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Journal of Chromatography A, 757 (1997) 41–49

JOURNAL OF CHROMATOGRAPHY A

Direct purification of lysozyme using continuous counter-current expanded bed adsorption

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Received 6 June 1996; revised 23 July 1996; accepted 2 August 1996

Abstract

We describe the application of a novel technique for the continuous counter-current chromatography of proteins. The unit operation has shown potential in extracting targeted species from unclarified feedstocks, delivering clarified streams of purified product. The adsorbent used in this equipment consists of a perfluorocarbon matrix, coated with poly(vinyl alcohol), and derivatized with the triazine dye Procion Red HE-7B. Purification of lysozyme from egg-whites and enriched bovine milk could be carried out continuously. The former was extracted in 90.5% yield at a rate of 7400 U/min, achieving a purification factor of 19.4. Lysozyme from the enriched milk sample was extracted continuously at a rate of 41 000 U/min, in 66.0% yield. The continuous product streams in both cases were fully clarified, thus enabling their direct application to a final polishing step, if desired.

Keywords: Continuous counter-current chromatography; Lysozyme

1. Introduction

With the ever-growing expansion of the biotechnology industry, it is becoming increasingly evident that there is still considerable scope for technological progress in the manufacturing of biological materials. Thus, in the production of a large proportion of diagnostic and therapeutic bio-chemicals, it has been shown repeatedly that the isolation and purification steps of the targeted molecule often prove to be the most problematic and expensive. These frequently account for up to 80% of the total manufacturing costs [1]. To counter these difficulties, a number of innovations have been proposed recently by biochemical engineers in the area of chromatographic matrix and contactor design. For example, Expanded Bed Adsorption (EBA) [2,3], and the development of increasingly sophisticated, synthetic, affinity-mimicking ligands [4–7], are now enabling impressively selective capture stages to be deployed just downstream of product fermentation and cell disruption [8,9]. Increasing interest is also being shown in developing unit operations capable of performing protein purification on a continuous basis, rather than in batch [10–12]. In recent work, we described a novel 4-stage system used to purify malate dehydrogenase (MDH) continuously from a crude homogenate of *Saccharomyces cerevisiae*, delivering a fully clarified product stream containing the targeted protein with a high yield and purification factor [13]. The adsorbent used consisted of a Procion Red HE-7B derivatized perfluorocarbon support.

In this work we illustrate further applications of this technology in the purification of hen lysozyme

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0021-9673/97/$17.00
PII S0021-9673(96)00680-2

from a solution of egg-whites, and from a mixture of lysozyme-enriched bovine milk. Lysozyme has a number of functions; for example, it is used as an additive to baby milk and as a component in ophthalmic preparations, and is also used in the treatment of ulcers and infections [14]. Because of the expanding potential of this enzyme, there is a necessity to develop a simple and effective technique for its purification, and these are the objectives of some of the work presented herein. More importantly, however, especially in the case of milk, this work further illustrates the suitability of the equipment for direct continuous purification from feedstocks typically used in the manufacture of recombinant proteins, where conventional packed bed techniques are likely to fail. Milk obtained from transgenic animals is one such source, and is gaining increasingly in potential.

2. Experimental

2.1. Chemicals and equipment

Hen lysozyme and powdered egg-whites were purchased from Sigma (Poole, Dorset, UK), as were *Micrococcus lysodeikticus* cells used in the spectrophotometric assay for lysozyme activity. Poly(vinyl alcohol) and all buffer salts used were purchased from Aldrich (Poole, Dorset, UK). Procion Red HE-7B and the solid perfluorocarbon support were kindly donated by Dr. C. Lowe (Institute of Biotechnology, University of Cambridge, UK). Coomassie assay reagents for the determination of protein concentration were purchased from Pierce (Rockford, IL, USA). All solvents used in this work were analytical grade, and were purchased from BDH (Poole, Dorset, UK). Bovine milk was obtained from local suppliers, and was 'virtually fat free' (1 mg/ml residual fat level).

2.2. Preparation of Procion Red HE-7B Perfluorocarbon Support

The procedure for the manufacture of the adsorbent is outlined in Owen et al. [15].

2.3. Protein preparation and assay

The 1.0 mg/ml egg-whites solution for continuous lysozyme extraction was prepared by dissolving the required mass of dried egg whites in 100 m*M* pH 9.2 sodium carbonate buffer. Similarly, for lysozyme extraction from enriched bovine milk experiments, the feedstock was made by dissolving the enzyme to 0.3 mg/ml in the milk, whose pH had previously been adjusted to 7.5 by the addition of dibasic and monobasic sodium phosphate (100 m*M*).

Total protein estimates for mixed-protein solutions in all experiments were carried out with the Pierce Coomassie protein assay reagent, using a BSA calibration curve. Lysozyme activity determination was carried out using the method described by Shugar [16], which involves measuring the rate of lysis of *Micrococcus lysodeikticus* by lysozyme. Results are expressed in units of enzyme activity, where 1 unit is defined as being that amount of enzyme causing a decrease in absorbance of 0.001 per min. Because of the strong dependence of the activity of the enzyme on the ionic strength of its environment, all samples to be assayed were brought by dilution to approximately 100 m*M* salts concentration, prior to assay. Data points plotted in Figs. 2–5 are obtained by multiplying the volumes collected in 20 min from the effluent streams by the concentrations and activities obtained spectrophotometrically.

2.4. Design aspects of the apparatus used in this work

Detailed description of the equipment, and its use in the continuous purification of malate dehydrogenase (MDH) from a crude *Saccharomyces cerevisiae* homogenate is described fully elsewhere [13]. However, a flowsheet illustrating the rationale of the overall unit is shown below in Fig. 1.

The four process streams enter their respective columns at the base, and then flow upwardly over the adsorbing material. The operating principle of the counter-current contactors is similar to that of expanded bed adsorption columns (EBA), in which upward plug-flow of the liquid causes fluidisation of the particles. This allows insolubles to pass freely through the ensuing voids. Because of the compara-

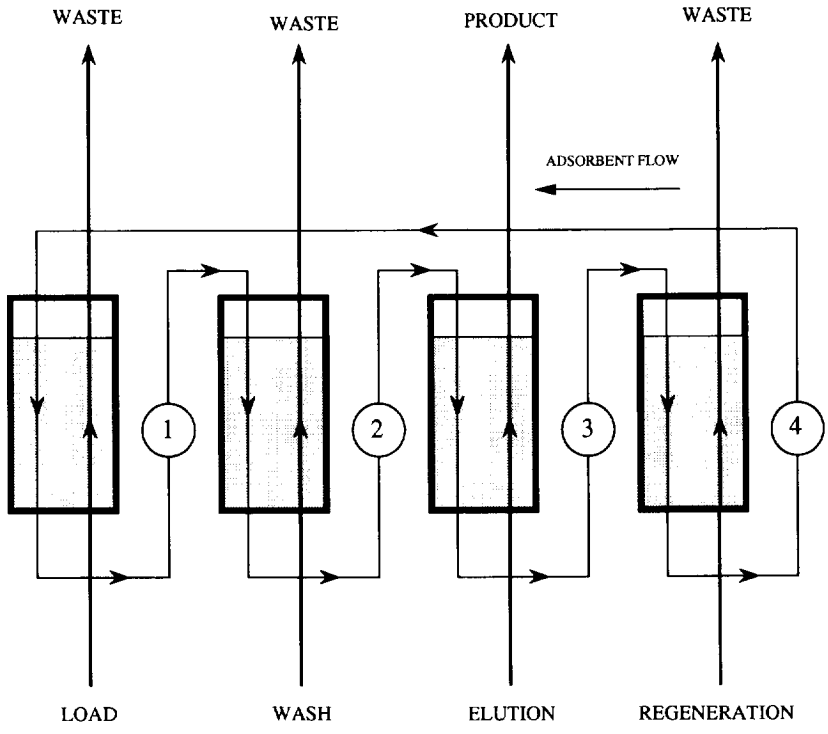

Fig. 1. Schematic of the four-stage counter-current arrangement. Four counter-current contactors are arranged in series and in a loop for continuous loading, washing, elution and re-equilibration of the transported adsorbent. Extraction of lysozyme takes place in the first stage, which is then delivered in purified form in the product stream exiting the third stage. In the second stage, adsorbent washing takes place, whereby particulate material and loosely bound proteins are washed off the perfluorocarbon particles. Following elution, re-equilibration of the adsorbent in the loading buffer then takes place in the fourth stage.

tively low liquid flow-rates involved, and the comparatively similar densities of the process solutions and the adsorbents concerned, the system operates in the incipient fluidisation mode, but with comparatively large fluidised-to-settled bed-height ratios. Consequently, there is little mixing of both the continuous and dispersed phases and so this extraction technique operates with an efficiency similar to that of conventional chromatographic columns rather than to conventional well-mixed fluidised beds. As this system is designed to be a truly continuous operation, loaded adsorbent is constantly removed from the base of one bed (to be processed in the subsequent stage) and adsorbent from the previous stage is simultaneously supplied at the top. Removal of adsorbent from the base of the beds, causes would-be suspended particles to rain down the columns in downward plug-flow, resulting in effective overall counter-current contacting. This principle is exploited in all four of the process stages, securing not only effective capture of the targeted protein in the first stage, but also highly efficient wash, elution and regeneration of the adsorbent in subsequent stages.

2.5. Four-stage continuous counter-current extraction of hen lysozyme from egg whites and milk

In all experiments the four-stage unit was filled with a total of about 210 ml of settled adsorbent. The adsorbent was distributed between the four stages to give approximately 30% of the total volume in each of the load and elution stages, and approximately 20% of the total volume in each of the wash and regeneration stages. Start-up consisted of initially fluidising the adsorbent in each of the four columns with their respective buffers at flow-rates as indi-

Table 1
Continuous counter-current extraction of lysozyme from a solution of egg-whites (Run A), feed and effluent characteristics

Run A Stage	Flow-rate in (ml/min)	Flow-rate out (ml/min)	Lysozyme flow (U/min)	Lysozyme activity (U/ml)	Total protein flow (mg/min)	Total protein concentration (mg/ml)	Lysozyme yield (loss) (%)	Specific activity U/mg	Purification factor
Fresh feed	5.6	(5.6)	8170	1460	5.60	1.0	(100)	1459	(1)
Load	5.6	6.59	120	18.2	3.78	0.57	1.5	–	–
Wash	7.7	6.75	220	33.0	1.49	0.22	2.7	–	–
Elution	8.2	8.29	7400	892	0.26	0.031	90.5	28340	19.4
Regeneration	8.3	8.46	285	33.7	0.068	0.008	3.5	–	–
Total	29.8	30.09	8020	–	5.56	–	98.2	–	–

Table 2
Continuous counter-current extraction of lysozyme from a lysozyme-enriched unclarified milk (Run B), feed and effluent characteristics

Run B Stage	Flow-rate in (ml/min)	Flow-rate out (ml/min)	Lysozyme flow (U/min)	Lysozyme activity (U/ml)	Total protein flow (mg/min)	Total protein concentration (mg/ml)	Lysozyme yield (loss) (%)	Specific activity U/mg	Purification factor	Turbidity (600 nm)
Fresh feed	7.3	(7.3)	62100	8500	111	15.3	(100)	558	(1)	6.850
Load	7.3	8.35	3230	387	72.5	8.69	5.2	–	–	1.917
Wash	12.4	12.11	5380	445	20.2	1.67	8.7	–	–	0.971
Elution	9.7	9.58	41000	4280	1.18	0.12	66.0	34800	62.4	0.017
Regeneration	8.4	10.02	2850	284	0.10	0.010	4.6	–	–	0.009
Total	37.8	40.06	52460	–	94.0	–	84.5	–	–	–

cated in Tables 1 and 2. After the adsorbent in each stage had expanded to a steady expanded bed height, adsorbent circulation between the stages was commenced by engaging peristaltic pumps. This was then immediately followed by switching the feed to the adsorption stage from buffer to the specified lysozyme-containing solution. Thus, lysozyme was bound to the adsorbent in the load stage before being delivered to the wash stage. In the wash stage the adsorbent was washed free of loosely adsorbed or non-adsorbed proteins and particulates. In the elution stage, the eluant stripped the lysozyme from the support and the stream exiting this stage constituted the product. The fourth stage served to regenerate the adsorbent by re-equilibration in the adsorption buffer.

The flow-rates of the fluidising medium to each of the four stages was maintained constant throughout each of the experiments. However, the rate of adsorbent delivery between subsequent stages of the system was periodically adjusted around an average of about 1.2 ml settled adsorbent/min to maintain bed heights of about 25 cm (±2 cm) or 15 cm (±2 cm) in the load and elution, and wash and regeneration columns, respectively. Higher adsorbent inventories were desirable in the former stages as these were considered to be more critical in optimising the yield of purified lysozyme.

Samples of all four streams exiting the system were collected over periods of exactly 20 min, and the volume, lysozyme activity, total protein content and turbidity of these were measured and recorded. From this data, the amounts of lysozyme measured, in activity units/min, and total protein (mg/min) could be calculated in the exit stream from each of the four stages.

2.6. Selection of binding, washing and elution conditions for lysozyme to the Procion Red HE-7B-PVA-perfluorocarbon support

We have shown in work documented elsewhere [15], that the capacity of this affinity adsorbent for lysozyme is highest at pH 9.2 (27 mg/ml in 100 m*M* carbonate buffer), so this pH was selected for the loading stage in the extraction experiments from egg-white. However, because of the strong pH influence on the stability of milk, milder (pH 7.5) alkaline conditions were used in the lysozyme extraction from lysozyme-enriched milk.

In both systems, the wash stage was operated at pH 7.0 (100 m*M* sodium phosphate). Elution was carried out with 1.5 *M* NaCl in 100 m*M* pH 7.0 sodium phosphate buffer, and adsorbent re-equilibration was carried out with the respective adsorption buffers.

3. Results and discussion

3.1. Run A: continuous purification of lysozyme from a solution of egg-whites

The system was started up as outlined above, with fluidising flow-rates as shown in Table 1. The specific activity of the feed stream was assayed at 1460 U/mg, and this was fed to the adsorption stage at 5.60 ml/min, corresponding to a loading rate of 8170 U/min. Profiles for lysozyme and total protein flow of the streams exiting the four stages are shown in Figs. 2 and 3. As can be seen from the graphs,

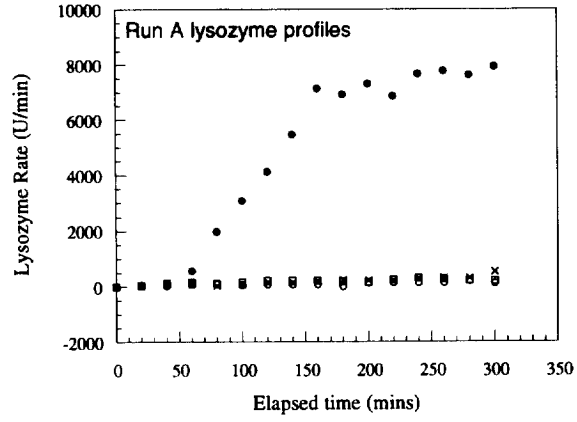

Fig. 2. Profiles of lysozyme activity rates delivered from the load, wash, elution and regeneration stages during the purification of lysozyme from egg-whites (Run A). Four-stage continuous counter-current lysozyme extraction from hen egg-whites was carried out under operating conditions described above. Thus, after initial fluidisation of adsorbent in the four stages with the respective buffer, lysozyme containing feed was subjected to the system via the adsorption stage. Then, circulation of adsorbent at approximately 1.2 settled ml/min around the four stages was commenced, to carry out continuous loading, washing, elution and regeneration of the material. (○)=load stage; (□)=wash stage; (●)=elution stage; (×)=regeneration stage.

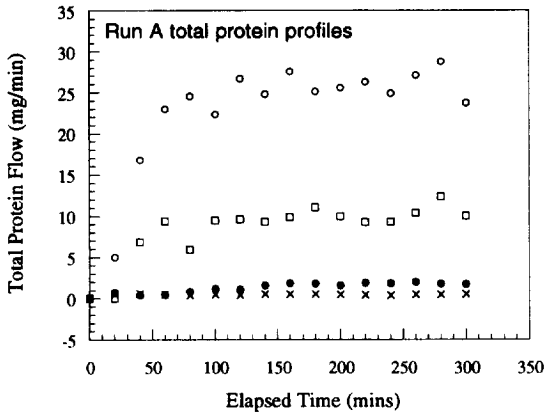

Fig. 3. Profiles of total protein rates delivered from the load, wash, elution and regeneration stages during the purification of lysozyme from egg-whites (Run A). Total protein profiles correspond to the lysozyme activity profiles of Fig. 2. (○)=Load stage; (□)=wash stage; (●)=elution stage; (×)=regeneration stage.

production of purified lysozyme reaches a steady state after approximately 160 min of operation. The lysozyme production rate of the elution stage at steady state is 7400 U/min in a total protein rate of 0.26 mg/min, giving a specific activity of the targeted enzyme of 28 300 U/mg. This corresponds to a purification factor of 19.4. At 90.5% the lysozyme yield in the product stream is very high. Losses in the adsorption stage flowthrough, and the wash and regeneration stages are minimal, as indicated in Table 1.

The total protein flow profiles for this experiment (Fig. 3) show similar characteristics in the approach to steady state as those of the lysozyme. Again steady-state protein flow is achieved in the elution stream after approximately 160 min from start-up. The graph illustrates the large decrease in the protein content of the product (elution) stream compared to that of the flowthrough.

Both Fig. 2 and Fig. 3 illustrate how the equipment was able to produce an approximately constant stream of product throughout the experiment. Turbidity measurements were not made on any samples in this experiment as the feed being dealt with in this system was essentially non-turbid.

Although this procedure was not optimised with respect to lysozyme productivity, we have illustrated how it could be used as a continuous process for purification of the enzyme. The classical method adopted in industry for its purification involves direct crystallisation from egg albumen at pH 9.5 by addition of 5% sodium chloride, typically achieving between 60% and 80% yield. However, the use of ion exchange and precipitation is now becoming the more widely used technique, and is somewhat more efficient [17]. For example, Fang Ming et al. [18] report the use of a novel ion exchanger which was capable of achieving an overall productivity of 0.21 mg protein/ml adsorbent/min (12.6 kg/m^3 h), though they did not report the specific activity of the enzyme in the product. Affinity chromatography has also been used for lysozyme purification using chitosan, but this method has proved to be prohibitively expensive [17].

The technique described here produced lysozyme with a specific activity of 28 300 U/mg in a single step, which compares well with the 28 340 U/mg of the pure enzyme obtainable commercially (see below). The specific productivity under the given operating conditions works out to approximately 1.23 μg/ml adsorbent/min, which is a comparatively low value compared to more conventional techniques such as that of Fang Ming et al. [18]. However, lysozyme is an exception in having such an uncharacteristically high p*I* value, and only few proteins can be purified effectively in a single step using techniques of low selectivity. Additionally, because of the high selectivity of the adsorbent, lower capacities are to be expected. Thus, we believe that the continuous counter-current nature of our proposed unit can show considerable advantages in a number of alternative purification challenges, and this is illustrated with the results of the model system illustrated above.

3.2. Run B: continuous purification of lysozyme from an enriched milk sample

The main aims of this experiment were again to assess the suitability of the novel contactors with an untreated feedstock, which is unlikely to be suited for direct application to a conventional fixed bed of adsorbent. As the potential of transgenic animals as a source of recombinant proteins increases, challenges associated with protein purification from milk are gaining in importance. Despite the absence of any cellular material in milk, conventional fixed-bed

chromatography can still not be used as an initial step in downstream processing, as bed occlusion is still likely to occur. Noppe et al. [19] report the use of Expanded Bed Adsorption (EBA) for the purification of equine lysozyme from skimmed equine milk, in conjunction with hydrophobic interaction chromatography (HIC). This two-step process resulted in purification to homogeneity, in 81% yield. Thus, they showed that the use of fluidised bed technology has considerable potential as an improved process for the purification of proteins from milk, as the feedstock does not require as much treatment before bioseparations can actually be effected.

Because of the affinity shown by the Procion Red HE-7B adsorbent for hen lysozyme, a sample of skimmed bovine milk was enriched with the enzyme with the aim of then re-extracting it in the novel unit.

Again the system was started up as outlined above, with fluidising flow-rates as shown in Table 2. Profiles for total protein and lysozyme flow of the streams exiting the four stages are shown in Figs. 4 and 5. Turbidity assays were also included in the measurements to assess whether the product stream was sufficiently clarified for final polishing using conventional chromatographic equipment. Average steady-state values obtained for this parameter are also given in Table 2. The profiles in Fig. 4 show that lysozyme production in this system reached an approximately constant rate after 120 min. Fig. 5

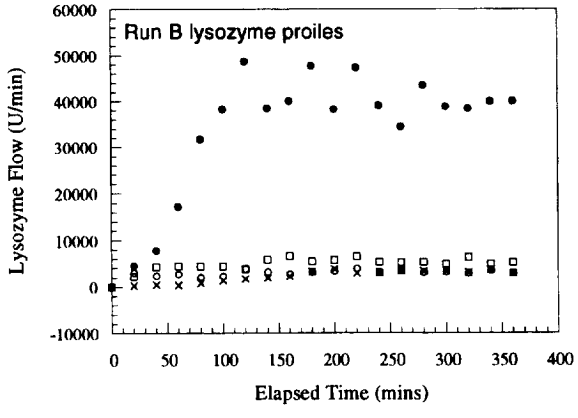

Fig. 4. Profiles of lysozyme activity rates delivered from the load, wash, elution and regeneration stages during the extraction of lysozyme from lysozyme-enriched bovine milk (Run B). For details, see legend to Fig. 2. (○)=Load stage; (□)=wash stage; (●)=elution stage; (×)=regeneration stage.

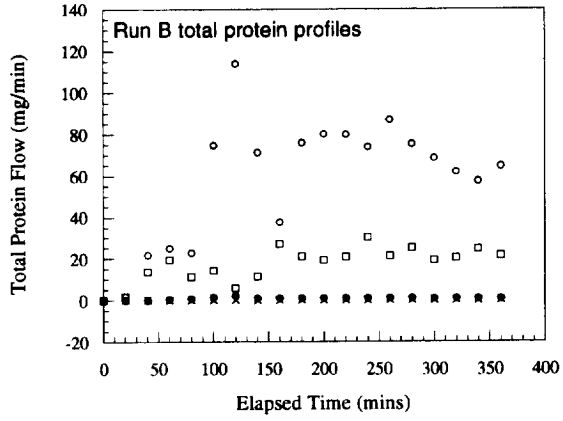

Fig. 5. Profiles of total protein rates delivered from the load, wash, elution and regeneration stages during the extraction of lysozyme from lysozyme-enriched bovine milk (Run B). Total protein profiles correspond to the lysozyme activity profiles of Fig. 4. (○)=Load stage; (□)=wash stage; (●)=elution stage; (×)=regeneration stage.

shows that despite constant total protein rates in the eluant (and regeneration) stream, protein rates exiting the load and wash stages showed more fluctuation. This stems from the fact that due to the turbidity of the milk, oscillations in the bed height of the adsorption stage were not always immediately detectable, and adjustments in adsorbent removal from this stage were, therefore, more substantial than was required in the run outlined above. Because of the milk present in the particle voids of adsorbent removed from the column, the larger swings in adsorbent flow had a concurrently enhanced effect on the total protein flow exiting the load and wash stages. This explains the less-steady nature of the profiles illustrated in Fig. 5.

After dissolution of the lysozyme in the buffered milk (pH 7.5) to 0.30 mg/ml, the specific activity of the feed stream was assayed at 36.7 U/mg with a total protein content of 15.3 mg/ml. This material was fed to the adsorption stage at 7.30 ml/min, corresponding to a loading rate of 4120 U/min. Lysozyme activity in the product stream at steady state was 4280 U/ml, with specific activity 34 800 U/mg. The average flow-rate of the product was 9.58 ml/min, thus giving a productivity of 41 000 U/min. From this data, it is clear that the lysozyme activity balance around the unit does not close. It is possible that lysozyme in the feed may have suffered

reversible inactivation, or may be otherwise engaged in cell lysis of other organisms in the milk, thus not displaying its entire catalytic activity when assayed. To obtain an idea of its activity in uninhibiting solutions, a 0.3 mg/ml solution from the same lysozyme source was made in a 100 m*M* sodium phosphate buffer pH 7.5, and also assayed. The activity of this sample was 8500 U/ml (28 300 U/mg), corresponding to a loading rate of 62 100 U/min. Based on this data, the specific activity of the lysozyme in the feedstock would have been 558 U/mg. The yield in the product stream would then be 66.0%, with a purification factor of 62.4. However, when compared to the rate of lysozyme exiting the four effluent stages (load, wash, elution and regeneration) the proportion of activity in the product stream was 78.1%. The steady-state turbidity data for the four streams exiting the system given in Table 2 confirm that the lysozyme product stream is essentially free of colloidal matter compared to the feed, and would, therefore, be suitable for processing in a final polishing step.

We have, therefore, shown in the above described procedures that hen-lysozyme can be extracted from very inhomogeneous solutions to yield a highly purified product, in a single step using the Procion Red HE-7B derivatized adsorbent. The specific activity of the purified lysozyme compared favourably with that of the commercially available enzyme in both cases (28 300 U/mg and 34 800 U/mg compared with 28 400 U/mg of the commercial product). These high degrees of purity were obtained despite the different adsorption conditions for the two experiments (pH 9.2 and 7.0, respectively) suggesting that the protein ligand interaction is likely to be more intricate than simply being an electrostatic-based attraction. Because the lysozyme was purified essentially to homogeneity in both runs, the different purification factors achieved reflect the fact that lysozyme was present in different proportions in the starting material, as opposed to stemming from differing purification protocols being carried out. The fact that widely different productivities were achieved in the two runs (8170 U/min against 62 100 U/min for runs A and B, respectively) was not a function of the changed adsorbent characteristics for the two cases, but was simply caused by the different concentrations of the lysozyme being used initially. In fact, feed streams of much higher lysozyme activity could have been subjected to the adsorption stage as the adsorbent loading did not approach maximum capacity in either case.

4. Conclusions

We have shown from the above that the four-stage technique can be used successfully for the extraction of proteins from egg-whites, and, more importantly, milk, which adds to its demonstrated success with preparations of homogenised *Saccharomyces cerevisiae* described in Owen et al. [13]. Thus, continuous product streams of purified target protein are generated from unclarified feeds, therefore having a clear advantage over conventional purification techniques, which are batch mode and rely on the prior inclusion of expensive clarification steps.

More specifically with the purification of lysozyme, we have demonstrated that the four-stage unit could purify the enzyme continuously in 90.5% yield from egg-whites and 66.0% yield from a preparation of lysozyme-enriched bovine milk. These corresponded to purification factors of 19.4 and 62.4, respectively, with productivities of 7400 U/min and 41 000 U/min.

The suitability of the four-stage unit with other unclarified feedstocks such as preparations of homogenised *E. coli* is currently being investigated.

Acknowledgments

R.O. would like to thank "The Royal Stud", Sandringham, Norfolk, UK, for donation of the horse milk.

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