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Superporous agarose as an affinity chromatography support

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Abstract

Superporous agarose beads were used as an affinity support in column chromatography. These beads characteristically possess two sets of pores, normal diffusion pores and flow pores, so-called superpores. The superpores, whose diameter is a substantial fraction of the particle diameter (i.e. 1/3 to 1/10 of the particle diameter), allow part of the chromatographic flow to pass through each individual bead. Consequently, significant improvement in mass transfer is observed in superporous beads as compared with homogeneous beads, especially at high flow-rates [Gustavsson and Larsson, J. Chromatogr. A, 734 (1996) 231–240.]

Superporous agarose beads and homogeneous agarose beads were each derivatized with two types of affinity ligands. A NAD^+ analogue was used for the purification of bovine lactate dehydrogenase and protein A was used for the adsorption of rabbit IgG. The performances of superporous beads and homogeneous beads were compared. Superporous bead columns derivatized with protein A and NAD^+ analogue could be operated 5 times and 3 times, respectively, as fast as corresponding homogeneous bead columns. © 1997 Elsevier Science B.V.

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1. Introduction

Affinity chromatography purifications are, whenever possible, carried out as an adsorption–wash–desorption process. Strong-binding ligands ensure efficient adsorption as well as no release of the target substance during the washing step. Finally, the desorption step is often achieved by abrupt and drastic change of the mobile phase composition. For example, lowering the pH by several pH units may completely abolish the protein–ligand interaction and thus effect the immediate elution of the protein in a concentrated form.

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The molecular binding of the protein to the ligand is often quite rapid and the limiting factor in such affinity systems is thus the diffusion of protein through the support material as shown in several reports [1,2]. An efficient way, of improving the adsorption rate is the use of support materials with smaller particle diameters. Small diameter supports, e.g. supports based on HPLC-grade silica, will decrease the diffusion distance and increase considerably the operational capacity at elevated flow-rates [3,4]. Extreme examples of methods to minimize the effects of diffusion are the use of non-porous, micron-sized or even sub-micron-sized supports [5,6]. In the last ten years new types of support materials with improved mass transfer properties,

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ascribed to intraparticle convection, have been presented [7–10]. The theoretical aspects of intraparticle convection related to catalysts [11–13] and to chromatographic supports [14–16] have been investigated by several groups.

In a previous article [17] we described a new type of support material, superporous agarose [18]. These beads are prepared by a double emulsification procedure and are characterized by two sets of pores, normal diffusion pores and flow pores, so-called superpores. These superpores, whose diameter is a substantial fraction of the particle diameter (i.e. 1/3 to 1/10 of the particle diameter), allow part of the chromatographic flow to pass through each individual bead. In this report we have used these new types of superporous agarose materials and evaluated their potential in affinity chromatography applications. The supports were derivatized with two types of affinity ligands. A NAD^+ analogue was used for the isolation of bovine heart lactate dehydrogenase and Protein A was used for the adsorption of rabbit IgG.

2. Experimental

2.1. Materials

Agarose powder (Sepharose quality) was a gift from Pharmacia Biotech AB (Uppsala, Sweden). Polyoxyethylenesorbitanmonooleate (Tween 80) and cyanogen bromide were obtained from Merck–Schuchardt (Munich, Germany). Sorbitane trioleate (Span 85) was purchased from Fluka (Buchs, Switzerland). Cyclohexane (puriss.) was obtained from Merck (Darmstadt, Germany). The NAD^+ analogue, N^6-[*N*-(2-aminoethyl)carbamoylmethyl]-NAD^+, was synthesized [19] according to a method similar to that reported for the corresponding 6-aminohexyl analogue [20]. The synthesis of the 6-aminohexyl analogue includes the following steps: alkylation with iodoacetic acid to give 1-carboxymethyl-NAD^+, rearrangement to N^6-carboxymethyl-NAD^+ and finally condensation with 1,6-diaminohexane to give N^6-[*N*-(6-aminohexyl)carbamoylmethyl]-NAD^+. The synthesis of the 2-aminoethyl analogue, used in this paper, is carried out in the same way, except that 1,2-diaminoethane is used instead of 1,6-diaminohexane in the last step. Rabbit IgG was purchased from Sigma (St. Louis, MO, USA). The protein assay used to determine protein contents was obtained from Bio-Rad Labs. (Hercules, CA, USA). Protein A was obtained from Perstorp Biolytica AB (Lund, Sweden).

2.2. Preparation of superporous agarose beads

Superporous agarose beads were prepared by a double emulsification procedure, as described previously [17].

2.3. Preparation of homogeneous agarose beads

The homogeneous agarose beads were prepared by emulsifying an agarose solution (6%, w/v) in cyclohexane containing Span 85 (4%, v/v), as described previously [17].

2.4. Preparation of lactate dehydrogenase extract

An extract of lactate dehydrogenase was prepared from bovine heart via disintegration, centrifugation and fractionating ammonium sulfate precipitation (30%–60% saturation), essentially as described previously [6]. The precipitated enzyme was dissolved in a limited amount of 0.05 *M* sodium phosphate buffer, pH 7 making the extract approximately 1 *M* with respect to ammonium sulphate. The extract had a protein content of 220 mg/ml and an activity of 300 U/ml (see Section 2.8).

2.5. Coupling of NAD^+-ligand to agarose beads

2.5.1. BrCN activation

Homogeneous and superporous agarose beads were activated with BrCN essentially as described by March et al. [21]. Typically, 25 g of agarose beads were suspended in 50 ml of cold (5°C) 1 *M* Na_2CO_3. 1.5 ml of a BrCN solution (0.50 g/ml in acetontrile) was added and the solution was stirred gently while the temperature was kept at 0–5°C by an ice water bath. After 1.5 min another 1.5 ml of the same BrCN solution was added to the reaction solution. After 4 min the reaction was terminated by washing the beads on a glass filter with 500 ml water (5°C), 100

ml 0.2 *M* sodium phosphate buffer pH 8.5 and 500 ml water (5°C).

2.5.2. Coupling of N^6-[N-(2-aminoethyl)carbamoylmethyl]-NAD^+

The activated agarose beads were suspended in 10 ml 0.2 *M* sodium phosphate buffer pH 8.5 (5°C) and 5 ml of a N^6-[*N*-(2-aminoethyl)carbamoylmethyl]-NAD^+ solution (20 mg/ml in water, 5°C) were added and the suspension was shaken. After 10 min the pH of the solution was adjusted from 8 to 8.5 and the reaction was continued for another 20 min with occasional shaking. Then the reaction was allowed to continue at room temperature for 16 h by rotating the reaction vessel "end-over-end". The gel beads were filtered off on a glass filter and suspended in 0.2 *M* glycine–NaOH pH 8.7 for 15 min to block the remaining active groups. Finally, the beads were washed with 500 ml water and 500 ml of 1 m*M* sodium acetate buffer pH 5. The beads were then stored at 4°C.

2.6. Coupling of protein A ligand to agarose beads

Homogeneous and superporous agarose beads were activated with BrCN essentially as described above, the main difference being the use of 0.1 g BrCN/g of gel and a final washing with 200 ml 0.2 *M* $NaHCO_3$ pH 9.5 (5°C), 200 ml water (5°C) and 200 ml 0.2 *M* $NaHCO_3$ pH 9.5 (5°C). Protein A (60 mg) was dissolved in 20 ml 0.2 *M* $NaHCO_3$ pH 9.5 (5°C). The activated agarose beads (10 g) were added to the Protein A solution and stirred "end-over-end" for 20 h at 5°C. The beads were then washed with coupling buffer and the remaining active CNBr groups were then blocked with 1 *M* ethanolamine, pH 9.0.

Protein A is a very stable protein and the beads could therefore be washed with strong agents: with 400 ml 0.1 *M* Na–acetate, 0.5 *M* NaCl, pH 4, with 400 ml 2 *M* urea, 0.5 *M* NaCl, with 400 ml 0.1 *M* $NaHCO_3$, 0.5 *M* NaCl, pH 10, and with 400 ml water. The beads were then stored at 4°C.

A semi-quantitative determination of the Protein A ligand in the agarose beads was made [22], according to the method of Bradford [23]. These measurements gave Protein A contents of approximately 3 mg/ml sedimented beads for both the superporous and homogeneous beads. The measurements were carried out using non-derivatized beads as blanks.

2.7. Chromatographic experiments

2.7.1. Purification of bovine lactate dehydrogenase

Superporous and homogeneous beads derivatized with the NAD^+ analogue were packed in 16-mm diameter columns, equipped with flow adapters, to a bed height of 50 mm. The mobile phase for the adsorption and wash step (0.05 *M* sodium phosphate buffer, pH 7, 1 m*M* EDTA, 1 m*M* mercaptoethanol, 25 m*M* sodium oxalate) was pumped with a positive displacement pump (Altex Model 110 A, Altex Sci., Berkeley, CA, USA) into the column via a 6-port valve (Valco, Houston, TX, USA) provided with a 50-ml superloop (Pharmacia Biotech AB, Uppsala, Sweden). Part of the effluent from the column was diverted with a separate, peristaltic, pump to a fraction collector (Gilson FC 203, for 96 well micro titer plates, Gilson Med. Electr., Middleton, WI, USA).

A 25-ml volume of the bovine lactate dehydrogenase extract, prepared as in Section 2.4, was mixed with 25 ml of the adsorption buffer and sodium oxalate was added to a final concentration of 25 m*M*. The sample was injected via the superloop and the eluate was collected at 2-ml intervals by the fraction collector for subsequent protein and activity analysis (see Section 2.8). After the adsorption–wash step, elution was carried out by injecting 50 ml of 0.05 *M* sodium phosphate buffer, pH 7 containing 1 m*M* EDTA, 1 m*M* mercaptoethanol and 1 m*M* NADH. The adsorption–wash step and the elution step were carried out at the same flow-rate.

2.7.2. Purification of rabbit IgG

Superporous and homogeneous beads derivatized with protein A were packed in 10-mm diameter columns, equipped with flow adapters, to a bed height of 40 mm. The columns were connected to an HPLC system (Pharmacia Biotech), including pump, 6-port valve with a 10-ml superloop, UV–Vis detector and recorder. The mobile phase for the adsorption step was 10 m*M* sodium phosphate buffer pH 8 containing 0.15 *M* NaCl. A 25-mg amount of rabbit IgG was dissolved in 10 ml of the adsorption buffer

and injected by the superloop. After the adsorption–wash step, elution was carried out by changing the mobile phase to 0.1 *M* sodium citrate–NaOH buffer, pH 3.5. The eluted proteins were detected at 300 nm, a wavelength giving a suitable detector response (the response at 280 nm was too high).

2.8. Protein and activity analysis

The lactate dehydrogenase activity was assayed by following the oxidation of NADH by pyruvate at 340 nm at room temperature. A 10-μl volume of suitably diluted fractions was added (multi-channel pipette) to 96-well micro titer plates containing 190 μl of an assay solution (4 m*M* NADH, 1 m*M* pyruvate, 1 m*M* EDTA, 1 m*M* mercaptoethanol, 0.05 *M* sodium phosphate buffer, pH 7). The decrease in the absorbance was measured using a micro titer plate reader (Multiskan MCC/340, Labsystem, Helsinki, Finland).

Protein content was analyzed according to the method of Bradford [23]. A 10-μl volume of suitably diluted fractions were added (multi-channel pipette) to 190 μl of the protein assay solution in 96-well micro titer plates. After 4 min incubation, at room temperature, the absorbance was measured using a micro titer plate reader at 595 nm.

3. Results and discussion

3.1. Preparation of adsorbent

The superporous beads used for the purification of bovine lactate dehydrogenase had a particle size between 300–500 μm, a superpore porosity of 50% (defined by the method of preparation and verified by size exclusion experiments with 0.5-μm latex particles as described in reference [17]) and an average superpore diameter of 50 μm (microscopy). The superporous beads used for the IgG adsorption had a particle size between 106–180 μm, a superpore porosity of 40% and an average superpore diameter of 30 μm. The beads used for the lactate dehydrogenase purification in particular were much larger than beads usually employed in affinity chromatography separations (a particle size in the range of 75–150 μm could be regarded as normal for preparative purposes). From earlier size-exclusion experiments and ion-exchange separations [17], it was expected that these very large beads should give a very low flow resistance and significantly, a satisfactory mass transfer thanks to the presence of superpores, which have been proven to carry part of the chromatographic flow [17].

The efficient mass transfer associated with superpores should also be an important aspect in connection with activation of the support and coupling of ligands, resulting in faster coupling times. It is also important to note that the superporous beads have the same chemical properties as ordinary agarose beads, since their method of preparation will not alter the basic agarose properties, for instance the porosity of the agarose (size-exclusion data, [17]) and the inertness towards proteins (size-exclusion data, [17]). Also, the stability over a large pH range was the same, as proven by the procedures carried out in this paper, (e.g. cyanogen bromide activation occurred at very high pH and elution of the antibodies occurred at very low pH), and by activation procedures used in the context of enzyme immobilization; (Gustavsson, unpubl.).

3.2. Chromatography

3.2.1. Purification of bovine lactate dehydrogenase

The affinity system chosen was support-bound NAD^+ which has affinity for many dehydrogenases. By making the extract 25 m*M* with respect to sodium oxalate, the specificity for lactate dehydrogenase was increased considerably [24]. Oxalate, NAD^+ and lactate dehydrogenase form a strong ternary complex, even in the presence of high concentrations of salts, such as 0.5 *M* ammonium sulphate. Therefore no dialysis of the enzyme preparation was carried out before application to the affinity columns. The presence of a high salt concentration during the loading step, followed by washing with low salt concentration, constitutes an additional but integral purification step.

The performances of homogeneous and superporous adsorbents were compared. To this end identical, short colums were packed with the respective adsorbent and used in a number of adsorption–wash–desorption cycles. The flow velocity was 3.0 or 9.9

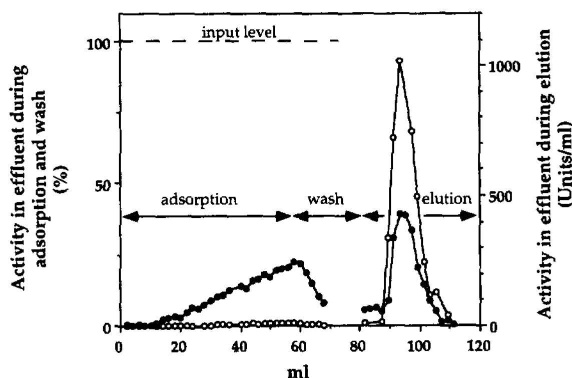

Fig. 1. Affinity chromatography purification of lactate dehydrogenase, at a flow velocity of 90 cm/h. Comparison between homogeneous agarose support (●) and superporous agarose support (○). Sample: 50 ml of a bovine lactate dehydrogenase extract. Affinity material: N^6-[*N*-(2-aminoethyl)carbamoylmethyl]-NAD^+ coupled to superporous and homogeneous agarose beads, both types with a particle diameter of 300–500 μm. Column size: 50×16-mm I.D. Elution was carried out with 1 m*M* NADH. Further details in Section 2.

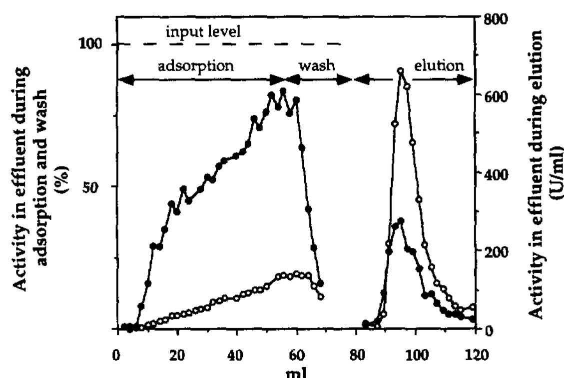

Fig. 2. Affinity chromatography purification of lactate dehydrogenase at a flow velocity of 300 cm/h. Comparison between homogeneous agarose support (●) and superporous agarose support (○). The same conditions as in Fig. 1.

ml/min, corresponding to 90 or 300 cm/h linear flow velocity respectively.

Fig. 1 shows the results at 3 ml/min. The superporous support adsorbs the enzyme quantitatively, while an early break-through occurred with the homogeneous adsorbent (approximately 15% of the enzyme was lost). The earlier break-through is easily understood in view of the very large particle size used here (400 μm, average particle size). The linear flow velocity (90 cm/h) with 400 μm particles corresponds to a reduced velocity of 2500 (assuming a diffusion coefficient for lactate dehydrogenase of $4 \cdot 10^{-7}$ cm^2 s^{-1} under the conditions used). It may be illustrative to calculate the equivalent flow velocity that gives a reduced velocity of 2500, for preparative scale particles (100 μm) and for high-performance particles (25 μm). The equivalent flow velocities would be as high as 360 and 1440 cm/h respectively.

Fig. 2 shows the result at 9.9 ml/min. In this case a similar breakthrough was observed also with the superporous material (approximately 15%, of the enzyme was lost). Thus the superporous bead column could be run more than 3 times as fast as the homogeneous bead column, before unacceptible losses occurred. Also, at the elution step, the superporous beads performed better than the homogeneous beads as judged from a higher specific activity (1.5–2 times higher specific activity). This probably reflects a more efficient wash step with superporous beads – faster equilibration due to shorter residual diffusion distances. It should be noted that the amount loaded in the chromatographic runs (7 500 U) corresponded to approximately one fifth of the maximal apparent static binding capacity, about 40 000 U for both types of adsorbent (measured in the presence of an excess volume of lactate dehydrogenase at a concentration of 90 U/ml; equilibration time 2 h). The observation that the static binding capacity was the same for both types of supports is somewhat unexpected considering that the superporous beads contains internal voids corresponding to 50% of their volume. A plausible explanation might be that the superpore structure diminishes the effects of pore blockage. Thus, it has been concluded that adsorbed proteins may partially block the pores in normal, porous particles, thereby making diffusive access to the inner parts of the particles difficult [25]. Such pore blockage will be more pronounced with large particles. Analogously, superporous particles would be expected to have a comparatively larger static capacity than homogeneous particles since from a diffusion point of view they behave as small particles (short diffusion distances). Thus, the present superporous particles could very well have the same static binding capacity as homogeneous particles in spite of their reduced gel content.

A closer examination of the profiles in Figs. 1 and 2 reveals that the added activity is not properly balanced by the eluted activity. Especially large deviation is seen with homogeneous particles at low flow-rate. The reason is unknown but could possibly be related to the pore blockage phenomenon or related to enzyme inactivation. However, this discrepency does not obscure the main conclusion of the experiment, namely that the superporous beads allow a considerably higher processing rate.

3.2.2. Purification of rabbit IgG

Fig. 3 shows the comparison between superporous and homogeneous supports in the adsorption of rabbit IgG. The beads were derivatized with protein A which binds IgG from most mammalian species, including a strong binding to rabbit IgG. The rabbit IgG sample was almost 100% pure, allowing a direct estimation of antibody adsorption from the detector response. The flow velocity was 0.5 or 2.5 ml/min, which corresponds to 40 or 190 cm/h linear flow velocity respectively. At 0.5 ml/min the superporous bead column adsorbed approximately 90% of the antibodies (a), while the homogeneous bead column only adsorbed approximately 65% of the antibodies (b). At 2.5 ml/min, the superporous bead column adsorbed approximately 80% of the antibodies (c) compared to the homogeneous bead column which only adsorbed 50% of the antibodies (d). Thus the superporous bead column adsorbed more efficiently at 2.5 ml/min than the homogeneous bead column at 0.5 ml/min, emphasizing the advantageous properties of the superpore concept.

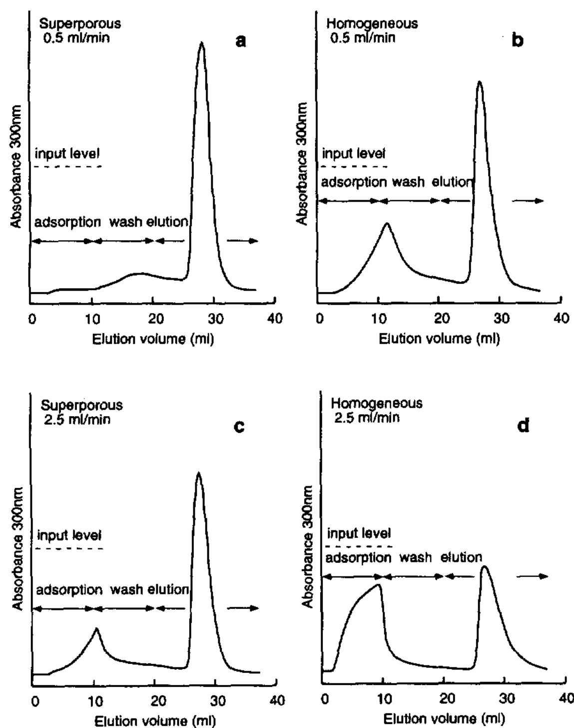

Fig. 3. Affinity chromatography adsorption of rabbit IgG. Comparison between superporous and homogeneous supports at two flow-rates. Sample: 25 mg of rabbit IgG dissolved in 10 ml of buffer. Affinity material: Protein A coupled to superporous and homogeneous beads, both types with a particle diameter of 106–180 μm. Column size: 40×10-mm I.D. Elution was carried out by changing the mobile phase to 100 m*M* sodium citrate–NaOH buffer, pH 3.5.

4. Conclusion

Superporous agarose materials have been shown to be well suited for affinity chromatography purposes. Superporous bead columns derivatized with protein A and NAD^+ analogue could be operated 5 times and 3 times, respectively, as fast as corresponding homogeneous bead columns. By using large superporous agarose beads many of the drawbacks of using small beads can be avoided, e.g. high back pressure which necessitates the use of expensive equipment and the clogging of columns etc. Other interesting affinity separations would possibly also benefit from the application of superporous materials, e.g. separation of microparticles such as viruses and organelles. The facile activation and coupling of ligand to the support also suggest the use of superporous agarose as a support for immobilized enzymes.

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