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Immobilized Metal Affinity Membrane Separation: Characteristics of Two Materials of Differing Preparation Chemistries

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ABSTRACT

The focus of this research is the investigation of protein retention by two fabricated membrane modules which differ by a spacing element. Results indicate that both membranes have an equivalent equilibrium protein absorption capacity, but the breakthrough curves differ drastically. The data imply that traditional views regarding ligand accessibility during chromatographic separation merit special attention in a membrane format.

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INTRODUCTION

Due to intrinsic limitations regarding physical characteristics of column packing (compressibility and other factors), separation via traditional affinity chromatography has moved toward systems with height-to-diameter ratios as small as technology permits (1). Such geometric configurations result in increased throughput, since for a given pressure drop the volume of fluid processed is inversely proportional to bed height. If this design strategy were extended ad infinitum, the ideal system would become one of infinitely small height, with a very large area accessible to flow. A method which closely mimics this ideality is a microporous-membrane-based chromatographic system. Several advantages of membrane chromatography systems have been touted, including reductions in process time, lower costs, and reduced diffusional limitations (2). These advantages primarily result from the exploitation of a microporous structure, leading to small diffusion pathways and increased convective transport of material to adsorption sites (3, 4). Membrane chromatography systems for protein recovery have been developed with different adsorption chemistries, including ion exchange, metal affinity, and triazine-based dyes (5). Promising results have been shown with model protein systems and with cellular lysates. Chromatography modules primarily have had dead-end flow conformation (stacks of membrane sheets) (1).

Pertinent to our work is the development of efficient membrane chromatography media with a separation motif based upon metal affinity (immobilized metal affinity membrane separation or IMAMS). Metal affinity is an attractive technique by which proteins may be purified; indeed, immobilized metal affinity chromatography (IMAC) and related techniques have successfully been used to recover target proteins which possess native or genetically enhanced metal binding characteristics (6, 7). Surprisingly, few investigations concerning the relationship between ligand coupling chemistry and protein adsorption have been described for IMAMS, despite the fact that it has been clearly demonstrated that variations in preparation chemistry affect performance characteristics (selectivity, metal ion capacity, protein adsorption, and chromatography) in IMAC (1, 8, 13). In particular, lack of retention for a protein known to interact favorably with IMAC media, low equilibrium capacity despite high ligand content, breakthrough curves that are unexpectedly nonideal, quick protein breakthrough, and inaccurate model predictions have been encountered in previous investigations of IMAMS (10, 11). Various theories to explain these phenenema have been postulated; however, most center about the issue of steric effects (12). Since selectivity toward as little as a single amino acid displayed on the protein surface can be exploited to achieve separation via metal affinity, a basic understanding of ligand acessibility would be of considerable importance in IMAMS (9).

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This paper reports on the preparation of two IMAMS media, and the characterization of their adsorption characteristics. These materials differ by the presence or absence of a model spacing element between the polymeric backbone and the chelating group, iminodiacetic acid (IDA). When charged with Cu(II), both materials adsorb model proteins similarly in equilibrium batch experiments; however, under dynamic conditions of flow their breakthrough characteristics are markedly different.

EXPERIMENTAL

Reagents for functionalization chemistry were primarily obtained from Aldrich Chemical Co. (Milwaukee, WI). Protein samples (hen egg white lysozyme L6876 or α-chymotrypsin C7762) for IMAMS were obtained from Sigma (St. Louis, MO). Microporous sheets with amine functional groups were obtained from Arbor Tech (Ann Arbor, MI). Concentration of protein in solution were determined by one of three methods: absorbance at 280 nm using a DU-640 spectrophotometer (Beckman, Fullerton, CA), high pressure liquid chromatography (HPLC), or size exclusion chromatography (BioRad, Hercules, CA). Elemental analysis was performed by Galbraith Laboratories (Knoxville, TN). IMAMS media Types I and II were prepared via the described chemistries at 60°C.

Membrane Preparation: Type I (No Spacer) and Type II (Spacer)

Type I media (no spacer) were prepared by a single reaction during which IDA chelating groups were prepared from the amine functionalities. Microporous sheets (4.00 g) were soaked in a mixture of chloroacetic acid (0.22 g) and 0.1 M $NaHCO_3$ buffer solution (250 mL). The reaction was allowed to proceed overnight with gentle agitation. After the reaction, the product was washed with Milli-Q grade water (200 mL). To charge the membrane with metal ions, a salt of the appropriate cation (e.g., $CuSO_4$) was dissolved in water (5 mg/mL). The media was then contacted with this solution and agitated for a minimum of 15 minutes. Metal-charged membranes were then rinsed thoroughly with Milli-Q grade water and equilibrated in buffer prior to use.

Type II media (spacer) were prepared in a two-step process which results in the coupling of IDA to the polymeric sheet via bis-oxiraine coupling chemistry. Microporous sheets (4.00 g) were soaked in a mixture of acetonitrile (50 mL) and 1,4-butanediol diglycidyl ether (1.66 mL). The reaction was carried out overnight with slight agitation at room temperature. The product of this first step was then placed in a mixture of 2.0 M $NaHCO_3$ (70 mL) and the disodium salt of iminodiacetic acid (1.52 g) overnight with gentle agitation. The

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membrane was then washed with Milli-Q grade water (200 mL) and charged with metal ions in the same fashion as Type I media.

Batch Protein Adsorption

A known weight of media (Type I or Type II) charged with Cu(II) and equilibrated in buffer (0.05 M $NaPO_4$ + 1.0 M NaCl, pH 7.5) was placed in 5 mL Teflon vials (Cole-Parmer, Niles, IL). Triplicate samples were incubated with varying concentrations of protein predissolved in 1–3 mL buffer with gentle agitation. HPLC size exclusion chromatography was used for analysis of buffer from each sample to determine the amount of bound protein in each sample.

Breakthrough Curve Determination

All breakthrough analyses were performed with a 1.0 mg/mL solution of protein in a 1.0 M NaCl and 0.05 M Na_2HPO_4 buffer at pH 7.5. The apparatus for breakthrough analysis (Fig. 1) consisted of a Teflon feed jar (Fischer Scientific, Pittsburgh, PA), a filter holder (Millipore, Boston MA), a Beckman DU-640 spectrophotometer, a pump (Pharmacia, Piscataway, NJ), a frequency counter (Cole-Parmer, Niles, IL), and a fraction collector. The procedure for performing a breakthrough experiment was the same for both media types and for control membranes with no functionalization. A 47-mm (diameter) circle was stamped from a medium sheet, and placed in the filter holder. After the system was filled with the buffer solution at 0.2 mL/min, the flow rate was increased to 0.5 mL/min. Feed was switched to a protein solution (1.0 mg/mL lysozyme or α-chymotrypsin), and the spectrophotometer was used to

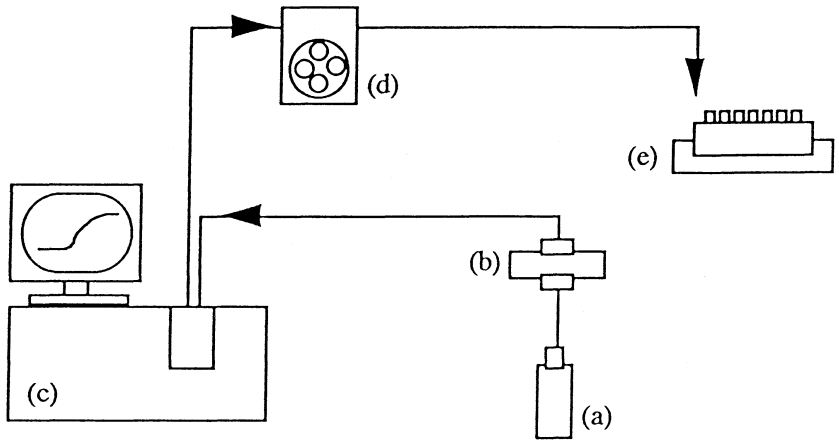

FIG. 1 Apparatus for breakthrough curve determination: (a) feed container, (b) membrane holder, (c) spectrophotometer, (d) pump, and (e) fraction collector.

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measure the downstream protein concentration at 280 nm. Experiments were allowed to run until maximum adsorption of protein onto the membrane was visually evident from the completion of the breakthrough curve.

RESULTS AND DISCUSSION

Metal affinity techniques utilized inexpensive metals bound to a stationary phase or a mass separating agent for protein purification. Techniques such as IMAMS or IMAC are quite specific toward proteins with surface-displayed histidine, since interactions between the chelated metal and the nitrogen atoms of the imidazole ring are the primary means by which protein is retained. In many instances a single histidine residue present on the surface can be exploited to achieve separation. This work focused on the characterization of two IMAMS materials by using the model proteins lysozyme and α-chymotrypsin. Both proteins have a single, exposed histidine: His 15 of lysozyme and His 57 of the active site of α-chymotrypsin are believed to be the amino acids primarily responsible for chelated metal–protein interactions (9, 14). IMAMS media prepared in this work differ by the incorporation of the spacing element 1,4-butanediol diglycidyl ether in the final structure. This commonly used spacing element was chosen due to the simplicity of its coupling chemistry.

Membrane Preparation

Figure 2 presents the final structures of Types I and II IMAMS media. Both media chelated divalent copper, as evident by a blue color change. Elemental analysis indicated that when charged with metal ions, each media type had a high metal content (800 μg/g membrane). Membranes displayed a five- to ten-fold higher Cu(II) content (0.9 wt%) when compared to other IMAMS media reported in the literature (2).

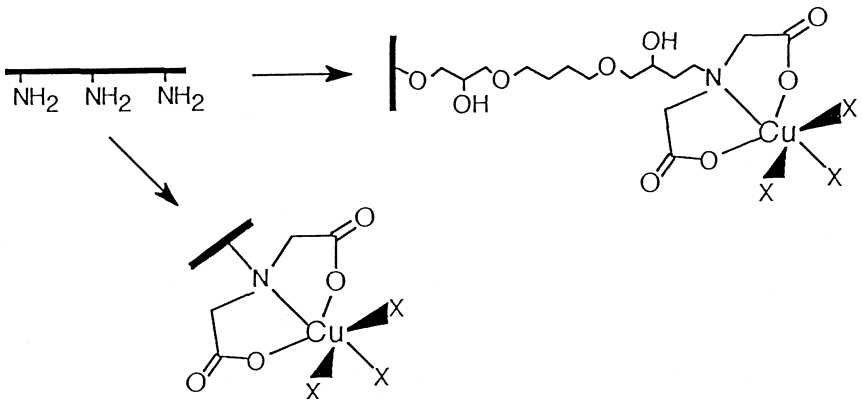

FIG. 2 Final structures for IMAMS materials (Type I and II).

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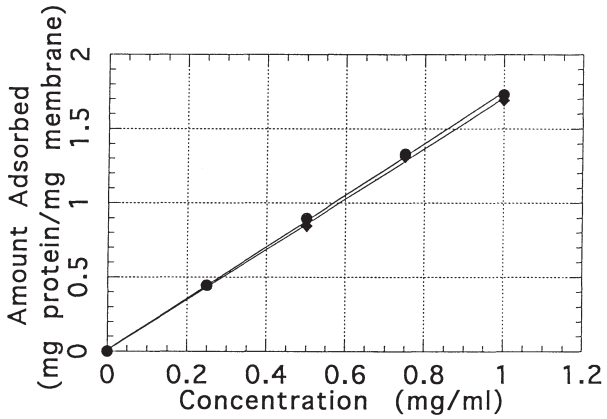

FIG. 3 Equilibrium adsorption of lysozyme for Types I and II media.

Adsorption Isotherms

Equilibrium adsorption of chicken egg white lysozyme between 0 and 1 mg/mL by both media is illustrated in Fig. 3. This range is representative of the range of concentrations employed during breakthrough curve determination. The observed adsorption isotherms were linear for Types I and II media over this concentration range and up 7.0 mg/mL. Least-squares analysis of the isotherms for each curve yielded values for the equilibrium dissociation constant (K_d) of 1.75 and 1.71 mL/mg (basis 0.15 mg of media).

Both media behaved similarly under equilibrium conditions, and the curves appeared to be non-Langmuirian over the concentration range of protein examined. Above 7.0 mg/mL, precipitation of protein was evident in several samples. Since the solubility of lysozyme in pure water is about 10 mg/mL, an upper concentration bound less than 10 mg/mL in high salt buffer can be expected for this experimental system. Although this solubility limit impared the ability to assess whether linear or saturation-type adsorption kinetics was truly characteristic of the material, it did not limit the characterization in dynamic experiments.

Breakthrough Curves

Figure 4 describes the average lysozyme breakthrough curves for Types I and II materials. Data were taken for each type of media using several membrane samples ($n = 3$), and curves were prepared by averaging adsorbance readings at discrete time intervals. Both materials exhibited the typical breakthrough curve characteristic of adsorptive materials; at early times the dimensionless concentration defined by (C/C_{inlet}) remained low, then increased toward a maximum value equal to unity. At capacity, each membrane binds

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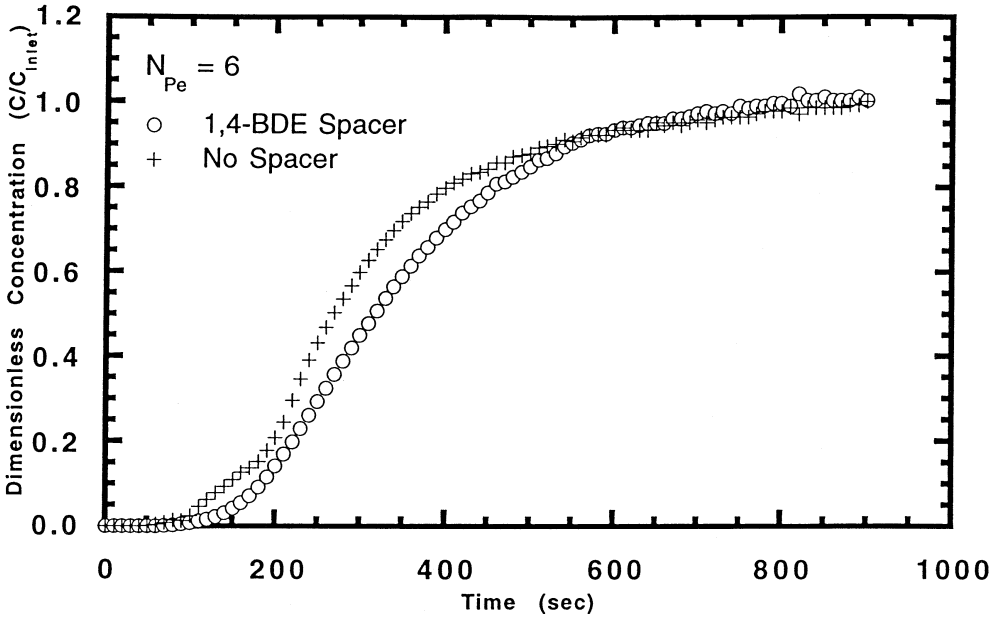

FIG. 4 Breakthrough curves for lysozyme adsorption (Type I and II media).

approximately 2.25 mg protein. On an area basis, this translates to a capacity of 0.13 mg/cm^2 material. Difference in performance was indicated primarily by the advanced breakthrough of lysozyme for Type I media relative to Type II. When the criteria of $C/C_{\text{inlet}} = 0.1$ was used to describe the point at which adsorption would typically be stopped during protein loading, inclusion of the spacing element permits additional loading time. Expanded breakthrough curves at early times are illustrated in Fig. 5, which show an additional load-

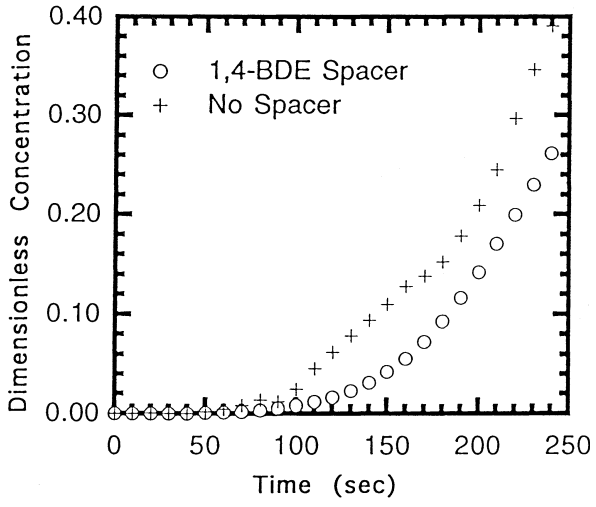

FIG. 5 Initial breakthrough curves for Types I and II media (lysozyme adsorption).

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ing time of approximately 50 seconds, which translates into 0.42 mg additional protein.

Figure 6 summarizes the ratio of loadings of lysozyme and α-chymotrypsin for different (C/C_{inlet}) ratios. For each protein, when C/C_{inlet} approaches unity, the ratio of protein bound by each membrane approaches 1:1. These data support the notion that both Types I and II behave similarly once equilibrium is attained, i.e., batch equilibrium or saturation under flow conditions. For the case of lysozyme, as C/C_{inlet} decreases toward 0.10, the disparity in the ratio of adsorbed protein is as high as 60%. Differences in loading ratios are more significant for the case of α-chymotrypsin. Indeed, if one examines the breakthrough curves for Types I and II media for this protein (Figs. 7 and 8), inclusion of a 14-atom spacing element is effective in promoting adsorption. Since α-chymotrypsin has previously been shown to interact with IMAC media of the same general characteristics, i.e., coupled IDA coupled to sepharose via diglycidyl ether, it is feasible that spacing elements designed for conventional chromatographic materials may not directly translate to a membrane setting in terms of dynamic adsorption characteristics either (14).

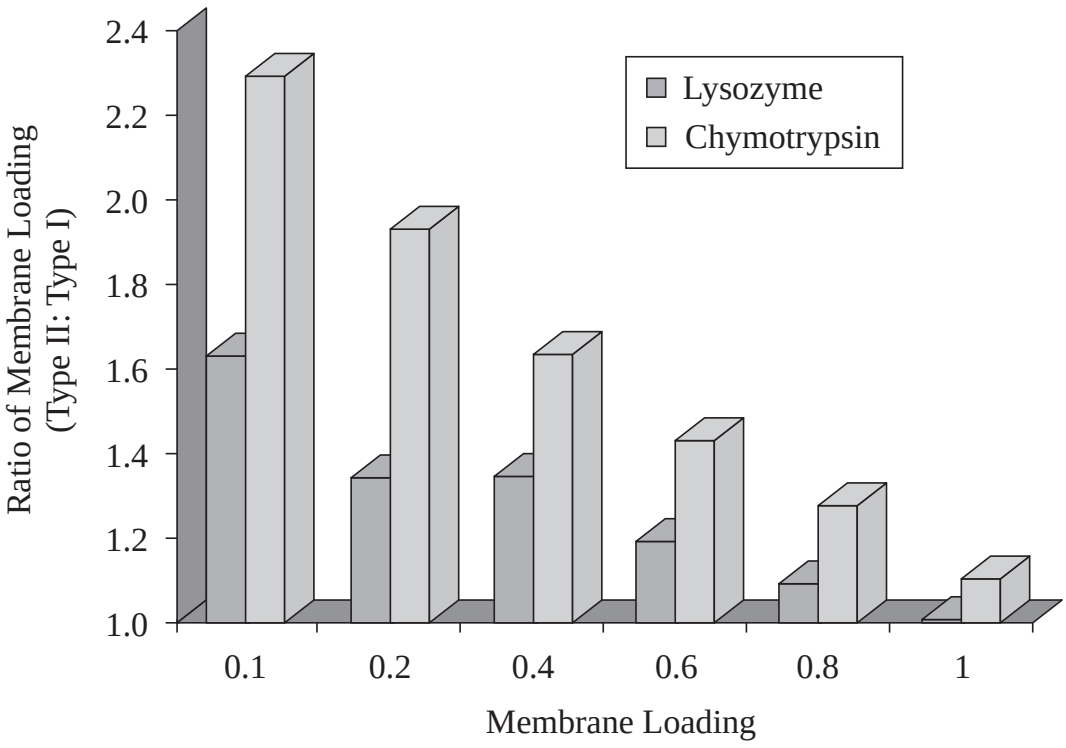

FIG. 6 Comparison of dynamic loading at various C/C_{inlet} for lysozyme and α-chymotrypsin adsorption.

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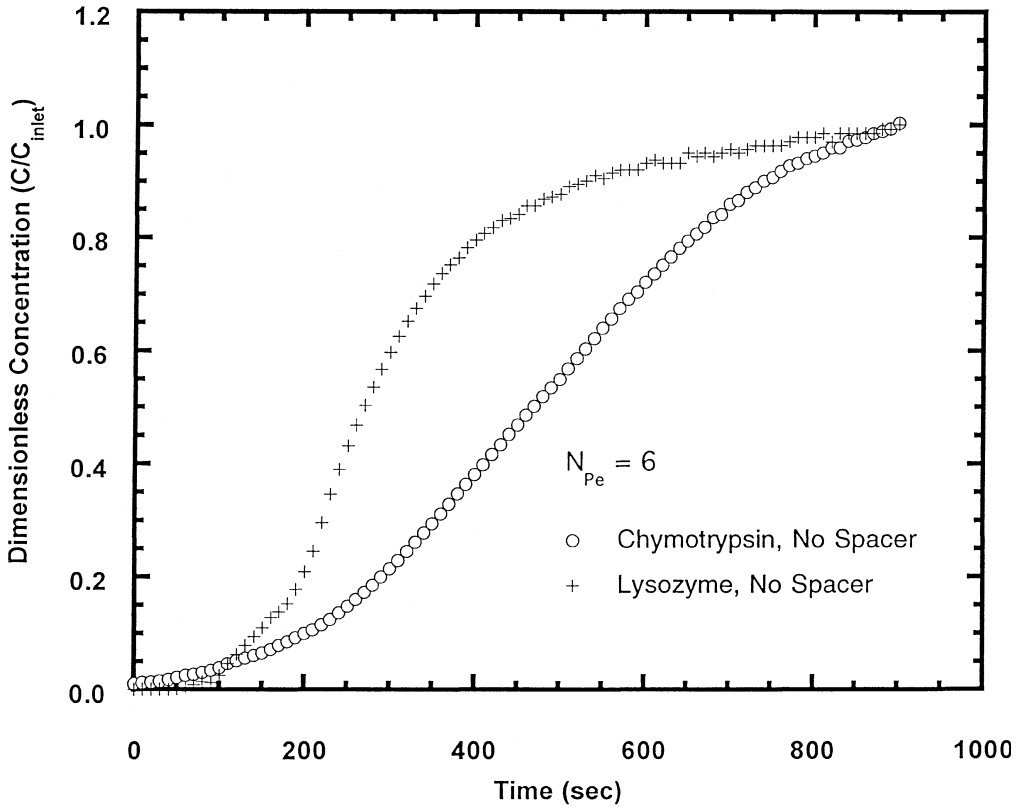

FIG. 7 Breakthrough curves for α-chymotrypsin adsorption on Type I media.

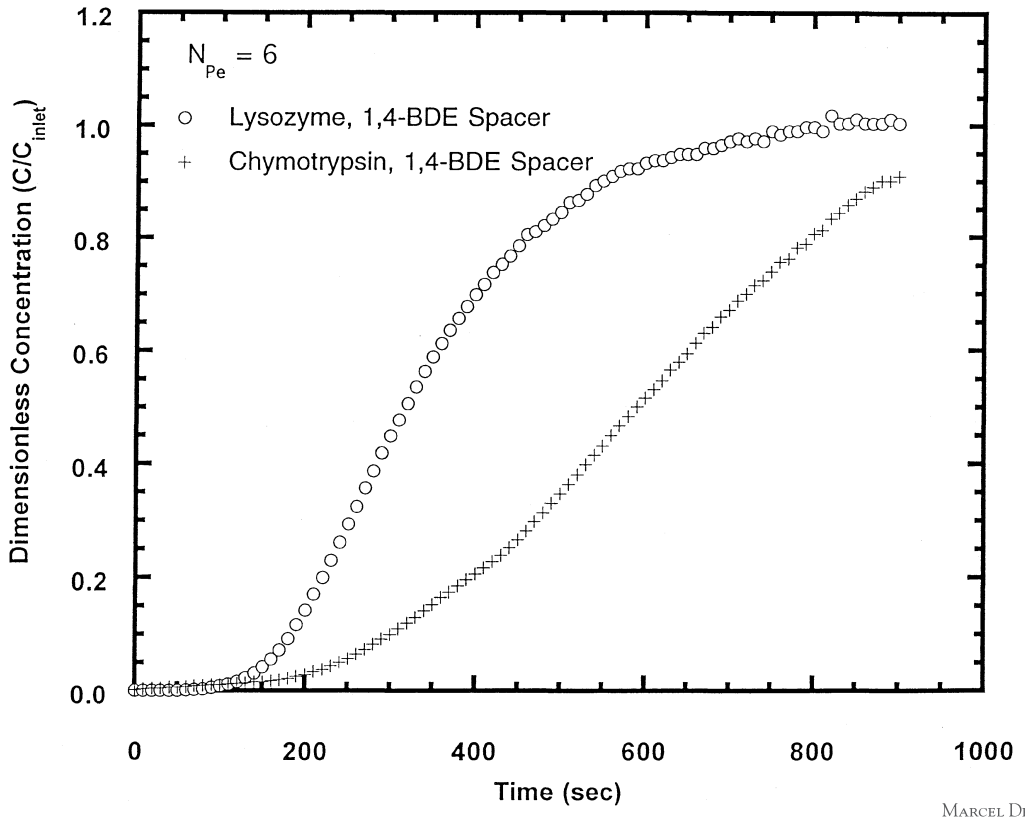

FIG. 8 Breakthrough curve for α-chymotrypsin adsorption on Type II media.

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CONCLUSIONS

The preparation of immobilized metal affinity membrane chromatography media with and without a model spacing element permitted the assessment of adsorption characteristics in equilibrium and dynamic settings. Experiments with lysozyme demonstrated that the inclusion of the spacing element had no observable effect on the equilibrium adsorption isotherm. When membranes were challenged in a flow configuration, however, the inclusion of the spacing element allowed for favorable adsorption, since both lysozyme breakthroughs were delayed. Results with a second protein, α-chymotrypsin, indicated that the relationship between binding residue solvent exposure and the medium's ability to project the chelating group away from the surface may be complicated in a membrane-based chromatographic system.

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