILIZED APL

Carrier	*COOH groups (μmole/ml)**	*Immobilized APL (μmole/ml)*	*First peak** Proteins (mg)*	*Second peak*	
				Proteins (mg)	*Specific activity (U/mg)*
Sepharose	10 –14	≈ 10	2.5	0.5	60
AB–MGG	7.8–10	≈ 7	2.55	0.45	61
AB–A6NH	7.7– 9.5	≈ 7	2.62	0.38	75

* The carboxylic functions were estimated using a technique developed in our laboratories[10].
** No LDH activity was detected.

4B and 1.1 μmole/ml for AB–MGG) we obtained similar results, both for the purification of commercial WGA and for the separation of the same lectin from a crude extract (Fig. 2 and Table II).

The major part of the inert proteins appear at the beginning of the diagram

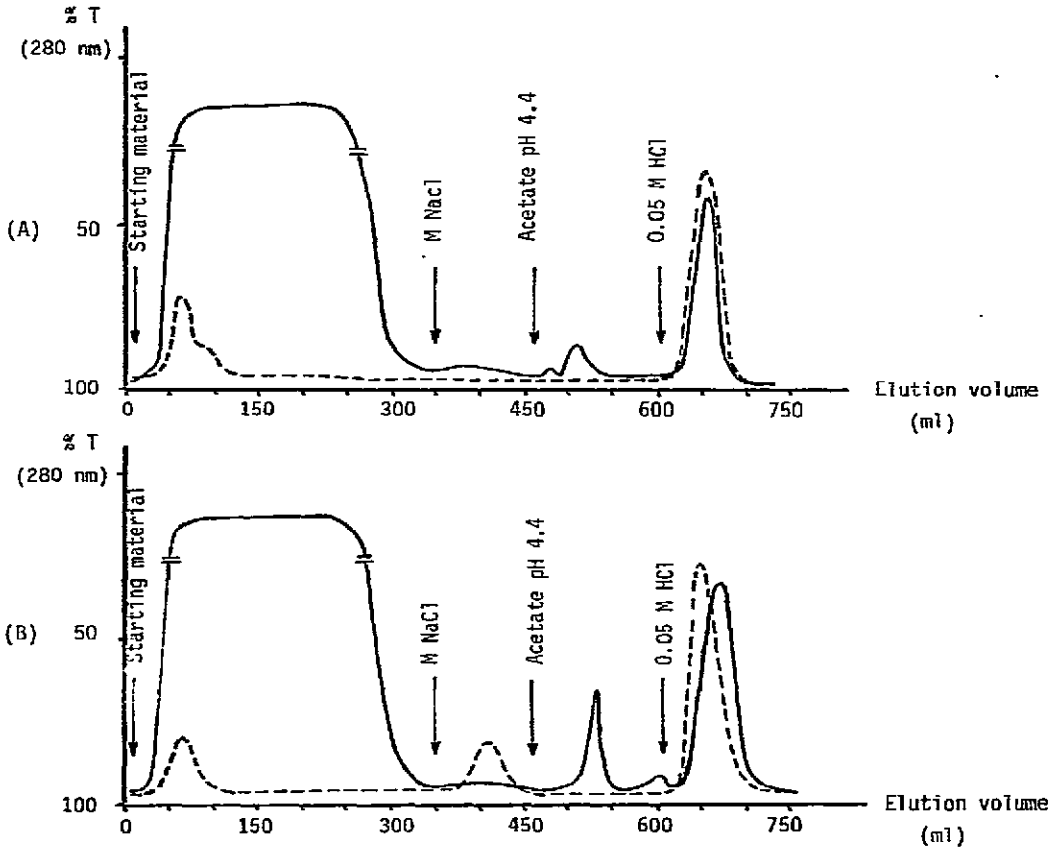

Fig. 2. Affinity chromatography of WGA on immobilized ABTAG. The two diagrams correspond to the use of derivatized Sepharose 4B (A) and AB–MGG (B). The continuous curves represent the results corresponding to the separation of WGA from a crude extract; the broken lines represent the purification of commercial WGA.

TABLE II

COMPARATIVE AFFINITY CHROMATOGRAPHIES OF WGA ON IMMOBILIZED ABTAG

Starting material	*Stage*	*Sepharose 4B*		*AB–MGG*	
		Proteins (mg)	*Activity (U/mg)**	*Proteins (mg)*	*Activity (U/mg)**
Commercial WGA	Deposit	10	128	10	128
	Elution (HCl)	8.1	256	8.6	128
Crude extract (wheat germ)	Deposit	1065	1	1065	1
	Elution (HCl)	8.5	256	8.3	256

* The agglutinating activity is defined by the limit dilution of a 1 mg/ml solution of WGA in Tris–HCl buffer, pH 8.5, which brings about an agglutination visible on rabbit red globules.

and represents *ca.* 10% of the total amount in the case of the commercial WGA, or over 99% in the case of a crude extract. The proteins eluted in minute amounts, using 1 *M* NaCl or 0.05 *M* acetate buffer, pH 4.4, did not show any appreciable agglutinating activity. We recovered all the WGA by percolating hydrochloric acid, and found that the purification rate was low for the commercial product and particularly high for the crude extract. In the latter case, the specific activity increased from 1 unit at the beginning to 256 units after purification.

DISCUSSION

LDH and WGA, two proteins with different origins and possessing two fairly distinct types of biological activity, can be purified easily by affinity chromatography on two new hydrophilic polyacrylic carriers, AB–MGG and AB–A6NH, carrying COOH groupings at the end of respectively hydrophilic and hydrophobic side-chains. In the particular case of the separation of LDH on immobilized APL, our experiments represent a new approach to this problem, since LDH is usually purified by affinity chromatography on immobilized analogues of NAD^+[11,12]. Owing to the low cost of APL (which is obtained easily in three stages from commercial L-phenylalanine), and taking into account the high yield with which it can be immobilized on polymers carrying carboxylic acid functions, this new effector should prove to be an excellent ligand for large-scale affinity chromatography of LDH.

On the contrary, the specific ligand of WGA that we used is more common and gave the expected results. We used it in order to test the new polyacrylic carrier, AB–MGG, and compared it with a standard carrier derived from Sepharose 4B. It is apparent that the fixation of a ligand on acid gels, such as AB–MGG, requires only one step, whereas with Sepharose 4B three or four stages are necessary.

The mixed polyosidic–polyacrylic functionalized copolymers AB–MGG and AB–A6NH[1] thus seem to be very useful carriers. The agarose present in the gels, which makes the beads rigid, does not run the risk of having its structure altered or degraded by drastic activation reagents. For instance, the copolymerization reactions of acrylic monomers are not likely to entail any deterioration in the structure of agarose, owing to the particularly mild operating conditions.

REFERENCES

1 E. Brown, A. Racois, E. Boschetti and M. Corgier, *J. Chromatogr.*, 150 (1978) 101.
2 P. Bouchard, Y. Moroux, R. Tixier, J. P. Privat and M. Monsigny, *Biochimie*, 58 (1976) 1247.
3 E. Brown and R. Joyeau, in preparation.
4 J. Porath, R. Axen and S. Ernback, *Nature (London)*, 215 (1967) 1491.
5 P. Cuatrecasas, *J. Biol. Chem.*, 245 (1970) 3059.
6 M. E. Rafestin, A. Obrenovitch, A. Oblin and M. Monsigny, *FEBS Lett.*, 40 (1974) 62.
7 I. P. Trayer, H. R. Trayer, D. A. P. Small and R. C. Bottomley, *Biochem. J.*, 139 (1974) 609.
8 H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
9 J. E. A. Salk, *J. Immunol.*, 49 (1944) 87.
10 E. Boschetti, P. Girot and M. Corgier, in preparation.
11 P. O'Carra and S. Barry, *FEBS Lett.*, 21 (1972) 281.
12 K. Mosbach, H. Guilford, R. Ohlsson and M. Scott, *Biochem. J.*, 127 (1972) 625.