Economic considerations important in the scale-up of an ovalbumin separation from hen egg-white on the anion-exchange cellulose DE92

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ABSTRACT

The scale-up of the separation of hen egg-white proteins has been investigated using Whatman DE92 anion-exchange cellulose. Having developed suitable chromatographic conditions, a maximum binding capacity of 100 mg protein/ml packed DE92 was determined in a 25-ml column. The process was scaled up 1000-fold and the influence of batch and column techniques on the chromatographic step assessed. Data indicate column processes to be more efficient than batch in the adsorptive stage.

INTRODUCTION

Ion-exchange chromatography is a major tool in the downstream processing of commercially important biopolymers. Being an adsorptive process relying on the electrical charge of the feedstock components, ion-exchange is a highly versatile and selective technique, often giving rise to a high degree of purification in a single step. Low-pressure ion-exchange media are traditionally based on polysaccharide supports including cellulose, agarose and dextran [1,2].

In order to make a process economically attractive and in many instances commercially viable, it is important to optimise throughput without adversely compromising product purity. There are several factors which directly influence throughput and these include capacity, kinetics and flow-rate. Cellulose is a macroporous sorbent and thus offers a very high protein binding capacity [3,4] and fast kinetics of adsorption/desorption [5,6] factors which influence both binding efficiency during the adsorptive process and chromatographic resolution in the des-

orptive process. In many instances process-time *i.e.* flow-rate becomes an influencing factor, and it is generally perceived that non-fibrous celluloses *i.e.* microgranular products are unable to support high flow-rates. We have confirmed this perception in studies where axial flow columns containing Whatman DE52 and QA52 were evaluated [4,7].

It has been reported that cellulose-based media exhibit fast flow performance in radial flow columns [8,9] and we have demonstrated that DE52 and QA52 support flow-rates up to 6-fold faster than in an axial flow column of identical volume whilst maintaining good chromatographic resolution [10,11].

We have previously investigated a number of parameters which affect the economics of process-scale column chromatography using Whatman QA52. These include studies on capacity and kinetics [5], feedstock composition [12], process optimisation [13] and scale-up [14,15].

In the present study we have extended these investigations to DE92, a recently developed fast-flowing anion-exchange cellulose, and have exam-

ined various scale-up parameters, in the separation of hen egg-white proteins in a three phase investigation. Firstly, the chromatographic conditions were developed at laboratory scale and shown to scale-up at least 1000-fold. Secondly, the maximum binding capacity of DE92 was determined using a suitable hen egg-white feedstock. Thirdly, the influence of batch *versus* column techniques were examined for the process-scale separation of hen egg-white proteins. This latter stage is complementary to a previously reported study using DE52 [3,16].

Hen egg-white is a natural protein-rich feedstock used commercially in the food processing and enzyme manufacturing industry. It is an ideal feedstock for process-scale ion-exchange studies since it is a freely available, multicomponent system which requires dilution and clarification in order for it to be effective for chromatography without fouling either the column components or chromatographic medium.

In food processing industries, for example, the feedstock volume is typically large. The key to economic success in these processes is optimal throughput. A means of improving throughput is reducing the process time and this can be achieved by increasing flow-rate in a column process or reduced handling/wash times in a batch process. In order to facilitate these improvements Whatman developed a range of anion- and cation-exchange media during 1990 based on fibrous cellulose, *i.e.* DE92, QA92, CM92 and SE92. These products may be operated at flow-rates 2-3-fold faster than the microgranular cellulosic media. In the present study we demonstrate that DE92 can be operated at high flow-rates in a process-scale system without loss of capacity or binding efficiency for the target proteins.

EXPERIMENTAL

Materials

Cell debris remover (CDR), DE52, DE92 and a PREP-25 column were obtained from Whatman Specialty Products (Maidstone, UK). Ovalbumin was obtained from Sigma (Poole, UK) Tris(hydroxymethyl)aminomethane (Tris) was obtained from Merck (Dagenham, UK). Sephadex G-25 medium was obtained from Pharmacia (Milton Keynes, UK). All other chemicals were of reagent grade. Fresh size 2 hen eggs were obtained from Barradale Farms (Headcorn, UK).

Feedstock preparation

Egg-whites were separated from 600 fresh hen eggs and diluted to 14% (v/v) with 0.025 M Tris–HCl buffer, pH 7.5 [17]. The egg-white suspensions were clarified using a total of 22 kg pre-equilibrated CDR in a batch mode. Spent CDR was removed by centrifugation through a 1.6 × 0.06 mm slotted screen (EHR 500 basket centrifuge, Robatel and Mulatier, Lyons, France) and the sample clarified by filtration through a grade 541 filter paper (Whatman Scientific, Maidstone, UK). The clear solution (200 l) containing *ca.* 10 mg total protein per ml was used for chromatography.

Chromatographic studies

Chromatographic development

Egg-white feedstock (7 ml) was applied to a DE92 column (15.5 cm × 1.5 cm I.D.) pre-equilibrated with 0.025 M Tris–HCl buffer, pH 7.5 [18], and non-bound material removed by washing with 0.025 M Tris–HCl buffer, pH 7.5 (50 ml). Bound material was eluted using a linear gradient of 0–0.5 M NaCl in 0.025 M Tris–HCl buffer, pH 7.5 (200 ml). The chromatography was carried out at a flow-rate of 2 ml/min.

A parallel experiment was carried out using DE92 packed in a PREP-25 column (16 cm × 45 cm I.D.) at a flow-rate of 2 l/min with a 1000-fold scale-up throughout.

Capacity determination

Egg-white feedstock was applied to a column of DE92 (15.5 cm × 1.5 cm I.D.) previously equilibrated with 0.025 M Tris–HCl buffer, pH 7.5 at a flow-rate of 2 ml/min over a period of 300 min by which time the absorbance of the eluate at 280 nm was similar to that of the feedstock. Non-bound material was removed by washing with 0.025 M Tris–HCl buffer, pH 7.5 (50 ml). Bound material was eluted with 0.025 M Tris–HCl buffer, pH 7.5 containing 0.5 M NaCl (100 ml). The flow-rate was maintained at 2 ml/min throughout.

Process-scale chromatography

DE92 (25 kg) was equilibrated with 0.025 M Tris–HCl buffer, pH 7.5. The ion exchanger was used with the egg-white feedstock (200 l) accordingly: (i) batch adsorption/batch desorption; (ii) batch adsorption/column desorption; (iii) column adsorption/column desorption. All procedures were carried out at room temperature (15–20°C).

(i) Batch adsorption/batch desorption. DE92 (25 kg) was stirred with the CDR-treated feedstock (200 l). The DE92 was collected by centrifugation and washed with 0.025 M Tris–HCl buffer, pH 7.5 (200 l). Bound material was eluted using 0.025 M Tris–HCl buffer, pH 7.5 containing 0.5 M NaCl (200 l). In this study elution was carried out on the centrifuge wall for half of the DE92 and in a stirred tank for the remainder of the DE92.

(ii) Batch adsorption/column desorption. DE92 (25 kg) was stirred with the CDR-treated feedstock (200 l). The slurry (8%, w/v) was pump-packed into a PREP-25 column (16 cm × 45 cm I.D.) at a pressure of *ca.* 10 p.s.i.

The bed was washed with 0.025 M Tris–HCl buffer, pH 7.5 (100 l) and bound material eluted using a linear gradient of 0–0.5 M NaCl in 0.025 M Tris–HCl buffer, pH 7.5 (200 l) at a flow-rate of 2.0 l/min.

(iii) Column adsorption/column desorption. DE92 (25 kg) was slurried to 14% (w/v) in 0.025 M Tris–HCl buffer, pH 7.5 and pump-packed into a PREP-25 column at a pressure of *ca.* 10 p.s.i. The egg-white feedstock (200 l) was loaded onto the column and non-bound material removed by washing with 0.025 M Tris–HCl buffer, pH 7.5 (100 l). Bound material was eluted using a linear gradient of 0–0.5 M NaCl in 0.025 M Tris–HCl buffer, pH 7.5 (200 l). A flow-rate of 2.0 l/min was maintained throughout.

Assays

Pooled fractions obtained at various stages of the chromatography were assayed for protein content using A_{280} measurement against standard solutions of ovalbumin.

Throughout the column procedures the effluent was monitored for absorbance at 280 nm and by conductivity.

Rechromatography of fractions

For each process-scale study samples of (a) egg-white feedstock, (b) non-bound material and (c) salt-eluted material were rechromatographed using DE52.

The salt-eluted material (c) was desalted by gel filtration through a column (12.5 cm × 1.0 cm I.D.) containing Sephadex G-25 medium, previously equilibrated with 0.025 M Tris–HCl buffer, pH 7.5.

The fractions were rechromatographed on a column (15 cm × 1.0 cm I.D.) containing DE52, previously equilibrated with 0.025 M Tris–HCl buffer, pH 7.5. Non-bound material was removed by washing with 0.025 M Tris–HCl buffer, pH 7.5 (25 ml) and bound material eluted using a linear gradient of 0–0.5 M NaCl in 0.025 M Tris–HCl buffer, pH 7.5 (100 ml). Flow-rate was maintained at 2 ml/min, and the absorbance at 280 nm and the conductivity of the eluent were continuously monitored.

RESULTS AND DISCUSSIONS

In our earlier studies on egg-white chromatography using DE52 we used a feedstock containing *ca.* 10 mg/ml total protein [3,16], as we considered this to be a typical and suitable working concentration of proteins. In the present study we used a similar feedstock for the evaluations of DE92. Under analytical loadings *i.e.* total protein loaded is < 5% of total capacity, a typical separation of egg-white proteins was seen for DE92 (Fig. 1a). The non-bound fraction consisted of two components, the basic protein lysozyme and the acidic protein conalbumin (mol. wt. 77 000 dalton). The adsorbed material contained low levels of conalbumin with the major component being ovalbumin (mol. wt. 45 000 dalton). The fibrous nature of the cellulose matrix in DE92 gives rise to significant size exclusion of larger molecules and this is evident by the very low levels of conalbumin which adsorb to the medium. Under identical conditions the conalbumin component is retained by both DE52 [3] and QA52 [7], which based on microgranular cellulose do not exhibit similar size-exclusion properties. Having developed a suitable chromatographic system using a laboratory column the separation was scaled-up
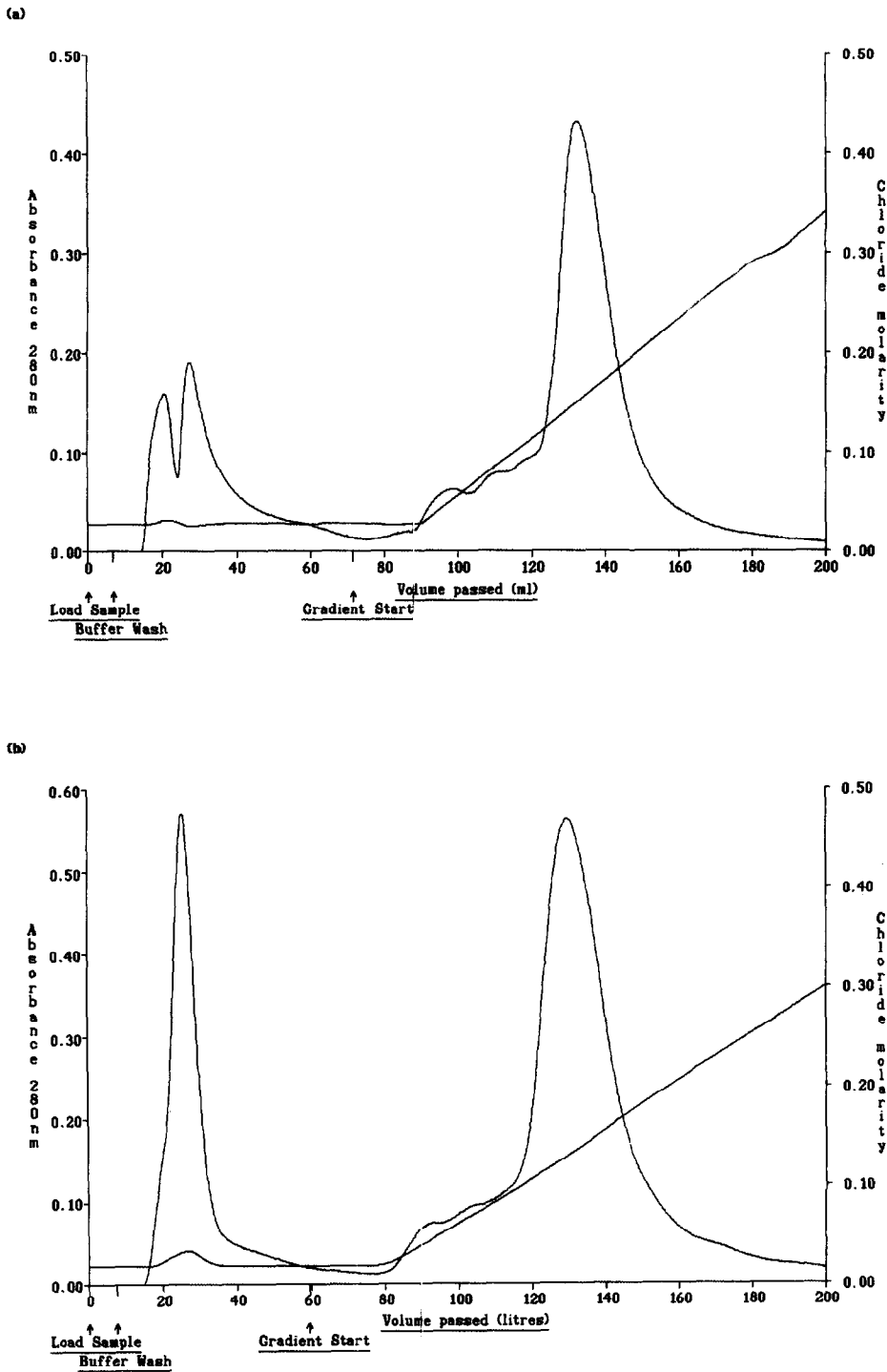
(a)

(b)

Fig. 1. Column chromatography of hen egg-white proteins on DE92 using 0.025 M Tris–HCl buffer, pH 7.5 at (a) laboratory-scale (15.5 cm × 1.5 cm I.D.) and (b) process-scale (16 cm × 45 cm I.D.).
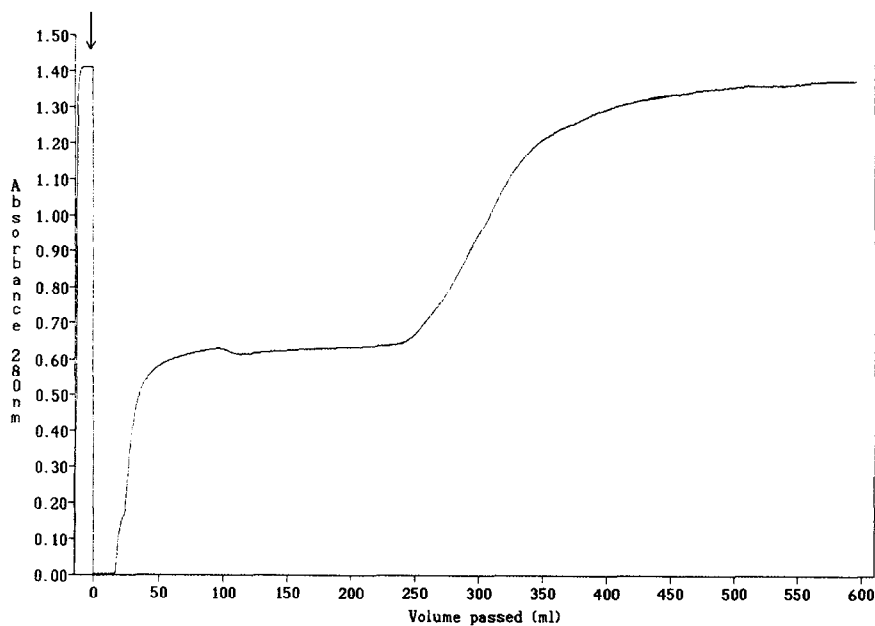
Fig. 2. Absorbance profile of column eluate during a saturation loading of DE92 with 10 mg/ml hen egg-white proteins using 0.025 M Tris–HCl buffer, pH 7.5. Absorbance of the feedstock is identified by the arrow.

1000-fold by increasing bed diameter 30-fold whilst maintaining column bed height and linear flow-rate (*ca.* 75 cm/h). This separation is represented in Fig. 1b and clearly the DE-92 process has scaled-up satisfactorily.

In order to determine the maximum capacity of the column for egg-white proteins, the feedstock (10.4 mg/ml) was applied to a laboratory column of DE92. Following a 610-ml loading of the column, the absorbance of the eluate at 280 nm was similar to the absorbance of the feedstock, indicating that a saturation loading had been achieved. The absorbance profile of the column eluate during this study is represented in Fig. 2. During the adsorption stage a non-linear stepped absorbance was observed. The shape can be attributed to components of the egg-white eluting from the column in order of ascending acidity due to protein–protein displacement etc., resulting in the ovalbumin component only being retained. Similar observations were reported for the treatment of goat serum with CDR [15]. Following the column loading and buffer wash, a total of 2.688 g protein were bound to the DE92 which reflects a capacity of 98 mg protein/ml packed column volume. Elution of bound protein with NaCl resulted

in recovery of 89%. In a similar experiment using DE52 a capacity of 158 mg protein/ml packed column volume was obtained with 98% recovery of bound protein [19].

In a process-scale separation, throughput is very critical and although it may be possible to operate a column of DE92 at a capacity of 98 mg/ml packed column volume, this is clearly an inefficient adsorptive process since only 66% of the total ovalbumin present in the feedstock bound to the DE92, based on an average ovalbumin content of 63.8% (w/w) of total egg-white protein [20]. In separations where the feedstock is freely available in excess then inefficient adsorption may be acceptable, but in many instances the value of the feedstock and/or target, coupled with increased process times and added effluent treatment outweigh any potential benefits of such a step. It would therefore be desirable to carry out the process under conditions where as close to 100% of the target binds to the medium during a single contacting operation.

In order to scale-up the chromatography while maintaining an efficient binding process, 200 l of egg-white feedstock was applied to 25 kg DE92, *i.e.* a loading of *ca.* 75 mg protein/ml packed column

TABLE I

PROTEIN CAPACITIES DURING PROCESS-SCALE CHROMATOGRAPHY OF EGG-WHITE PROTEINS

Run	Adsorption mode	Desorption mode	Feedstock total protein (g)	Stage of chromatography	Total protein (g)		Binding efficiency (%)
					In mobile phase	Adsorbed to DE92	
1	Batch	Batch centrifuge wall	1846[a]	Loading Wash Elution	256 29 572	667 638 66	69.1
2	Batch	Batch stirred tank		Loading Wash Elution	256 27 609	658 631 22	68.4
3	Batch	Column	1965	Loading Wash Elution	571 98 1097	1394 1296 199	66.0
4	Column	Column	1945	Loading Wash Elution	426 113 1421	1519 1406 —	72.3

[a] Split into 2 lots after loading.

volume, which is submaximal loading as determined previously (Fig. 2). In this study we have compared batch and column techniques under similar process-chromatography conditions. The loading capacity data for these studies are summarised in Table I. In order to determine the efficiency of the adsorptive process with respect to total feedstock proteins a binding efficiency is calculated according to eqn. 1:

Binding efficiency (%) =

$$\frac{\text{mass of protein adsorbed}}{\text{mass of protein loaded}} \cdot 100 \quad (1)$$

The batch loading data indicate that *ca.* 68% of applied protein is adsorbed to the DE92 and this compares to a binding efficiency of *ca.* 72% for column loading. These observations are consistent with those obtained using DE52 [3,16] and are expected since a batch step is in fact a simple equilibrium process and can be regarded as having a theoretical plate count of one. On the other hand, a column has a higher plate count and consequently will be more efficient during the loading procedure.

The elution stages were efficient in all cases and are consistent with our previous studies on DE52 [3,16].

The data indicate that *ca.* 55 mg protein bound per ml (g) of DE92 from a loading of *ca.* 75 mg total proteins/ml. Based on an ovalbumin content of 63.8% (w/w) of total egg-white protein [20] then assuming 100% adsorption of the ovalbumin (*ca.* 48 mg/ml), only small amounts of other proteins would be expected to have bound to the DE92. This is confirmed in the rechromatography of various fractions obtained during the process. The egg-white feedstock gives a typical chromatogram on DE52 (Figs. 3a, 4a, 5a and 6a). The lysozyme component does not bind to the column and the two adsorbed components elute in the order conalbumin followed by ovalbumin. In each adsorption-process the lysozyme and conalbumin constituents did not bind to the DE92 whereas the ovalbumin was totally bound (Figs. 3b, 4b, 5b and 6b). Chromatograms of the pooled-bound material, following desalting, are represented in Figs. 3c, 4c, 5c and 6c. In each case the proteins bound to the DE92 appear not to be significantly different and as suggested above are predominantly ovalbumin.

In any chromatographic process there are two key factors which affect the success and economics of the separation. Firstly the binding efficiency of the medium for the target during a single adsorptive cycle and secondly the purity of the product. Bind-
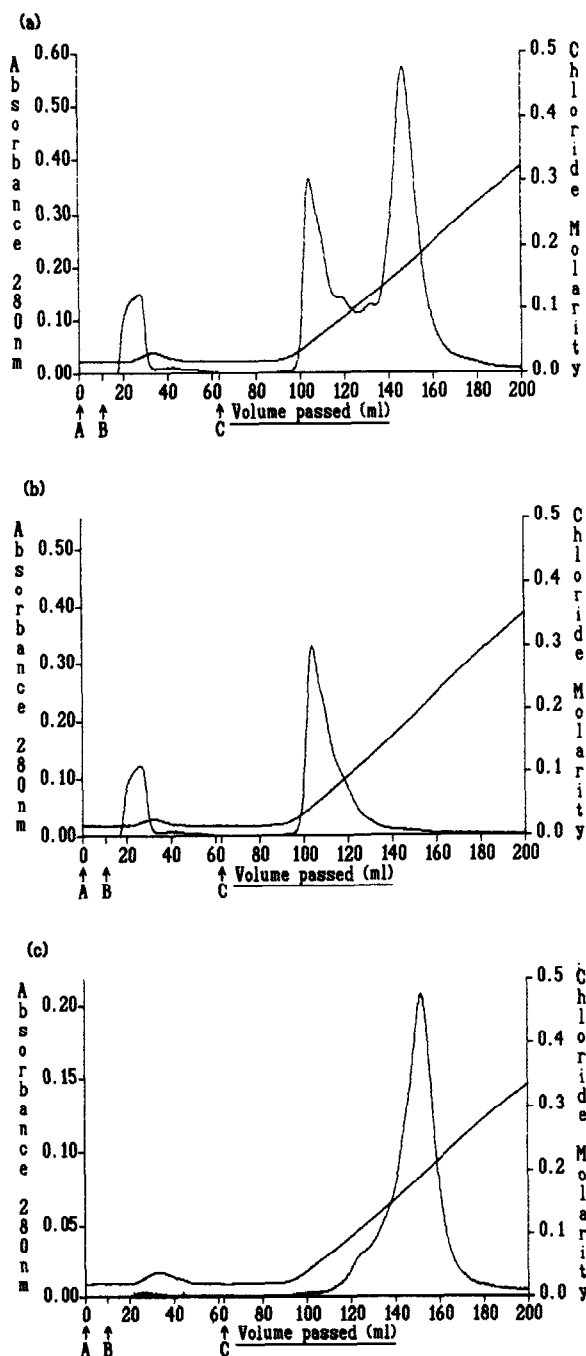
Fig. 3. Rechromatography using DE52 of individual fractions eluted during chromatography of hen egg-white proteins on DE92 following batch adsorption/batch centrifuge wall desorption using 0.025 M Tris–HCl buffer, pH 7.5. (a) Egg-white feedstock, (b) non-bound material, (c) salt-eluted material. A = Load sample; B = buffer wash; C = gradient start.
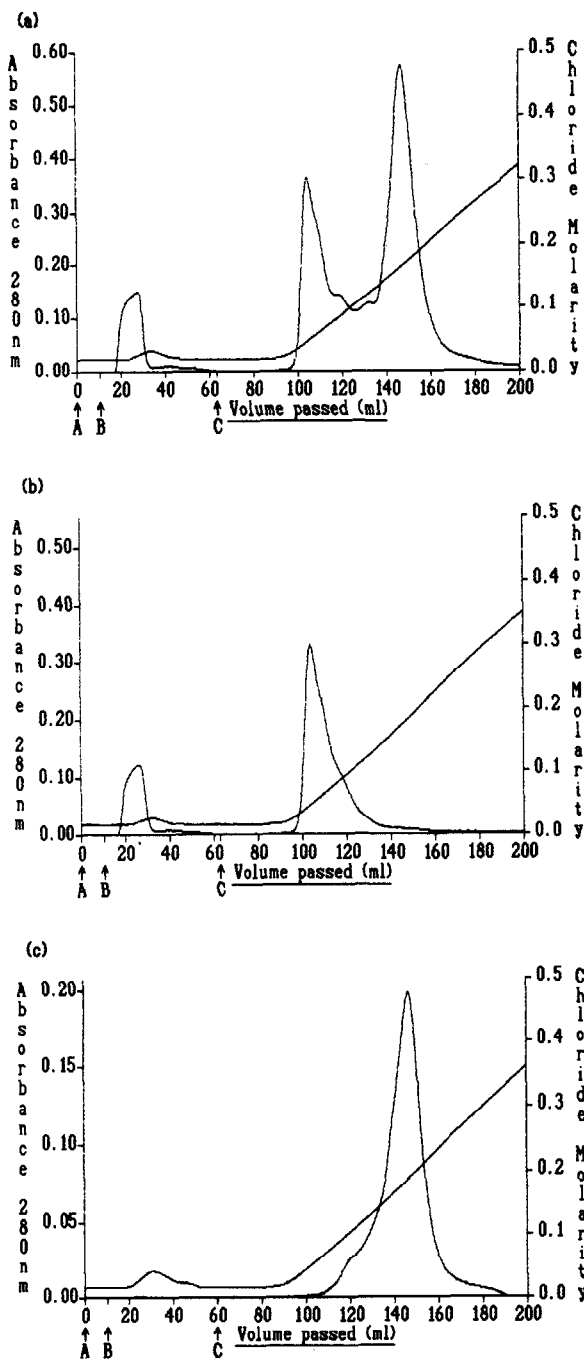
Fig. 4. Rechromatography using DE52 of individual fractions eluted during chromatography of hen egg-white proteins on DE92 following batch adsorption/batch stirred tank desorption using 0.025 M Tris–HCl buffer, pH 7.5. (a) Egg-white feedstock, (b) non-bound material, (c) salt-eluted material. A = Load sample; B = buffer wash; C = gradient start.

ing efficiency is dependent on the contact time between the target and ion-exchange medium and this is affected by flow-rate and the kinetics of adsorption. Product purity is generally dependent on the elution conditions and desorption kinetics of the medium. Since the DE92 was overloaded with protein such that the ovalbumin component displaced conalbumin and other weakly acidic components, the requirements for highly controlled elution and fast kinetics are reduced. This is confirmed in Figs. 3c and 4c where the step eluted bound material appeared to be very similar to the gradient eluted material Figs. 5c and 6c. Had the DE92 not been overloaded with egg-white feedstock *e.g.* reducing the feedstock volume to *ca.* 50 l then all acidic components would have been expected to adsorb to the medium. Consequently the gradient eluted material would give a significantly greater purity fraction than the bulk step eluted material.

Whatman ion-exchange celluloses exhibit fast kinetics of adsorption [5,6] but as discussed above are often used at low flow-rates. A typical linear flow-rate for DE52 would be *ca.* 30 cm/h using a process-scale axial column [3]. In the present study DE92 was used at a linear flow-rate of *ca.* 75 cm/h, the fibrous nature of the medium facilitating its fast flow properties. We have demonstrated that a protein separation using a natural feedstock can be effected using DE92 and this can be scaled-up from the laboratory to process-scale. Using *ca.* 56% of the total egg-white protein binding capacity of the DE92, the separation could be carried out at process-scale using either batch or column adsorption/desorption techniques with a very high binding efficiency. It is evident that column loading is more efficient than batch although the latter is potentially more time efficient especially in separations where large volumes of dilute feedstock may be involved.

In many production separations the requirement is for 100% recovery of a high value target protein with less regard for capacity of the medium. Consequently slow adsorption kinetics may be tolerated since binding efficiency is of low importance. When binding efficiency does become important, for example, where a competitor product may be available at a comparable cost, then throughput optimisation becomes a key factor. In these separations adsorption/desorption kinetics and flow-rate become determinants in media selection. Whatman
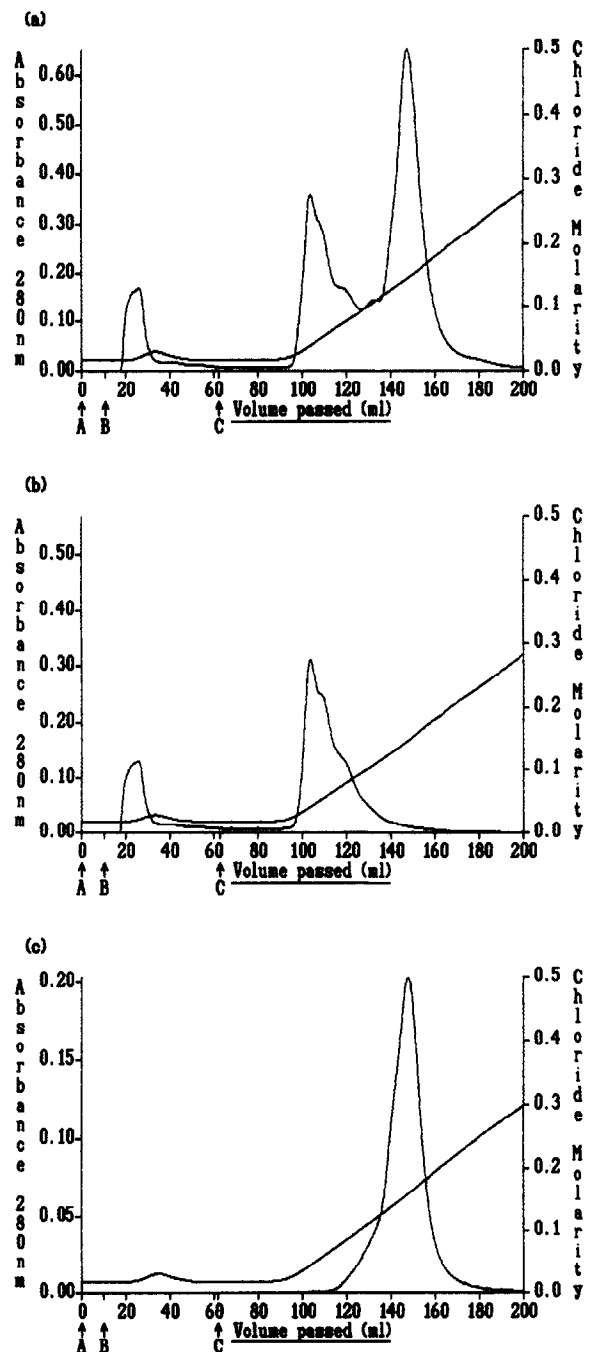

Fig. 5. Rechromatography using DE52 of individual fractions eluted during chromatography of hen egg-white proteins on DE92 following batch adsorption/column desorption using 0.025 *M* Tris–HCl buffer, pH 7.5. (a) Egg-white feedstock, (b) non-bound material, (c) salt-eluted material. A = Load sample; B = buffer wash; C = gradient start.
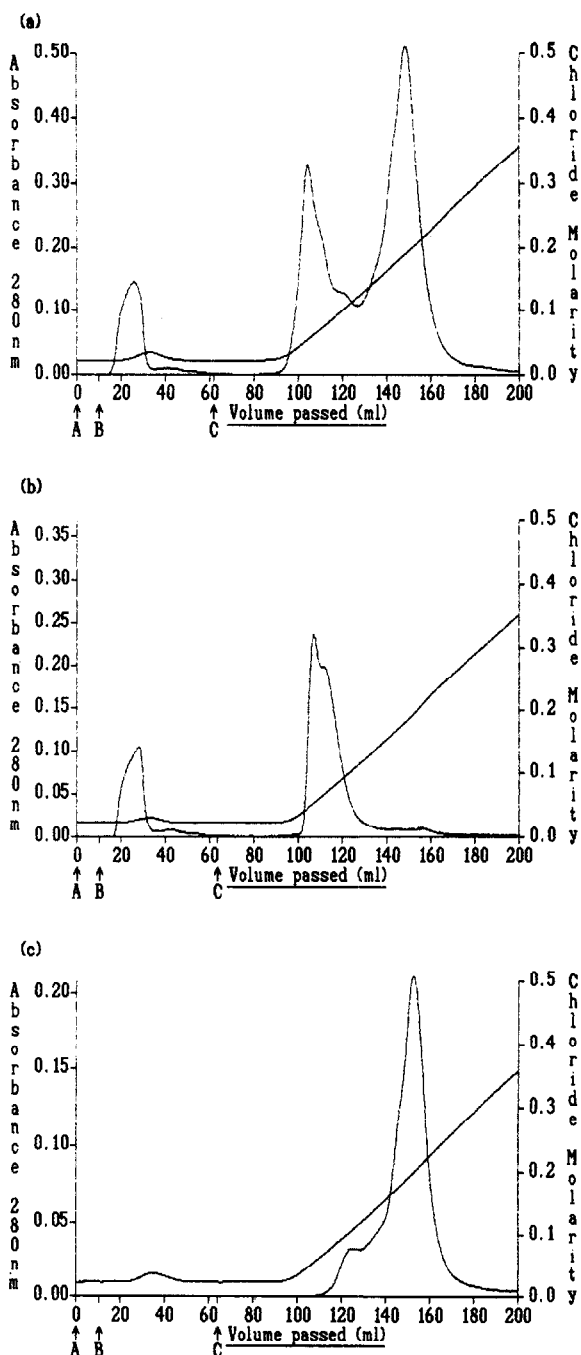
Fig. 6. Rechromatography using DE52 of individual fractions eluted during chromatography of hen egg-white proteins on DE92 following column adsorption/column desorption using 0.025 *M* Tris–HCl buffer, pH 7.5. (a) Egg-white feedstock, (b) non-bound material, (c) salt-eluted material. A = Load sample; B = buffer wash; C = gradient start.

ion-exchange celluloses offer fast binding kinetics and consequently are effective in providing high binding capacity and efficiency while ensuring good product purity. Dependent on the process requirements then either microgranular products, *i.e.* DE52, CM52, etc, or fibrous products, DE92, CM92, etc, may be applicable. The selection of either of these media types will depend on the nature of the target molecule, *i.e.* molecular weight or feedstock volume which could influence flow-rate, process time etc. The data in this study taken with our previous evaluations of DE52 [16] and QA52 [15] demonstrate that Whatman ion-exchange celluloses are suitable for process-scale protein separations in a range of manufacturing industries, where product yield and purity are key economic considerations.

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