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Superporous agarose, a new material for chromatography

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Abstract

This paper reports on a new type of spherical agarose chromatography particles characterized by two sets of pores, normal diffusion pores, characteristic of all agarose materials and very wide pores, so-called superpores or flow pores. These superpores allow part of the chromatographic flow to pass through each individual particle, which gives improved mass transfer, especially in situations where diffusion is the limiting factor for the overall performance of a chromatographic separation. The particles were prepared by a double emulsification procedure. Observations under a microscope and size-exclusion chromatography were used in order to demonstrate pore flow. The chromatographic behaviour of the new particles was as efficient as that of homogeneous particles which were several times smaller. The agarose particles were derivatized with polyethyleneimine and used for an ion-exchange chromatographic separation of three model proteins. As expected from a perfusion material, the superporous beads resolved the protein mixture more efficiently than homogeneous beads of the same size.

Keywords: Stationary phases, LC; Perfusion chromatography; Agarose, superporous; Flow pores

1. Introduction

Various chromatography steps are important in most purification schemes for biological macromolecules, such as proteins. A large number of reports and overviews confirm this [1,2]. Prominent examples are ion-exchange chromatography, affinity chromatography and size-exclusion chromatography. For several decades most chromatographic packing materials intended for separation of biomolecules have been polysaccharide-based for good reasons. These materials, e.g. crosslinked dextran and agarose, are available in suitable porosities, are generally inert towards proteins and are stable against hydrolysis. Furthermore, they withstand strong solutions of sodium hydroxide, which is a cheap and very efficient all-purpose sanitizing agent in industrial operations. Polysaccharide packings are also readily derivatized with groups that improve their selectivity, e.g. ion-exchange groups and bioaffinity ligands. The main drawback of polysaccharide materials is their poor mechanical strength. For standard packings (particle diameter 0.1–0.2 mm) the mechanical stability is hardly a problem. Such packings should be run at a low flow-rate, which gives little mechanical strain, to allow for the slow diffusion in the large particles. However, if HPLC-type separations are attempted in order to achieve improved chromatographic efficiency and thereby improved throughput, much smaller particles are needed. Such particles, when operated under optimal conditions, give such a high flow resistance that agarose particle

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beds will collapse. For HPLC separations, mechanically strong particles of silica or polystyrene in particular have therefore become choice materials. Silica and polystyrene in their native forms are poorly suited to protein separations, but a number of chemistries have been worked out to give them suitable surface properties [3–5].

Several years ago, Nilsson et al. [6] developed a special type of gelatin particle, that proved to be an excellent support for growing animal cells. The particles were prepared by a double emulsification procedure, giving particles with large cavities that increased the surface area for growing cells. A closer study using electron microscopy showed also some interconnected cavities. The idea was then suggested [7] that similar particles might be constructed, in which all cavities were interconnected to a continuous network of pores. If such particles were used in a chromatographic bed, one could expect part of the chromatographic flow to pass through the interior of each particle, which should significantly improve the chromatographic efficiency. We therefore started the development of these new particles and concentrated our efforts on agarose, which was considered a choice base material for chromatography of biomolecules.

In this report we describe a new support material which combines the desirable properties of traditional agarose supports and HPLC-type materials. The new support is prepared from agarose and has a fairly large particle size. Significantly, the support material contains two sets of pores, normal diffusion pores characteristic of all agarose materials and very wide pores, so-called superpores or flow pores. A chromatographed substance is transported by flow in these superpores to the interior of each individual particle, leaving only short distances to be covered by slow diffusion processes. In this way the new particles should exhibit chromatographic efficiency equal to homogeneous particles that are several times smaller. The principle of pore flow and its profound influence on chromatographic efficiency (perfusion chromatography) has been demonstrated previously by Afeyan et al. [8,9], primarily for polystyrene-based supports. Several groups have also developed theories of perfusion chromatography, mainly Rodrigues et al. [10,11], Liapis et al. [12,13] and Afeyan et al. [8].

2. Theory

A general measure of chromatographic efficiency is the plate height (HETP). The smaller the plate height the more efficient is the separation process. The following relationship [14] takes into account the various contributions to the total plate height (HETP).

$$\mathrm{HETP} = Au^{0.33} + \frac{B}{u} + Cu + Eu \qquad (1)$$

Where u is the linear velocity of the mobile phase and the terms respectively relate to eddy diffusion + mobile phase mass transfer (A), longitudinal diffusion (B), stagnant mobile phase diffusion (C) and binding kinetics (E). The term Cu, relating to mass transfer in the pores, is dominant in most realistically arranged chromatographic separations, i.e. when the separation is diffusion controlled. In such situations Eq. 1 simplifies to

$$\mathrm{HETP} = Cu \qquad (2)$$

The term Cu can be expanded [14]

$$\mathrm{HETP} = \frac{C'(d_p)^2 u}{D} \qquad (3)$$

Where C′ is a constant (under a certain set of conditions), d_p is the particle diameter, u is the linear velocity of the mobile phase and D is the diffusion coefficient. Thus, the chromatographic efficiency is very much dependent on the particle size and it is easy to understand the benefits of pore flow. For instance, if chromatographed molecules are transported by flow into the interior of a superporous agarose particle (Fig. 1A), only a short distance remains to be covered by diffusion.

Thus, as a very rough approximation, d_p in Eq. 3 should no longer denote the particle diameter but the distance between flow pores, which should lead to a considerable reduction of the HETP value. It is immediately obvious that the performance of superporous particles could at best be close to that of

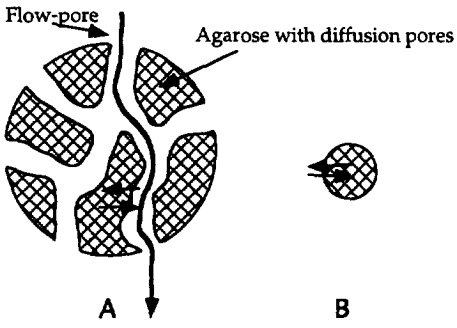

Fig. 1. Illustration of the diffusion distance in (A) a superporous bead and (B) a small homogeneous bead.

homogeneous particles with a diameter equal to the distance between the superpores (Fig. 1B).

3. Experimental

3.1. Materials

Agarose powder (Sepharose quality) was a gift from Pharmacia (Uppsala, Sweden). Polyoxyethylene sorbitanmonooleate (Tween 80), sodium borohydride and 2,2′-azobis(2-methylpropionitrile) (AIBN) were obtained from Merck-Schuchardt (Munich, Germany). Sorbitane trioleate (Span 85) and ethylene glycol dimethacrylate (EDMA) were purchased from Fluka (Buchs, Switzerland). Cyclohexane (puriss.) and sodium azide were purchased from Merck (Darmstadt, Germany). Cibacron Blue 3G-A, 1,4-butanediol diglycidyl ether (70%) and all proteins used in this study were purchased from Sigma (St. Louis, MO, USA). Polyethyleneimine (50% solution) was obtained from BDH (Poole, UK). Uniform polystyrene particles (0.5 μm) were purchased from Seradyn Particle Technology (Indianapolis, IN, USA).

3.2. Preparation of superporous agarose particles (typical procedure)

Superporous agarose particles were prepared essentially as described in the Swedish patent application 9200827-5. One hundred ml of an agarose solution (6%, w/v) was prepared by heating a suspension of agarose in water to 95–100°C in a microwave oven, and keeping it at that temperature for 1 min. During the warm-up period, care was taken to keep the agarose powder well suspended with occasional shaking. The solution was transferred to a thermostatted (60°C) stirred glass reactor and a mixture containing 3.0 ml of Tween 80 and 50 ml of cyclohexane (60°C) was added. The mixture was emulsified by stirring at 1000 rpm for 4 min (emulsion 1). A thermostatted solution (60°C) containing 300 ml of cyclohexane and 12 ml of Span 85 was added. The reactor was stirred at 600 rpm. After 1 min the reactor thermostat setting was changed to 25°C. When the temperature decreased below approximately 40°C, the agarose solidified into superporous spherical particles. The particles were isolated on a sieve and washed with water, ethanol–water (50:50, v/v) and finally water. The particles were sized wet with graded metal screens.

3.3. Preparation of homogeneous agarose particles (typical procedure)

One hundred ml of an agarose solution (6%, w/v), prepared as described in Section 3.2 was emulsified (stirring speed 600 rpm) with 200 ml of cyclohexane containing 8 ml Span 85 in a thermostatted (60°C) glass reactor for 3 min. The reactor thermostat was then adjusted to 25°C. The homogeneous agarose particles that were formed were collected, washed and sized as described in Section 3.2.

3.4. Dyeing of beads with Cibacron Blue 3G-A

To facilitate light microscopy studies, a few beads of each type were dyed with Cibacron Blue 3G-A according to the method of Dean and Watson [15].

3.5. Preparation of ion-exchange particles

Superporous agarose beads and homogeneous agarose beads were activated with bis-oxirane according to the method of Sundberg and Porath [16]. Both types of beads were then reacted with polyethyleneimine according to the method of Sluyterman and Wijdenes [17]. The ion-exchange capacity of each material was determined by titration with 0.1 *M* NaOH from pH 7 to 11. The ion-exchange capacity was 160 μmol/ml for the superporous beads and 220 μmol/ml for the homogeneous beads.

3.6. Preparation of superporous membranes for microscopy

Samples of emulsion 1, prepared as described in Section 3.2 were poured on a warm (60°C) glass Petri dish. After 10 s the Petri dish was transferred to an ice bath and the emulsion solidified to an approximately 3 mm thick membrane of superporous agarose. The membrane was washed with water, ethanol–water (50:50, v/v) and again with water. Thin slices suitable for observation under a microscope were prepared with the aid of a microtome or using a razor blade. The slices were studied using a Nikon Labophot-2 light microscope.

3.7. Preparation of a superpore structure replica for electron microscopy visualization

Emulsion 1, prepared as described in Section 3.2, was poured into a glass column, (1 cm I.D.), thermostatted to 60°C in a water bath. The glass column was brought to room temperature to solidify the agarose. The superporous gel thus obtained was trimmed to a length of 1.5 cm and packed in a glass column, (1 cm I.D.), equipped with flow adapters. The organic phase in the superpores was removed by pumping water, ethanol–water (50:50, v/v) and finally water through the column.

Then, the superpores of the gel were filled with a polymerizable monomer solution, by pumping a mixture of ethylene glycol dimethacrylate (52.4 mmol) and AIBN (0.76 mmol) in 16 ml of toluene through the column. The column inlet and outlet were stoppered and polymerization was initiated with ultraviolet light (366 nm, low pressure mercury lamp) at 4°C for 16 h. After polymerization, the gel cylinder was removed from the column and the agarose phase melted away in a boiling water bath. The superpore structure replica thus obtained was dried and fixed to a grid using Leit-C glue (Neubauer, Germany). The replica was then sputter-coated with gold (20 nm thick layer) and was studied using an ISI 100 A scanning electron microscope at 15 kV.

3.8. Chromatographic experiments

Superporous beads were compared with homogeneous beads in size-exclusion chromatography experiments. The beads were packed in 16 mm diameter columns equipped with flow adapters. The columns were integrated with HPLC equipment (Kontron or Pharmacia-LKB), including pump, injection valve or autoinjector, UV–Vis detector and recorder. The mobile phase was 0.10 *M* sodium phosphate buffer, pH 7. The samples, dissolved in the mobile phase, were injected (50 μl or less) and chromatographed at different flow-rates and their elution profiles recorded. HETP data were computed using the formula [14]:

$$\mathrm{HETP} = \frac{L}{5.54\left(\frac{t_R}{W_{1/2}}\right)^2} \quad (4)$$

Where L is the column length, t_R is the retention time and $W_{1/2}$ is the width of the band at half-height. Extra-column band broadening was usually less than 10% of the total band broadening and was corrected for. Appropriate data for corrections were conveniently obtained by injecting very small samples into empty columns, whose flow adapters had been pushed against each other.

Superporous beads were also compared with homogeneous beads in an ion-exchange protein separation experiment. The ion-exchange beads, prepared as described in Section 3.5, were packed in 5 mm diameter glass columns equipped with flow adapters of steel (work-shop construction). The ion-exchange columns were integrated with HPLC equipment (Pharmacia-LKB) as above.

4. Results and discussion

4.1. Observation of superpores by microscopy

Fig. 2a shows a micrograph of superporous beads mixed with homogeneous agarose beads, both dyed with Cibacron Blue to improve visibility. The beads were immersed in water and illuminated from below. Both types of beads had a particle size of 300–500 μm. The superporous beads had a superpore size of about 30 μm, and a nominal superpore porosity of 40%, i.e. 40% of the bead volume was superpores according to the recipe followed when preparing the beads. The speckled appearance of the superporous beads is explained by the fact that light was absorbed

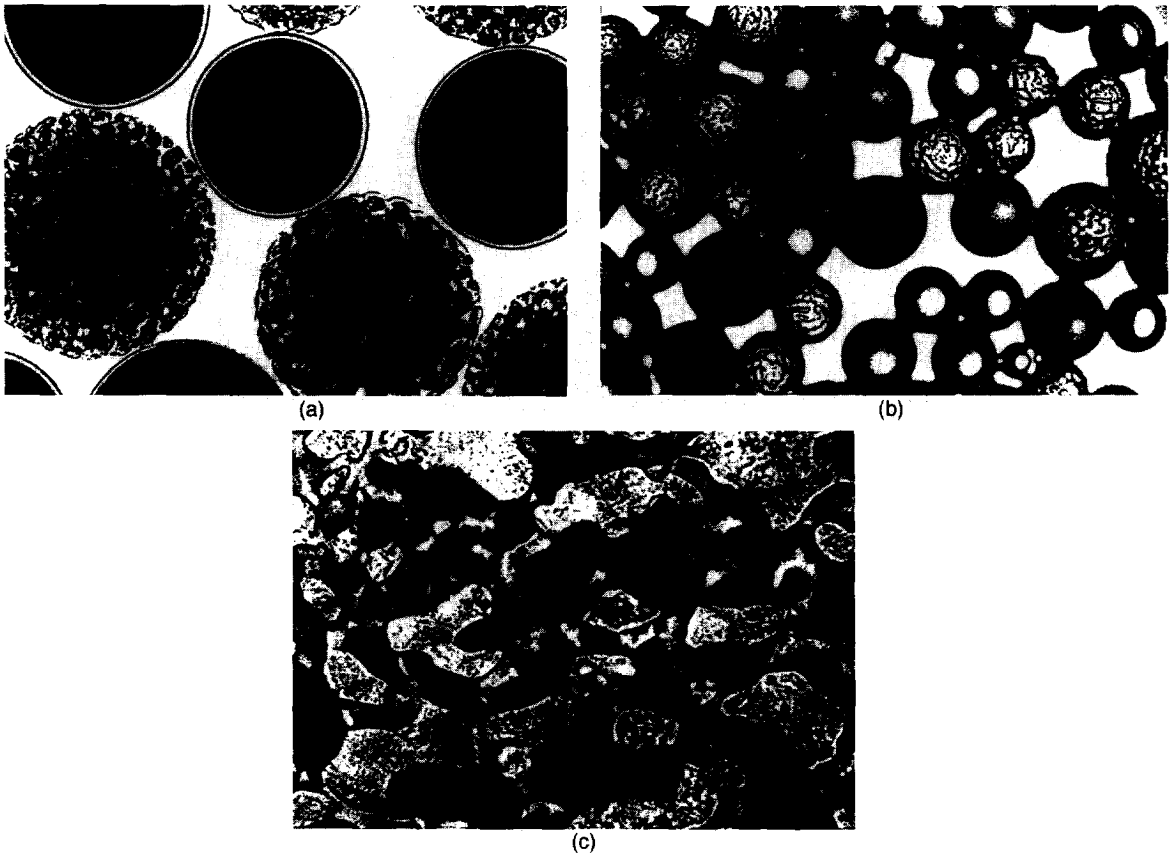

Fig. 2. (a) Micrograph of superporous beads and homogeneous beads. The halo around the homogeneous beads is a diffraction effect. (b) Micrograph of semi-dry superporous beads and homogeneous beads. (c) Micrograph of a superporous gel. The light areas constitute the top surface of the membrane. The darker areas constitute the superpores.

only by the coloured agarose and not by the superpores (which contained water). Fig. 2b shows the same type of preparation as in Fig. 2a, but here the beads are semi-dry. Note the dented appearance, which is due to air beginning to enter the pore openings. Fig. 2c shows a micrograph of a superporous gel slice. The gel was cast as a 3 mm thick membrane as described in Section 3.6 and then sliced with a microtome to give a flat upper surface. The illumination is from below. Note the fairly even distribution of meandering superpores which is very important for an even flow through the material. The width of the superpores was about 50 μm.

4.2. Electron microscopy

Fig. 3 shows scanning electron micrographs of a superpore structure replica, obtained by filling the superpores in a superporous gel with acrylic monomers, polymerizing and finally melting away the original agarose phase. The figures give a picture of an evenly distributed network of superpores with diameters of between 25 and 75 μm.

4.3. Direct observation of pore flow in individual superporous beads

Pore flow was directly observable under the microscope (Fig. 4). A large (0.35 mm) superporous bead was sucked into an HPLC-type PTFE tubing and then studied under the microscope. By using a micro manipulator, a thin glass capillary could be placed on selected places on the bead and could deliver water which was sucked into the pores by the applied vacuum.

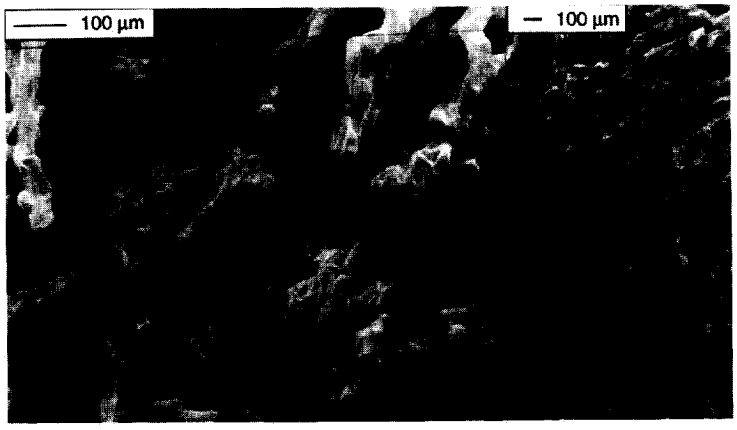

Fig. 3. Scanning electron micrograph of a superpore structure replica at two magnifications. Details for preparing the superpore structure replica are given in the Section 3.7. The empty space between the structures seen in the figure originates from the melted agarose phase.

4.4. Direct observation of pore flow in cast, superporous membrane samples

Superporous membranes, about 3 mm thick, were prepared with different pore widths. Circular membrane discs were punched out and the discs were placed on a glass filter funnel attached to a water suction pump. By applying a slight vacuum, it was easy to pass large volumes of water through the membrane which provided very convincing evidence of pore flow. Similarly, it was easy to pass fine particles through the membrane (0.5 μm latex particles and yeast cells). Furthermore, membranes with large superpores (>50 μm) allowed the passage of water without any vacuum. The slight pressure generated by applying a few mm of water head caused cross-membrane transport. Wide pore membranes could readily be drained of water by applying a vacuum. In this way the superpores became filled with air. This was not possible with membranes with narrow superpores. Full vacuum rapidly sucked the head of free water through the membrane leaving the membrane surface dry, after which all flux ceased abruptly, indicating that the bubble point for the pores was larger than about 1 bar [18].

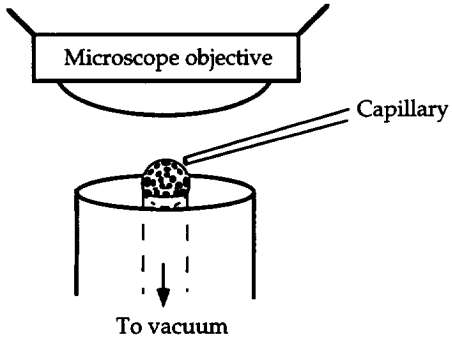

Fig. 4. Direct observation of pore flow in individual superporous beads.

4.5. Size-exclusion chromatographic evidence of pore flow

4.5.1. Size-exclusion chromatographic verification of superpore volumes

Superporous particles were compared with homogeneous agarose particles of the same size in size-exclusion chromatography experiments, in order to verify that the superpores were operative also under chromatographic conditions. Two columns were packed with superporous particles and homogeneous particles, respectively. Both types of beads had a particle diameter of 0.4 mm. The bed volume for both types of adsorbents was 31 ml. The elution volume for latex particles (0.5 μm) differed as expected for the two types of particles. The elution volume for the homogeneous agarose particles was thus about 12.5 ml, which is close to the expected value for the void volume in a column with 60% packing density. Significantly, the elution volume with the superporous material was about 8.5 ml

larger, which thus is a measure of the superpore volume. The superporous particles used in the experiment were designed to have a superpore porosity of 50%, which is in good agreement with the experimental value of 46%. The elution peaks for the latex particles were narrow and the HETP values calculated from them were fairly independent of flow-rate, which is expected for excluded, non-diffusing substances.

4.5.2. HETP experiments

A number of size-exclusion chromatography experiments were carried out to characterize the properties of superporous beads. The beads differed with respect to superpore porosity, particle size and agarose content. In all cases, reference runs were carried out with homogeneous agarose beads. Fig. 5 shows the results with a low-molecular-mass substance, sodium azide. The difference in performance between homogeneous and superporous particles is only moderate and may be explained by the fact that low-molecular-mass substances have rather high diffusion coefficients (around 10^{-5} cm^2 s^{-1}). The flow range (up to 2.5 cm/min) was rather high considering the large particles used.

Fig. 6 shows the results for the same particles but

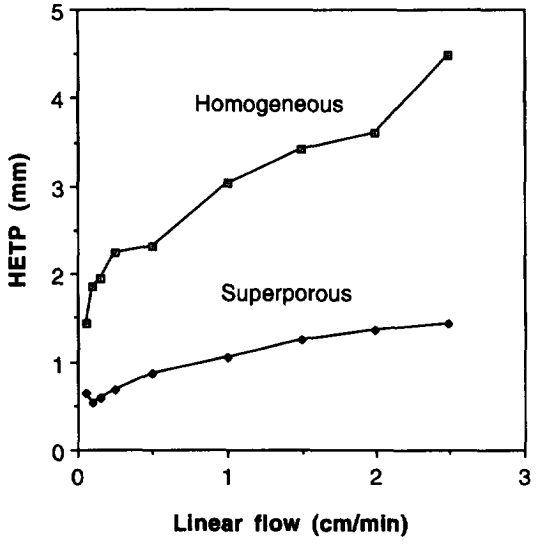

Fig. 5. Size-exclusion chromatography of a low-molecular-mass substance (sodium azide). HETP as a function of flow-rate. Particle size was 0.40 mm.

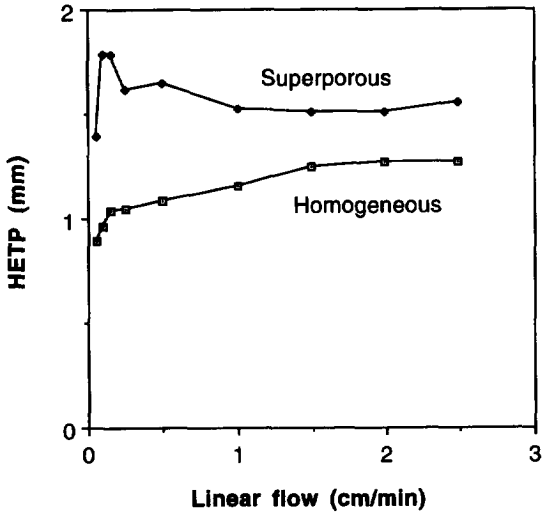

Fig. 6. Size-exclusion chromatography of a high-molecular-mass substance (latex polymer). HETP as a function of flow-rate. Particle size was 0.40 mm.

with a high-molecular-mass latex polymer (about 0.5 μm particle size). The plate height is fairly constant for the whole flow range for both particles (there is some scatter in the data). The finding that the plate height is independent of the flow-rate is completely in line with what can be expected theoretically. Latex particles will have free access to the superpores but will be totally excluded from the diffusion pores in the agarose matrix and thus there should not be any band broadening at increased flow-rates.

So why, in this case, are the superporous particles less efficient than the homogeneous agarose particles? A reasonable explanation is that since diffusion does not play any significant role here, there is no benefit to gain from the superpores, at least not related to the third term in Eq. 1, which covers mass transfer in the stagnant mobile phase (Section 2). On the contrary, the superpores constitute an additional volume in which the high-molecular-mass polymer might be dispersed. Also, the superpores are certainly not perfect in the sense that the flow velocity inside the beads is the same as that outside the beads.

As expected, the most convincing improvements with superporous gels were achieved with proteins. A protein such as bovine serum albumin (M_r 68 000) diffuses much more slowly than salts such as sodium azide, and since it will be partly excluded from the

agarose polymer, its effective diffusion coefficient will be even lower. Thus, the diffusion controlled domain will already be entered at low flow-rates. Under such conditions, the superpores should be especially valuable since they diminish the diffusion distances.

Fig. 7 shows this very clearly. The plate height for the superporous particles increased only moderately at increased flow-rates, while it very soon reached very high values for the homogeneous material. At flow velocities above 0.5 cm/min extremely asymmetric peaks were obtained with the homogeneous particles, making HETP calculations meaningless.

Fig. 8 gives further experimental support for the efficiency of the superporous agarose particles. Here, the performance of smaller superporous particles (0.14 mm) was compared with that of homogeneous particles of different sizes, including ones that were three-times smaller. The particles were prepared by a slightly different protocol than before. Thus, all particles were prepared from 7.5% agarose. The superporous particles had a nominal porosity of 33% instead of 50%. Again, the trend in behaviour is very clear. At low flow-rates the diffusion rate in homogeneous particles is sufficient to give a low plate height and a performance similar to that of the superporous particles. At high flow-rates, the superporous particles are much more efficient due to the pore flow and the resulting reduced internal diffusion distances.

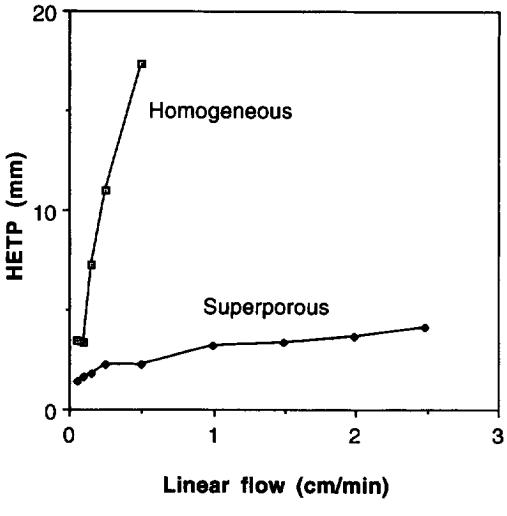

Fig. 7. Size-exclusion chromatography of bovine serum albumin. HETP as a function of flow-rate. Particle size was 0.40 mm.

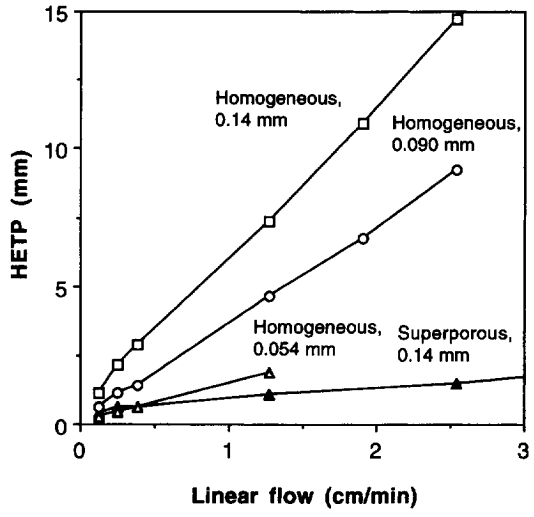

Fig. 8. Size-exclusion chromatography of bovine serum albumin. HETP as a function of flow-rate for different particles.

Except at very low flow-rates the large (0.14 mm) superporous particles performed better than the almost three-fold smaller (0.054 mm) homogeneous particles. The comparison between the two types of particles could not be completed at higher flow-rates, since the back pressure for the small homogeneous particles became so high that the bed collapsed. The pressure was not measured accurately during the runs, but should in theory be about seven times higher for the smaller particles [19].

4.6. Ion-exchange chromatography

Superporous beads were compared with homogeneous beads in an ion-exchange separation of a mixture of three model proteins, ovalbumin, bovine serum albumin and β-lactoglobulin (Fig. 9). As could be expected from a perfusion material, the superporous bead column showed an improved resolution of the three proteins compared to the homogeneous bead column. Thus, the superporous bead column distinguished between the two isomers of β-lactoglobulin. The greater advantage of the superporous column at higher flow-rates is characteristic of perfusive systems.

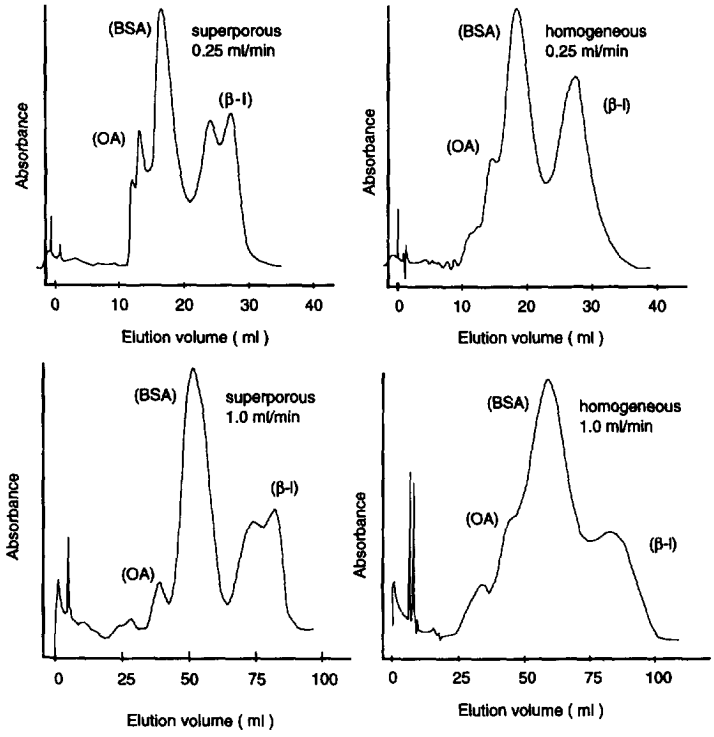

Fig. 9. Ion-exchange chromatographic comparison between homogeneous and superporous supports at two elution rates. Sample, 1 ml protein mixture containing 0.25 mg bovine serum albumin (BSA), 0.25 mg ovalbumin (OA) and 0.25 mg β-lactoglobulin A and B (β-l) in starting buffer; Ion-exchanger, polyethyleneimine–agarose, superporous and homogeneous beads, both types with a particle diameter of 75–106 μm; column size, 3 × 0.5 cm I.D.; starting buffer, 20 m*M* Tris–HCl, 0.02 *M* NaCl, pH 7.0; end buffer, 20 m*M* Tris–HCl, 0.6 *M* NaCl, pH 7.0. The gradient started at 0 min and was completed at 100 min. The proteins were detected at 280 nm.

5. Conclusions

We have prepared a new type of agarose particles characterized by two sets of pores: (1) normal diffusion pores characteristic of all agarose materials, and, (2) very wide so-called superpores that allow part of the chromatographic flow to pass through each individual particle.

Such a pore flow gives improved mass transfer, especially in situations where diffusion is the limiting factor for the overall performance. Thus, the benefits of superporosity were most clearly demonstrated for larger particles and at high flow-rates. Size-exclusion and ion-exchange chromatography were used for demonstrating the perfusion properties of the new material. The superporous agarose should also be suitable as a base for other separation materials, e.g. affinity supports and as a support material for immobilized enzymes.

Acknowledgments

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