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Reproducibility of physical characteristics, protein immobilization and chromatographic performance of 3M Emphaze Biosupport Medium AB 1☆

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Abstract

3M Emphaze Biosupport Medium AB 1, a polymeric azlactone-functional support, provides for rapid and efficient coupling of proteins for uses such as affinity chromatography. The support is easy to use, with no preactivation or additional reagents required. It rapidly immobilizes proteins from solutions between pH 4 and 11.

Materials used in chromatographic processes need to be reproducible, in order to provide uniform performance. The following characteristics have been monitored in order to evaluate the reproducibility of Emphaze Biosupport Medium: particle size distribution, pore size distribution, surface area, bed volume, protein exclusion limits, pressure and flow performance, protein coupling and affinity performance.

1. Introduction

The development of 3M Emphaze Biosupport Medium AB 1 began in the mid-1980s when a group of synthetic chemists and biochemists at 3M began to collaborate on the use of azlactone-based polymers for covalently coupling biomolecules to polymeric supports [1–6]. The azlactone chemistry provides a stable reactive group for easy and versatile coupling of nucleophilic molecules, as illustrated in Fig. 1.

The initial developmental work with azlactone media focused on the reactive azlactone chemistry, and the evaluation of immobilized biomolecules [7–12]. The next step was to incorporate this reactive azlactone chemistry into a robust support which was suitable for chromatographic applications. The final stage of development

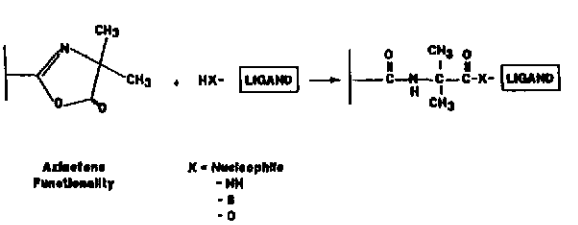

Fig. 1. Azlactone reaction chemistry, the covalent coupling of a nucleophile to vinyldimethylazlactone.

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involved scaling-up the synthetic steps to obtain a manufacturing process which produced uniform, fully functional product with each lot.

This product, 3M Emphaze Biosupport Medium AB 1, is a porous co-polymer of vinyldimethylazlactone and methylenebisacrylamide. The support is spherical, with an average bead diameter of 60 μm, as illustrated in Fig. 2. Emphaze AB 1 is supplied in a stable, dry form. Coupling of biomolecules is accomplished by addition of dry AB 1 to a solution which contains the biomolecule. As the beads swell in this solution, the covalent binding of biomolecules occurs. Typically the coupling reaction is complete within one hour, and the hydrated volume is approximately 8 ml of support per gram of dry media. Emphaze AB 1 contains over 30 μmol of reactive sites per ml of support, and can couple over 35 mg of protein per ml of swollen gel.

Emphaze AB 1 is stable in both aqueous and non-aqueous solutions, and buffer or solvent switching causes negligible, if any, changes in volume to occur. This feature permits elution and cleaning of the support with harsh solutions, the coupling of hydrophobic ligands in non-aqueous solvents, and also non-aqueous immobilized enzyme work.

This article focuses on the final stage of product development, and describes the physical and biochemical properties of Emphaze AB 1 which were evaluated in order to demonstrate the run-to-run uniformity of the manufacturing process.

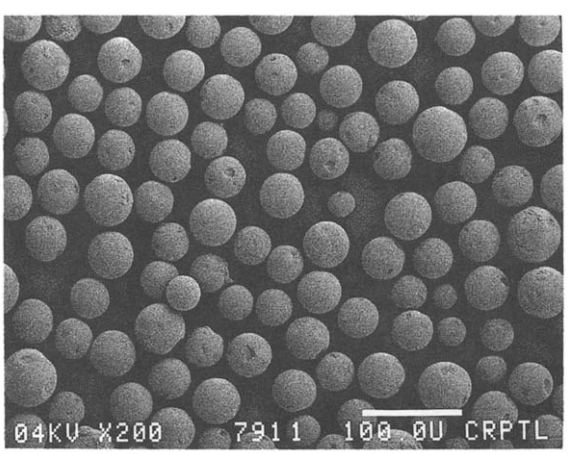

Fig. 2. Photomicrograph of 3M Emphaze Biosupport Medium AB 1.

2. Experimental

2.1. Materials

Stationary phases

Twenty seven different lots of 3M Emphaze Biosupport Media AB 1 (3M Company, St. Paul, MN, USA) were used as sample media for the following series of physical and chemical analyses.

Chemicals and solvents

Sodium chloride, monobasic and dibasic sodium phosphate, boric acid, D(+)-glucosamine hydrochloride, 2-(N-cyclohexylamino)ethanesulfonic acid (CHES) buffer, sodium dodecyl sulfate (SDS), ethanolamine, guanidine hydrochloride, cytochrome *c*, lysozyme, horse heart myoglobin, carbonic anhydrase, ovalbumin, fetuin, bovine serum albumin, alkaline phosphatase, human immunoglobulin, catalase, apoferritin, thyroglobulin, blue dextran (M_r 2 000 000), and λ phage DNA (M_r 30 000 000) were purchased from Sigma (St. Louis, MO, USA). "BCA" reagent for colorimetric protein analysis was obtained from Pierce (Rockford, IL, USA). Potassium chloride was obtained from J.T. Baker (Phillipsburg, NJ, USA). Trisodium citrate, sodium sulfate and sodium pyrophosphate were obtained from Mallinckrodt (Paris, KY, USA). Sodium hydroxide was obtained from EM Science (Gibbstown, NJ, USA). Sodium nitrite was obtained from Matheson, Coleman & Bell (Norwood, OH, USA). Recombinant Protein A was purchased from Repligen (Cambridge, MA, USA). Tissue culture fluid consisted of ATCC HB-124 DB9G8 (murine anti-insulin IgG_{2ak}) cultured in 10% fetal calf serum with 90% RPMI-1640 (Gibco, Grand Island, NY, USA). Liquid nitrogen was obtained from Oxygen Services (St. Paul, MN, USA). A Barnstead water-treatment system (Dubuque, IA, USA) produced ultra-pure water.

2.2. Equipment

Particle size measurements were performed with a Coulter LS-100 light-scattering particle size analyzer (Hialeah, FL, USA), which was equipped with a hazardous fluid module. A Micromeretics ASAP-2400 porosimeter (Norcross, GA, USA) was used to obtain pore size and surface area measurements. Graduated centrifuge tubes (15 ml, part 25319-15, Corning, Corning, NY, USA) and a tabletop centrifuge (Beckman Model TJ-6, Palo Alto, CA, USA) were used in determining hydration volumes. Flow parameters were measured with a system which was controlled and monitored by a Vectra ES-12 personal computer (Hewlett-Packard, Avondale, PA, USA). The other system components included a Model QV-0 Lab Pump (Fluid Metering, Oyster Bay, NY, USA) which was able to operate at flow-rates between 4 and 40 ml/min, a 0 to 1.3 MPa pressure transducer (Model PX941-200GI; Omega Engineering, Stamford, CT, USA), a Model DAS-8/AO data acquisition and control interface board (Keithly MetraByte, Taunton, MA, USA), and a 10 cm × 1 cm Waters AP-1 glass column (Milford, MA, USA).

Exclusion volume measurements were made with an HPLC system which consisted of the following components: a Spectra-Physics Model SP8750 pump (San Jose, CA, USA), a Spectra-Physics Model SP8780XR autosampler, a Kratos Spectroflow 757 UV monitor (Ramsey, NJ, USA), a Spectra-Physics Model SP4270 integrator and Spectra-Physics Spectra Station software. Polyether ether ketone (PEEK) columns (150 × 4.6 mm) were obtained from Alltech (Deerfield, IL, USA). The protein coupling analyses employed a Hewlett-Packard Model 8452A diode array spectrophotometer for absorbance measurements. Hema-Tek rocker plates were obtained from Miles (Elkhart, IN, USA), Labquake rotary mixers were obtained from Labindustries (Berkeley, CA, USA) and Vortex Genie-2 mixers were obtained from Scientific Industries (Bohemia, NY, USA). Tissue culture fluid was cultured in an Acusyst-P hollow fiber reactor (Endotronics, Minneapolis, MN, USA) and filtered through a 0.45-μm Pellicon cassette (Millipore, Milford, MA, USA). Affinity chromatography was performed on a Waters Delta Prep 3000 liquid chromatograph, which was controlled by Waters Maxima software. Polyacrylamide gel electrophoresis was performed with a Model PS 2500 power supply and model SE 250 gel apparatus (both from Hoefer Scientific, San Francisco, CA, USA), 10–20% gradient gels (Daiichi Pure Chemicals, Tokyo, Japan), and PRO Blue Stain (Integrated Separation Systems, Natick, MA, USA).

2.3. Procedures

Measurement of particle size

Prior to the analysis of samples, a control sample of garnet particles with an average diameter of 15 μm (Coulter LS Control G15) was analyzed to verify proper instrument operation. Analyses of hydrated Emphaze AB 1 samples were conducted by adding sufficient sample (20%, v/v, in filtered water) to produce 8 to 12% obscuration of incident laser light. The resulting diffraction pattern was analyzed to obtain a particle size distribution using a mathematical model which accounted for the refractive indices of the suspending fluid and the particles.

Measurement of pore size and surface area

To prepare samples for porosimetry measurements, 150 to 200 mg of Emphaze AB 1 dry beads were evacuated on the degas manifold of the ASAP 2400 at 100°C and less than 10 Mtorr (1 Torr = 133.322 Pa), until the instrument detected no further removal of volatiles. Samples were then transferred to the analysis manifold for automated measurement of the adsorption and desorption isotherms using nitrogen gas.

Measurement of hydration volume

Approximately 250 mg of Emphaze AB 1 dry beads were hydrated in calibrated, graduated centrifuge tubes by the addition of 10 ml of phosphate-buffered saline (PBS: 150 m*M* sodium chloride, 25 m*M* sodium phosphate) pH 7.4, and then mixing for 60 min on a rocker plate or rotary mixer. The beads were settled by centrifu-

gation at 1330 *g* for 90 min, and the bed volume in the graduated centrifuge tube was noted. The hydration volume for the sample is obtained by dividing this bed volume by the sample mass.

Measurement of flow parameters

Samples were prepared for analysis by hydration of 1.2–1.5 g of Emphaze AB 1 in 20 ml of PBS, pH 7.5. After mixing the bead slurry on a rocker plate for 30 min, the sample was centrifuged at 1330 *g* for 10 min. The volume of supernatant was then adjusted to obtain a 75% (v/v) slurry concentration. The Waters AP-1 column was primed with 1 ml of buffer, and then 11 ml of bead slurry was added to the column through the attached packing adapter. Buffer was allowed to flow from the column until the packing adapter was empty. Flow was then stopped, and the slurry was allowed to settle for 30 min. The column inlet assembly was then installed, and the inlet frit was carefully positioned at the level of the settled beads. The system was then operated at a flow-rate of 4 ml/min (306 cm/h) for 5 min to pack the bed further, and the inlet was again adjusted to the level of the settled beads. The analysis program was then initiated. The flow-rate was varied from 4 to 40 ml/min (306 to 3056 cm/h), in 4 ml/min increments, for 5 min per increment. The system pressure was monitored continuously.

Size-exclusion chromatography

A hydrophilic stationary phase for size-exclusion chromatography was prepared by coupling glucosamine to samples of Emphaze AB 1 Biosupport Media. Emphaze beads (0.8 to 0.9 g) were added to a buffered glucosamine solution (20 mg/ml of glucosamine hydrochloride, 1.2 *M* sodium sulfate, 50 m*M* sodium pyrophosphate at pH 8.5). The slurry was dispersed by vortex mixing, then mixed at room temperature overnight on a rocker plate. The derivatized Emphaze beads were then rinsed twice with four bed volumes of PBS, pH 7.4, and finally slurry packed into PEEK columns (150 × 4.6 mm) for exclusion volume analyses. The mobile phase (100 m*M* sodium sulfate, 50 m*M* sodium phosphate, 50 m*M* boric acid at pH 7.0) was also used to prepare 1 mg/ml solutions of the size exclusion solutes: sodium nitrite, cytochrome *c*, lysozyme, myoglobin, carbonic anhydrase, ovalbumin, fetuin, bovine serum albumin, alkaline phosphatase, human immunoglobulin, catalase, apoferritin, thyroglobulin, blue dextran, and λ phage DNA (50 μg/ml). The flow-rate for size-exclusion analysis was 0.25 ml/min. The size-exclusion solutes were injected individually, in order to avoid merged peaks. A composite chromatogram of four size-exclusion test solutes is shown in Fig. 3. The large particle size, as well as some non-specific interactions, have caused considerable band broadening.

The elution volume for sodium nitrite represented the total permeable volume, V_P, and the elution volume for λ phage DNA represented the total exclusion volume, V_E. The pore volume of the packed column, V_{pore}, is the difference between the total permeable volume and the total exclusion volume. The accessible pore volume for a given solute, V_{solute}, is the difference between the solute's retention volume, V_R, and the exclusion volume. The percentage of the pore volume which is accessible to a given solute was calculated as follows:

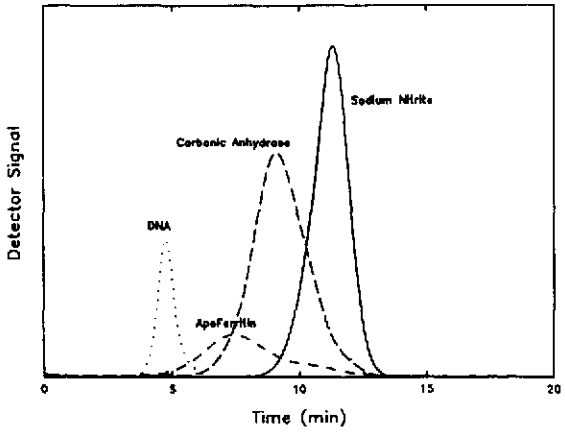

Fig. 3. A composite chromatogram for four size-exclusion reference solutes.

$$\text{Solute accessibility } (\%) = 100(V_{\text{solute}}/V_{\text{pore}}) = 100[(V_R - V_E)/(V_P - V_E)]$$

since elution volume = elution time (t) × flow-rate, then dividing the numerator and denominator by flow-rate transforms the above formula to:

$$\text{Solute accessibility } (\%) = \frac{(t_R - t_{DNA})}{(t_{\text{nitrite}} - t_{DNA})} \cdot 100 = 100K_{av}$$

This relationship was then used to evaluate the exclusion limits and permeability of the Emphaze media by size-exclusion chromatography.

Measurement of protein coupling

Emphaze AB 1 samples (28–32 mg) were hydrated with 3 ml of myoglobin reagent solution (5 mg/ml myoglobin, 1.0 *M* sodium citrate, 0.1 *M* CHES, pH 9.0) and then mixed on a rocker plate or rotary mixer at room temperature for 60 to 70 min. A 5-ml volume of 1% (w/w) SDS was then added to the sample tube, and the mixing was continued for an additional 30 min to remove non-specifically bound myoglobin. The slurry was centrifuged at 1330 *g* for 5 min to settle the beads, and the supernatant was sampled for spectrophotometric measurement at 532 nm (A_{super}). The spectrophotometer was blanked with respect to a solution which contained citrate–CHES buffer (3 ml) plus 1% SDS (5 ml). The absorbance of a myoglobin reference solution (A_{ref}), which contained 3 ml of myoglobin reagent solution and 5 ml of 1% SDS, represented the amount of myoglobin available for coupling. The difference between the reference and supernatant absorbances was proportional to the amount of myoglobin coupled to the bead sample, as follows:

$$B\ (\text{mg/g}) = (A_{\text{ref}} - A_{\text{super}})CV/[WA_{\text{ref}}]$$

where B is the amount of myoglobin coupled, C is the concentration of the myoglobin reference solution, V is the amount of myoglobin reagent solution per sample and W is the sample mass.

Purification of IgG by affinity chromatography

An affinity support for the purification of murine IgG from tissue culture fluid was prepared by coupling recombinant Protein A to 3M Emphaze Biosupport Medium AB 1. Emphaze beads (18.72 g) were added to a 1-l bottle which contained 555 ml of a buffered Protein A solution (2.03 mg/ml of Protein A, 0.85 *M* sodium citrate, 100 m*M* 3-(N-morpholino)propanesulfonic acid (MOPS) at pH 7.5). The bottle was then rocked for 1 h at room temperature. After the coupling reaction, the slurry was filtered on a glass fritted Buchner funnel, porosity D, and the filtrate was used to determine the amount of Protein A that was coupled to the Emphaze media. The Protein A beads were then resuspended in 600 ml of quenching solution (1.0 *M* ethanolamine, 25 m*M* sodium pyrophosphate, pH 7.5), rocked for 5 h, filtered as above, then resuspended in 400 ml of PBS for 20 min. The washing was completed by filtering the PBS from the slurry and then gradually rinsing the filtercake with 1.8 l of PBS. The washed Protein A beads were then slurried in PBS and stored at 5°C before use. The BCA colorimetric protein assay was used to determine the amount of Protein A which was coupled to Emphaze AB 1, based upon the difference in Protein A concentration between the initial and filtrate solutions. The affinity support contained 7.7 mg of Protein A per ml of media. A 1-ml volume of the Emphaze–Protein A support was then slurry packed into a Waters AP-1 glass column (1.0 cm I.D.) to a final bed height of 1.3 cm.

The isolation of murine IgG was begun after equilibrating the affinity column with PBS. The column was loaded at 1 ml/min (76 cm/h) with 25 ml of filtered (0.45 μm) tissue culture fluid which contained murine IgG. The affinity column was then washed, eluted, cleaned and reequilibrated at 3 ml/min (229 cm/h). The wash and reequilibration buffer was PBS, pH 7.2. The elution buffer was 100 m*M* sodium citrate, pH 2.5, and the cleaning buffer was 3.0 *M* guanidine hydrochloride, 20 m*M* sodium phosphate, pH 7.2. The eluted IgG was quantified by spectrophotometric analysis at 280 nm, and its purity

was confirmed by polyacrylamide gel electrophoresis.

3. Results and discussion

3.1. Particle size

Typical particle size distributions for seven lots of Emphaze AB 1, as measured by the light-scattering particle size analyzer, are illustrated in Fig. 4. A photomicrograph of representative Emphaze AB 1 medium is presented in Fig. 2. The average particle diameter was 62 μm, and 85% of the particles ranged in diameter from 45 to 81 μm. The reproducibility of these three particle size parameters for 27 production lots of Emphaze AB 1 is summarized in Fig. 5. The relative standard deviation (R.S.D.) for the average particle size measurement was 2.0%, and the R.S.D.s for the 5th and 90th percentiles were 1.7 and 3.5%, respectively.

3.2. Pore size and exclusion volumes

A typical pore size distribution for dry Emphaze AB 1 beads, as measured by nitrogen porosimetry, is shown in Fig. 6. The cumulative pore distribution data, shown in Fig. 7, indicates that over 70% of the pore volume for Emphaze AB 1 is within pores that have pore diameters greater than 300 Å.

Since the dry support swells approximately twofold upon hydration, the accessible pore volume of the hydrated media may be somewhat different from that of the dry support. Size-exclusion chromatography was performed with Emphaze AB 1 media in order to obtain a practical perspective of the porous structure of hydrated support. The size-exclusion data in Fig. 8 shows that apoferritin, a protein with a molecular mass of 443 000, had access to 40% of the bead pore volume. IgG, with a molecular mass of 150 000, had access to 57% of the bead pore

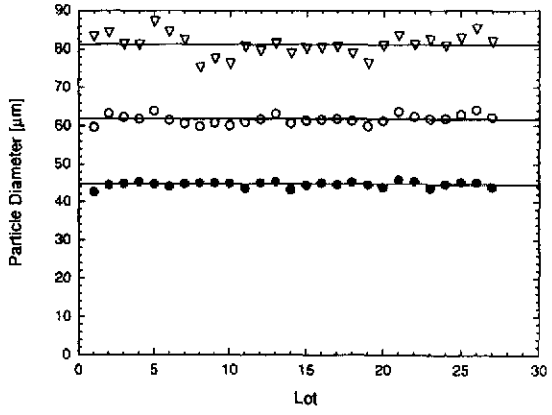

Fig. 5. Lot-to-lot reproducibility of particle size parameters: ▽ = 90th percentile; ○ = mean diameter; ● = 5th percentile.

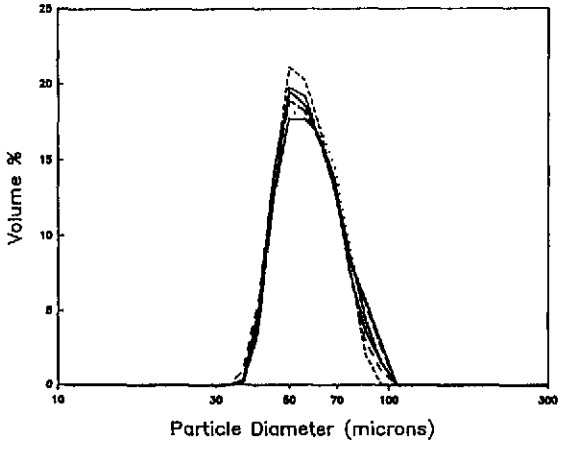

Fig. 4. Particle size distribution (of seven lots) for 3M Emphaze Biosupport Medium AB 1.

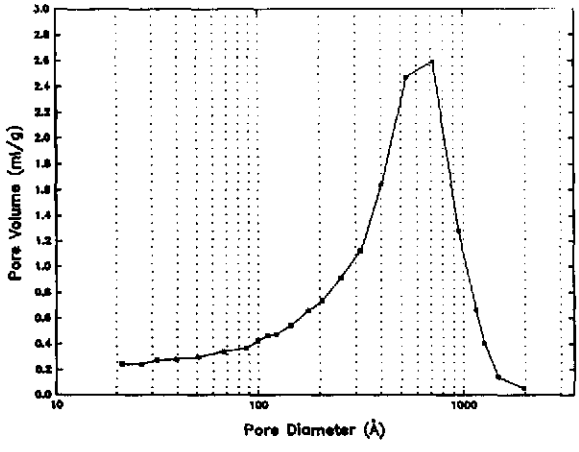

Fig. 6. Desorption pore size distribution for 3M Emphaze Biosupport Medium AB 1.

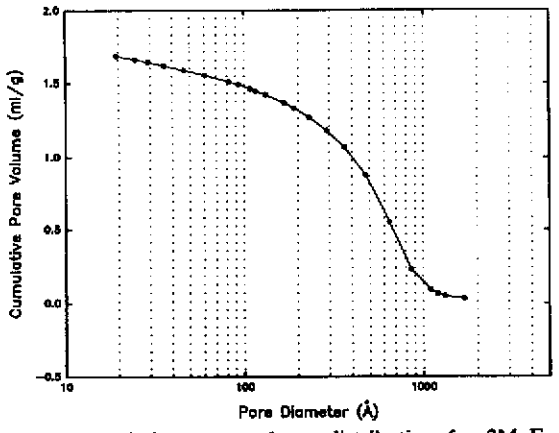

Fig. 7. Cumulative pore volume distribution for 3M Emphaze Biosupport Medium AB 1.

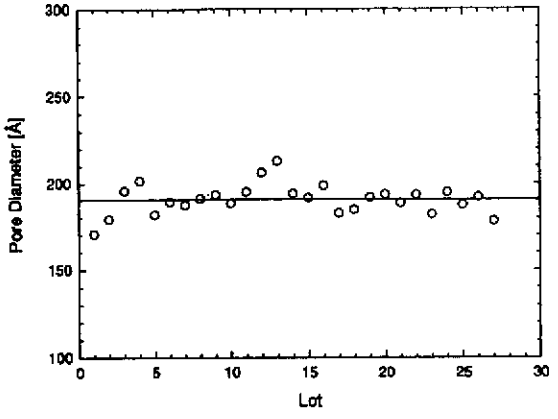

Fig. 9. Lot-to-lot reproducibility of pore diameter.

volume, and carbonic anhydrase, with a molecular mass of 29 000, had access to 73% of the bead pore volume. The values for the mean pore diameter for 27 lots of dry Emphaze AB 1 are reported in Fig. 9. The R.S.D. for the mean pore diameter was 4.6%. The mean pore diameter of 190 Å is less than the pore diameter which represents the greatest pore volume (700 Å, from Fig. 6), because calculation of the mean pore value is influenced by the relatively large population of small diameter pores which contribute very little to the total pore volume. Also note that average pore size values which are obtained by nitrogen porosimetry are typically smaller than those from mercury porosimetry. This is because nitrogen is smaller than mercury, and thus it can obtain data from smaller pores.

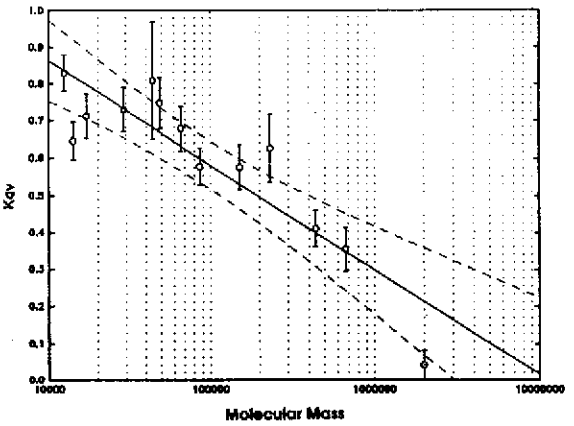

Fig. 8. Size-exclusion performance for proteins (molecular mass): cytochrome *c* (12 400), lysozyme (14 000), myoglobin (16 900), carbonic anhydrase (29 000), ovalbumin (44 000), fetuin (48 700), bovine serum albumin (66 000), alkaline phosphatase (86 000), human immunoglobulin (150 000), catalase (232 000), apoferritin (440 000), thyroglobulin (670 000), blue dextran (2 000 000), λ phage DNA (30 000 000).

3.3. Surface area

A typical surface area distribution for dry Emphaze AB 1 media is presented in Fig. 10. This graph displays the cumulative pore surface area as a function of pore diameter. Approximately 25% of the total surface area is contained in pores having diameters greater than 300 Å,

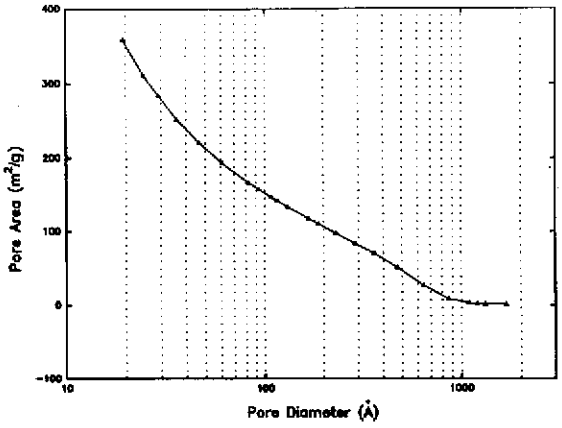

Fig. 10. Cumulative desorption pore surface area distribution.

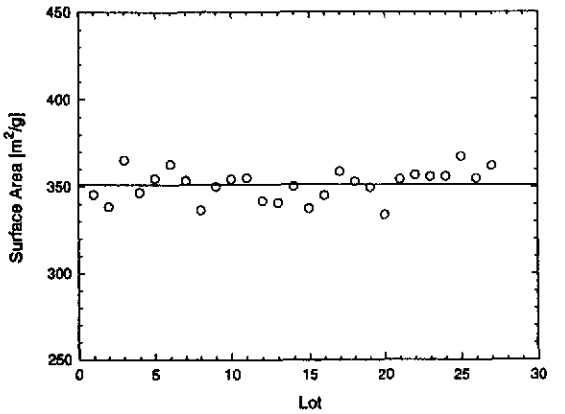

Fig. 11. Lot-to-lot reproducibility of surface area.

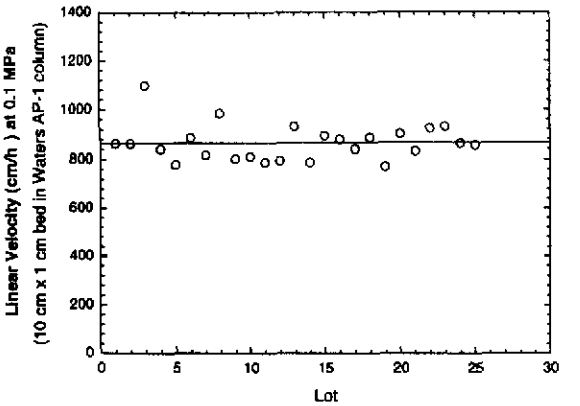

Fig. 13. Lot-to-lot reproducibility of flow-rate at 0.1 MPa.

and 41% of the total surface area is contained in pores having diameters greater than 100 Å. The reproducibility of bead surface area for Emphaze AB 1 medium is summarized in Fig. 11. For 27 production runs the mean surface area was 351 m^2/g, with an R.S.D. of 2.5%.

3.4. Hydration volume

The reproducibility of hydration volume values for Emphaze AB 1 media is displayed in Fig. 12. The mean hydration volume for 27 production runs was 8.3 ml per gram of dry media. This value varied by 3.3% over these bead lots.

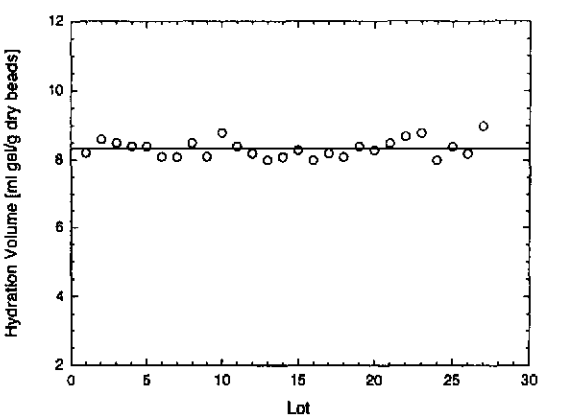

Fig. 12. Lot-to-lot reproducibility of hydration volume.

3.5. Flow parameters

The reproducibility of flow-rate at 0.1 MPa is reported in Fig. 13. For 27 production lots of beads the flow-rate at 0.1 MPa averaged 865 cm/h, with an R.S.D. of 8.5%.

3.6. Protein coupling

The reproducibility of protein coupling performance by Emphaze AB 1 was evaluated with myoglobin, a reasonably priced protein with a visible color to facilitate a protein coupling assay. The Emphaze AB 1 samples were challenged with a sufficient amount of myoglobin to attain a 50% coupling efficiency of the myoglobin. For eight production runs, myoglobin coupling performance averaged 30 mg of myoglobin per ml of media, with a lot-to-lot (R.S.D.) of 7.9%, as shown in Fig. 14.

3.7. Purification of IgG from ascites by Protein A affinity chromatography: reproducibility of separations using Protein A-modified Emphaze AB 1

A single column which contained Protein A-modified Emphaze AB 1 was used to isolate murine IgG from tissue culture media, and the affinity support was then cleaned with 3.0 *M* guanidine hydrochloride after each separation.

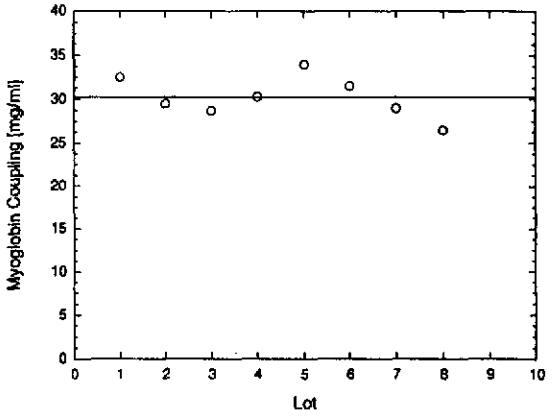

Fig. 14. Lot-to-lot reproducibility of protein coupling performance.

Fig. 15 summarizes the performance of this affinity support over 139 purification cycles. The lot-to-lot R.S.D. was 5.2%, and the Protein A-modified Emphaze AB 1 media retained 99% of its specific binding capacity after 139 cycles.

4. Conclusions

The physical and biochemical performance characteristics for 27 production lots of 3M Emphaze Biosupport Medium AB 1 were monitored in order to evaluate the reproducibility of manufacturing this chromatographic support. The physical attributes, such as particle size distribution, pore size distribution, surface area, hydration volume, and flow-rate at 0.1 MPa, as well as the biochemical attributes, such as protein coupling and affinity purifications, all exhibited excellent lot-to-lot reproducibility, with R.S.D.s ranging from 1.7 to 8.5% for these parameters.

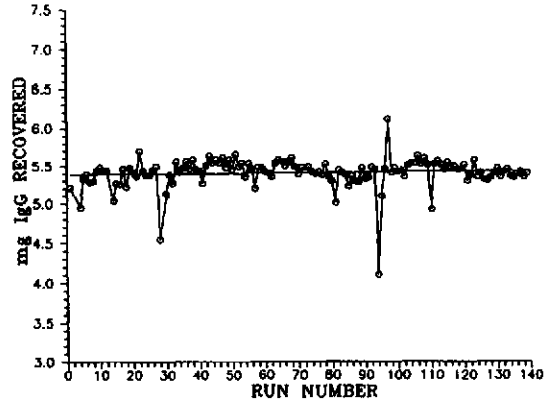

Fig. 15. Run-to-run reproducibility of IgG purification by protein A-modified Emphaze AB 1. O——O = Concentration by A_{280}.

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