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Enhanced performance of expanded bed chromatography on rigid superporous adsorbent matrix

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Abstract

Rigid spherical macroporous adsorbent beads with surface hydroxyl groups were prepared by cross-linking of cellulose. These beads had diameter in the range 100–200 μm and a mean pore size of about 3 μm with about 60% pore volume. The matrix (bulk density ~1600 $kg\,m^{-3}$) could be expanded into a stable bed and used for protein chromatography. Chromatographic runs were performed on a 10 mm diameter column under non-retaining and retaining conditions on the prepared matrix (called Celbeads) and performance of the runs was measured in terms of the height equivalent to a theoretical plate (HETP). The HETP curves in both packed and expanded bed modes followed profiles typical of macroporous adsorbents, i.e. increasing and levelling with velocity. Unimpaired performance of the matrix at increasing flow-rates permitted expanded bed elution of adsorbed solutes without loss of efficiency in terms of purification factor and product concentration. As a model system, Celbeads was used to purify lactate dehydrogenase from porcine muscle homogenate by dye-affinity chromatography. The prepared matrix provided about 100 theoretical plates per meter for the enzyme system at a linear flow velocity of 1.27 $cm\,min^{-1}$ in an expanded bed elution mode, and gave enzyme yields of 100% with a purification factor of 31 using an optimized procedure. The adsorbent could be cleaned in place with 5 *M* urea and used repeatedly without loss of performance.

Keywords: Affinity sorbents; Efficiency; Expanded bed chromatography; Stationary phases, LC; Lactate dehydrogenase; Enzymes

1. Introduction

Adsorptive and chromatographic separations are employed as high resolution steps in late stages of downstream processing of biomolecules. Increasing efforts have been made over the last decade to devise techniques that can isolate and purify target molecules from its ''crude'' broth in quickest possible way. Expanded bed chromatography is a technique that not only isolates and purifies target proteins on a preparative scale directly from ''crude'' broths containing particulate suspended matter, it also makes this possible in a much lesser time than that required by conventional adsorption chromatography [1–3]. A large number of reports have appeared that have successfully demonstrated the utility of the concept of adsorption in expanded bed and have used adsorbents that have specific properties for expanded bed operation [4,5]. There are, however, a number of facts that emerge from the literature on expanded bed

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purifications. All workers have used column sizes typical of preparative scale operations. Application of expanded bed technology on laboratory scale column needs to be explored for enabling quicker laboratory scale separations. Further, almost all workers report adsorption carried out in upflow expanded bed mode and elution carried out in downflow packed bed mode. In cases wherein speedy product isolation is essential, it may be desirable that not only is expanded bed adsorption carried out at highest possible flow-rate, but that the elution is also performed at high flow-rate. However, use of high mobile phase flow-rate is known to adversely affect column efficiency in packed bed mode. Hjorth et al. [6] have reported some data on elution in expanded bed mode and concluded that the band width is considerably diluted and hence expanded bed elution is not advisable. In this report we present data on expanded bed dynamics using indigenously developed rigid macroporous cross-linked cellulose beads, and show thereby that with use of macroporous matrix, high flow-rates can be used without adversely affecting column efficiency in both adsorption and elution modes, in packed as well as expanded bed operations. Further, the prepared matrix can be used in preparative as well as laboratory scale columns and thus also holds promise for both small scale laboratory purifications and for protocol scouting experiments.

Performance of a chromatographic process is measured in terms of the height equivalent to a theoretical plate (HETP) under given operating conditions. The smaller the HETP the more efficient the separation procedure both in terms of product concentration and resolution from other molecules. Dependence of HETP on superficial velocity u_o, is adequately described by the celebrated Van Deemter equation [7] as

$$\text{HETP}, H = A + B/u_0 + Cu_0 \quad (1)$$

where the three terms of the equation account for dispersion, molecular diffusion and intraparticle effects respectively, and the constants *A*, *B* and *C* are system and operation dependent parameters. In most practical cases in protein chromatography, molecular diffusion has negligible contribution to the HETP. The most significant contribution to HETP comes from intraparticle effects which include film mass transfer resistance, pore diffusion resistance and adsorption/desorption rates. Reduction in particle size and use of non-porous adsorbent can lead to substantial reduction in HETP. However, while smaller particle size results in unacceptable pressure drops especially for gel based supports, non-porous matrix will result in greatly reduced adsorption capacity of the matrix. Afeyan and coworkers [8,9] demonstrated that efficiency of a packed adsorption column can be increased by use of macroporous matrix that allows finite intraparticle convection. Macroporous adsorbents that permit intraparticle flow provide an elegant way to reduce mass transfer resistances and have been shown to exhibit a plateauing of the HETP curve with velocity and hence provide small HETP values even at high flow-rates [10]. While a few macroporous adsorbents are available in the market, none are suitable for use as affinity chromatography matrix for preparative scale applications especially for expanded bed chromatography. The approach adopted in the present work was therefore to improve expanded bed protein adsorption and elution by developing adsorbent particles that are macroporous, have particle diameter in the range of 100–200 μm and possess high particle density. While macroporority may ensure intraparticle convective flow; small and heavy particles offer small diffusion lengths and permit higher interstitial bed expansion velocities, respectively to result in efficient and stable expanded bed operation that will allow speedy bioseparations.

A new type of superporous (pore size: ~3 μm) rigid beaded adsorbent, based on cross-linked cellulose, was prepared and was found to give a stable expanded bed over a large velocity range. Dynamic performance of the matrix was evaluated. The developed adsorbent showed negligible non-specific adsorption and a good density of surface hydroxyl groups for activation and use as an affinity matrix. Cibacron Blue 3GA, a pseudo-affinity dye ligand popularly used in bioseparations, was covalently immobilized on the developed matrix (hereinafter called Celbeads), and the affinity matrix was used for purification of lactate dehydrogenase (LDH) directly from porcine muscle homogenate. Purification was performed by adsorption in expanded bed mode, while elution was carried out in both expanded and

packed bed modes. The adsorbent could be fully regenerated by cleaning in place procedure like washing with 1 *M* NaCl or 5 *M* urea.

2. Experimental

2.1. Materials

Macroporous cellulose beads (Celbeads) were prepared according to the procedure for which patent application has been filed. Streamline DEAE was purchased from Pharmacia, Uppsala, Sweden. Streamline DEAE is a composite cross-linked agarose gel based anion-exchanger adsorbent especially designed for expanded bed operations. Cibacron Blue 3GA was procured from Sigma, while the Bio-Rad protein assay kit was from Bio-Rad Lab., Hercules, USA. Divinylsulphone was purchased from Lancaster Synthesis, Lancashire, UK. Sodium salt of pyruvic acid and β-nicotinamide adenine dinucleotide in reduced form (NADH) were procured from Loba Chemie, Mumbai, India. Fat free porcine muscle was purchased from Mafco, Mumbai, India. All other chemicals were of analytical grade and were purchased from local suppliers.

2.2. Characterization and chemical derivatisation of Celbeads

2.2.1. Bead characterization

Celbeads was characterised for the properties requisite of an adsorbent for protein purifications. Shape and size of Celbeads was analysed on an IP PLUS Image Analyser. Porosity and surface area analysis was carried out by mercury porositimetry. Bead porosity was also measured by gel filtration experiments on a 10 mm diameter Bio-Rad ECONO column using different molecular mass markers. A number of proteins were tested on Celbeads for non-specific adsorption.

Suitability of Celbeads for expanded bed operation was investigated by bed expansion experiments on the set-up described below. Bed expansion studies were carried out for Streamline DEAE and Celbeads with deionised water by varying inlet flow-rate.

2.2.2. Immobilization of cibacron blue on celbeads

Cibacron Blue 3GA is a reactive triazine dye widely used in affinity purification of proteins. Cibacron Blue 3GA is known to bind, in a fairly specific manner, to NAD nucleotide binding enzymes like dehydrogenases and is hence commonly employed for their purification [11]. Cibacron Blue 3GA was covalently coupled to Celbeads using the standard procedure reported for hydroxyl group containing supports [12]. Amount of dye immobilized was calculated by spectrophotometrically measuring the balance unbound dye at 610 nm after the immobilization.

2.2.3. Preparation of anion-exchanger Celbeads

For use as a weak anion-exchange matrix, Celbeads was activated with divinylsulfone (DVS) and reacted with ethylenediamine using the reported method for hydroxyl supports [13]. The excess active groups were blocked by suspending the beads in 2-mercaptoethanol for 2 h at room temperature. The beads were finally washed thoroughly with 1*M* sodium chloride solution and water. Celbeads thus prepared exhibited weak anion exchange capacity. This matrix will be hereafter referred to as Celbeads-EDA. These beads were used as adsorbent for bovine serum albumin (BSA) at pH 7.0 in 50 m*M* Tris–HCl buffer. Adsorption isotherm for BSA on Celbeads-EDA was determined and is given in Fig. 1 It was also experimentally determined that the BSA bound could be quantitatively eluted in the same buffer containing 0.5 *M* sodium chloride.

3. Column experiments

All column experiments were performed on the experimental setup that consisted of a 250 mm×10 mm inner I.D. glass column, equipped with two 140 mm long BioRad ECONO adjustable flow adapters modified with 90 μm stainless steel wire mesh flow distributors in place of traditional porous polymer distributor. The bottom adapter was connected to an Alitea XV peristaltic pump while the top adapter was connected to an on-line Hitachi UV–Vis 1100 spectrophotometer which in turn was connected on to an ISCO Retreiver II fraction collector. The absorbance readings at the spectrophotometer were recorded on a

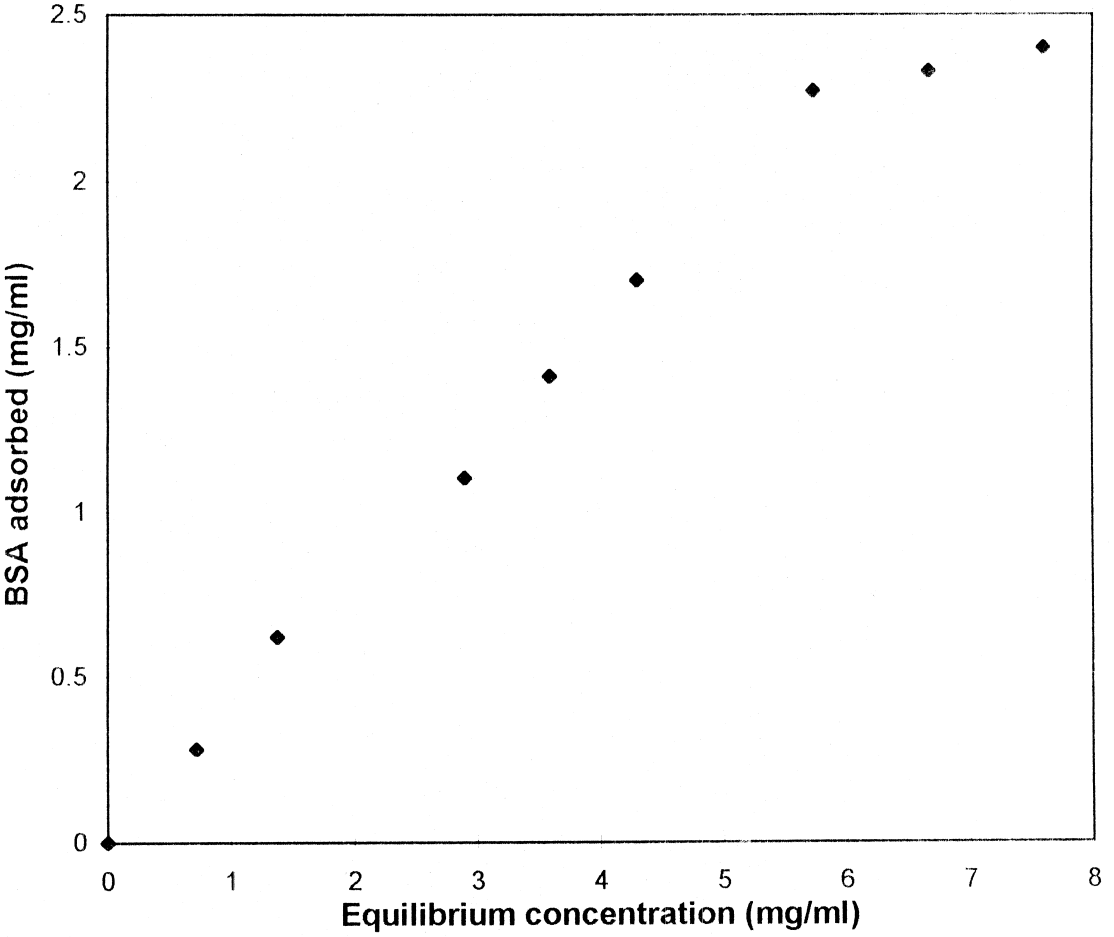

Fig. 1. Adsorption isotherm for BSA on Celbeads-EDA at 30°C in 50 m*M* Tris–HCl buffer pH 7.0.

continuous chart recorder. In all the experiments throughout, the settled bed height of adsorbent was maintained at 110 mm and all experiments were performed in upflow mode. Pressure drop across the packed bed of Celbeads and 100 μm spherical glass beads was measured using a manometer filled with carbon tetrachloride. The pressure drop across the bed of glass beads was found to follow Ergun's equation.

Chromatographic experiments under non-retaining conditions were performed in both packed and expanded bed mode on Celbeads. Separate runs were made with each of the three biomolecules tyrosine (molecular mass ~181), papain (molecular mass ~23 000) and bovine serum albumin (molecular mass ~66 000), all used as 4 mg ml^{-1} aqueous solutions. While tyrosine was loaded in 50 m*M* Tris–HCl buffer at pH 7.0, papain and BSA were loaded in the same buffer containing 1 *M* NaCl to ensure non-retaining conditions on pre-equilibrated adsorbent. Chromatographic peaks were obtained at flow velocities ranging from 0.64 cm min^{-1} to 6.4 cm min^{-1} in both packed and expanded modes by injecting sample as a plug in the inlet flow line. The chromatograms obtained on the chart recorder showed that the elution bands were almost symmetrical but for a slight tailing in the end. The volume of the injected sample was varied from 0.5 ml to 4 ml. Fig. 2 shows typical chromatographic peaks obtained in the experiments on 110 mm packed bed under non-retaining conditions at different flow-rates for 4 ml injection volume. Total plate number N for a run

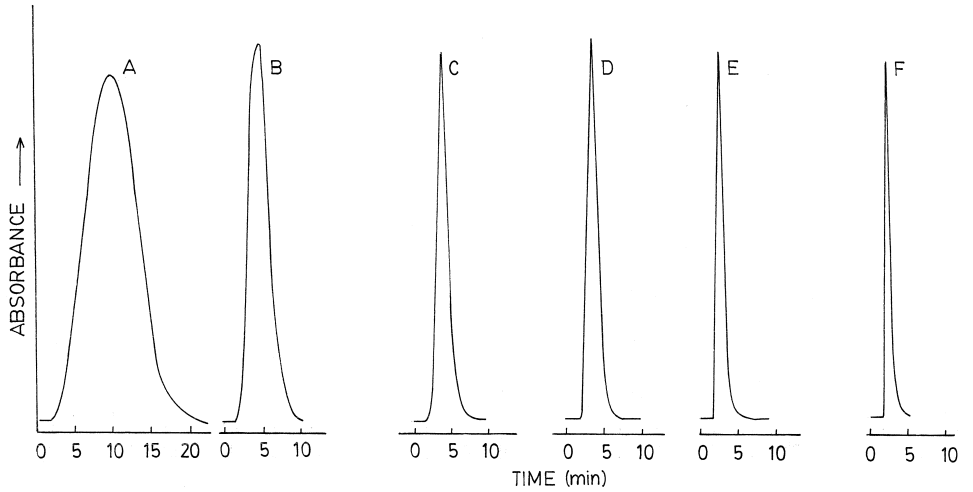

Fig. 2. Chromatographic peaks for BSA in 50 m*M* Tris–HCl buffer pH 7.0 containing 1 *M* NaCl under non-retaining conditions in a 110 mm Celbeads column at different flow-rates: (A) −0.5 ml min^{-1}, (B) −1.2 ml min^{-1}, (C) −2.0 ml min^{-1}, (D) −2.5 ml min^{-1}, (E) −3.5 ml min^{-1}, (F) −5.0 ml min^{-1}.

was calculated by measuring the retention time and peak width at half peak height and using the following relation [14];

$$N = 5.54 \cdot \left(\frac{t_R}{w_{1/2}} \right)^2 \quad (2)$$

where t_R is the retention time and $w_{1/2}$ is the peak width at half the peak height. The operating bed height divided by N gave height equivalent to theoretical plate. HETP was determined as a function of superficial velocity for each of the three molecules. It was found that HETP determined at a given flow-rate was independent of the injected sample volume in the range 0.5 ml to 4 ml.

Experiments were also performed by loading 4 ml of 4 mg ml^{-1} BSA in 50 m*M* Tris–HCl buffer, pH 7.0 on Celbeads-EDA in packed bed and expanded bed mode till breakthrough and exhaustion (measured as absorbance at 280 nm on spectrophotometer). The bed was washed well with working buffer, and then the BSA eluted in 0.5 *M* NaCl in the same buffer. All three steps of protein loading, wash and elution were performed at same flow-rate. The entire operation was carried out at different flow-rates in the range 0.64 cm min^{-1} to 6.4 cm min^{-1}. The HETP values for different flow-rates were calculated in the manner described above.

4. Chromatographic Procedure for purification of LDH from porcine muscle homogenate

Porcine muscle was cleaned of fibrous tissue and fat, suspended in ice cold 50 m*M* Tris–HCl buffer at pH 8.0 and then homogenized in a house hold mixer for 15 min. The homogenate obtained was centrifuged at −5°C in a Sigma 6K10 centrifuge at 9000 *g* and the supernatant obtained was filtered through a pad of glass wool on a Buchner funnel to give the crude extract. This extract was stored at −20°C in suitable aliquots and thawed prior to use. The broth after thawing had an enzyme activity of about 200 Units of LDH ml^{-1} and a total protein content of 13 mg ml^{-1}. LDH activity was assayed according to the reported procedure based on oxidation of NADH in a reaction converting pyruvate to lactate [15]. The amount of enzyme that utilized 1 μmol of NADH per min per ml reaction mixture under standard assay conditions was taken as 1 unit. Total protein content was measured spectrophotometrically by using the BioRad protein assay kit with bovine serum albumin as standard.

Cibacron Blue immobilized Celbeads (7.5 ml settled volume) was poured as a suspension in the working buffer (50 m*M* Tris–HCl buffer pH 8.0) into the column from the top. The column was equilibrated in working buffer at 1 ml min^{-1} and 20

ml of crude muscle extract was passed through the column at 1 ml min^{-1}. To remove residual crude, suspended matter and substances physically adhering to the adsorbent, working buffer containing 0.15 *M* KCl was passed through the adsorbent bed at same flow-rate till eluate protein content dropped to baseline on spectrophotometer. Elution with 0.5 *M* KCl in working buffer was performed in packed bed mode as well as expanded mode at 1 ml min^{-1}.

5. Frontal chromatography analysis

The main objective of the present work was to highlight the efficiency of macroporous adsorbent matrix in terms of HETP at high flow-rates. It is known that dynamic capacity of an adsorbent is also a function of flow-rate, and decreases with increasing flow-rate. To estimate the effect of flow-rate on dynamic adsorbent capacity BSA (as 4 mg ml^{-1} solution in 50 m*M* Tris–HCl buffer at pH 7.0) was applied till breakthrough and exhaustion on Celbeads-EDA and Streamline DEAE beds. Experiments were done at two velocities on both the adsorbents at 1.53 cm min^{-1} and 3.8 cm min^{-1}. The amount of BSA adsorbed was calculated by balance and designated as the bed capacity in respective cases.

6. Results and discussion

6.1. Celbeads

Image analysis of the prepared adsorbent matrix indicated that the adsorbent beads were spherical in shape and fell in a size range of 100–200 μm. Rigid and porous structure of Celbeads permitted porosity and surface area analysis by mercury porositimetry and gave average pore radius as 33 500 Å and pore volume was 59.8%. The surface area estimated was 26.4 $m^2 g^{-1}$. Thus the beads had a fairly large average pore size (about 3 μm) and a decent pore volume. Gel filtration experiments with blue dextran, BSA and papain all gave a pore volume of 59% indicating an almost mono-disperse pore size distribution. Non-specific adsorption was attempted for several proteins in batch mode and found to be negligible in all cases. For example, 1 ml of Celbeads equilibrated in 5 ml of 160 mg BSA ml^{-1} at pH 7.0 resulted in less than 30 μg BSA adsorbed per millilitre of Celbeads.

Experimental (in 10 mm diameter column) and reported (in 250 mm diameter column) bed expansion for Streamline DEAE is shown in Fig. 3 plotted as degree of bed expansion against flow-rate, where the degree of bed expansion is defined as ratio of expanded bed height to zero velocity static bed height [1]. The bed expansion behaviour of Streamline DEAE can be seen to be same in the two columns. This is in accordance with the generally accepted rule that the wall effect on bed expansion behaviour is negligible so long as the column diameter to particle diameter ratio is more than 20 [16].

Thommes et al. [17] have stated that for negligible effect of walls on residence time distribution the ratio should be more than 100. Experiments were carried out with wire mesh as flow distributor and also with the standard porous polymer distributor from BioRad, to record breakthrough curves of BSA under non-retaining conditions in packed and expanded bed modes on the 10 mm diameter column. Fig. 4 shows the breakthrough curves for BSA on Streamline DEAE under non-retaining conditions obtained with the two distributors at a linear flow velocity of 2.5 cm min^{-1} on the column in packed and expanded bed modes. It can be seen that breakthrough profile in both cases was independent of the flow distributor and that the wire mesh and the porous polymer distributor gave identical residence time distribution. Similar runs were also made with tyrosine and the data compared to that reported by Karau et al. [18] who quantified residence time distribution in terms of Bodenstein number, *Bo* ($u_0.L/D_a$, where u_0 is the superficial velocity, L the bed height and D_a the axial dispersion coefficient) obtained by acetone pulse injections in a 20 mm diameter column filled with Streamline DEAE. On a 14 cm settled bed height column the reported *Bo* was about 80 in the velocity range of 3–8 cm min^{-1}. This value was found comparable to the *Bo* value of about 67 obtained in this work on a settled Streamline DEAE bed of 11 cm in the 10 mm diameter column with tyrosine at a velocity of 2.5 cm min^{-1}. While use of Bodenstein number in cases involving pore diffusion effects is erroneous, the comparison

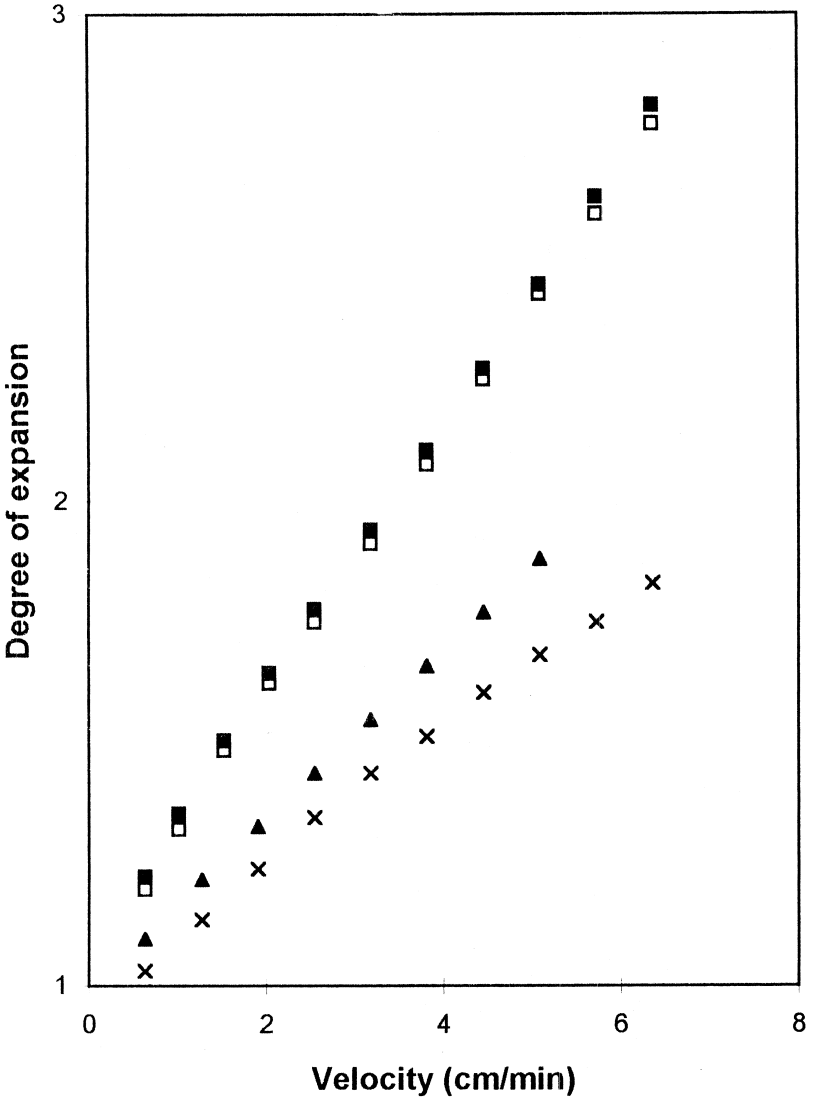

Fig. 3. Bed expansion characteristics in distilled water: ■ Streamline DEAE (reported); □ Streamline DEAE (measured in 10 mm glass column); ▲ Celbeads; × Celbeads-CB.

nevertheless indicates that a 10 mm diameter column gives same residence time distribution as a 20 mm diameter column at a given mobile phase velocity. Thus both, bed expansion and residence time distribution experiments proved that the 10 mm diameter column designed and employed in the work could function satisfactorily as a true expanded bed column.

Fig. 3 also includes bed expansion data with Celbeads and Cibacron Blue immobilized Celbeads (Celbeads-CB). Both gave particulate and stable expansion behaviour that fitted the Richardson-Zaki relation. The degree of expansion for Celbeads at a given velocity is lower than that of Streamline DEAE on account of higher bulk density of Celbeads (~1600 $kg\,m^{-3}$). Reported density of Streamline DEAE is 1200 $kg\,m^{-3}$. Higher bulk density implies that higher flow-rates are required for bed expansion, and while higher velocities adversely affect performance of gel type or microporous matrices, it is

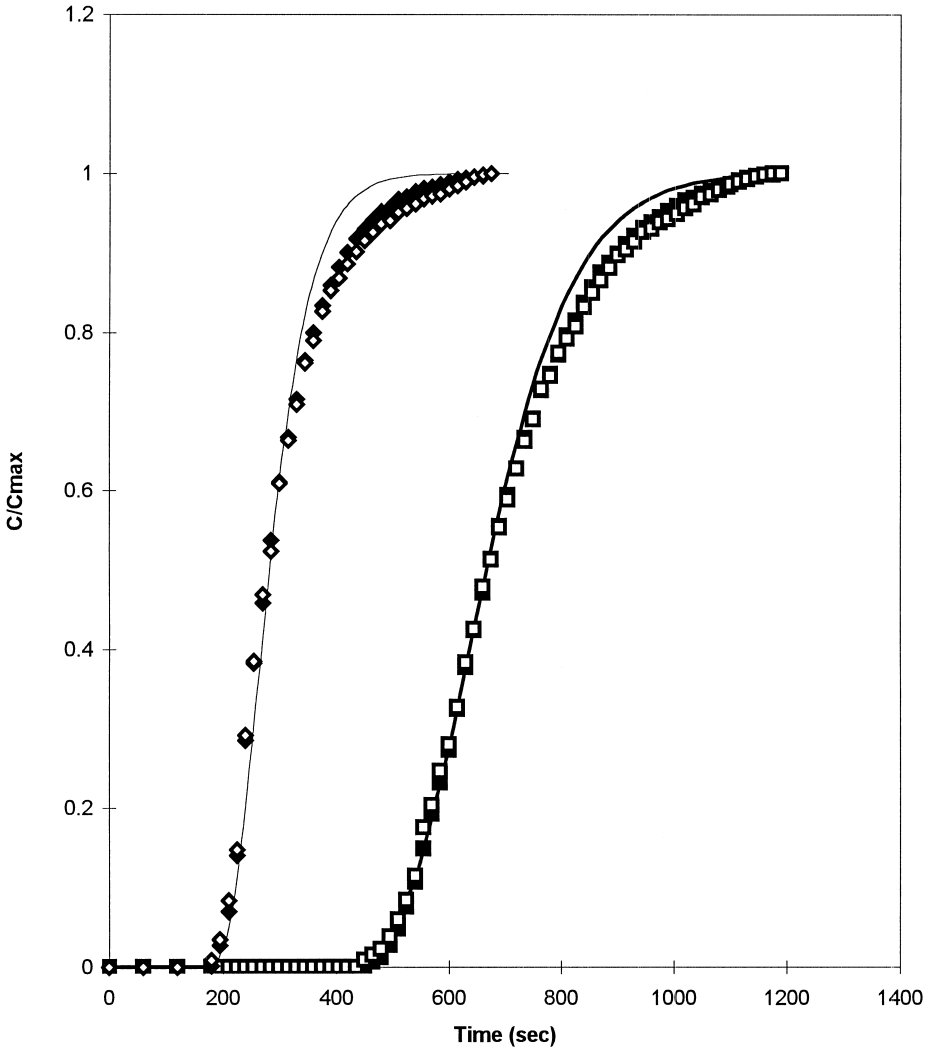

Fig. 4. Breakthrough curves for BSA on Streamline DEAE under non-retaining conditions at 2.5 cm min^{-1} in 50 m*M* Tris–HCl buffer pH 7.0 containing 1 *M* NaCl: ◇ packed bed mode, porous polymer distributor; ♦ packed bed mode, steel wire mesh; □ expanded bed mode, porous polymer distributor; ■ expanded bed mode, steel wire mesh. Continuous lines show predicted breakthrough curves for respective cases with wire mesh flow distributors.

shown in this work that performance of a macroporous matrix does not get seriously affected with increasing velocity.

Cibacron Blue was immobilized on Celbeads using protocol applicable to adsorbents with surface hydroxyl groups. It was found that the dye could be satisfactorily immobilized and the prepared Celbeads-CB had a dye loading of 7.4 μmol CB ml^{-1} beads resulting from the procedure that used 10 mg CB loaded per millilitre of settled adsorbent. The dye bound well to the matrix and no leakage was detected during elution or cleaning procedure. Maximum capacity of the prepared Celbeads-CB for LDH from muscle crude was found to be 120 U ml^{-1} absorbent when equilibrated with undiluted broth.

6.2. HETP analysis

As stated earlier in the Experimental section, HETP values calculated from the finite pulse in-

jections was found to be independent of sample volume from 0.5 to 4 ml. The accuracy of the method was tested by estimation of HETP for Streamline DEAE under non-retaining conditions for BSA and using the HETP values to predict the breakthrough profiles shown in Fig. 4. The HETP values were found to be 1.3 cm and 8 cm for packed and expanded bed respectively. The predicted breakthrough profiles are shown as continuous lines in Fig. 4. It can be seen that there is reasonable agreement between the predicted and experimental curves for both packed and expanded bed runs. Fig. 5 shows variation in HETP with velocity for packed bed runs on Celbeads under non-retaining conditions for the three different molecules. The plot was identical for all three molecules and HETP was found to increase with velocity till a critical value of 1.5 cm min^{-1} and then levelled off to an $H_{plateau}$ of about 0.5 cm. This variation of HETP with velocity is reported to be typical of macroporous adsorbents and the critical velocity, u_{oc} is the velocity at which convective flow begins in the macropores [8]. Vega et al. [19] reported u_{oc} for POROS Q/M macroporous adsorbent from Perseptive Biosystems, UK (0.8 μm average pore size) to be about 15 cm min^{-1} which is ten times higher than obtained with Celbeads. Celbeads can therefore be expected to give lower HETP values at high flow-rates, than possible with gel

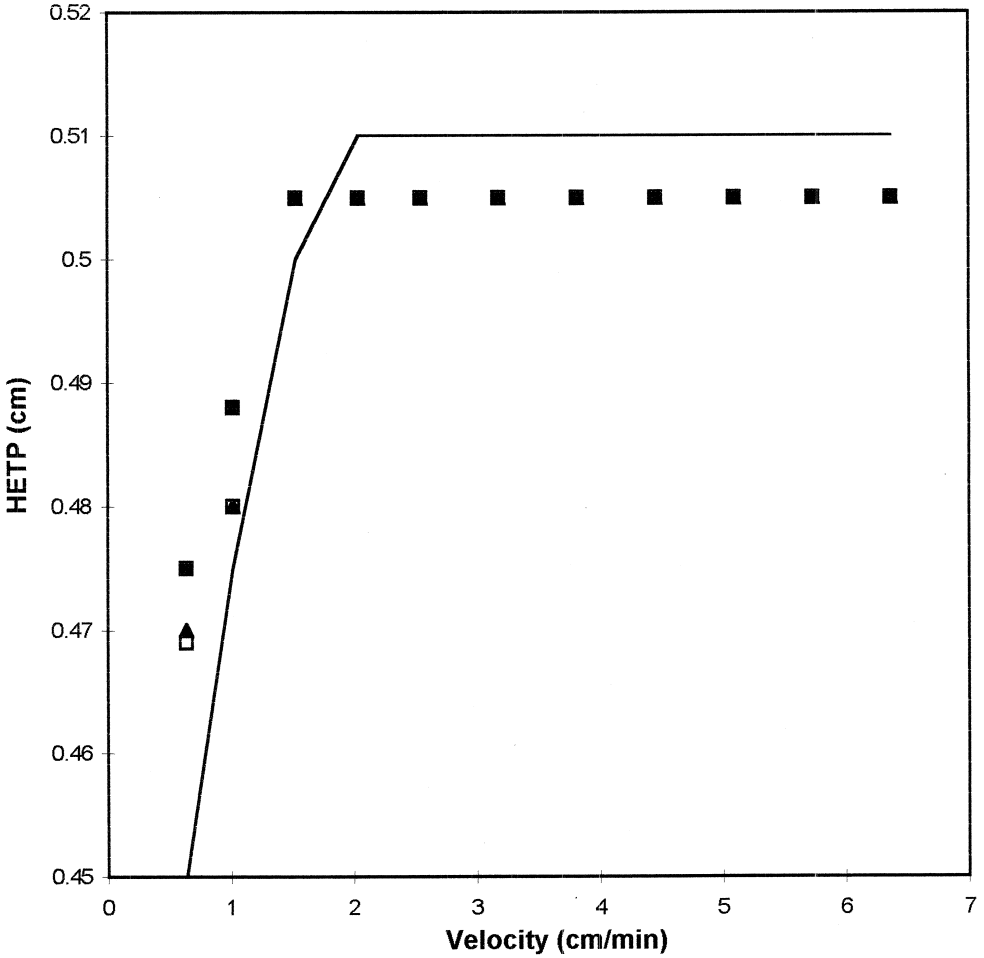

Fig. 5. Height equivalent to theoretical plate variation with superficial velocity in packed bed mode on Celbeads under non-retaining conditions: ■ tyrosine; □ papain; ▲ BSA. Solid line shows the predicted HETP for BSA.

adsorbents or adsorbents with small pores. Fig. 6 depicts HETP variation with velocity obtained in expanded bed for the three molecules BSA, papain and tyrosine on Celbeads under non-retaining conditions. Expanded bed operation showed an HETP behaviour similar to that in packed bed except that u_{oc} shifted to a higher value (3.7 cm min^{-1}) and the average $H_{plateau}$ increased from about 0.5 cm to 3.0 cm.

The height equivalent to theoretical plate (HETP) for a chromatographic column packed with macroporous particles was related to system and operation parameters by Rodrigues et al. [10] using a modified form of Van Deemter equation

$$H = A + \frac{B}{u_0} + Cf(\lambda)u_0 \quad (3)$$

wherein $f(\lambda)$ is a function of λ, the intraparticle Peclet number defined as

$$\lambda = \frac{v_0 l}{D_e} \quad (4)$$

where v_0 is the convective velocity inside the pores and l is the half thickness of a slab particle. The function $f(\lambda)$ accounts for the intraparticle transport by virtue of enhanced effective diffusivity $\tilde{D}_e$ in macroporous particles which was related to the plain

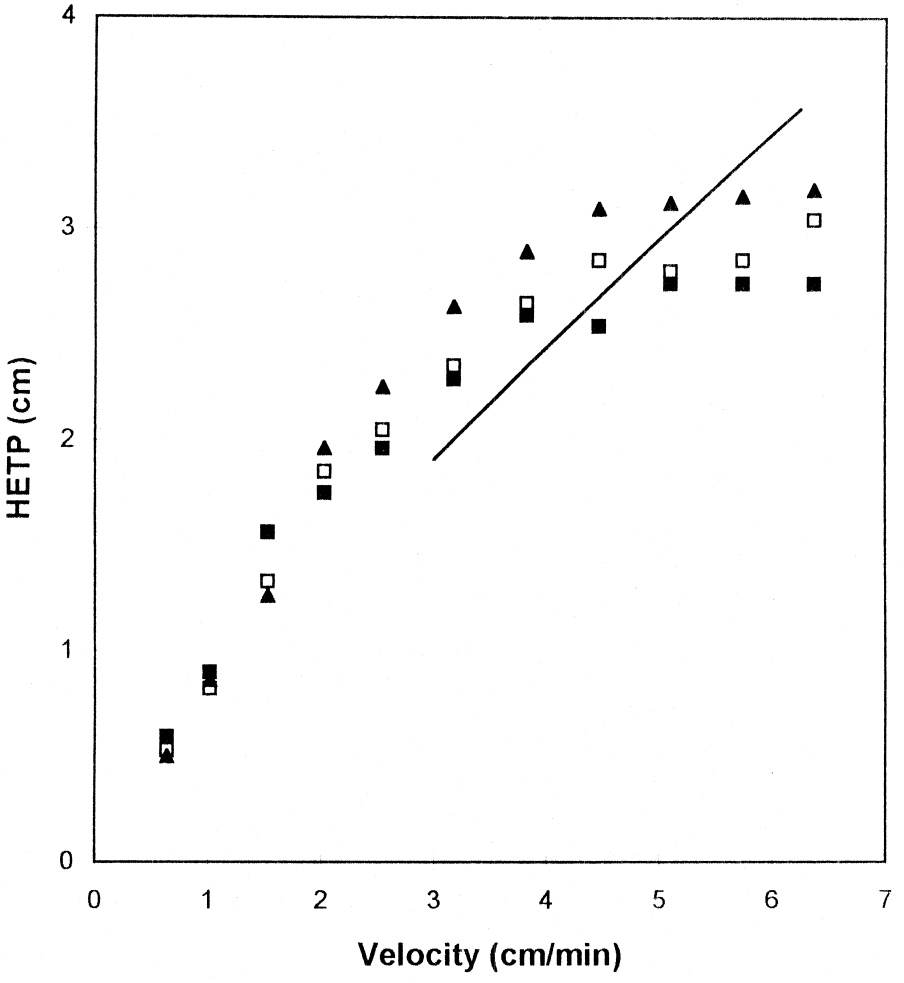

Fig. 6. Height equivalent to theoretical plate variation with superficial velocity in expanded bed mode on Celbeads under non-retaining conditions: ■ tyrosine; □ papain; ▲ BSA. Solid line is the predicted HETP.

effective pore diffusivity $D_{e,}$ by Rodrigues et al. [20] as

$$\tilde{D}_e = D_e \cdot \frac{1}{f(\lambda)} \quad (5)$$

The function $f(\lambda)$ was derived and is given as

$$f(\lambda) = \frac{3}{\lambda} \cdot \left(\frac{1}{\tanh \lambda} - \frac{1}{\lambda} \right) \quad (6)$$

At low flow-rates, when there is no intraparticle flow, λ is small and $f(\lambda)$ is unity and the modified Van Deemter equation transforms to the classical Van Deemter equation. At high flow-rates when the intraparticle flow is finite, λ is high and $f(\lambda) = 3/\lambda$. For macroporous particles facilitating intraparticle flow, HETP was shown by Rodrigues et al. [21] to be given by the following relation

$$H = 2d_p + \frac{1}{30} \cdot \frac{v}{(1+v)^2} \cdot \frac{\epsilon_p}{\epsilon_b} \cdot \frac{d_p}{D_e} \cdot f(\lambda) u_0 \quad (7)$$

where ϵ_b is the bed porosity, ϵ_p the intraparticle porosity, d_p the particle diameter, D_e the intraparticle effective diffusivity, and v is defined as

$$v = \frac{1-\epsilon_b}{\epsilon_b} \cdot \epsilon_p$$

Eq. (7) at high flow-rate (when HETP becomes constant) was shown to result into the following relation for plateau HETP

$$H_{plateau} = 2d_p + \frac{3}{5} \cdot \frac{v}{(1+v^2)} \cdot \frac{\epsilon_p}{\epsilon_b} \cdot \frac{B_b}{B_p} \cdot d_p \quad (8)$$

where B_p is the particle permeability and B_b the bed permeability defined by

$$\frac{-\Delta P}{L} = \frac{\eta}{B_b} \cdot u_0 \quad (9)$$

where $\Delta P/L$ is the pressure drop across the bed and η is the fluid viscosity.

The bed permeability was estimated from the slope of the plot of pressure drop against velocity for Celbeads (Fig. 7). The average B_b was found to be $4.0 \cdot 10^{-8}$ cm ^{2}. Using the B_b calculated, the experimental $H_{plateau}$ value of 0.5 cm, and ϵ_p and ϵ_b values of 0.58 and 0.25 respectively, the particle permeability B_p for Celbeads was calculated from Eq. (8) and was $2.03 \cdot 10^{-9}$ cm ^{2} which is about 100 times higher compared to reported particle permeability of $1.5 \cdot 10^{-11}$ cm^2 for Poros Q/M [21]. The value of the effective diffusivity D_e for Celbeads was calculated from the initial slope of the plot of HETP vs. velocity for packed bed (Fig. 5). The calculated value of D_e was found to be $1.35 \cdot 10^{-6}$ cm^2 s^{-1} which being almost the molecular diffusivity for BSA means that even in low velocity region the Celbeads provides no extra pore diffusional resistance on account of decreased $D_{e.}$

In the present experiments HETP in expanded bed was noted to be higher than in packed bed at same velocities. This can be explained on the basis of higher axial dispersion and lower intraparticle velocities in expanded bed. Pressure drop across an expanded bed is equal to the mass of the bed and given by

$$\frac{-\Delta P}{L} = g(1-\epsilon_b)(\rho_p - \rho_l) \quad (10)$$

where g is the gravity, ρ_p the particle density, and ρ_l the liquid density. Since the bed expands with increasing velocity, bed permeability also increases with velocity whereas the particle permeability may not be expected to change. For any given state of bed expansion the bed permeability could be calculated, and using Eq. (9), HETP was calculated for different flow-rates and is plotted as a solid line in Fig. 6. It can be seen that while the order of magnitude of HETP agrees well with that obtained in the experiments, the experimentally observed plateauing of HETP is not predicted. The reason for plateauing of HETP in expanded beds is not apparent and investigation on this aspect is currently in progress.

The role of extra large pores in Celbeads was expected to be also dominant under retaining conditions where the effect of intraparticle interactions term in the Van Deemter equation is more significant. In typical protein chromatography, proteins are first adsorbed and then eluted sequentially by change of pH, ionic strength or composition of buffer. The band widths and their resolution under elution conditions determines the efficiency of separation on the column. Experiments were therefore conducted to determine HETP under elution conditions for a protein that was first adsorbed to breakthrough and exhaustion. Fig. 8 shows the HETP variation with velocity obtained on Celbeads-EDA wherein BSA

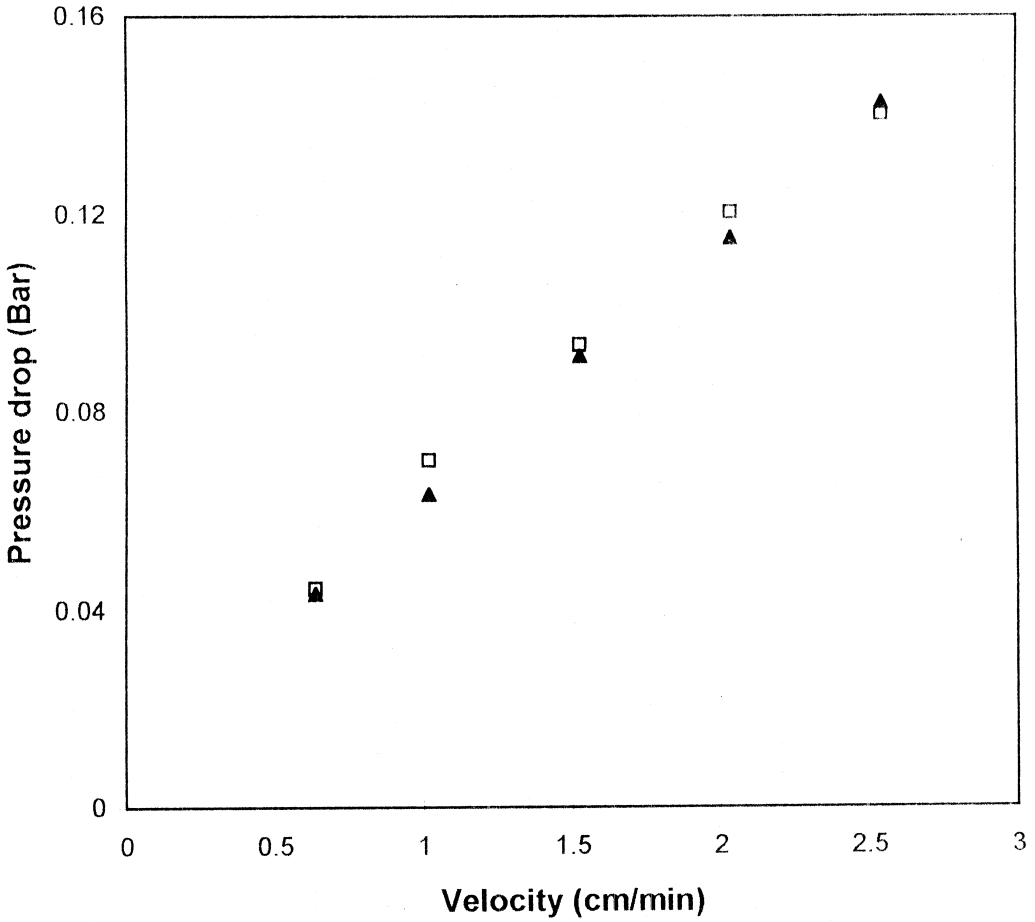

Fig. 7. Pressure drop across 110 mm packed bed as a function of superficial velocity for □ Celbeads; ▲ Glass beads (100 μm).

was loaded, retained and eluted in expanded bed and packed bed modes. Interestingly, both expanded bed and packed bed elutions followed the same pattern of HETP variation and the u_{oc} apparently decreased to about 2 cm min^{-1}. In packed bed case, HETP under adsorption–elution conditions can be seen to be higher than that obtained under non-retaining conditions (Fig. 5). In salt elutions, whereas progressing salt front and high eluting solute concentration lead to sharpening of the band, kinetics of desorption tends to broaden it. The sum total of these effects was therefore observed as moderate increase in HETP for packed bed elution when compared to HETP in non-retaining conditions. The situation for the expanded bed case is however opposite and HETP in adsorption–elution conditions (Fig. 8) was much lower than that in non-retaining conditions (Fig. 6). This result is difficult to explain and is currently under further investigation. There is no data in the literature on HETP for expanded bed elutions since almost all of the reported work on expanded beds uses elution in packed bed mode. Nevertheless, reasonably low values and plateauing of HETP indicates that it should be possible to elute adsorbed proteins in expanded bed mode at high flow-rates without seriously affecting the column efficiency. This was proven by adsorbing lactate dehydrogenase from porcine muscle homogeante on

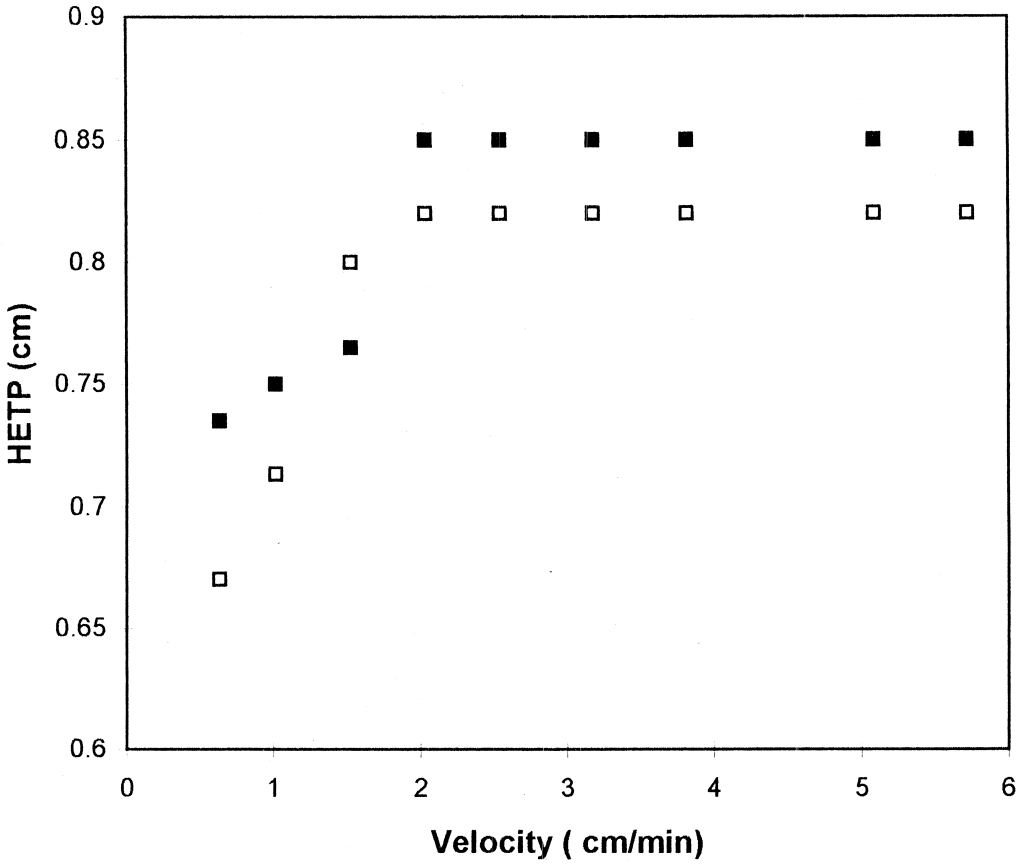

Fig. 8. Height equivalent to theoretical plate variation with superficial velocity in packed bed mode and expanded bed mode for BSA in 50 m*M* Tris–HCl buffer pH 7.0 on Celbeads-EDA under retaining conditions: □ packed bed; ■ expanded mode.

Celbeads-CB in expanded bed and eluting the enzyme in upflow packed bed as well as expanded bed mode at same flow-rate.

6.3. Purification of lactate dehydrogenase

Figs. 9 and 10 present the chromatograms obtained for purification of LDH with loading done in expanded bed mode and elution performed in both packed and expanded bed modes. Table 1 gives details of the performance of the entire operation under packed bed and expanded bed elution conditions. The whole operation of loading, wash and elution took about 60 min in both cases. Expanded bed elution gave a purification factor of 31 while packed bed elution purified the enzyme 26-fold. The chromatograms and eluted activities and protein concentrations indicate that column performance in both expanded and packed bed elution was same in terms of HETP and the eluted band containing LDH in each case comprised of six fractions of 3 ml each. The average HETP value for the operation was calculated to be about 1 cm thus giving 100 plates per meter column height.

The adsorbent after LDH elution, was washed in expanded bed mode at a flow velocity of 0.6 cm min^{-1} with 5 *M* urea solution in the working buffer. This could desorb all the bound protein from the matrix and fully restore the adsorption capacity of Celbeads-CB. The matrix was used five times

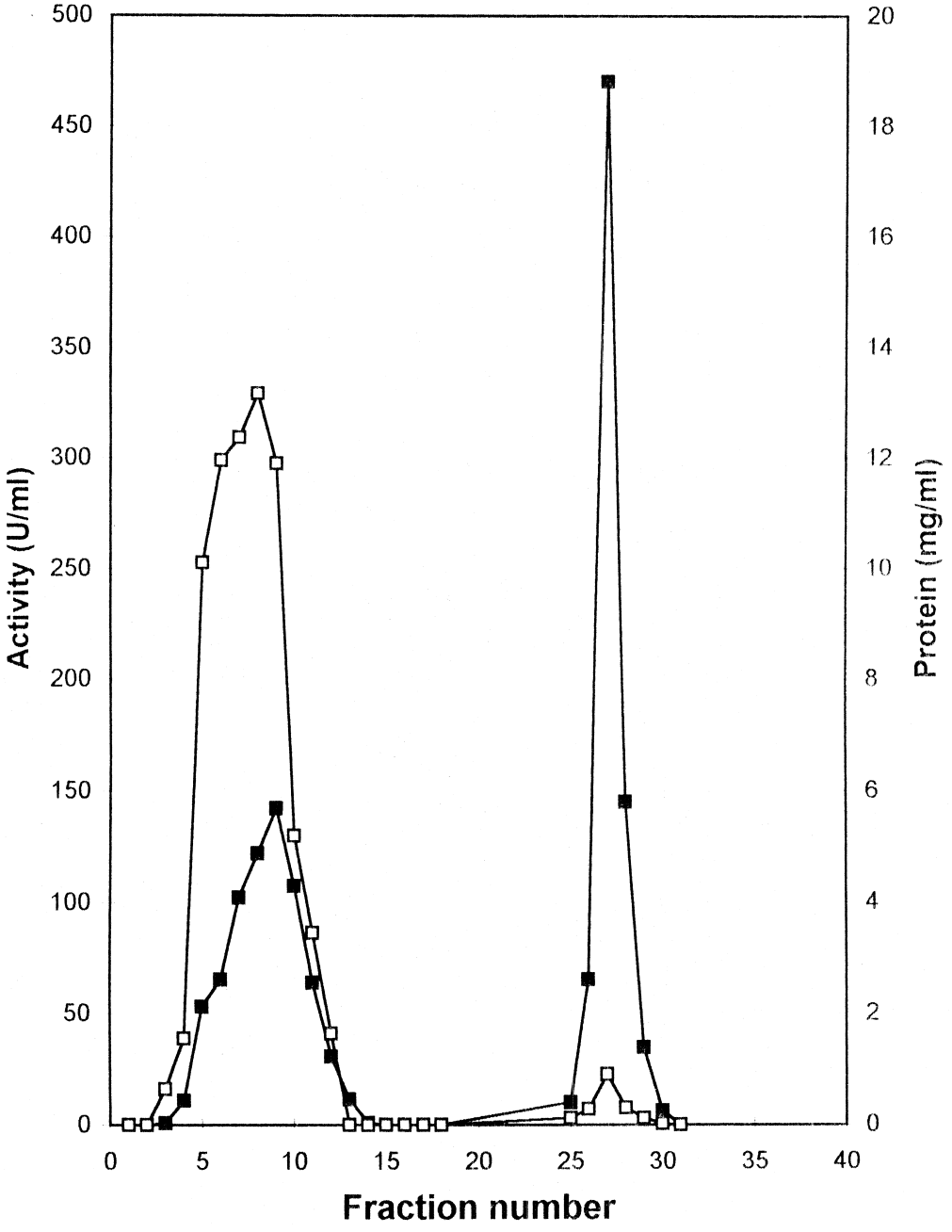

Fig. 9. Chromatography of porcine muscle homogenate on Celbeads-CB. Elution in a packed bed mode: ■ activity U ml^{-1}; □ protein content mg ml^{-1}.

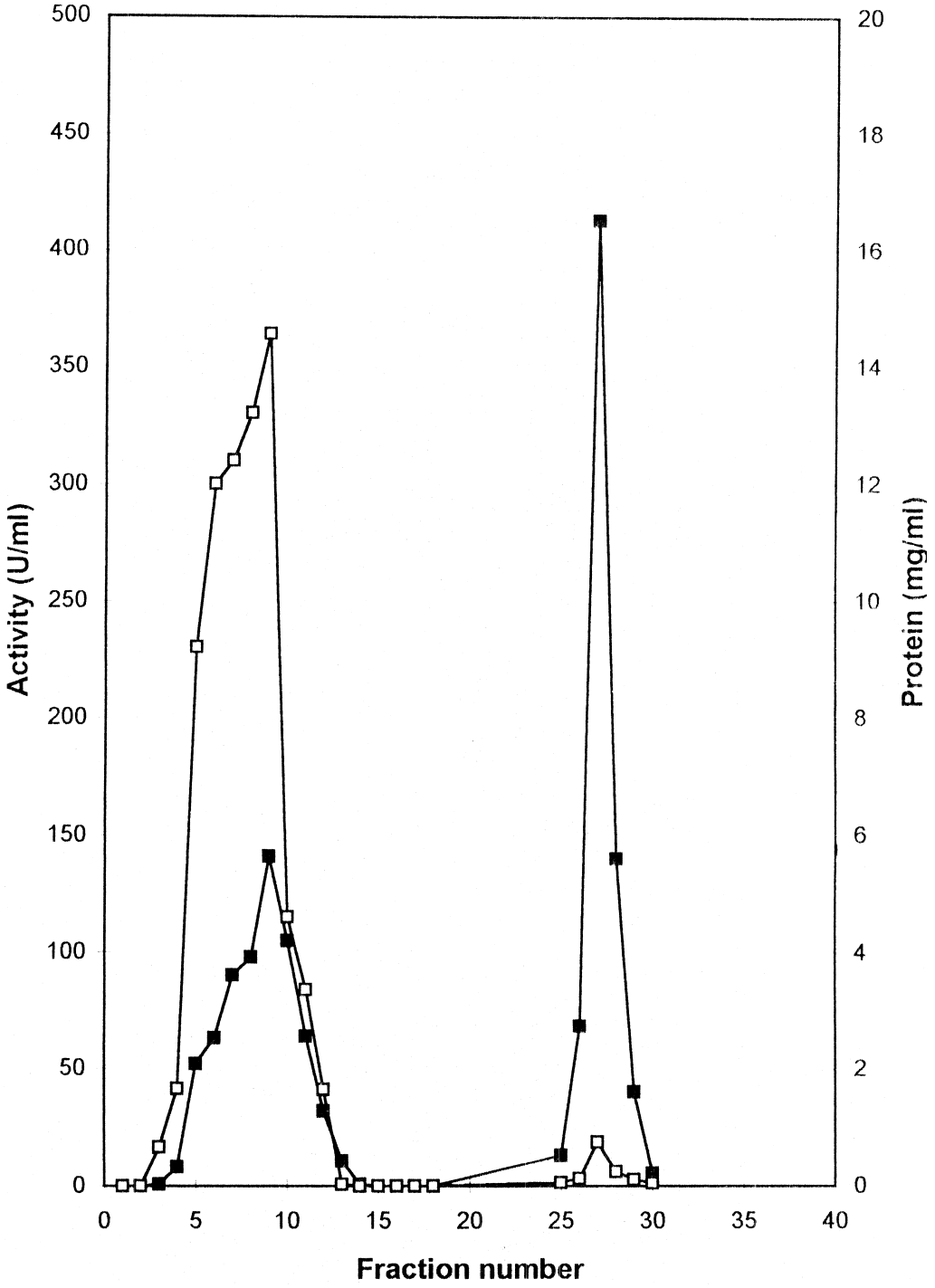

Fig. 10. Chromatography of porcine muscle homogenate on Celbeads-CB. Elution in an expanded bed mode: ■ activity U ml^{-1}; □ protein content mg ml^{-1}.

Table 1
A typical run for purification of L-lactate dehydrogenase from porcine muscle extract

Data	Packed bed elution	Expanded bed elution
Settled bed height	95 mm	95 mm
Crude specific activity	15.4 U mg^{-1}	15.4 U mg^{-1}
Loading flow-rate	1 ml min^{-1}	1 ml min^{-1}
Expanded bed height	108 mm	108 mm
Total crude loaded	20 ml	20 ml
Total activity loaded	4064.6 U	4064.6 U
Total protein loaded	263.6 mg	263.6 mg
Activity adsorbed	2020	2062
Protein adsorbed	47.6 mg	43.1 mg
Elution flow-rate	1 ml min^{-1}	1 ml min^{-1}
Eluted activity	2205 U	2129.6 U
Eluted protein	5.4 mg	4.4 mg
Eluted specific activity	408.4 U mg^{-1}	484.0 U mg^{-1}
Purification factor	26.5	31.4

without removal from the column and using the 5 *M* cleaning-in-place procedure. There was no loss of performance of the matrix over the five cycles used.

6.4. Dynamic capacity

Table 2 presents the results of dynamic capacity measurement for expanded bed adsorption of BSA on both Streamline DEAE and Celbeads-EDA, at two operating velocities in packed and expanded bed modes. There was a larger reduction in capacity of the Streamline adsorbent at high velocities as compared to Celbeads in both packed bed and expanded bed adsorptions. Thus while there was a 17% reduction in capacity in packed mode at higher velocity for Streamline DEAE, there was a 37% reduction in capacity in expanded mode. However for Celbeads there was a 10% reduction in capacity in packed bed and a 20% reduction in capacity in expanded mode at higher velocity. The prepared matrix therefore showed better dynamic capacity retention with increasing velocity.

Table 2
Influence of flow velocity on breakthrough capacity for BSA on Streamline DEAE and Celbeads

Experimental condition	Flow velocity (cm min^{-1})	Capacity mg ml^{-1}	
		Streamline DEAE	Celbeads
Packed bed	1.53	56.00	0.96
	3.82	46.67	0.86
Expanded bed	1.53	51.15	0.91
	3.82	34.30	0.77

7. Conclusions

The following conclusions are drawn from the presented work:

(a) Celbeads was developed as a rigid, superporous spherical beaded adsorbent with surface hydroxyl groups for protein chromatography. The native adsorbent matrix showed negligible non-specific adsorption and could be used as a near ideal support for affinity chromatography. The matrix could be successfully modified to a low capacity weak anion-exchanger and a dye affinity matrix with immobilized Cibacron Blue. Celbeads showed stable and particulate bed expansion in a 10 mm diameter column and could be used as expanded bed adsorbent matrix for protein purification.

(b) It was seen that a 10 mm diameter column with a bed height to diameter ratio of 11, and equipped with suitable flow adaptors, performed as a true expanded bed with both the commercial adsorbent and Celbeads. A small column of this kind can be effectively used for method scouting using lesser quantities of adsorbent and extract.

(c) Superporous Celbeads when expanded gave performance similar to one observed with such adsorbents in packed beds where HETP first increases and then levels off with increasing mobile phase velocity. Calculations based on existing theories for superporous adsorbents indicated that Celbeads contained large pores and provided negligible added pore diffusion resistance in both packed and expanded bed modes at all velocities used. Further, HETP on Celbeads under similar conditions was independent of solute molecular mass up to 66 000.

(d) The observed plate height, HETP, on Celbeads was same for expanded and packed bed elutions and remained constant with increasing velocity. More than 100 theoretical plates were possible per meter of both packed and expanded bed of Celbeads at all flow velocities in the range 0.6 to 6.4 cm min^{-1}. Celbeads-CB as a dye affinity adsorbent was used to

purify lactate dehydrogenase from porcine muscle homogenate using expanded bed adsorption. Enzyme elution carried out at high velocity in both packed bed and expanded bed modes gave almost identical performance. It was also noted that macroporous adsorbent resulted in a lesser loss of dynamic adsorption capacity with increasing velocity as compared to gel type adsorbents.

(e) There are two observations noted in this work that need explanations and for which further work is warranted. It is not clear why should there be a plateauing of HETP with velocity in expanded beds of macroporous beads. Secondly, a lower value of HETP with Celbeads under adsorption–elution conditions than in non-retaining conditions in expanded bed needs logical explanation. Work is underway to find solutions to these problems.

8. Symbols

A	eddy diffusion term in the Van Deemter equation, cm
B	longitudinal diffusion term in the van Deemter equation, cm ^{2} s^{-1}
B_b	bed permeability, cm^2
B_p	particle permeability, cm^2
C	particle interaction term in the Van Deemter equation, s
d_p	particle diameter, cm
D_e	effective diffusivity, cm^2 s^{-1}
$\tilde{D}_e$	augmented diffusivity, cm^2 s^{-1}
H	height equivalent to a theoretical plate, HETP, cm
l	half thickness of the slab, cm
L	bed length, cm
N	plate number
ΔP	bed pressure drop, bar
t_R	retention time, min
u_0	flow velocity, cm s^{-1}
u_{oc}	critical velocity, cm s^{-1}
v_0	intraparticle convective velocity, cm s^{-1}
$w_{1/2}$	peak width at half the peak height, min

Greek symbols

ϵ_b	bed porosity
ϵ_p	particle porosity
λ	Intraparticle Peclet number
ρ_p	particle density
ρ_l	liquid density

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