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Biochemical separations by continuous-bed chromatography

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Abstract

Innovations in column-packing media for biomolecule purification have progressed from large spherical, porous polysaccharide beads to advanced polymeric supports. Continuous-bed technology is a radical new technology for chromatography based on the polymerization of advanced monomers and ionomers directly in the chromatographic column. The polymer chains form aggregates which coalesce into a dense, homogeneous network of interconnected nodules consisting of microparticles with an average diameter of 3000 Å. The voids or channels between the nodules are large enough to permit a high hydrodynamic flow. Due to the high cross-linking of the polymer matrix, the surface of each nodule is nonporous yet the polymeric microparticles provide a very large surface area for high binding capacity. This paper will demonstrate the properties and advantages of using a continuous bed support for high resolution biomolecule separations at high flow-rates without sacrificing capacity.

Keywords: Continuous beds; Stationary phases, LC; Proteins

1. Introduction

Over the past two decades, most laboratory- and process-scale chromatographic separations of proteins have been performed using macroporous beads of silica and agarose as well as with beads produced from polymers such as methacrylate and polystyrene divinylbenzene [1]. For polymeric supports, the multistep production process is time-consuming as it involves the polymerization itself, sizing of the beads, and derivatization of the beads. Once the beads have been produced, they must be packed into columns in a manner providing the maximum number of theoretical plates plus excellent peak symmetry. Given such a multistep process for column production, some batch-to-batch variation in column performance must be expected.

Our search for a new type of media offering the best chromatographic has resulted in the development of continuous bed chromatography. The patented continuous bed chromatography technology was invented by Hjertén at the University of Uppsala in Sweden [2–4]. The continuous bed matrix is comprised of a polymeric monolith that is formed in a chromatographic column tube. This eliminates the extra step of column packing. The polymerization conditions are optimized so as to create no pores but the exposed surfaces are highly convoluted resulting in high surface areas for high protein binding. The ionic ligand is incorporated into the polymer during reaction as a reactive monomer. Columns based on continuous bed technology overcome many of the

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problems associated with traditional beaded supports by providing high resolution at high flow-rates and high binding capacity.

This paper describes some of the features of a new line of ion-exchange columns (UNO columns) based on continuous bed technology.

2. Experimental

2.1. Materials

The experiments were performed using the BioLogic HR or HRLC 5000 HPLC chromatography systems (Bio-Rad Labs., Hercules CA, USA). Bovine IgG, lysozyme, chicken egg ovalbumin, adenosine, adenosine 5′-monophosphate, adenosine 5′-diphosphate adenosine 5′-triphosphate, were purchased from Sigma. UNO Q1 (quaternary amine) and S1 (sulfonic acid), 3.5×0.7-cm, continuous bed columns were purchased from Bio-Rad Labs. The continuous bed matrix was prepared similar to that described in Ref. [3]. Mono Q column was purchased from Pharmacia (Piscataway, NJ, USA).

2.2. Scanning electron microscopy (SEM) and mercury porosimetry sample preparation

Continuous bed column was washed with 10 column volumes of deionized water followed by 5 column volumes of methanol. The polymer was removed from the column tube and dried under vacuum at 60°C for 2 h. The gel was sputtered with gold and examined using a model SX-40 scanning electron microscope (Topcon). Mercury porosimetry analysis was performed by Micromeritics (Norcross, GA, USA).

3. Results and discussion

In a departure from traditional column manufacturing, the UNO chromatographic bed is formed directly in the glass tube. The reactive solution contains a mixture of monomer, cross-linker, ionomers and initiator is polymerized within the column tube to form a nonwater-soluble polymer. Both anion and cation-exchangers are formed in a similar manner using different ionomers.

The resulting polymer forms a dense network of polymer bundles which are highly convoluted. This provides a large surface area for effective protein binding as shown in a scanning electron micrograph of the continuous bed polymer in Fig. 1. The nodules range in diameter between 0.2 and 0.5 μm.

The continuous bed polymer was analyzed by mercury intrusion porosimetry in a dried state. As shown in Fig. 2, the continuous bed matrix is nonporous in nature and has an interstitial distance of 3.0 μm, which roughly corresponds to the interstitial distance of packed 10 μm beads. The surface area of the cation-exchange polymer was 10 m^2/g.

Subsequent to polymerization, the continuous bed is soft and compresses under buffer flow. To access high flow velocities typically used for biochromatography, the bed is mechanically compressed between 30–60% of the initial polymer bed height. The amount of compression required is based on the desired maximum flow-rate, polymer formulation, buffer viscosity, and column aspect ratio. The UNO columns were optimized for use with medium pressure chromatography systems and have the pressure-flow characteristics shown in Fig. 3.

Column efficiency is primarily controlled by the size of the packing particles, viscosity of the buffer, length of the column and flow-rate. Typically, the efficiency of a column is demonstrated using a relationship between plate height and flow-rate. Column matrices that promote fast solute mass transfer (e.g. nonporous supports) exhibit constant

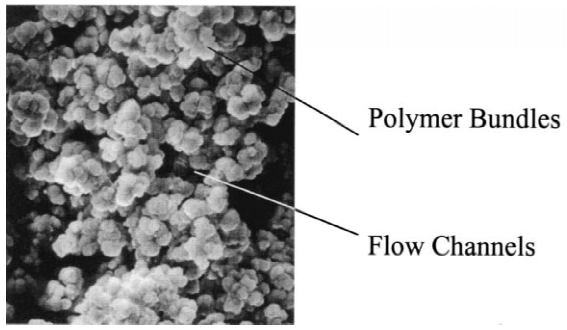

Fig. 1. Scanning electron microscope of continuous bed anion-exchange gel. Magnification is 1.95 kV. Average nodule size is 0.5 μm.

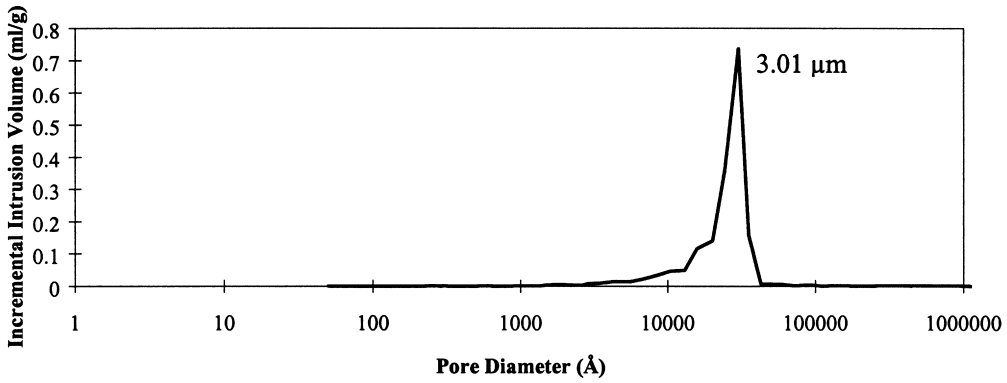

Fig. 2. Mercury porosimetry analysis of dried continuous bed polymer. The mean channel diameter was 3.01 μm.

plate heights above the diffusional flow-rate range. As shown in Fig. 4, continuous bed Q and S columns, exhibit fast mass-transfer properties through the linear velocity range of 200–1000 cm/h for ovalbumin and lysozyme, respectively.

There are many methods for determining the protein capacity of a given support. The actual method used is dependent on the type of result required. Frontal adsorption analysis determines the dynamic protein capacity of a support under flow conditions. If fast mass transfer is occurring, then the breakthrough point for a given protein will be constant independent of flow-rate. If slow mass transfer is occurring, then the breakthrough point will occur earlier as the flow-rate is increased. The continuous bed S1 column exhibits fast mass transfer and had a constant 10% breakthrough capacity of 41 mg IgG throughout 0.5 to 9 ml/min as shown in Fig. 5.

Functional column capacity can be measured by injecting increasing amounts of a complex mixture of protein and determining where overload conditions start to dramatically affect resolution [5]. The complex mixture capacity is typically lower than the dynamic binding capacity for a single protein because the weakly charged proteins are prematurely displaced from the ligand by strongly charged proteins. As shown in Fig. 6, up to 20 mg of yeast enzyme extract can be injected onto the S1 column with little loss in resolution.

With traditional column packing, the retention time reproducibility within a batch is difficult to maintain since each column is packed individually. Continuous bed columns are simultaneously polymerized providing very high reproducibility as shown in Fig. 7.

Batch-to-batch reproducibility during column manufacturing is maintained since each batch of the

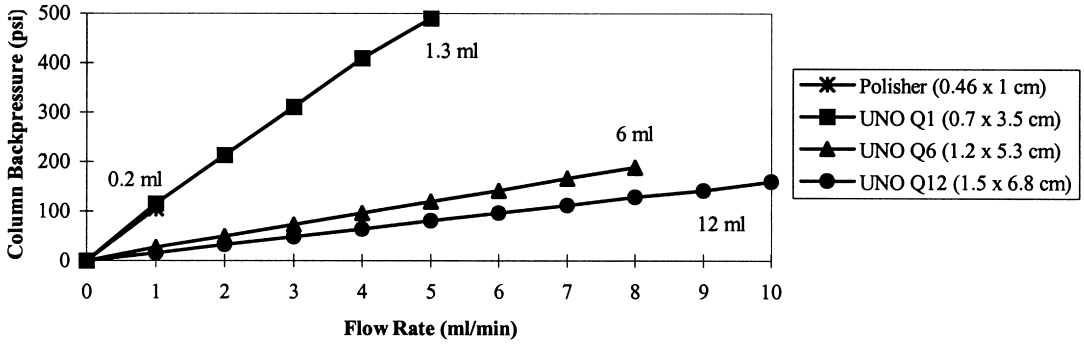

Fig. 3. Pressure/flow performance of continuous bed anion-exchange columns in 20 m*M* Tris (pH 8.1).

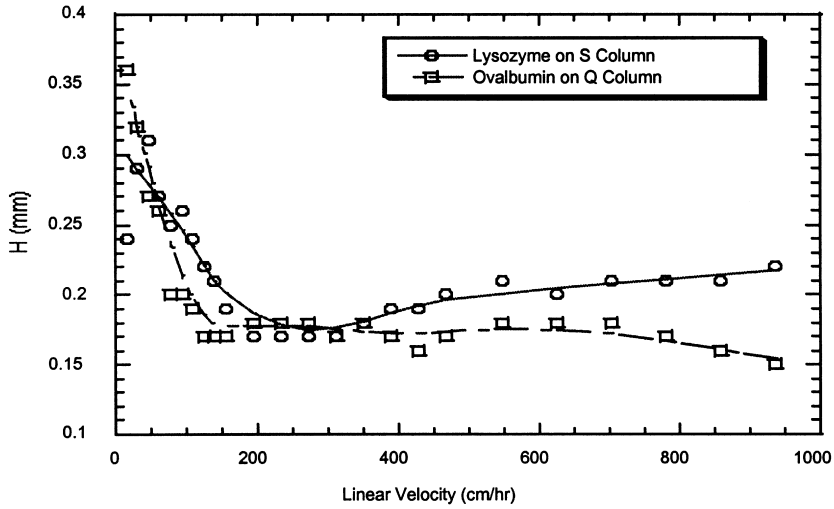

Fig. 4. Effect of flow-rate on plate height for ovalbumin and lysozyme on continuous bed Q1 (3.5×0.7 cm) and S1 columns (3.5×0.7 cm), respectively. The injection size was 20 μl of a 1 mg/ml solution. The buffer for the Q1 column was 20 m*M* Tris+1.0 *M* NaCl, pH 8.0 and for the S1 column was 50 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES)+0.5 *M* NaCl, pH 6.5.

mother liquor is prepared prior to addition to the column tubes. Twenty-three UNO Q1 production batches resulted in retention time R.S.D.s for adenosine, AMP, ADP and ATP of 1.30%, 1.30%, 0.90% and 0.77%, respectively.

Crude protein samples can foul columns and reduce performance by reducing resolution and increasing back pressure. Some performance can be recovered by washing the column with sodium hydroxide. However, some column chemistries are not compatible with long-term base exposure due to hydrolysis of the ligand from the support. A Q1 continuous bed column was exposed to 0.01 *M* NaOH for 45 h and the effect on retention time for a

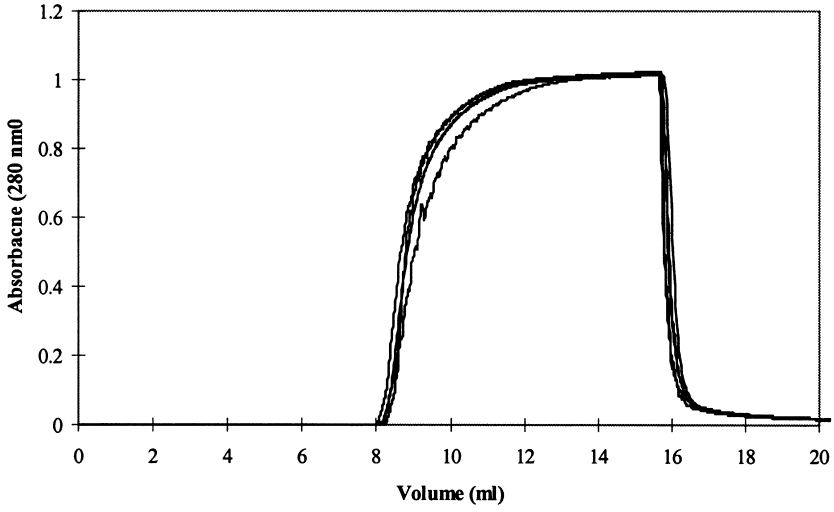

Fig. 5. Breakthrough profiles for bovine IgG on continuous bed S1 column in 50 m*M* sodium acetate, pH 5.0, at 0.5 ml/min (78 cm/h), 1 ml/min (156 cm/h), 3 ml/min (468 cm/h), 6 ml/min (936 cm/h) and 9 ml/min (1404 cm/h).

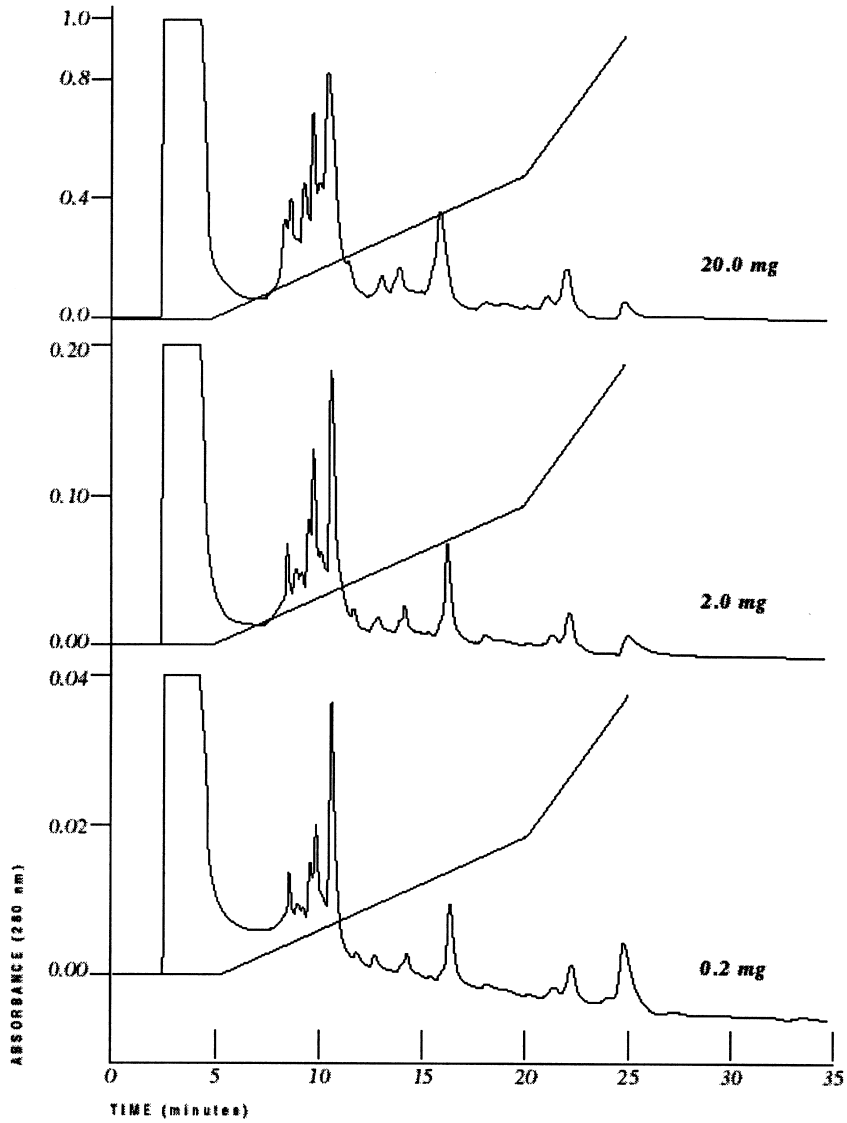

Fig. 6. Comparison of loading yeast enzyme extract on continuous bed S1 column. Buffer A: 50 m*M* sodium phosphate, pH 7.0; buffer B: A+1 *M* NaCl; gradient 0% B for 5 min, 0–35% B in 15 ml, 35–100% B in 15 min, flow-rate 1 ml/min.

Table 1
The effect of exposure to 0.01 *M* NaOH on the retention times for proteins on the UNO Q1 column

Time exposure to 10 m*M* NaOH (h)	Volume of 10 m*M* NaOH exposure (ml)	Retention time (min) (% change)			
		Myoglobin	Conalbumin	Ovalbumin	STI
0	0	0.64	2.31	2.98	3.65
4	1000	0.66 (0%)	2.32 (0%)	2.92 (−2%)	3.56 (−2%)
45	8800	0.64 (−5%)	2.27 (−2%)	2.73 (−8%)	3.33 (−9%)

0.01 *M* NaOH was cycled through the column at 4 ml/min. Column was equilibrated into 20 m*M* Tris, pH 8.1 and the protein standard was separated using a 0–0.5 *M* NaCl gradient in 5 min at 4 ml/min.

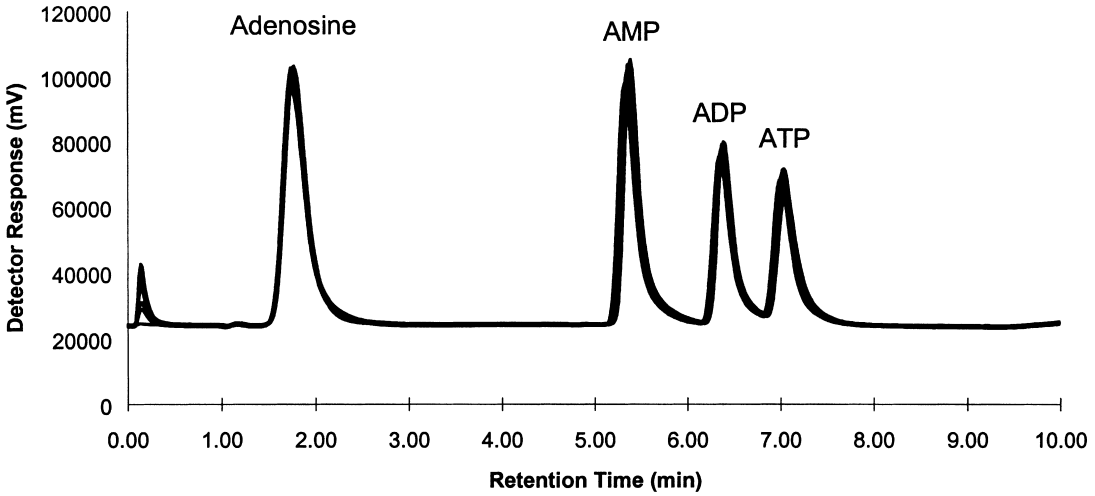

Fig. 7. Retention time comparison of 12 columns from one batch of continuous bed Q1 columns. The R.S.D.s for adenosine, AMP, ADP and ATP is 0.52%, 0.31%, 0.25% and 0.23%, respectively. Buffer A: 20 m*M* Tris, pH 8.2; buffer B: A+1.0 *M* NaCl; gradient: 0% B for 1 min, 0–30% B in 5 min, 30% B in 2 min, 30–0% B in 2 min. Injection size: 100 μl of 0.5 mg/ml of each component.

mixture of purified proteins was evaluated (Table 1). In the worst case, only a 9% decrease in retention time was observed after exposure to 8000 column volumes of 0.01 *M* NaOH.

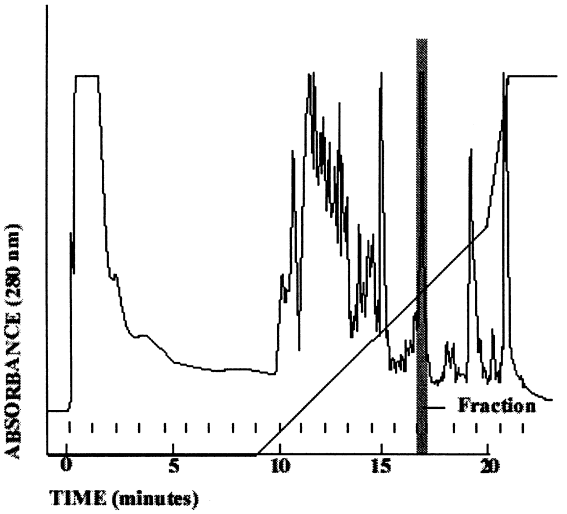

Fig. 8. Separation of *Xenopus laevis* oocytes extract on UNO Q1 column. Load: *Xenopus laevis* oocytes 40–50% $NH_4(SO_4)_2$ fraction, 6 mg protein; buffer A: 50 m*M* Tris, 2 m*M* EDTA, 1 m*M* dithiothreitol (DTT), pH 7.4; buffer B: A+1 *M* NaCl, pH 7.4; gradient: 0–60% B in 50 ml; flow-rate: 4.5 ml/min; absorbance at 280 nm.

4. High-resolution separations

Continuous bed columns exhibit high-resolution separations with complex samples as shown in Fig. 8. The sample was a preprocessed extract from *Xenopus laevis* oocytes containing two elongation factor complexes, p30–p47 and p30–p36–p47 that were separated on an UNO Q1 column at 4.5 ml/min [6]. Fig. 9 shows the sodium dodecyl sulfate–polyacrylamide gel electrophoretic (SDS–PAGE) analysis of the collected fractions and lanes 14 and

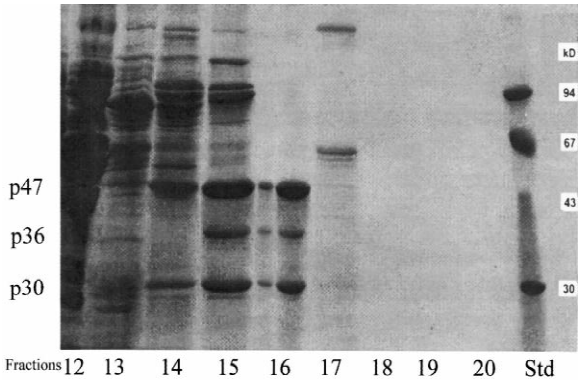

Fig. 9. SDS–PAGE gel of UNO Q1 fractions from *Xenopus laevis* oocytes extract. Fraction 14 contains the p30–p47 complex and fraction 16 contains the p30–p36–p47 complex.

16 contain, respectively, the p30–p47 and p30–p36–p47 complexes. The traditional purification scheme to separate the complexes required chromatography on a 10-μm monodispersed bead anion-exchange column, a heparin column and a DEAE membrane column. Continuous beds' high selectivity, resolution and flow properties provided rapid fractionation of the two elongation factor complexes on a single column in one step.

5. Protein activity vs. flow-rate

Phosphoinositide 3-kinase has proven difficult to purify in an active form when overexpressed in various expression systems [7]. Consequently, for continued biochemical studies of the phosphoinositide pathway, a routine purification protocol for phosphoinositide 3-kinase from rat liver has been established. The protocol involved multiple steps including size exclusion, hydroxyapatite and ion-exchange chromatography.

Fig. 10 shows chromatograms illustrating the use of an UNO Q1 column to partially purify a cytosolic phosphoinositide 3-kinase from a rat liver homogenate at 0.5 and 2.5 ml/min with no loss in resolution and protein activity.

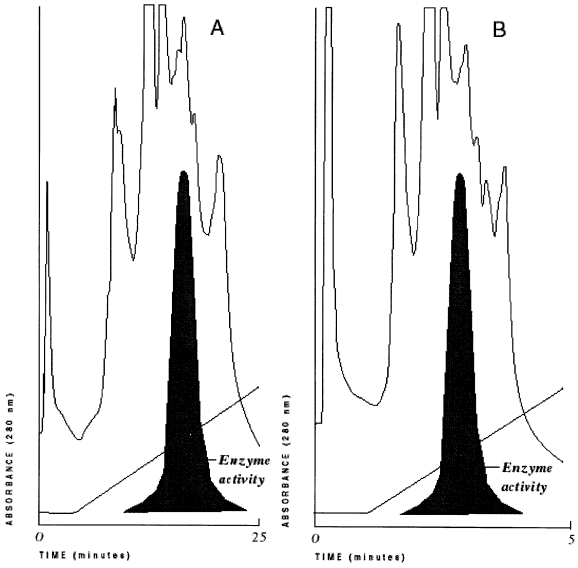

Fig. 10. Separation of cytosolic phosphoinositide 3-kinase from a rat liver homogenate on 10-μm monodispersed bead column at 0.5 ml/min and UNO Q1 at (A) 0.5 and (B) 2.5 ml/min. Load: 15 ml; buffer A: 40 m*M* triethanolamine, pH 7.6; buffer B: A+1 *M* KCl; gradient: 0–35% B over 20 ml; absorbance at 280 nm. Activity assayed by phosphoinositide 3-kinase by enzymatic conversion of phosphoinositide to phosphoinositide 3-phosphate.

6. Conclusions

Continuous bed chromatography matrix, supplied as the UNO Q and S columns, is a new type of media that overcomes some of the problems associated with traditional beaded supports by providing high resolution and good binding capacities at high flow-rates. The unique manufacturing process provides high batch-to-batch reproducibility and enhanced column lifetimes.

Acknowledgements

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