Egg-White Protein Fractionation Using New Weak Anion-Exchange Resins Based on Poly(Glycidyl Methacrylate-co-Ethylendimethacrylate). Preparation and Characterization

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Abstract

A new formulation for a poly(glycidyl methacrylate-co-ethylendimethacrylate)-based resin with a 11:9 proportion of monomer to crosslinker is developed and amino-functionalized in order to obtain new particulate materials suitable for egg-white protein fractionation. Functionalization is carried out using three different chemical reagents: diethylamine (DEA), DEA–tetrahydrofuran (THF) (1:1), and concentrated ammonia. The ammonia- and DEA–THF-treated polymers are used to fractionate egg-white proteins, in particular lysozyme and ovalbumin, by anion-exchange chromatography in packed column experiments, the latter resin showing better performances. Finally, both supports, working at semipreparative scale and step-gradient elution, separate pure ovalbumin with a yield of 83%.

Introduction

In recent years, new technologies and synthetic chromatographic supports for protein separation and purification have been developed (1–7). Over the last decade, organic polymeric supports have received a great deal of attention, especially those based on styrene–divinylbenzene, glycidyl methacrylate–ethylene dimethacrylate, methacrylate–trialylisocianurate–divinylbenzene, and dimethylaminostyrene–ethylenglycol–dimethacrylate, among others (8–10). These polymers are usually prepared by either suspension or emulsion polymerization techniques (11). Amino-functionalized methacrylate-based polymers, both in the form of monolithic rods or as particulate separation media, were found to behave as good chromatographic supports for anion exchange of proteins (12,13). However, particulate materials show high backpressures when the size of the particles is reduced so as to obtain good resolution in proteins separation. One way of improving the diffusion flow rate inside the particles is to use macroporous particulate polymers, which allow better accessibility and thus increase the efficiency of the columns at relatively high flow rates.

One of the target fields for the application of these supports is the chromatographic separation of proteins from byproducts of the food industry (14–18). Different features must be considered in the design of polymeric particulate chromatographic supports for protein recovery and purification, such as porosity, granulometry, and mechanical resistance. Moreover, because the larger particles accumulate near the walls and the smaller ones towards the center during the column filling process, it is also very important to employ particles as uniform in size as possible (19–21).

Among the different byproducts from the food industry, one of growing interest is the egg white obtained from eggshells by centrifugation after shell breaking operations in processing plants (22–24), in view of the fact that the disposal of this byproduct creates environmental problems. Furthermore, considering that 10% (w/w) of an egg is shell and that approximately 1 g of egg white may remain attached to the shell after breaking, this constitutes an important amount of egg white in all egg processing plants. Approximately 12% (w/w) of egg white is solids and 85% is proteins (25), with a valuable application in fields such as biochemistry, pharmacy, or nutrition.

It is obvious that both economic and environmental factors make the recovery of this byproduct an interesting field to be explored, and it is likely that the obtainment of proteins from food industry byproducts will increase in the future. Because of the fact that proteins are electrically charged at physiologic pH, one of the most efficient techniques to achieve their separation is via ion-exchange chromatography.

The aim of this work was to study the chromatographic properties of new separation supports based on particulate amino-functionalized poly(glycidylmethacrylate-co-ethylen dimethacrylate)

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resins for anion-exchange chromatography of egg-white proteins. Physical characterization of the resins was also carried out. It was found that the physical and chromatographic properties of the polymers were highly dependent on the functionalization conditions.

Experimental

Materials

Polymer preparation

The polymer was synthesized in situ using a 40% (v/v) mixture of a monomer solution of 55% glycidyl methacrylate–45% ethylen-dimethacrylate (Aldrich, St. Louis, MO) as cross-linking agent, with 60% (v/v) cyclohexanol (Aldrich) as porogenic reagent, and then adding azoisobutyronitrile (Fluka, Buchs, Switzerland), 1% weight with respect to monomers, as polymerization initiator. Experimental protocols are referred in the literature [Paredes et al. (12) and Suarez et al. (13)]. After extraction from the tubes, the resin cylinders were milled in an Omron Sysdrive Ball Mill. The particles thus obtained were sieved selecting sizes between 100 and 250 µm for the experimental runs. Once the material had been milled and classified, it was washed twice with 50 mL of tetrahydrofuran (THF) (Panreac, Barcelona, Spain) in a stirred batch reactor during 3 h to eliminate the excess of cyclohexanol, then filtered and dried. (The particulate polymer will be referred as sample P1.)

Particulate polymer functionalization

Amination of the epoxide groups of the particulate polymer was carried out using three different functionalization procedures. For sample P2, 30 g of P1 material was manually packed in a 30- × 1.15-cm glass column (Amicon, Stonehouse, U.K.) and a 30% ammonia solution (Panreac ACS) was then pumped through the column at a flow rate of 0.2 mL/min using a Masterflex 7554-60 pump during 14 h at 50°C. The aminated resin was washed by pumping distilled water through the column at the same flow rate for 6 h. The particles were then dried at 60°C and sieved again in order to select those with sizes between 100 and 250 µm. For sample P3, 30 g of P1 material was treated in a batch reactor with 100 mL of diethylamine (DEA) (Panreac) at 40°C during 24 h. For sample P4, 30 g of P1 material was treated in a Roto-vapor device with 100 mL of a mixture of DEA–THF (50:50) at 50°C for 10 h at 50 rpm.

Samples P3 and P4 were successively washed for 3 h with 100 mL of methanol (Panreac), methanol–water (50:50), and water at room temperature. They were then filtered, dried at 60°C for 6 h, and sieved again to remove the particles whose size may have decreased during the amination reaction.

Samples characterization

Particle size distribution of the particulate resins was obtained using a Malvern Instruments Mastersizer laser granulometer (Worcestershire, U.K.). The quantity of amino groups was determined by elemental analysis using a PerkinElmer 240B elemental analyzer (Norwalk, CT). Specific surface areas and mesopore size distributions were calculated from N_2 adsorption/desorption isotherms at 77 K, obtained using a Micrometrics ASAP 2010 instrument (Atlanta, GA). Macroporosity was studied by mercury intrusion porosimetry with a CE Instruments mercury porosimeter Pascal 240, operating in a pressure range of between 0.1 and 200 MPa (CE Instruments, Milan, Italy). Scanning electron micrographs of the resins were taken with a Jeol 2000 EXII electron microscope operating at 10 kV (Jeol, Peabody, MA). The density of the polymers was also calculated by pycnometry.

Experimental procedures

Packed column experiments

The particulate polymers were packed in a 7.5- × 0.4-cm stainless steel column using a slurry packing technique with a single-piston Shandon device at 350 bar (Thermo, Pittsburgh, PA). The solvent employed, both for slurry and charge, was methanol (Panreac). The slurry was previously prepared by ultrasonicating a 5% suspension of particulate resin in 20 mL of methanol for 10 min.

Bed-flow characterization

The resistance to flow rate was measured by determining backpressure in a 7.5- × 0.4-cm stainless steel column using an Äkta fast-protein liquid chromatography (FPLC) device from Amersham Biosciences (Uppsala, Sweden). Permeability was calculated using Darcy's equation (21):

$$\mu = k \frac{\Delta P}{nL} \qquad \text{Eq. 1}$$

where µ is the linear velocity of the mobile phase in the column; ΔP is the backpressure on the column; η is the viscosity coefficient of the liquid, which was measured using an Ostwald viscosimeter; and L is the column length.

In order to determine the axial dispersion coefficient (D_{ax}), the column was placed in an Äkta FPLC chromatograph in order to carry out stimulus-response assays under nonretention conditions (26). These conditions are imposed by passing a solution consisting of 0.3M NaCl (Panreac) in 10mM Tris-HCl buffer, (pH = 7.6) (tris-hydroxymethylaminomethane, United States Biochemical, Cleveland, OH) at different flow rates. In all these assays, 100 µL of solution containing 1 g/L of ovalbumin in the NaCl–buffer solution was injected in the column. The absorbance at 280 nm was then measured at the column outlet with a Shimazdu Rd UV-1203 UV–vis detector (Kyoto, Japan).

Chromatographic characterization

Before being charged with protein, the column was equilibrated with a 0.3M NaCl in 10mM Tris-HCl buffer solution (pH = 7.6). The column was then washed with 10mM Tris-HCl solution (buffer A) until removal of NaCl, saturated with protein (ovalbumin), and then washed with distilled water to extract the unretained protein. The specifically retained protein was then eluted with 0.3M NaCl in 10mM Tris HCl solution (buffer B).

Dynamic capacity was calculated by approximate integration of the breakthrough curve obtained with ovalbumin. Useful capacity was calculated at an absorbance value of 10% of the maximum value. Prior to other studies, it was verified in batch experiments that more than 95% of the maximum retention capacity of the resins was reached in less than 15 min for each protein under

study, showing good kinetics for the retention process.

The equilibrium constant was calculated using the constant separation factor isotherm (27), which allows the study of nonlinear equilibrium isotherms to be generalized:

$$q_i = \frac{KQ_iC_e}{[C_m + (K-1)C_e]} \quad \text{Eq. 2}$$

where K is the equilibrium constant, q_i is the amount of substance adsorbed, Q_i is the maximum amount of substance adsorbed, C_m is the equilibrium maximum concentration of adsorbate, and C_e is the values of equilibrium concentrations for different concentration conditions.

Egg-white protein fractionation

The chromatographic properties of the aminated resins were tested using trypsin inhibitor II, ovalbumin grade 3, and lysozyme (all purchased from Sigma, St. Louis, MO) as standard proteins. The protein mixture was prepared by dispersing them, 1 mg/mL, in 0.01M Tris-HCl buffer (pH 7.6). On the other hand, the samples of hen egg white were prepared by diluting 10-fold with 0.01M Tris-HCl buffer containing 10mM β-mercaptoethanol (19). In all cases, the samples were gently stirred for 12 h at 4°C and then filtered through 0.45-µm cellulose–acetate filters.

The egg-white proteins were separated in an Äkta FPLC device (Amersham Biosciences) at 0.4 mL/min flow rate using NaCl 0.3M gradient in Tris HCl 10mM (pH = 7.6). Identification of the proteins was carried out by sodium dodecyl sulfate (SDS)–poliacrylamide electrophoresis (PAGE) using a Mini Protean 3 Slab Cell (Bio-Rad Laboratories, Hercules, CA), employing 6.8% acrylamide gels for concentration and 14% for separation. The injected volume of the samples was 15 µL, with approximately 2 µg/mL protein concentration.

Results and Discussion

Particulate polymer characterization

The retention capacity of an ion-exchange chromatographic support depends on the number of ionizable functionalities that behave as ion-exchangeable groups. However, the accessibility of these binding sites to biomolecules is controlled by the surface area and porosity of the material. The control of pore size distribution is also critical in order to improve the diffusion flow rate, and it was found that changes in pore size and pore volume can cause substantial changes in intraparticle permeability (28).

Particles size distribution

The importance of using particles as uniform in size as possible so as to increase the efficiency of the columns has already been reported (19–21), for this reason the polymer particles sieved between 150 and 250 µm were analyzed in a Mastersizer laser granulometer (Malvern Instruments). The polymer particles (sample P1) have a narrow unimodal particle size distribution with values for the particle volume mean diameter [D(4,3)] of 227 µm and for the median diameter [D(v,0.5)] of 218.83 µm.

Around 2/3 of the ammonia-functionalized polymer particles (sample P2) have diameters between 100 and 250 µm. The particle size distribution is also unimodal with a value of the particle volume mean diameter [D(4,3)] of 179.67 µm, and the value of the median diameter being 177.15 µm. This means that treatment with ammonia favors a uniform disaggregation of the polymer particles.

The particle size distribution is quite symmetric for samples P1 and P2 but not for the DEA-functionalized materials, which show an almost bimodal distribution curve with values of D(4,3) and D(v,0.5) of 105 and 75 µm, considerably lower than those shown by the nonfunctionalized and the ammonia-treated polymers. The stirring of the resin during functionalization causes fragmentation of the polymer particles, leading to a wide particle size distribution. The use of smaller particles for column packing caused important flow problems; therefore, the functionalized polymer particles were always sieved again.

Polymer functionalization

The amount of amino groups fixed on the resin by amination of the polymer epoxide groups was determined by elemental analysis. Table I summarizes the elemental analysis of carbon, hydrogen, and nitrogen for the four samples. It can be seen that the DEA–THF-functionalized material (sample P4) has the higher amino groups content. The presence of THF as solvent seems to increase the accessibility of the reacting functional groups, thus favoring the amination of the epoxide groups of the polymer. This method of functionalization is more effective, allowing us to predict a better retention capacity for this resin.

Physical properties

Brunauer–Emmet–Teller method surface area and mesopore size distribution were calculated from nitrogen adsorption-desorption isotherms at 77 K (29). None of the isotherms show enhanced nitrogen uptake or hysteresis in the low pressure range, indicating the absence of microporosity. The mesoporosity of the samples was analyzed using the method of Barret, Joyner, and Halenda (30) applied to the desorption branch of the isotherms. The pore size distribution curves of all samples show a unique peak (pb1), which indicates unimodal distribution of pore diameters. BET surface areas and the values of the most frequent pore diameters (D_{pmax}) obtained from the diameter distribution curves are given in Table I. It can be seen that the ammonia-functionalized resin has the highest specific surface area and the lowest value of the most frequent pore diameters. The development of this mesoporosity is probably caused by additional crosslinking in the polymer during the treatment with ammonia introducing epoxide groups, which implies a lower retention capacity for this resin because proteins cannot access smaller

Table I. Textural Parameters of Samples Determined from Adsorption–Desorption Isotherms at 77 K

Sample	SBET (m^2/g)	D_{pmax}(nm)	%C	%H	%N
P1	36	16.5	58.97	6.90	0
P2	63	12.9	48.43	7.07	1.74
P3	49	15.1	58.05	7.60	1.33
P4	40	15.5	42.84	5.50	1.85

pores. Treatment with DEA increases the polymer BET surface area from 36 to 49 m^2/g, and the value of the most frequent pore diameter decreases slightly. On the other hand, the specific surface area and mesoporosity of the DEA–THF-functionalized polymer remains almost unchanged, which means that this treatment does not significantly alter the mesoporosity of the polymer.

Gas adsorption measurements are widely accepted means of assessing surface area and mesoporosity but are not adequate for measuring porosity of large pore solids (i.e., those with pore widths larger than 50 nm). Mercury intrusion porosimetry allows an accurate measure of pore volumes to be obtained, and although it does not give accurate values of the surface area for those solids containing pores with widths smaller than 50 nm, it is a very useful technique for studying the porosity of large pore materials. The pore size distribution profile of the nonfunctionalized polymer shows a bimodal pore size distribution, with a peak (pb1) at 11 nm, in the mesopore range, and a second peak (pb1) at 2000 nm, the cumulative pore volume being 2.85 cm^{-3}/g. The polymer treated with ammonia (Figure 1) shows a small peak (pb1) at 10 nm, the value of the most frequent pore radius being 5000 nm and the cumulative volume 8.1 cm^3/g, most of the pores radii being in the range of 200 to 5000 nm. The pore size distribution profile of the polymer treated with DEA–THF has a peak (pb1) at 5000 nm, most of the pores have radii between 100 and 5000 nm. The cumulative pore volume of this sample is 12 cm^{-3}/g, which is approximately 50% higher than the value corresponding to the ammonia-treated polymer and almost 6-fold the value obtained for the nonfunctionalized polymer; very large pore volumes are an indication that the porosity is also caused by interparticle spacing. It may be concluded that amination of the polymer modifies its porous structure, thus favoring the formation of wider macropores.

Scanning electronic microscopy images of the polymers confirm the variation in particle and pore sizes after functionalization (Figures 2A–C). The electron micrographs show the presence of submicron-sized grainy particle agglomerates with different pore diameters (pb1). Treatment with concentrated ammonia yields a more homogeneous microscopic topography, but functionalization with DEA favors the formation of larger particle aggregates, as well as wider pores and interparticle cavities.

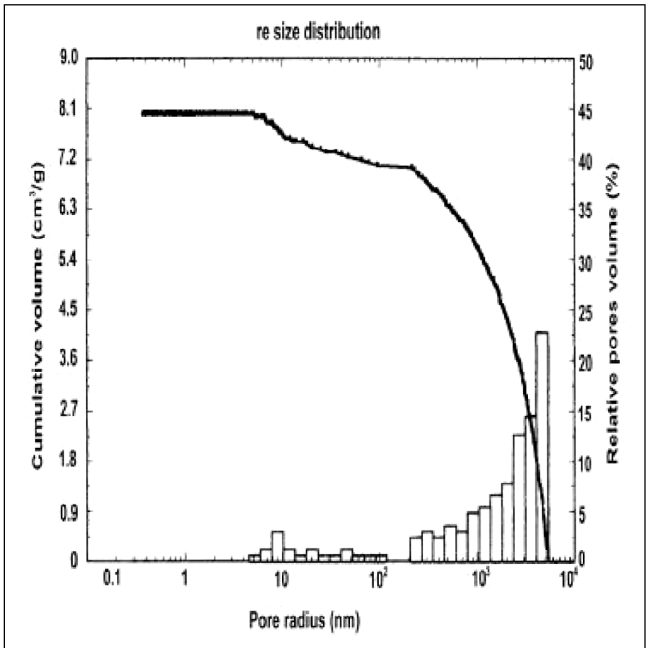

Figure 1. Pore-size distribution profile of the ammonia treated polymer measured by mercury intrusion porosimetry.

Packed column experiments

The chromatographic properties of ammonia- and DEA–THF-functionalized materials were studied in packed column experi-

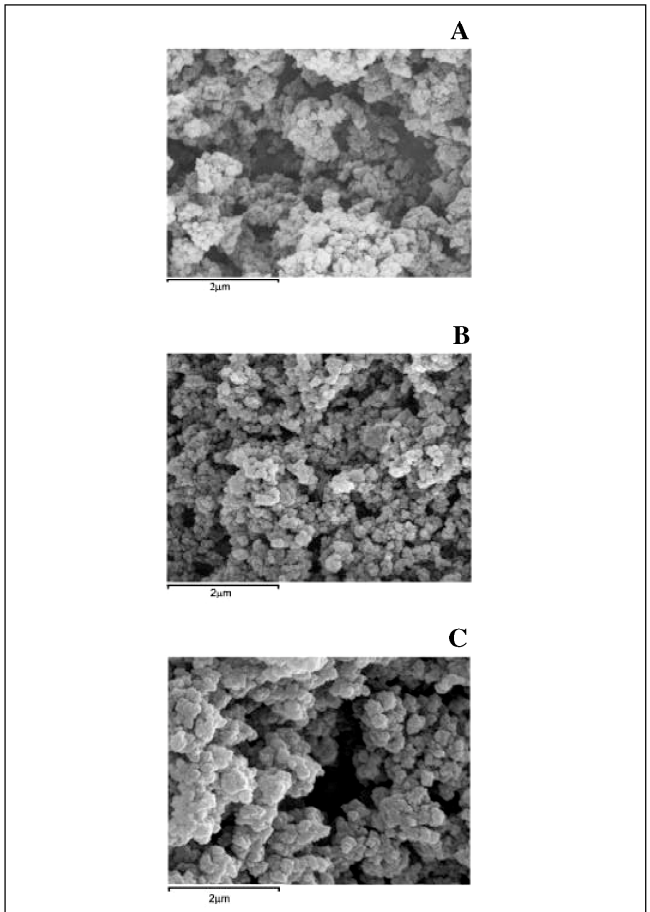

Figure 2. Scaning electronic microscopy images: (A) nonfunctionalized polymer, (B) ammonia treated polymer, and (C) DEA–THF functionalized polymer, (magnification × 3000).

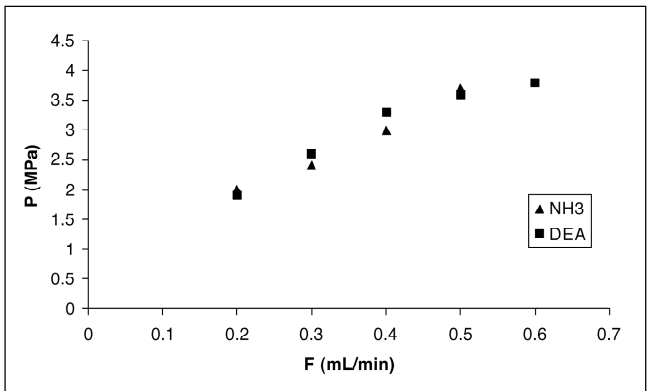

Figure 3. Column backpressure versus flow rate representation for the ammonia and DEA–THF-treated resins.

ments. The chromatographic behavior of the polymer aminated with pure DEA was not studied its lower content in amino groups.

The representations of backpressures versus flow rate for both columns are shown in Figure 3. For a given flow rate, the DEA–THF-functionalized material presents higher backpressure, a consequence of the formation of smaller polymer particles during the functionalization process.

Permeability was obtained from Darcy's equation (21), representing μ versus DP/hL, being L = 7.5 cm and η = 0,0117 dyn·cm^{-2}·s. The values obtained for the permeability coefficient (k) were 1.505 × 10^{-10} cm^2·s for the ammonia-functionalized resin and 1.812 × 10^{-10} cm^2·s for the polymer treated with DEA–THF. As expected, considering its porous structure, the permeability of the latter is better.

Values of the axial dispersion coefficient (D_{ax}) obtained at different flow rates for both resins are represented in Figure 4. Those corresponding to the DEA–THF-functionalized material at different flow rates are lower and more stable, thus allowing the prediction of better separation qualities for this column in a wide range of working conditions.

The height equivalent to a theoretical plate (HETP) was calculated under nonretention conditions and is given by the expression:

$$HETP = \frac{L}{N} \qquad \text{Eq. 3}$$

where L is the column length and N is the plate number given by the expression:

$$N = (t_R / \sigma)^2 \qquad \text{Eq. 4}$$

where t_R is the sample retention time and σ is the standard deviation of the corresponding Gaussian curve. The HETP values obtained for the DEA–THF-treated material are 30% lower than those corresponding to the polymer functionalized with ammonia, with HETP values of around 0.29 cm for a flow rate of 0.5 mL/min in the former case and 0.38 for the same flow rate for the latter material. This confirms better separation qualities for the resin aminated with DEA–THF, possibly attributable to the lower particle size of the DEA-functionalized resin.

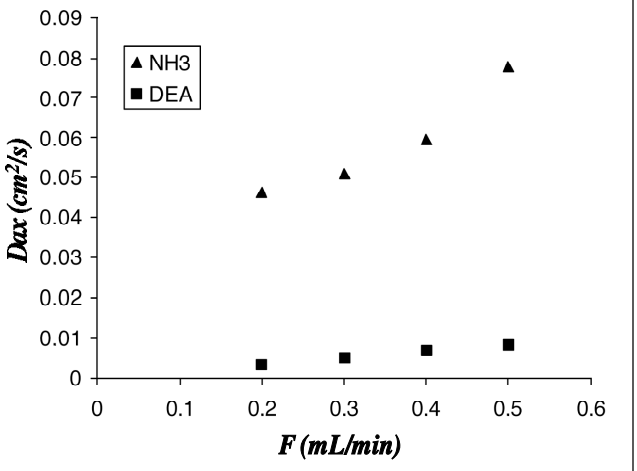

Figure 4. Values of the axial dispersion coefficient (D_{ax}) obtained at different flow rates for both resins.

Chromatographic characterization

Values of ovalbumin equilibrium concentration versus total amount of adsorbed protein for both columns are represented in Figure 5. Retention capacities and equilibrium constants (K), calculated from these curves using the constant factor isotherm, are summarized in Table II. The DEA–THF-functionalized material presents a very good retention capacity, almost 5-fold that of the ammonia-treated material, as should be expected considering their respective porous structures. The presence of wider macropores favors the accessibility of protein molecules to the resin surface. Furthermore, this resin has a higher content in amino groups, which behave as protein attaching sites.

The ammonia-functionalized polymer presents a lower retention capacity but a higher value for the equilibrium constant, obtained from the constant factor isotherm. This isotherm presents an almost rectangular shape with the maximum capacity reached for low liquid concentrations (~ 0.25 mg/mL). This behavior (not high capacity and rectangular shape isotherm) suggests that surface adsorption of protein takes place because the

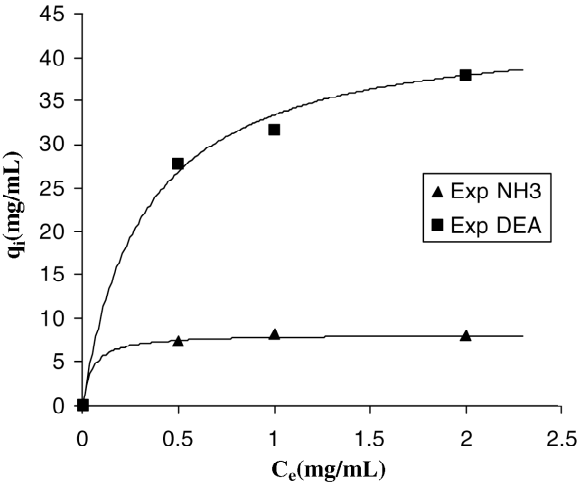

Figure 5. Equilibrium isotherms of ovalbumin for both functionalized resins. Equilibrium concentration in the solution (C_e) versus equilibrium concentration of adsorbed protein in the resin (q_i).

Table II. Retention Capacities and *K* Values (Obtained from the Constant Factor Isotherm) for Both Columns

Parameter	Ammonia-funct. resin P2 sample	DEA–THF-funct. resin P4 sample
Total retention capacity (mgOVA/mL resin)	8	38
Useful capacity (10%) (mgOVA/mL resin)	7.5	36
K	41.67	7.27

lower size of the mesopores of this resin impedes the penetration of proteins into the narrower pores.

Egg-white protein separation

Once the resins had been physically characterized, their separation performances were tested using a mixture of proteins present in egg white. Two sets of experiments were carried out: initially the chromatographic supports were used to separate mixtures of commercial egg-white proteins (supplied by Sigma), and subsequently, proteins were fractionated from natural egg white.

Separation of commercial egg-white proteins

A mixture of commercial lysozyme, ovalbumin, and trypsin inhibitor, containing 1 mg/mL of each one, was separated with both columns in order to test their separation performances. Figure 6A shows the chromatogram of the protein mixture obtained with the ammonia-functionalized resin. The buffer used for equilibration of the column was Tris-HCl 10mM (buffer A), pH 7.6. For elution, a solution of 0.3M NaCl in Tris-HCl 10mM (buffer B) was used. The gradient went from 0% to 100% of buffer B in 120 min at a flow rate of 0.4 mL/min. Figure 7 shows the chromatogram obtained with the DEA–THF-functionalized resin under the same experimental conditions. Five main peaks were obtained with the resin functionalized with ammonia and six with the DEA–THF-treated material. Both resins present similar chromatographic behavior, the resolution of the peaks is poor because of the high particule size, but this can be easily improved using particules of small size to fill the columns. For the two compared materials, better relative resolution was achieved with the latter one.

In order to identify the peaks, chromatographs of the isolated commercial proteins were obtained; Figures 6B–D show the results for lysozime, trypsin inhibitor, and ovalbumin, respectively. It can be observed that the samples are not completely pure, resulting in the presence of different peaks in the chromatograms. Electrophoretic analyses were also carried out; the molecular weights of every band were calculated from the reference front (R_f) of the standards in the gel. Table III summarizes the results obtained.

Peak 1 of both chromatograms (Figures 6A and 7) belongs to a protein with a molecular weight of 14,400 Da, corresponding to lysozyme, which is unretained by the resins because of its isoelectrical point (pI 10.7). It should be noted that the pH of the buffer was 7.6 and that under these conditions lysozyme has a positive charge and is not retained by the anionic resins but appears in the void volume. Peak 2 consists of a main protein of approximately 41,000 Da, which may correspond to trypsin inhibitor (31) with a small amount of ovotransferrin (76,000 Da). This peak presents considerably better resolution in the ammonia-functionalized polymer than in the DEA–THF-treated resin. Peak 3 has a calculated molecular weight of 36,500 Da, which corresponds to a globulin (31). Peak 4 corresponds to different proteins in both columns. In the DEA–THF column, this peak belongs to the same globulin as Peak 3, although in the ammonia column it corresponds to ovalbumin, with a molecular weight of 45,000 Da. Peak 5 of both columns and Peak 6 of the DEA–THF column, with molecular weights of 45,000 Da, correspond to ovalbumin. Finally, at the end of the gradient (100% of buffer B), two peaks were obtained, probably corresponding to subunits of either ovomucin or ovoglobulins (43,000 Da) (31). This is more clearly observed with the DEA–THF-functionalized resin. It can thus be seen that both materials are able to fractionate the main proteins of egg white.

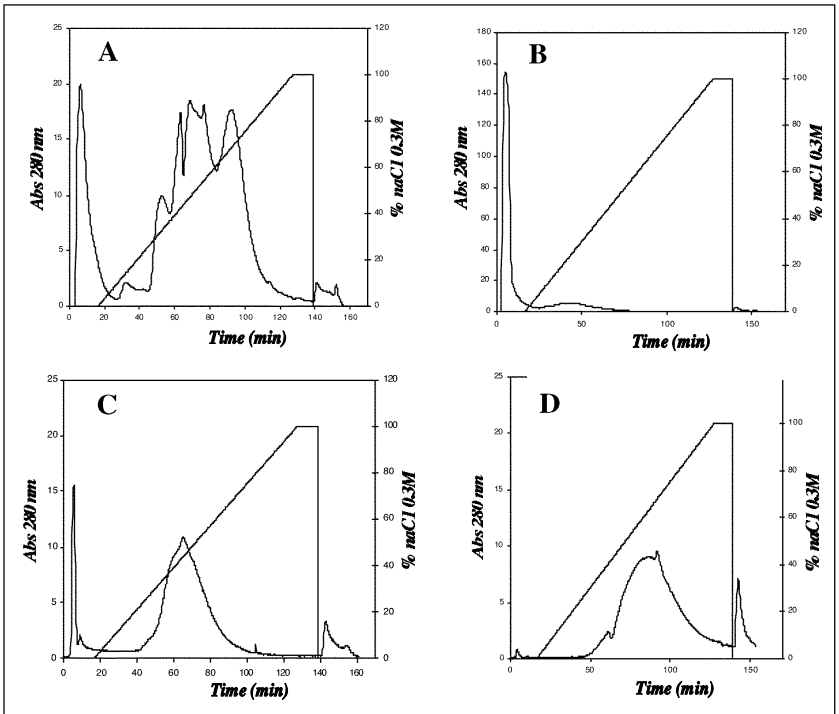

Figure 6. (A) Chromatogram of commercial egg white proteins separated using the ammonia functionalized polymer. Experimental conditions: column, resin packed in a 7.5- × 0.4-cm stainless steel column; temperature, 25°C; flow rate, 0.4 mL/min; UV detection, 280 nm; and gradient, 0–100% in 120 min. Mobile phases: buffer Tris-HCl 10mM; and buffer B, 0.3M NaCl in Tris-HCl 10mM (A). Chromatogram of lysozyme at the same experimental condictions (B). Chromatogram of trypsin inhibitor (C). Chromatogram of ovalbumin (D).

In order to verify the efficiency of the supports to fractionate proteins from natural samples of egg white, different experiments were carried out. The chromatographic behavior of both resins was found to be similar. Separation of natural egg-white proteins was carried out in a column of 7.5 × 0.4 cm using a continuous gradient going from 0% to 60% of buffer B in 120 min. The fractionation achieved with the natural egg white was similar to those obtained with the commercial isolate proteins, presenting the same number of main peaks and the same fractionation order in the chromatogram.

Separation of natural egg-white proteins at semipreparative scale using a step gradient

In light of the good behavior of the DEA–THF-aminated polymer for the separation of egg-white proteins at laboratory scale, and in particular ovalbumin, the fractionation at semipreparative scale of the proteins contained in dried egg-white sam-

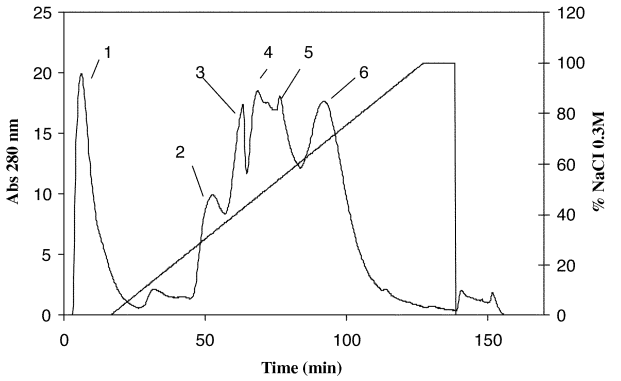

Figure 7. Chromatogram of commercial egg-white proteins separated with DEA–THF-treated polymer. Experimental conditions: column, resin packed in a 7.5- × 0.4-cm stainless steel column; temperature, 25°C; flow rate, 0.4 mL/min; UV detection, 280 nm; and gradient, 0–100% in 120 min. Mobile phases: buffer A, Tris-HCl 10mM; and buffer B, 0.3M NaCl in Tris-HCl 10mM. Peak identification: (1) lysozyme; (2) trypsin inhibitor, (3,4) small globulin, and (5,6) ovalbumin.

Table III. Molecular Weights Corresponding to Peaks of Figures 7A and 8 Calculated from R_f of the Commercial Egg-White Proteins in Gel

Peak	Approx. molecular weight (Da) for DEA–THF column	Approx. molecular weight (Da) for NH3 column
1	14,400	14,400
2	41,000	41,000
3	36,500	36,500
4	36,500	45,000
5	45,000	45,000
6	45,000	

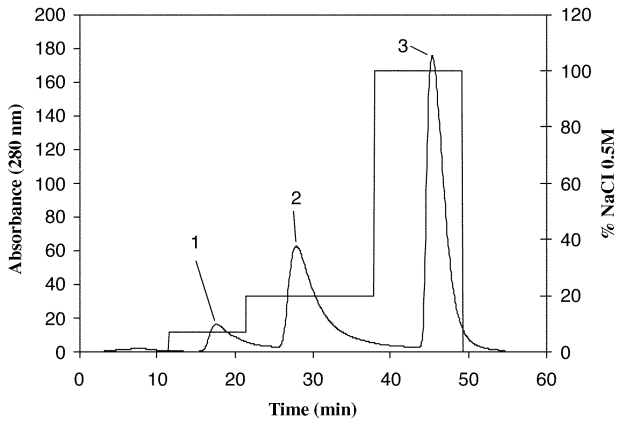

Figure 8. Chromatogram of natural egg-white proteins obtained with the DEA–THF resin at semipreparative scale using step gradient elution Experimental conditions: column, resin packed in a 3- × 3.2-cm borosilicate column; temperature, 25°C; flow rate, 18 mL/min; UV detection, 280 nm; and step gradient. Mobile phases: buffer A, Tris-HCl 10mM; and buffer C, 0.5M NaCl in Tris-HCl 10mM. Peak identification: (1) protein mixture in low concentration; (2) lysozyme, ovotransferrin, and ovalbumin impurities; and (3) pure ovalbumin.

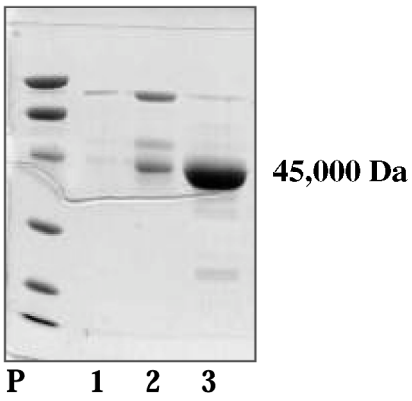

Figure 9. SDS–PAGE gel electrophoresis of natural egg-white proteins separated with the DEA–THF resin at semipreparative scale using step gradient elution. Strip P corresponds to a standard mixture of six proteins of known molecular weights. The different numbers of the strips in the gel correspond with the number of the peaks shown in the chromatogram of Figure 8.

ples working with step gradients was studied in order to simplify the operation using isocratic pumps. The main objective of these experiments was to obtain purified ovalbumin. Experiments were carried out using a borosilicate glass column (32-mm i.d.) loaded with 23 mL of DEA–THF-functionalized resin. The injected samples contained 5 mg/mL of dried egg white, a sufficient amount of proteins to fill 90% of the resin capacity, in Tris-HCl 10mM (pH 7.6). Elution was carried out with a solution of NaCl 0.5M in Tris-HCl 10mM (buffer C). These conditions and the salt concentration for each step were chosen after prior testing and taking into account the results obtained previously with the small column. The obtained chromatogram is presented in Figure 8. SDS–PAGE electrophoresis of the obtained peaks is shown in Figure 9. Strip P corresponds to the molecular weight of standards consisting of a mixture of six proteins of known molecular weights (phosphorilase 97.4 KDa, bovine seroalbumin 66.2 KDa, ovalbumin 45.0 KDa, carbonic anhidrase 31.0 KDa, trypsin inhibitor 21.5 KDa, and lysozyme 14.4 KDa).

At the first step of elution, carried out with a solution containing 20% v/v of buffer C in buffer A, the proteins start eluting in low concentrations (peak 1 in Figure 8, strip 1 in Figure 9). A second step using 60% v/v of buffer C in buffer A extracts a protein mixture containing ovotransferrin, lysozyme, and impurities of ovalbumin (Peak 2 in Figure 8, strip 2 in Figure 9). Finally, with a third step of elution using 100% of buffer C, practically pure ovalbumin was obtained (Peak 3 in Figure 8, strip 3 in Figure 9). This protein was then dialyzed and dried, obtaining 38.2 mg of solid. As the average quantity of ovalbumin in the egg white is approximately 48 mg, a yield of 83% of recovered ovalbumin was obtained.

Conclusion

Poly(glycidyl methacrylate-co-ethylendimethacrylate) was obtained by "bulk" polymerization of a novel mixture of monomers (55% glycidyl methacrylate and 45% ethylen-

dimethacrylate). Amination of the epoxide groups of the particulate polymer was carried out using three different chemical reagents: concentrated ammonia, DEA, and 50% (v/v) DEA in THF, the last one being found to be more effective, as it presents the higher content in amino groups. The ammonia- and DEA–THF-functionalized particulate polymers were able to fractionate commercial and natural egg-white proteins by anion exchange chromatography, particularly lysozyme and ovalbumin. The latter resin showed a good retention capacity for ovalbumin, 38 mg/mL of resin, and better fractionation performance because of its higher content in amino groups and wider porous cavities. Finally, DEA–THF material working with step gradient elution at semipreparative scale enabled the recovery of practically pure ovalbumin, achieving a yield of 83%.

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