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Superporous agarose beads as a hydrophobic interaction chromatography support[1]

Per-Erik Gustavsson[a], Anders Axelsson[b], Per-Olof Larsson[a,*]

[a]*Department of Pure and Applied Biochemistry, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, S-221 00 Lund, Sweden*

[b]*Department of Chemical Engineering 1, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, S-221 00 Lund, Sweden*

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Abstract

Superporous agarose beads were used as a support for hydrophobic interaction chromatography. These beads have large connecting flow pores in addition to their normal diffusion pores. The flow pores, which are approximately one fifth of the overall diameter of the superporous agarose beads, were earlier shown to give the beads improved mass transfer properties relative to homogeneous agarose beads (Gustavsson and Larsson, *J. Chromatogr. A*, 734 (1996) 231–240). Superporous agarose beads and homogeneous agarose beads of the same particle size range (106–180 μm) were derivatized with phenyl groups. The properties of the superporous beads were then compared with the homogeneous beads in the separation of a mixture of three model proteins (ribonuclease A, lysozyme and bovine serum albumin) at various superficial flow velocities from 30 to 600 cm/h. The superporous beads gave satisfactory separation at flow velocities five times higher than was possible for homogeneous beads. The performance of the two types of beads was also compared in the purification of lactate dehydrogenase from a beef heart extract at a superficial flow velocity of 150 cm/h. The superporous beads performed considerably better, leading to twice the purification factor and twice the concentration of the desired product. The results were interpreted using the theoretical treatment given by Carta and Rodrigues (Carta and Rodrigues, Chem. Eng. Sci., 48 (1993) 3927).

Keywords: Stationary phases, LC; Flow pores; Agarose, superporous; Ribonuclease A; Lysozyme; Bovine serum albumin

1. Introduction

Hydrophobic interaction chromatography (HIC) is a well established bioseparation technique in laboratory-scale as well as industrial-scale purification of proteins [1,2]. In industry, HIC is usually employed in the intermediate purification steps, with typical particle sizes for the sorbents of 30–100 μm [2]. In analytical separations, particle sizes of 5–30 μm are more common. Popular hydrophobic ligands include butyl, octyl and phenyl groups. The proteins are generally adsorbed to the hydrophobic stationary phase at high salt concentration, with the driving force for adsorption being a displacement of ordered water molecules around the proteins and the ligands which leads to an increase in entropy [3]. The adsorbed proteins are then usually eluted with a

*Corresponding author.

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decreasing salt gradient which weakens the hydrophobic interactions.

The performance (e.g. resolution and capacity) of chromatography columns in protein separations has increased considerably in recent years, especially at elevated flow-rates. One line of development is the design of new packing materials with enhanced mass transfer properties, ascribed to intraparticle convection [4–7]. The theoretical aspects of intraparticle convection related to chromatographic supports have been investigated by several groups [6–11].

In previous articles [5,12,13] we have described a new type of agarose material, so-called superporous agarose. The superporous agarose beads have large connecting flow pores with sizes in the range 1/4 to 1/20 of the overall bead diameter. These pores have been shown to carry part of the chromatographic flow [13], giving an improved performance in ion-exchange and affinity chromatography separations of proteins compared with homogeneous beads of the same particle size [5,12]. In this paper we have evaluated the performance of these superporous agarose beads in hydrophobic interaction chromatography applications.

2. Experimental

2.1. Materials

Agarose powder (Sepharose quality) was a gift from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Polyoxyethylenesorbitan monooleate (Tween 80) and sodium borohydride 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). Lysozyme from chicken egg white and ribonuclease A from bovine pancreas were obtained from Sigma (St. Louis, MO, USA). Bovine serum albumin was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Glycidyl phenyl ether was obtained from Acros Chimica N.V. (Geel, Belgium). The protein assay kit used to determine the protein content was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Sepharose CL-4B and Phenyl Sepharose CL-4B were obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden).

2.2. Preparation of superporous agarose beads

Superporous agarose beads were prepared by a double emulsification procedure, as described previously [5]. The superporous beads used in this study had a particle size between 106–180 μm, a superpore porosity of 40% (defined by the method of preparation and verified by size exclusion experiments with 0.5-μm latex particles as described in reference [5]) and an average superpore diameter of 30 μm (microscopy). The distance between the superpores was estimated to be approximately 45 μm. In a packed bed with a bed porosity of 40%, the superpore fluid velocity in these beads was found to be on average 17% of the interstitial fluid velocity [13]. Further the tortuosity (τ) of these superpores was estimated to be 1.2 [13].

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 [5].

2.4. Coupling of phenyl ligand to agarose beads

The phenyl ligand was coupled to homogeneous and superporous agarose beads essentially as described by Sundberg and Porath [14], (see discussion in Section 3.1). The water in 20 ml of sedimented agarose beads was exchanged for a water–dioxane (1:1) solution by washing on a glass filter funnel. The washed beads were transferred to an Erlenmeyer flask and a water–dioxane (1:1) solution (20 ml) containing sodium borohydride (28 mg) was added. One molar aqueous NaOH (10.5 ml) was added and the reaction vessel placed on a rotary shaker. After 5 min, glycidyl phenyl ether (5 ml) was added, and the reaction allowed to proceed at room temperature for 24 h. The beads were then washed with a water–

dioxane mixture (1:1, 500 ml) followed by water (500 ml). The beads were then stored at 4°C.

2.5. Quantitative determination of the phenyl ligand

The degree of phenyl group substitution was determined by UV spectrophotometry according to the method of Johansson and Nyström [15]. Phenyl substituted and unsubstituted beads which acted as a control were dried and then hydrolysed using concentrated hydrochloric acid (20°C, 10 min). After dilution with methanol, a UV spectrum was recorded between 230 and 340 nm using the hydrolysates from unsubstituted beads as blanks. The degree of substitution was then calculated using the molar absorptivity for 2-phenoxyethanol of 1760 $M^{-1}\,cm^{-1}$ at 270.5 nm [15]. A UV spectrum of hydrolysed Phenyl Sepharose CL-4B with a known phenyl content (0.91 μmol/g dry weight according to the manufacturer) was also recorded as a general check of the methodology. Here Sepharose CL-4B was used as a blank.

2.6. Preparation of lactate dehydrogenase extract

An extract of lactate dehydrogenase was prepared from bovine heart (250 g) via disintegration, centrifugation and fractionating ammonium sulphate precipitation (30%–60% saturation), essentially as described earlier [16]. The precipitated enzyme was dissolved in 450 ml of 0.02 *M* sodium phosphate buffer, pH 7 making the extract approximately 0.35 *M* with respect to ammonium sulphate (conductivity measurements). The extract had a protein content of 12 mg/ml, determined according to the method of Bradford [17], and an activity of 82 U/ml.

2.7. Enzyme assay

The lactate dehydrogenase activity was assayed by following spectrophotometrically (340 nm) the oxidation of NADH by pyruvate at room temperature. Twenty microlitres of suitably diluted fractions were added to 2 ml of an assay solution (0.16 m*M* NADH and 1 m*M* pyruvate in 0.05 *M* sodium phosphate buffer, pH 7.0).

2.8. Chromatographic experiments

2.8.1. Separation of model proteins

Superporous and homogeneous beads derivatized with the phenyl ligand were packed in 16-mm diameter glass columns, equipped with flow adapters, to a bed height of 40 mm. The columns were integrated with HPLC equipment (Amersham Pharmacia Biotech AB, Uppsala, Sweden), including pumps, injection valve, UV–Vis detector and recorder. For flow-rates up to 5 ml/min, the 2150 pump was used and for higher flow-rates (up to 50 ml/min) the HiLoad P-50 pump was used. The separations were carried out by gradient elution at room temperature. The ammonium sulphate gradient profiles were measured by a conductivity meter (type CDM 2d, Radiometer, Copenhagen, Denmark) on ten times diluted 1-ml fractions obtained by a fraction collector.

2.8.2. Purification of lactate dehydrogenase

Superporous and homogeneous beads derivatized with the phenyl ligand were packed into 16-mm diameter glass columns, equipped with flow adapters, to a bed height of 40 mm. The columns were integrated with HPLC equipment as before. Ammonium sulphate (0.76 g) was added to the bovine lactate dehydrogenase extract (5 ml) which was prepared as in Section 2.6, giving a final concentration of 1.5 *M* with respect to ammonium sulphate. The sample was injected and the eluate was collected at 2-ml intervals by a fraction collector. The collected fractions were analysed for lactate dehydrogenase activity (see Section 2.7).

3. Results and discussion

3.1. Preparation of adsorbent

Table 1 shows the phenyl content for the different beads as measured by the UV spectrophotometric method (see Section 2.5). The table shows that the phenyl content of the superporous and the homogeneous beads is approximately the same per mg of dry gel and is approximately half of the value of the commercially available Phenyl Sepharose CL-4B. This difference can be explained through the details

Table 1
Dry weight and phenyl contents of HIC beads

Bead type	Dry weight (mg/ml sedimented bed)	Phenyl content	
		Dry weight content (μmol/mg)	Sedimented bed content (μmol/ml)
Superporous	24	0.42	10
Homogeneous	36	0.38	14
Sepharose CL-4B	27	0.81	22

of the particular phenyl coupling protocol used, which is different from the standard protocol [18], where glycidyl ethers are coupled to the agarose support in an organic solvent using boron trifluoride ethyl etherate as a catalyst. In order to avoid possible shrinkage or distortion of the superpore structure when exposed to or transferred from pure organic solvents, the coupling method of Sundberg and Porath was used [14], where diglycidyl ethers are coupled to agarose support at high pH in aqueous solution. In order to increase the solubility of the glycidyl phenyl ether, we employed an aqueous dioxane solution (1:1).

The dry weight of the different bead types was also determined to allow comparison of the phenyl contents per ml of sedimented bed. As can be seen from Table 1, the dry weight of the superporous beads is lower than the dry weight of the homogeneous beads. This is explained by the superpore porosity of the superporous beads, which leads to a corresponding decrease of the phenyl content per ml of sedimented bed compared with the homogeneous beads. The measured phenyl content of the Phenyl Sepharose CL-4B (0.81 μmol/mg dry gel) agreed well with the manufacturer's value of 0.91 μmol/mg dry gel for this particular batch.

3.2. Chromatography

3.2.1. Separation of model proteins

Fig. 1 shows the comparison between the superporous beads and the homogeneous beads in the

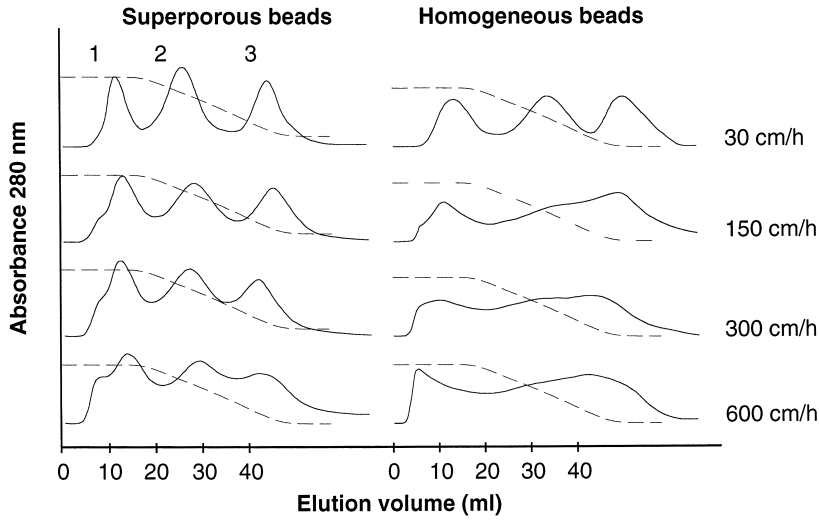

Fig. 1. Hydrophobic interaction chromatography comparison between superporous and homogeneous supports. Sample: 1 ml of protein mixture containing 0.25 mg ribonuclease A (1), 0.13 mg lysozyme (2) and 0.25 mg bovine serum albumin (3) in starting buffer. Support: Superporous and homogeneous beads derivatized with phenyl groups, both types with a particle diameter of 106–180 μm. Column size: 40×16-mm I.D. Starting buffer: 20 m*M* sodium phosphate, 1.5 *M* $(NH_4)_2SO_4$, pH 7.0. End buffer: 20 m*M* sodium phosphate, 0.5 *M* $(NH_4)_2SO_4$, pH 7.0. The descending salt gradient started after 10-ml elution volume and ended after 40-ml elution volume. The proteins were detected spectrophotometrically at 280 nm.

hydrophobic interaction chromatography separation of three model proteins. At a superficial flow velocity of 30 cm/h the two adsorbents showed a similar performance, giving approximately the same resolution of the three proteins. However, the superporous adsorbent gave slightly sharper elution peaks, which is attributed to the shorter diffusion distances present in these beads as compared to the homogeneous beads.

Running the separation at a five times higher superficial flow velocity (150 cm/h) led as expected, to greater differences between the two adsorbents. At this flow velocity the superporous adsorbent still gave a satisfactory resolution of the proteins, owing to the presence of superpores, while the homogeneous adsorbent showed poor resolution of lysozyme and bovine serum albumin. Ribonuclease A was still well separated from lysozyme and bovine serum albumin on the homogeneous adsorbent due to the fact that ribonuclease A was only slightly retained with the adsorption buffer used. At yet higher flow velocities (300 and 600 cm/h) the superporous adsorbent showed a slower decrease in the resolution of the proteins and a smaller breakthrough than the homogeneous adsorbent.

The reason that the proteins eluted later with the homogeneous adsorbent is probably due to the fact that the homogeneous adsorbent contained a higher concentration of phenyl ligands (see also the discussion in Section 3.1). It should be pointed out that the greater retention of the proteins on the homogeneous column was not as a result of a different gradient profile in the homogeneous column compared to the superporous column, a situation which might be anticipated at high flow velocities when the superporous beads should be more rapidly equilibrated with the progressing gradient. The measurements of the ammonium sulphate gradient profiles showed that the same gradient profiles (within the accuracy of measurements) were obtained for the superporous column and the homogeneous column at all flow velocities investigated.

3.2.2. Theoretical evaluation of the model protein separation

Due to the rather high fraction of intraparticle fluid velocity in the superpores (17% of the interstitial fluid velocity [13]) and the rather long diffusion distances between the superpores (≈45 μm (30 μm×60/40)), the decreasing resolution of the superporous column with increasing flow velocity may be explained by diffusional resistance in the agarose phase between the superpores. An elegant analysis of the mass transfer properties of superporous particles (gigaporous particles, permeable particles) has been made by Rodrigues et al. [19,20] and Carta et al. [7]. The problem is approached by studying the combined effect of internal diffusion and internal convection by introducing an enhancement factor [$1/f(\lambda)$] and a convection-augmented diffusivity (D_e). Although physically incorrect we call this the convective diffusivity to make a clear distinction from the normal effective diffusivity (D'). By calculating the enhancement factor, the convective diffusivity can be calculated from the effective diffusion coefficient by [21]:

$$D_e = \frac{D'}{f(\lambda)} \tag{1}$$

where the function $f(\lambda)$ is given by the following equation [21]:

$$f(\lambda) = \frac{3}{\lambda}\left(\frac{1}{\tan h\lambda} - \frac{1}{\lambda}\right) \tag{2}$$

Fig. 2 shows $f(\lambda)$ as a function of the intraparticle Peclet number (λ) and is rewritten from Carta and Rodrigues [21]. The intraparticle Peclet number describes the ratio between the mass transfer by convective flow and the mass transfer by diffusion within the superporous particle:

$$\lambda = \frac{u_{pore} R_p}{3D'} \tag{3}$$

where u_{pore} is the intraparticle superficial velocity and R_p is the radius of the superporous particle and D' is the effective diffusivity in the superpores. The definition is perhaps even more clearly understood by explaining the intraparticle Peclet number as the ratio between the internal diffusion time (t_D) and the intraparticle convection time (t_C):

$$t_D = \frac{R_p^2}{D'} \tag{4}$$

$$t_C = \frac{3R_p}{u_{pore}} \tag{5}$$

Low intraparticle Peclet numbers are obtained when the convection is low and attains zero when

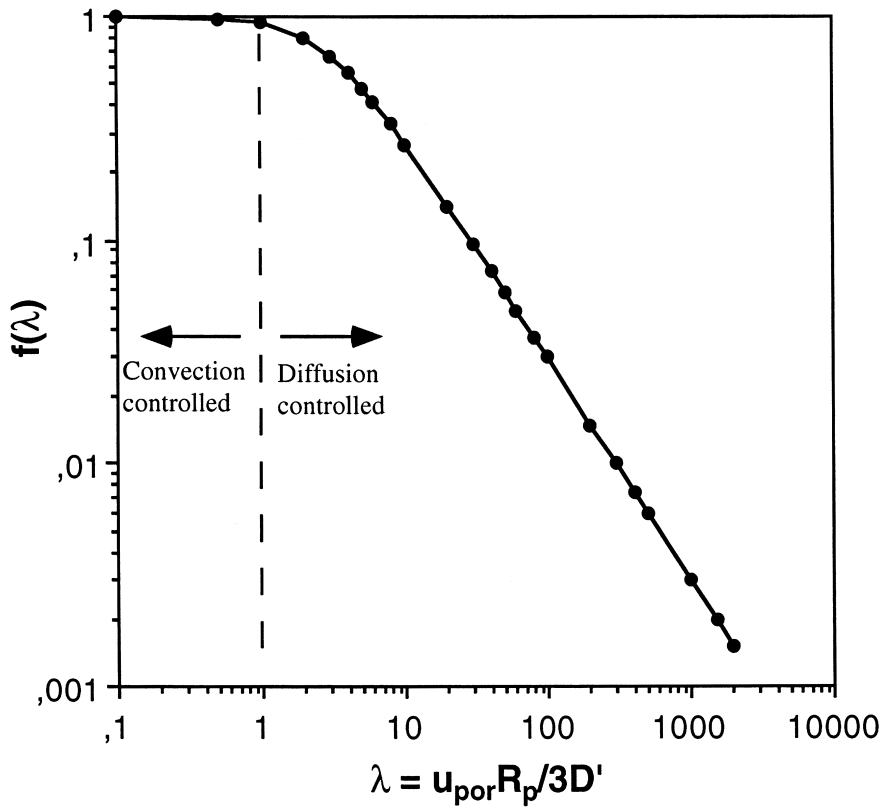

Fig. 2. $f(\lambda)$ as a function of the intraparticle Peclet number.

convection is absent. The enhancement factor at this point is equal to one and the particle performs as a normal diffusive particle with no convective pores at all. When the intraparticle Peclet number is increased by introducing superpores, the intraparticle velocity u_{pore} increases, resulting in an increasing enhancement factor $[1/f(\lambda)]$. By multiplying the effective diffusivity with the enhancement factor the convective diffusivity is obtained. The dramatic increase of the mass transfer rate is demonstrated by the logarithmic scale of the axis in Fig. 2. An efficient superporous particle is obtained when high intraparticle Peclet numbers are obtained. Here the convective transport is much larger than the diffusive transport. Thus, as the diffusion process is the slow one this is said to control the mass transfer rate. Still it must be remembered that the total transport is totally dominated by the convection at very large intraparticle Peclet numbers. At large intraparticle Peclet values the function $f(\lambda)$ attains asymptotically $3/\lambda$, which means that the convective diffusivity is proportional to the intraparticle velocity, u_{pore}.

The way of approaching the mass transfer case in the particle described above is very similar to the effectiveness factor and Thiele modulus concept used in connection with immobilised catalysts discussed in the literature already in the 1930's [22]. Even the mathematics is similar ending up with the same Fig. 2 as above with the effectiveness factor instead of the function $f(\lambda)$ on the y-axis and the Thiele modulus instead of the intraparticle Peclet number on the x-axis. The Thiele modulus describes the ratio between the kinetic rate (instead of convective flow) and the diffusive rate. The right hand side of Fig. 2 is said to be diffusion controlled as the reaction kinetics is sufficiently fast not to act as a bottleneck for the mass transfer. In the same way the left hand side of Fig. 2 is said to be kinetically controlled where the diffusion is sufficiently fast not to decrease the total mass transfer.

As mentioned above, Fig. 2 could be used to analyse the performance of the superporous particle by calculating the intraparticle Peclet number. Carta and Rodrigues [21] have extended their theoretical analysis to give an analytical expression by which the existence of diffusion hindrance caused by the gel distance between the superpores can be investigated. Eq. (6) describes in fact the asymptotic line in the right hand part of Fig. 2:

$$F \gg \left(\frac{20(1-\varepsilon_p)D''\phi}{\varepsilon\tau D' v} \right)^{1/2} \quad (6)$$

where F is the ratio between the particle superficial velocity and the bed superficial velocity, ε_p is the superpore void fraction of the bead, ε is the bed void fraction, D'' and D' are the diffusivity of the solute in the smaller diffusion pores and the superpores respectively, τ is the tortuosity of the superpores, v is the reduced velocity as defined in Eq. (7) [21] and ϕ is as defined in Eq. (8) [21]:

$$v = \frac{2R_p u}{D} \quad (7)$$

$$\phi = \frac{\varepsilon_p^3}{\varepsilon^3} \frac{(1-\varepsilon)^2}{(1-\varepsilon_p)^2} \quad (8)$$

where u is the interstitial fluid velocity and D is the free diffusivity of the solute.

When F is much larger than the value of the expression [Eq. (6)], then the mass transfer in the particle is limited by the diffusion in the regions between the superpores. The expression was developed by Carta and Rodrigues assuming gel particles consisting of agglomerated subparticles. In the present case the gels have a slightly different design, but it is assumed that the approach anyhow should be reasonably valid. Further, the expression [Eq. (6)] was derived for a strongly retained solute assuming linear equilibrium. In the present case we assume these conditions to be fulfilled. Since in our case BSA (Fig. 1) was the most strongly retained solute, we will calculate the values in Eq. (6) obtained for this protein at the different reduced velocities in Fig. 1. Examination of Eq. (6) reveals that the value obtained will decrease as the reduced velocity is increased; however, it should be remembered that our superporous particles have a fixed value of $F = 0.17$ independent of the flow-rate. Taking the free diffusivity of BSA at 20°C to be 5.9×10^{-7} cm^2 s^{-1} [8] and R_p to be 71.5 μm, the reduced velocities obtained for BSA in Fig. 1 can be calculated using Eq. (7), and are displayed in Table 2. Using the following experimentally determined values $\varepsilon_p = 0.4$, $\varepsilon = 0.4$ and $\tau = 1.2$ for the superporous column (see Section 2.2), and taking the diffusivity of BSA in 6% agarose beads to be 2.5×10^{-7} cm^2 s^{-1} [23], the reduced velocities obtained for BSA can then be used to calculate the corresponding value on the left hand side of Eq. (6). The estimation of the diffusivity of BSA in the superpores can be difficult but can be assumed to be close to the free diffusivity value as the diameter of the superpores (30 μm) are so much bigger than the diameter of the BSA molecules. Therefore, the diffusivity of the BSA molecules in the superpores can be estimated to just be decreased by the tortuosity of the superpores giving a D' value of $5.9\times10^{-7}/1.2 = 4.9\times10^{-7}$ cm^2 s^{-1}. The calculated values obtained from the expression in Eq. (6) is given in Table 2 together with the calculated values of the intraparticle Peclet number (Eq. (3)) obtained for BSA in Fig. 1.

The high intraparticle Peclet numbers displayed in Table 2 implies that the superpores in the superporous agarose beads used in this study are sufficiently large enough not to give any convective flow limitations for BSA at the flow velocities used in Fig. 1. The superporous agarose beads should behave close

Table 2
Calculated parameters obtained from the conditions in Fig. 1

Superficial flow (cm/h)	Reduced velocity	F value from the expression in Eq. (6)	F value for superporous beads	λ
30	505	0.16	0.17	7
150	2525	0.07	0.17	34
300	5050	0.05	0.17	69
600	10 100	0.04	0.17	138

to that of smaller beads with a diameter equal to the diffusion distances between the superpores (≈45 μm). Furthermore, this distance gives diffusional hindrance in the gel between the superpores, limiting the chromatographic process. This is evident from the calculated values in Table 2. Since the superporous agarose beads used in Fig. 1 have an *F* value of 0.17, it is clear that diffusional hindrance exists for the BSA molecule at superficial velocities above 30 cm/h in Fig. 1. This implies that even better separation could be achieved by using superporous particles with superpores closer together.

3.2.3. Purification of lactate dehydrogenase

Fig. 3 shows the purification of lactate dehydrogenase from a crude extract by the superporous adsorbent. The purification factor was 5.7 and the activity yield was 82%. The purification factor is in the range of what can be expected from hydrophobic interaction purification protocols for crude extracts of LDH [24]. Fig. 4 shows the same purification but with the homogeneous adsorbent. In this case, the purification factor was 2.7 and the activity yield was 87%. The superficial flow velocity in both cases was 150 cm/h. The improved purification factor observed with superporous beads is interpreted as a result of the superior properties of the superporous beads, during sample loading and washing as well as gradient elution. A comparison of Figs. 3 and 4 clearly shows that the superporous beads allow a more extensive resolution of the protein sample (more details in the UV trace) as well as elution of the lactate dehydrogenase in a narrower band.

4. Conclusion

We have prepared and evaluated superporous agarose beads in hydrophobic interaction chromatography applications and compared their performance with homogeneous beads of the same particle size. The superporous bead column could resolve a mixture of three model proteins at a much higher flow velocity than the homogeneous bead column. The performance of the two types of beads were also compared in the purification of lactate dehydrogenase from beef heart, where an increase in the purification factor as well as a reduction in eluate

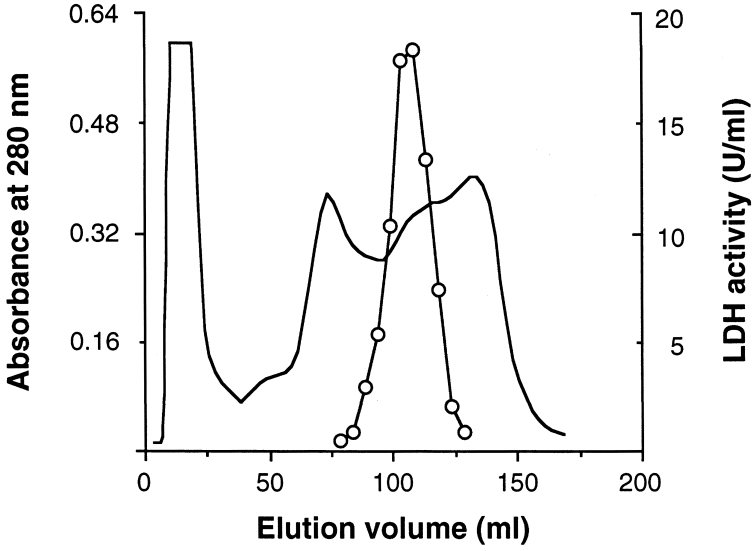

Fig. 3. Hydrophobic interaction chromatography purification of lactate dehydrogenase with superporous support. Support: Superporous beads derivatized with phenyl groups, with a particle diameter of 106–180 μm. Sample: 5 ml of an bovine lactate dehydrogenase extract in starting buffer. Column size: 40×16 mm I.D. Starting buffer: 20 m*M* sodium phosphate, 1.5 *M* $(NH_4)_2SO_4$, pH 7.0. End buffer: 20 m*M* sodium phosphate, pH 7.0. Flow-rate: 5 ml/min (150 cm/h). The descending salt gradient started after 20-ml elution volume and ended after 140-ml elution volume. The proteins were detected spectrophotometrically at 280 nm.

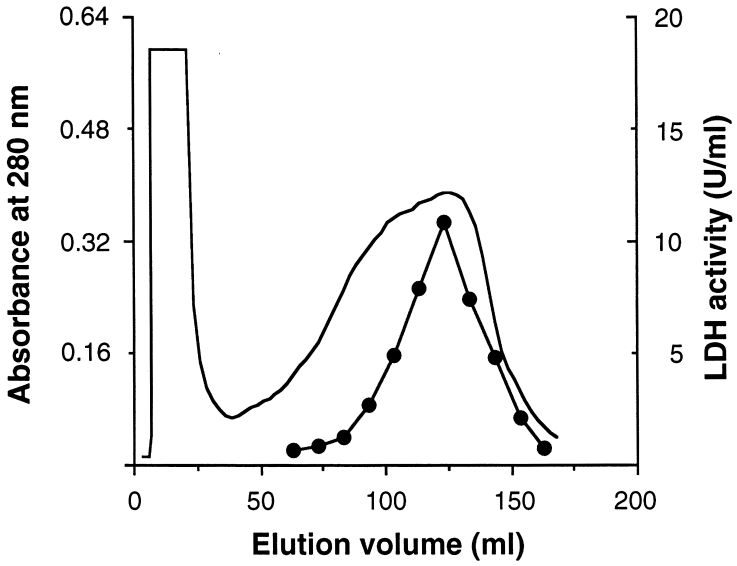

Fig. 4. Hydrophobic interaction chromatography purification of lactate dehydrogenase with homogeneous support. Support: Homogeneous beads derivatized with phenyl groups, with a particle diameter of 106–180 μm. The conditions were the same as in Fig. 3.

volume was achieved with the superporous column compared to the homogeneous column. Comparison of the results obtained with calculations based on the theoretical approach by Carta and Rodrigues [21] indicates that the performance of superporous agarose beads at high frow velocities is limited by residual diffusion distances between the superpores, i.e. even better performance could be expected from particles with superpores located closer together.

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