THE USE OF SPHERON AS A MATRIX FOR AFFINITY CHROMATOGRAPHY OF NAD-DEPENDENT DEHYDROGENASES

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The paper compares several methods of coupling common ligands of dehydrogenases, *viz.* N^6-[(6-aminohexyl)carbamoylmethyl]-AMP and N^6-[(6-aminohexyl)carbamoylmethyl]-NAD, to a hydrophilic macroporous glycolmethacrylate gel, Spheron. The affinants coupled best to a CNBr-activated gel and to a gel with hydrazide groups (after activation with nitrous acid). The affinity properties of gels based on Spheron and on Sepharose 4B were similar (the stability and separation efficiency were almost identical, the binding capacity and the recovery of dehydrogenase activity were somewhat better with the Sepharose). The materials based on Spheron were used in several separation experiments, *viz.* separation of lactate dehydrogenase from albumin, separation of lactate dehydrogenase from alcohol dehydrogenase under different conditions and separation of isoenzymes of lactate dehydrogenase. Spheron 300 with a coupled affinant was also employed in an attempt to purify a crude alcohol dehydrogenase.

Affinity chromatography, as a biospecific separation method, has in recent years been finding an ever increasing use in the isolation of enzymes and other proteins from biological materials. In the separation of NAD-dependent dehydrogenases (but also kinases and other enzymes), materials very useful for affinity chromatography appear to be such as contain covalently bound (immobilized) "common" ligands, generally a coenzyme or its fragments[1]. Rather versatile are especially derivatives of NAD and AMP having a longer chain (spacer), with an aliphatic amino group, attached to the adenine ring[1-3]. Such substances can quite readily be fixed to a suitable matrix, thus forming suitable materials for affinity chromatography of dehydrogenases. The matrices thus far used for this purpose have been of the agarose type (especially Sepharoses), less frequently cellulose, glass and other materials[1].

The objective of the present study was to ascertain whether Spherons, being rather hydrophilic glycolmethacrylate gels, can also be employed as matrices for affinity chromatography of NAD-dependent dehydrogenases (in the use of the above-mentioned common ligands as affinants). These materials were successfully used both for the immobilization of enzymes and other proteins, and for affinity chromatography[4-6]. Some properties of these materials proved very advantageous in the experiments (considerable rigidity, good hydrodynamic properties, chemical stability, resistance to microorganisms and a wide choice of processes of gel activation for the binding of an affinant). As to the utility of Spherons for the affinity chromatography of dehydrogenases, rather little is known yet[7].

EXPERIMENTAL

The Spherons used (particle size 63—100 µm, unless otherwise stated) were gifts from Dr M. Smrž (Lachema, Brno, Czechoslovakia); tested were: the primary, non-substituted gels (Spheron 300 and Spheron 1 000, differing by the exclusion limits — see further), Spheron CH 1 000 (particle size 100—200 µm, containing c. 0·3 mmol hydrazide groups per gram of dry weight), Spheron Epoxy 300 (0·6 mmol epoxide groups/g) and Spheron HMDA 1 000 (with bound hexamethylenediamine, 0·5 mmol/g, particle size 100—200 µm).

Sepharose 4B was a product of Pharmacia Fine Chemicals (Sweden), lactate dehydrogenase (LDH, EC. 1.1.1.27) ex rabbit muscle was from the firm Koch-Light (England), LDH ex beef heart from the firm Boehringer (F.R.G.). Alcohol dehydrogenase (ADH, EC. 1.1.1.1.) was isolated from horse liver as described[8] (one preparation was purified and contained about 40% of the enzyme, another was heat-denatured and less purified, containing about 1% of the enzyme). Human serum albumin and coenzyme of NAD were products from the firm Imuna (Czechoslovakia); NADH and AMP were Reanal products (Hungary), pyrazole was from the firm Aldrich (U.S.A.). The rest were Lachema chemicals (Czechoslovakia).

N^6-[(6-aminohexyl)carbamoylmethyl]-AMP (*I*) and N^5-[(6-aminohexyl)carbamoylmethyl]-NAD (*II*) were obtained from AMP and NAD, adhering to described procedures[2,3,9]; purity of these affinants, determined by HPLC[9,10], ranged about 90%.

Activation of Spheron 300, Spheron 1 000 and Sepharose 4B with cyanogen bromide was carried out essentially as described in [4,11]: 5 g of a wet material, suspended in 5 ml of water, was activated with 0·1 g of cyanogen bromide for 15 min under cooling in an ice-water bath, while pH was kept to 11. The activated gel was washed with aqueous $NaHCO_3$ (0·1 mol l^{-1}, pH 8·5) and agitated for 24 h at 4°C with 5 ml of the same solution containing 25 µmol of an affinant (*I* or *II*). The gel was then washed with 0·1 mol l^{-1} $NaHCO_3$, water, 1·0 mol l^{-1} NaCl and water again.

In the coupling of an affinant to Spheron with hydrazide groups (Spheron CH 1 000) the procedure was essentially as described in[4]: 5 g of a wet gel was suspended in 2 mol l^{-1} HCl, and 40 ml of 2% $NaNO_2$ was added under cooling with ice. Following the activation (15 min), the gel was washed with cold 0·1 mol l^{-1} $NaHCO_3$ (pH 8·5) and agitated 20 h at 4°C with 5 ml of the same solution with 25 µmol of an affinant. The gel was washed as described above.

Spheron with bound hexamethylenediamine (Spheron HMDA 1 000) was activated with glutardialdehyde[12] (5 g of a wet gel + 5 ml 15 mmol l^{-1} glutardialdehyde in 0·1 mol l^{-1} Na-phosphate buffer pH 7). The activation was allowed to proceed 4 h at room temperature under gentle agitation. The activated carrier was washed with the phosphate buffer and agitated 15 h at room temperature with 5 ml of this buffer containing 25 µmol of an affinant. The gel was washed as described above.

Spheron Epoxy 300 (wet, 5 g), having been washed, was agitated with 5 ml of a buffer (Na-phosphate buffer, 0·1 mol l^{-1}, pH 7, or $NaHCO_3$, 0·1 mol l^{-1}, pH 9) containing 25 µmol of a ligand. The coupling was allowed to proceed for 60 h at room temperature. The gel was then washed as described above. The amount of an immobilized affinant was determined in all cases from the difference in content of the affinant in the solution before and after the coupling reaction (including the solutions used for washing the gel); these differences were ascertained by measuring the absorbances of the solutions at 266 nm (absorption maximum of the two affinants; *cf.* refs[2,3]).

The affinity materials were tested in columns c. 1 × 3 cm containing 2 ml of the swollen gels. The mobile phase was the Na-phosphate buffer pH 7; 0·1 mol l^{-1}, the flow rate was kept with a peristaltic pump (Pharmacia, Sweden) at 0·1—0·15 ml/min. The samples (0·1 ml) applied in model experiments contained 0·5—1 mg of albumin and 5—25 units of LDH, or c. 0·1 unit of ADH (*i.e.* the quantity of albumin was over an order of magnitude greater than that of the

dehydrogenase; *cf.* values of specific activity in refs[13,14]). A gradient elution (usually with an increasing concentration of NADH or NaCl in the Na-phosphate buffer) was carried out in an apparatus Bio Rad (U.S.A.). Using a fraction collector FCC 60 (Laboratorní přístroje, Czechoslovakia), 0·5— or 1 ml fractions, cooled with ice, were taken and evaluated spectrophotometrically for protein concentration (280 nm) and enzymic acitivity (340 nm). The activity of LDH was determined as described in[13], the activity and purity of ADH according to[14]. The spectrophotometric data were measured employing an apparatus Cary 118 (Varian, U.S.A.) or Specord (Zeiss, G.D.R.). After the separation experiments the affinity materials in the columns were washed with 2 mol l^{-1} NaCl and equilibrated with the starting buffer.

RESULTS AND DISCUSSION

Preparation of an Affinity Material

To prepare a Spheron matrix with coupled ligands *I* or *II* we used several methods, recommended by the manufacturer[5] or proved useful in other cases[1-4,11,12]. The results of these experiments are compared with those obtained with Sepharose 4B activated with cyanogen bromide in the conventional way (Table I). The tested methods of activation and coupling appear to differ in efficiency. In the activation with cyanogen bromide, Spheron and Sepharose gave comparable results. The yield of coupling was good in either case (with Spheron even somewhat higher), but under the conditions of the experiment more affinant got coupled to the Sepharose dry weight. This apparent discrepancy was due to a greater swelling capacity of Sepharose

TABLE I

Comparison of different ways of linking N^6-[(6-aminohexyl)carbamoylmethyl]-AMP (*I*) and N^6-[(6-aminohexyl)carbamoylmethyl]-NAD (*II*) to Spheron and Sepharose. The activation of the matrices and the coupling of the affinants is described in Experimental (25 μmol of a ligand per 5 g of wet gel). The given values are averages from two parallel experiments; GA denotes glutardialdehyde

Matrix	Activation	Ligand	Amount of bound ligand, μmol/g of dry weight	Yield of coupling %
Sepharose 4B	CNBr	*I*	24	22
Sepharose 4B	CNBr	*II*	20	19
Spheron 300	CNBr	*I*	6	30
Spheron 300	CNBr	*II*	4	20
Spheron CH 1 000	HNO_2	*I*	5	24
Spheron HMDA 1 000	GA	*I*	10	49
Spheron Epoxy 300 (pH 9)	—	*I*	2	10
Spheron Epoxy 300 (pH 7)	—	*I*	<2	<10

than that of Spheron (in the experiments listed in Table I the weight of the wet gel was constant, which means that the amount of the Sepharose matrix was smaller than the amount of the Spheron matrix). The method of a direct coupling of an affinant to Spheron with epoxide groups (in which no activation of the material is necessary) appears unsuitable in this case, since even prolonged treatment of the material with the affinant failed to bring about any significant immobilization. For this reason the Spheron Epoxy materials were not tested any more. The coupling to Spheron CH (containing hydrazide groups, converted to azide groups by HNO_2) was comparable, under the given conditions, to the binding of the affinant to the non-substituted gel after activation with CNBr. Out of the methods given in Table I, the most efficient one was the coupling to Spheron HMDA (containing amino groups) after activation with glutardialdehyde. The attained extents of modification of the gels (Table I) were not so high as those reported in ref.[7], where different matrices for affinity chromatography of dehydrogenases are also compared. This may be due to the fact that the authors[7] used a very efficient method of activation of matrices by reaction with sulphonyl chlorides.

Testing of Affinity Materials

The affinity gels prepared were packed into small chromatographic columns (see Experimental), to verify that they really had the properties of affinity materials. The experiments were carried out with model mixtures of a pure dehydrogenase (LDH or ADH) and albumin. It was found that the non-modified gels (Sepharose 4B, Spherons 300 and 1 000) were not capable of separating these proteins under the given experimental conditions; they were almost completely eluted with the void volumes of the columns (the gel-filtration effects were negligible). With the columns containing the non-substituted matrices all the activity of LDH or ADH was eluted even with the starting buffer (see further). An example of a model separation on an affinity material (Spheron 300 with affinant *I*) is shown in Fig. 1. All the materials tested (the first five affinity matrices given in Table I, Spheron 1 000 with *I* or *II* bound after activation with CNBr) behaved similarly in these experiments. LDH and/or ADH (a preparation of about 40% purity, see Experimental) were sorbed in the columns, whereas albumin was eluted even with the starting buffer (Fig. 1). Elution of the enzymes was observed after application of a continuous or discontinuous gradient of NADH or NaCl. With ADH it proved better to use the coenzyme, with LDH either method was equally suitable. In eluting LDH with the coenzyme there were some differences depending on the isoenzyme composition of this dehydrogenase (with LDH ex rabbit muscle the elution required c. 0·3 mmol l^{-1} NADH, with LDH ex beef heart most activity was eluted with a concentration of only 0·1 to 0·15 mmol l^{-1}). The material obtained by coupling *I* to Spheron HMDA, activated with glutardialdehyde, exhibited no properties of an affinity material (failed to

retain the dehydrogenases applied), although it had the highest content of the bound ligand of all the Spherons tested (Table I).

This may have been due to glutardialdehyde giving rise to polymeric structures[15], which leads to diminution of the matrix porosity and, consequently, inaccessibility of the bound affinant to the enzymes. The immobilization after activation with glutardialdehyde seems more suitable for macromolecules (see *e.g.*[12]) than for the rather small molecules of affinants *I* and *II*.

Next experiments were carried out with Spheron 300, to which the affinant *I* and/or *II* was bound after activation with CNBr. These materials were compared with Sepharose 4B having the same affinants after an analogous activation.(The fact that Spheron 1 000 behaved like Spheron 300 in these experiments had been found before.)

The capacity for LDH of the column packed with Spheron was about 25 units, the one packed with Sepharose had a capacity about 30% higher, though its content of the affinant was about 35% lower. This may have been caused by a worse accessibility of the affinant in the Spheron to LDH (the exclusion limits of Spherons 300 and 1 000 are 260 000–700 000 and 800 000 to 5 000 000 respectively, ref.[5], whereas that of Sepharose 4B is higher than 3 000 000, ref.[16]; the molecular mass of LDH is about 140 000, ref.[17]). The capacities of the materials with the immobilized affinant *II* were similar; in either case they were c. 50% higher in the use of 5 $mmol\,l^{-1}$ pyruvate in the starting buffer. This was due to the pyruvate forming ternary complexes with LDH and NAD, in which the coenzyme is bound more formly than in a binary complex built of LDH or NAD only[17].

The effect of temperature on the separation properties was investigated with Spheron 300 and affinant *II*. It turned out that the separation of 10 units of LDH from 1 mg of albumin at 4°C and at 25°C was practically identical. In view of this fact all the elementary testing experiments were carried out at the same temperature. Since in most of the model experiments the times of separation were relatively short (usually not much longer than 2 h) the denaturation of the enzymes was not marked. The recovery of activity of the dehydrogenases eluted from the columns was good; in an application of 10 LDH units about 80% of the activity was eluted from Spheron with coupled *I* or *II*, Sepharose with the same affinants gave even higher yields (up to 95%). The results were similar even in testing the non-substituted materials. The somewhat lower recovery from the Spheron matrix may have been due to a moderate denaturation of the enzyme on this material. The affinity materials in the columns were kept in 0·1 $mol\,l^{-1}$ Na-phosphate buffer pH 7 at 4°C and were used in about 20 working cycles (most of them taking around 3 h, including the washing and equilibration) at room temperature. The binding capacities of the columns with the immobilized *II* deteriorated rapidly after 4 to 5 months (with both Spheron and Sepharose), in 6 months most of the materials were of no use. With the affinant *I* the gels proved to be more stable; the columns could be used for 30 to 40 working cycles in the course of 6 to 8 months. The storing stability of the two kinds of affinity material at 4°C and at −20°C was comparable.

Examples of Use of the Affinity Materials

The affinants *I* and *II* coupled to Spheron 300 were also employed in more difficult cases than separation of the dehydrogenases from albumin (Fig. 1). Using a column of Spheron 300 with the affinant *II* we attempted resolution of a mixture of LDH and ADH by applying a gradient elution with NADH, since affinity of ADH to this coenzyme is greater than that of LDH[18]. As can be seen from Fig. 2*a* the separation was not satisfactory. We tried to make use of the fact that the presence of pyrazole enhances the affinity of NAD to ADH[19], but not to LDH. In a gradient elution with increasing concentration of NADH and simultaneously decreasing concentration of pyrazole in the mobile phase the separation of the two enzymes was better, their elution order being reversed (Fig. 2*b*).

Spheron 300 with the affinant *I* was used in attempted resolution of the isoenzymes of LDH ex rabbit muscle. The experiments were based on the fact that the individual isoenzymes of LDH considerably differ in affinity to NADH[17,18]. In a gradient

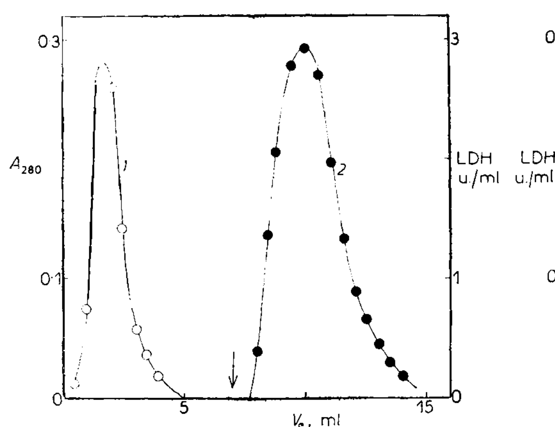

FIG. 1

Resolution of a model mixture of albumin (0·5 mg) and LDH ex rabbit muscle (10 units) on Spheron 300 with affinant *I*. For conditions see Experimental; *1* absorbance at 280 nm, *2* enzymic activity of LDH; the arrow designates the start of application of NADH (0·3 mmol l^{-1}) in the elution buffer, V_e the elution volume

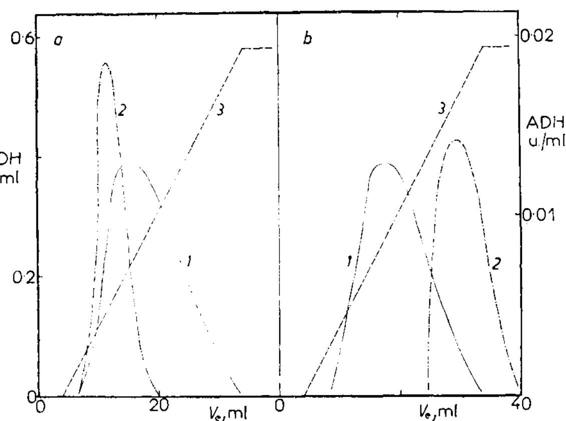

FIG. 2

Separation of LDH (*1*, 5 units) from ADH (*2*, 0·15 unit) on Spheron with affinant *II*. For conditions see Experimental; *3* gradient; the starting buffer in *a* was 0·1 mol l^{-1} Na-phosphate pH 7, in *b* the same buffer with 0·1 mmol l^{-1} pyrazole. The final buffer in either case was 0·1 mol l^{-1} Na-phosphate pH 7 with 0·3 mmol l^{-1} NADH. The activity of ADH in 1-ml fractions of experiment *b* was measured after removal of pyrazole by dialysis against 0·1 mol l^{-1} Na-phosphate buffer pH 7

elution with NADH five peaks of enzyme activity were obtained (Fig. 3). Since the last three were inhibited by urea (2 mmol urea/l) it can be deduced that they represented the isoenzymes M_4, HM_3 and H_2M_2. The isoenzymes specific for the heart muscle, *i.e.* H_4 and H_3M, are not much inhibited in this medium[20]. They are eluted first because they have the highest affinity to NADH[18].

An analogous separation had been attained in the use of the Sepharose matrix with the affinant *I* and a concave elution gradient of NADH[21]. The linear gradient used in our experiments seems to be the reason why the separation of the peaks (Fig. 3) is worse than in ref.[21]. In the case of Spheron 300 with the affinant *II* and a gradient elution with NADH (Fig. 2*a*) the LDH isoenzymes were not separated, probably because the isoenzymes specific for the heart muscle have much higher affinities to both NADH in the eluant and to NAD fixed to the matrix[18].

The column with Spheron 300 and the affinant *II* was also used in an attempt to purify a crude preparation of ADH, containing only c. 1% of the enzyme and some foreign proteins. This was applied, in an amount corresponding to about 0·1 unit of ADH, to a column of the standard size (see Experimental) and a gradient elution with NADH was started (Fig. 2*a*). In contrast to more purified ADH preparations, whose comparable activity got trapped in the column quantitatively, in the present case partial elution of the enzymic activity was effected even with the starting buffer. In application of a gradient of NADH, only c. 40% of the enzymic activity was eluted and the purification was rather low (about twofold). With the use of pyrazole (Fig. 2*b*) the purification was about threefold. The purification effect of Sepharose 4B with the same affinant was an order of magnitude higher.

The low capacity of Spheron with coupled *II* for purification of a crude ADH may be due to the foreign proteins binding non-specifically to the matrix, thus reducing the capacity of the material. The sorbed impurities may also enhance denaturation of the enzyme bound in the column. The latter interpretation is supported by the observation that with purer preparations

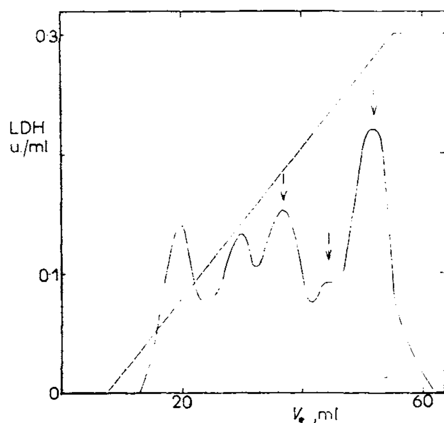

FIG. 3

Attempted separation of LDH isoenzymes on Spheron 300 with affinant *I*. For conditions see Experimental; 8 units of rabbit muscle LDH was applied, (— — —) concentration gradient of NADH (final concentration 0·3 mmol l^{-1} in 0·1 mol l^{-1} Na-phosphate buffer pH 7). The arrows denote the peaks in which the enzymic activity was strongly suppressed by adding 2 mol l^{-1} urea to the reaction mixture

of ADH the denaturation was much lower in this column and the results were better if the sample first passed through a column packed with non-substituted Spheron. It can be expected that Spheron matrices with hydrophilic saccharide residues, now being developed[22], will lend themselves better to more complex biological mixtures than the materials tested in this study.

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