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Immobilised metal ion affinity chromatography purification of alcohol dehydrogenase from baker's yeast using an expanded bed adsorption system

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Abstract

Alcohol dehydrogenase (ADH) from solutions of homogenised packed bakers' yeast has been successfully purified using immobilised metal-ion affinity chromatography in an expanded bed. Method scouting carried out using pure ADH solutions loaded onto 5-ml HiTrap columns charged with Zn^{2+}, Ni^{2+} and Cu^{2+} and eluted using 0–50 m*M* EDTA gradient found that charging with Zn^{2+} gave the highest recovery and the lowest EDTA concentration required for elution. These results were used to develop a protocol for the expanded bed system and further tested using clarified yeast homogenate loaded onto XK16/20 packed beds (approximately 30 ml) packed with Chelating Sepharose FastFlow matrix in order to determine the optimum elution conditions using EDTA. The ADH was found to elute at 5 m*M* EDTA and the dynamic and total binding capacities of Streamline chelating for ADH were found to be 235 U/ml and 1075 U/ml matrix, respectively. Expanded bed work based on a step EDTA elution protocol demonstrated that ADH could be successfully eluted from unclarified homogenised bakers' yeast diluted to 10 mg/ml total protein content with a recovery of 80–100% that was maintained over five consecutive runs with a vigorous clean-in-place procedure between each run.

Keywords: Immobilised metal ion affinity chromatography; Expanded bed adsorption; Yeast; Alcohol dehydrogenase; Enzymes

1. Introduction

Purification of proteins and enzymes from whole cell fermentation broths or cell homogenates has traditionally necessitated multiple steps such as centrifugation and microfiltration to yield a clarified feedstock for application to conventional packed bed chromatography systems. These steps add to the cost and time of the overall process, as well as resulting in a loss of product at each step [1,2].

The expanded bed system purifies products in an identical way to a packed bed chromatography system, but while the bed is expanded particulates in the feedstock e.g., cells and other debris can pass between the matrix beads, and samples do not need to be clarified prior to application to the bed. Initial attempts to purify products using fluidised beds utilised standard packed bed chromatography ma-

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trices [3,4], but the low density of the beads meant that only very low liquid linear velocities could be used without over-expansion of the bed, thus limiting the systems productivity. In addition the beds were frequently found to be unstable when expanded, creating regions of turbulent liquid flow rather than plug flow and thus increasing process times and buffer volumes required while decreasing the purification of the product upon elution. However with the development of purpose-designed densified adsorbents such as the Streamline system of adsorbents and columns. Streamline systems consist of a base matrix (highly cross-linked 6% beaded agarose containing crystalline quartz to increase the density of the beads) with ligands attached in a similar manner to conventional chromatography matrices. The average density is 1.2 g/ml. The particle size range from 100 mm to 300 mm with an average size of 200 mm. Columns are equipped with suitable flow distributors designed to produce stable plug flow. With these systems higher linear flow-rates and stable expansion charcteristics can now be obtained [5,6]. This has enabled expanded bed adsorption to develop into a very useful downstream processing tool. Purification using the Streamline system has been demonstrated for a wide variety of products using both ion-exchange [7] and dye-ligand [8] matrices. Table 1 gives examples of expanded bed purifications currently noted in the literature. Although these publications detail a wide variety of systems, the use of homogenised yeast represents a particularly severe challenge. Such a feedstock displays many characteristics associated with industrial products including a high debris concentration and fouling by nucleic acids. In practice ion-exchange matrices challenged with such materials tend to give lower levels of resolution than for well-defined feedstocks. By contrast, immobilised metal-ion affinity chromatography (IMAC) has found widespread use in the high resolution separation of proteins from crude feedstocks and may give the potential for a more effective separation system and thus higher resolutions and purification factors than ion-exchange chromatography.

A typical traditional route for the purification of an intracellular enzyme from whole cells compared to an expanded bed route for the same purification is shown in Fig. 1. In this case the enzyme of choice was alcohol dehydrogenase (ADH) from pressed baker's yeast. Previous investigations have successfully purified the enzymes glucose-6-phosphate dehydrogenase (G6PDH) [7] and ADH [20] from the same source, suggesting that the material is a convenient source for this work.

IMAC utilises metal ions bound to the surface of a stationary phase [9] (an agarose-derived matrix) via a chelating ligand forming ''dentate'' bonds of electron donation [in this case the tridentate ligand iminodiacetic acid (IDA)]. In almost all cases the metal ions used in this process are the first row transition metals (nickel, zinc and copper). The metal ions listed are well known for their ability to form complexes with oxygen and nitrogen ligands and hence will associate with IDA. The parts of a protein that are known or most likely to bind to the metal ion are the side chains of histidine, cysteine and tryptophan, along with the amino terminal and possibly some backbone nitrogens [21]. Because of

Table 1
Examples taken from literature of expanded bed processes previously studied, including yields and purification factors where available

Mechanism	Organism	Isolated from	Product	% Yield	Purification factor	Ref.
Dye affinity	*S. cerevisae*	Unclarified homogenate	PFK 1	39.1	5.2	[10]
Anion exchange	*E. coli*	Fermentation broth	ZZ-M5 fusion protein	>90	N/A	[11]
Dye affinity	*S. cerevisae*	Unclarified homogenate	MDH	89	113	[12]
Dye affinity	*S. cerevisae*	Unclarified homogenate	G-6-PDH	94	172	[12]
Anion exchange	*Candida kefyr*	Fermentation broth	Inulinase	93	5.8	[13]
Anion exchange	*S. cerevisae*	Unclarified homogenate	G-6-PDH	77	17	[12]
Anion exchange	Transgenic livestock	Clarified milk	Human rProtein C	N/A	N/A	[14]
Anion exchange	*E. coli*	Fermentation broth	Periplasmic rEndotoxin A	N/A	N/A	[15]
Ion exchange	*E. coli*	Unclarified homogenate	Soluble protein	N/A	N/A	[16]

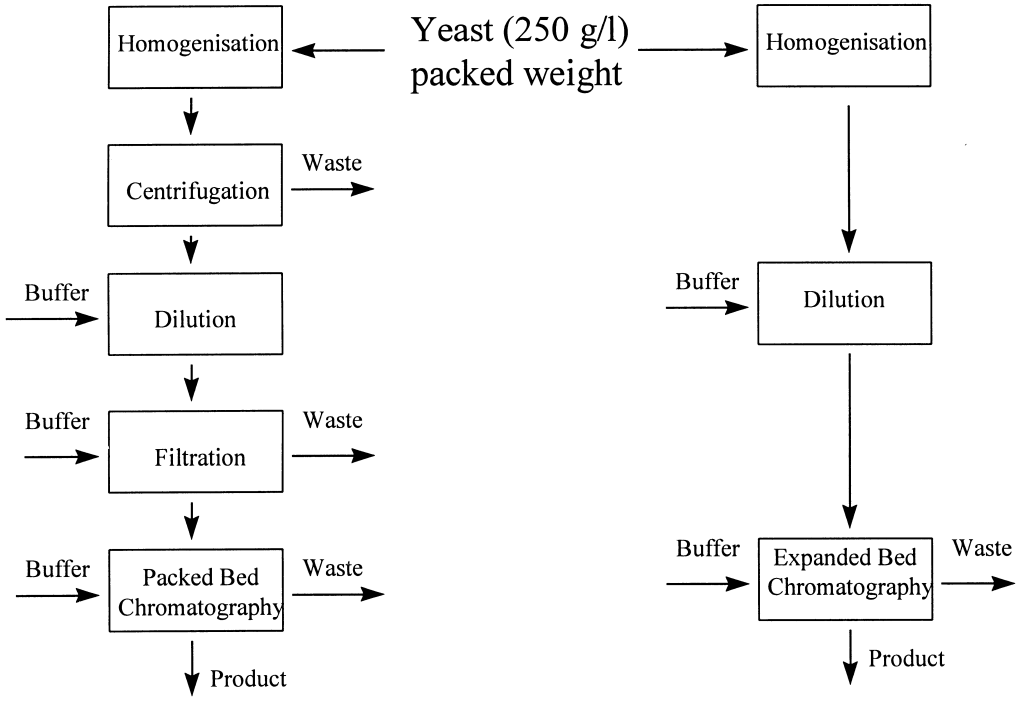

Fig. 1. Comparison of different stages involved in expanded bed and packed bed processes.

the presence of several histidines in yeast ADH, it is reasonable to expect that it will bind strongly to a charged IMAC matrix.

Elution from an IMAC matrix is normally carried out in one of three ways [17]; by lowering the pH of the system, addition of competing agents such as imidazole or histidine, or finally by the addition of a strongly chelating agent such as EDTA. In this case pH elution is not a favourable option since although a drop to a pH of 4 should be sufficient to release ADH [18], the enzyme activity would be impaired at such a value, reducing the process yields. Competitive elution or the use of EDTA would be the methods of choice in this situation – in this paper we report on the use of EDTA elution, since EDTA does not interfere with the standard methods of monitoring the system. Both histidine and imidazole interefere with the system monitoring at UV 280 nm since both adsorb at this frequency, and imidazole was shown to affect the protein assays of the feedstock (average protein concentration 10 mg/ml feed) by 5–10% when imidazole was added in the concentration range 0–5 *M*. Since the Bio-Rad assay reagent used measures average histidine levels to calculate protein concentration, then histidine as an elution agent will interfere heavily with assay results.

2. Experimental

2.1. Materials

Packed bed chromatography was carried out using 5-ml Chelating HiTrap columns and XK16/20 glass columns (1.6 cm diameter, 15 cm packed matrix height) packed with approximately 30 ml of Chelating Sepharose FastFlow, all provided by Amersham Pharmacia Biotech (Uppsala, Sweden) on a Pharmacia fast protein liquid chromatography (FPLC) system consisting of an LCC-500 controller, two P-500 pumps and a Frac-100 fraction collector. The output from the column was monitored for UV absorbance at 280 and 254 nm, and these values logged using a PE Nelson 900 interface and Turbochrom v4 (both Perkin-Elmer Nelson Systems, CA, USA).

The expanded bed separations were carried out using a Streamline 25 expanded bed (2.5 cm diameter, 15 cm settled matrix height) filled with 75 ml of Streamline chelating matrix (gifts from Amersham Pharmacia Biotech). Peristaltic pumps (Model 505-S, Watson-Marlow, Cornwall, UK) were used for buffer and feed application. UV absorbance at 280 and 254 nm was monitored using the system described above.

For both sets of experiments, fractions collected were analysed for ADH activity and protein concentration. These analyses were conducted using a Uvikon 922 UV–Vis spectrometer (Kontron Instruments, Milan, Italy).

Baker's yeast was obtained in the form of blocks (DCL, Clackmannanshire, UK). All other chemicals used were laboratory grade (Sigma–Aldrich–Fluka, Poole, UK).

2.2. Off-line ADH assay

ADH activity was determined following the method of Bergmeyer [22]. ADH catalyses the reaction below:

$$\text{Ethanol} + \beta\text{NAD}^{+} \overset{\text{ADH}}{\leftrightarrow} \text{Acetaldehyde} + \beta\text{NADH} + \text{H}^{+}$$

The formation of acetaldehyde from ethanol may be monitored by measuring the increase in absorbance at 340 nm due to the formation of βNADH. The reaction mixture consisted of 600 m*M* ethanol (Fluka), 1.0 m*M* glutathione (Sigma), 0.62 m*M* semicarbazide HCl (Sigma), 1.8 m*M* NAD^{+} (Sigma) in 50 m*M* Tris·HCl (Sigma) buffer at pH 8.8. Semicarbazide inhibits the reverse reaction.

One unit of enzyme activity is defined as the amount of ADH necessary to catalyse the conversion of 1 μmol of ethanol to acetaldehyde per minute at 25°C. All assays were performed in 1 cm path length cuvettes and the reaction was started by the addition of the enzyme. Potassium dihydrogenphosphate buffer (100 m*M*, pH 6.5) was used to dilute samples to produce a linear change in absorbance less than 0.5 AU/min. Assays were performed in duplicate with a reproducibility of ±5%.

2.3. Total protein assay

Total protein concentration was determined using the Bradford assay [23]. The assay is based on the shift in absorbance from 465 to 595 nm which occurs when Coomassie Blue G-250 dye binds to proteins in acidic solution. The colour change from pale orange to blue is relatively linear and thus permits quick and easy protein determination. In this study a commercially available dye was used (Bio-Rad Protein Assay reagent, Bio-Rad, Hemel Hempstead, UK).

Samples to be assayed were diluted to within the range 0.01 to 1.0 mg/ml protein using potassium dihydrogenphosphate buffer (100 m*M*, pH 6.5). Reagent and dilute sample were added to the cuvette and the absorbance at 595 nm read after 10 min. A standard curve produced using bovine serum albumin (Sigma) for each batch of dye reagent, enabled protein determination to be made directly from the absorbance reading of the unknown sample. Assays were performed in duplicate with a reproducibility of ±5%.

2.4. Methods

For initial testing, pure ADH (Sigma, 340 units/mg solid, EC 1.1.1.1) at 2 mg solid/ml buffer was used. For later trials, bakers' yeast was disrupted in a high-pressure homogeniser (Lab 60, APV, Crawley, UK) fitted with a restricted orifice discharge valve for five discrete passes at 500 bar and 5°C C and then solid KH_2PO_4 and NaCl added to make the solution up to the concentration of buffer A (0.02 *M* KH_2PO_4–0.5 *M* NaCl). This was clarified using a Beckman J2-MI centrifuge (Beckman Instruments, CA, USA) at 16 000 *g* for 30 min. For the expanded bed work, unclarified homogenate as before was diluted to 10 mg overall protein/ml homogenate in order to ensure consistent load conditions between runs and also to lower the viscosity of the feed to a level where bed height was not greatly increased by the change from buffer to feedstock.

Initial trials were carried out using a 5-ml HiTrap in order to determine the binding and elution characteristics of pure ADH to immobilised Zn^{2+}, Cu^{2+} and Ni^{2+} ions. From the results of these trials, an XK16/20 packed bed was used to determine if the

behaviour of ADH in clarified yeast homogenate differed from that of pure ADH. Finally the chosen protocol was run in replicate on the Streamline 25 expanded bed in order to ensure reproducibility of results.

For all the experiments the feed was loaded at 200 cm/h and elution was carried out at 100 cm/h to ensure consistency on scale-up. In the case of the expanded bed elution was carried out in a downwards direction with the bed in packed mode to attempt to minimise the ADH peak width and hence improve purification and concentration of the product.

2.5. Determination of binding and elution characteristics

A 5-ml sample of metal ions in a 5000 ppm solution was loaded onto the column from a superloop. The column was then washed in buffer A (0.02 *M* KH_2PO_4–0.5 *M* NaCl, 10 column volumes). Pure ADH solution (5 ml) was loaded onto the column followed by another wash in buffer A (five column volumes). A gradient elution of 0 to 100% of buffer B (buffer A plus 0.05 *M* EDTA) was then carried out over six column volumes. This procedure was repeated using all three metals and in duplicate.

2.6. Packed bed experiments

The effect of other components in homogenised yeast on the elution conditions for ADH determined in the HiTrap trials was studied. This was done using a Pharmacia XK16/20 column packed to a bed height of 15 cm with Chelating Sepharose FastFlow matrix. This was then loaded with one column volume (approximately 30 ml) of Zn^{2+} ions in solution at 5000 ppm. After washing (five column volumes buffer A) one column volume of clarified yeast homogenate diluted to 10 mg/ml protein content was loaded onto the column. Again the column was washed in buffer A and a step elution protocol was run using EDTA concentrations of 0.003, 0.006 and 0.05 *M* in buffer A. Samples taken at 2-min intervals throughout the load, wash and elution were assayed for ADH activity and protein concentration.

2.7. Expanded bed experiments

Following the packed bed method scouting, several runs were carried out using the Streamline 25 expanded bed system. The layout of the system used is shown in Fig. 2. The column was filled with 75 ml of Streamline chelating matrix. This was fully expanded in both water and buffer A in order to determine the expansion characteristics of the matrix. In addition residence time distribution experiments (RTDs) were carried out using a step change from buffer A to buffer A plus 0.25% (v/v) acetone, monitored using UV 280 nm absorbance. This was conducted in order to check the stability of the bed on expansion and to ensure that the flow through the bed was approximately plug.

Prior to loading the yeast homogenate, the matrix was activated by loading 75 ml of 5000 ppm Zn^{2+} in solution. The bed was expanded at a linear velocity of 200 cm/h in buffer A until the height was stable (typically 0.5 h). Yeast homogenate, diluted to an overall protein concentration of 10 mg/ml, was loaded onto the column until the ADH activity in the outlet stream reached 5% of the activity in the load sample (5% breakthrough). The bed was then washed, again at 200 cm/h, until the UV 280 nm absorbance returned to the baseline level. The wash was then stopped and the bed allowed to settle. The direction of flow was then reversed, and the bound ADH eluted at 100 cm/h using the following protocol:

1 column volume buffer A
2 column volumes buffer B (A + 0.003 *M* EDTA)
3 column volumes buffer C (A + 0.006 *M* EDTA)
2 column volumes buffer D (A + 0.05 *M* EDTA)

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The system was cleaned after being re-expanded in buffer A. The initial clean-in-place (CIP) procedure consisted of a water wash and then 10 column volumes of 1 *M* warm NaOH. (approximately 50°C), then a second water wash followed by 20% (v/v) ethanol for storage. Following further trials, a more rigorous CIP procedure was adopted using 1 *M* NaOH followed by 30% (v/v) isopropanol, 25%

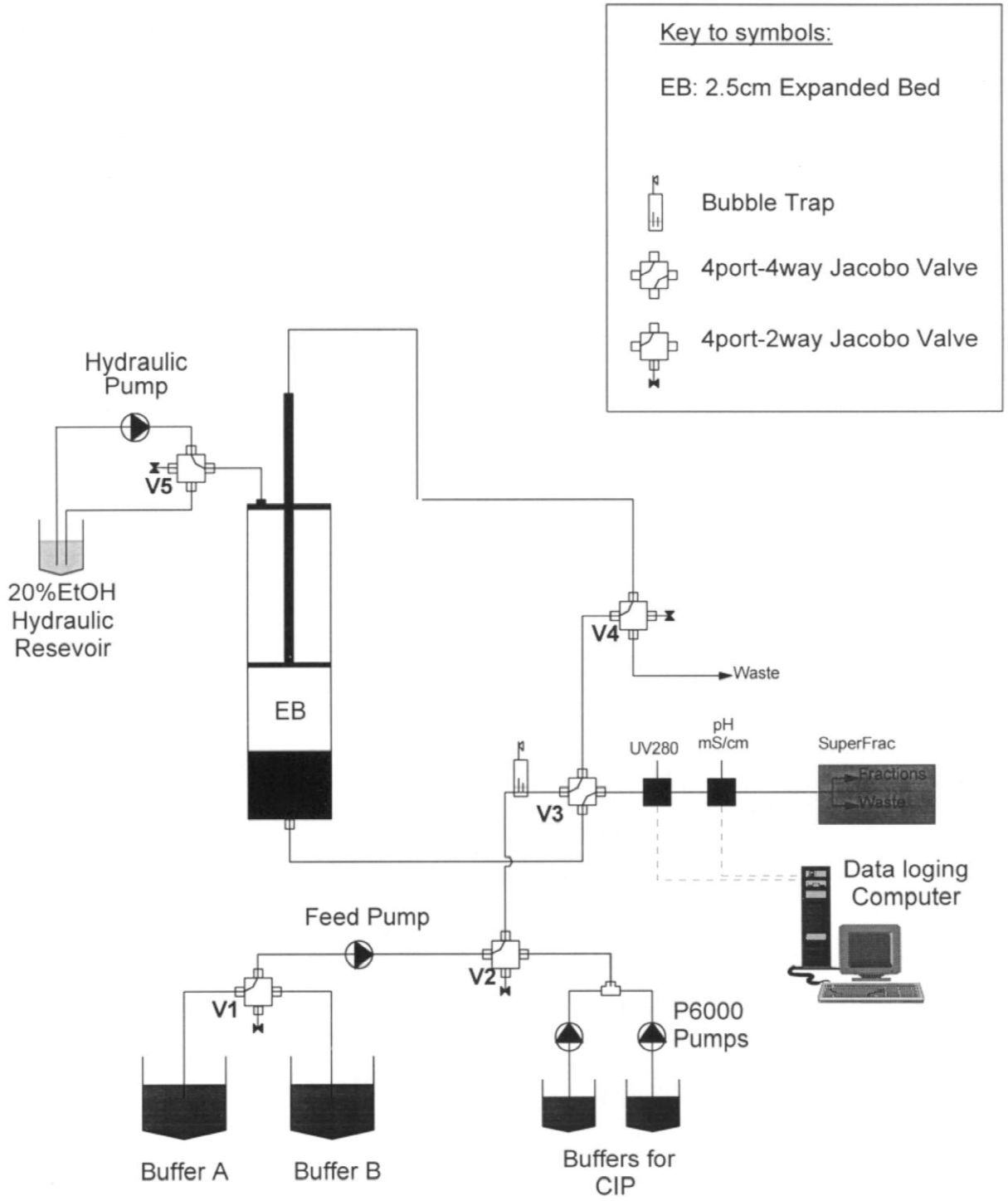

Fig. 2. Schematic layout of expanded bed system showing valves, piping and monitoring equipment [20].

(v/v) acetic acid and then 20% (v/v) ethanol, with water washes in-between each step.

Trials were carried out in triplicate to ensure reproducibility of results.

3. Results

3.1. Determination of binding and elution characteristics

Using Zn^{2+} as the immobilised metal ADH was eluted at approximately 5 m*M* EDTA and could be recovered in excess of 90% of loaded activity. In contrast, using Ni^{2+} the ADH could only be recovered at less than 50% of the total loaded activity, and was eluted at approximately 15 m*M* EDTA. The ADH could not be eluted from the Cu^{2+} charged column before the Cu^{2+} ions themselves were stripped from the column by the chelating action of the EDTA. On the basis of these results it was decided that both larger scale packed bed trials and expanded bed work would be carried out using immobilised Zn^{2+} ions.

3.2. Packed bed experiments

The elution trace from the small packed column showed ADH eluted during the 0.006 *M* EDTA step, demonstrating that any effects created by components in clarified yeast homogenate other than ADH did not significantly affect the elution conditions required. Consequently this elution regime was considered suitable for experiments using the Streamline 25 expanded bed with unclarified homogenised yeast.

The total binding capacity of the Streamline chelating matrix were estimated with 30 ml of the matrix packed in an XK16/20 column. In addition dynamic binding capacity was calculated in the Streamline 25 column in expanded mode using frontal analysis techniques at 50% total ADH breakthrough (clarified yeast homogenate at 10 mg/ml total protein content) using a flow-rate of 200 cm/h. The dynamic capacity of the bed was calculated as the number of units of ADH per ml of settled matrix bound before breakthrough of ADH occurs [19] and was be estimated from the amount of enzyme contained in the fractions between 50% UV 280 nm breakthrough and 50% ADH breakthrough. In this case the dynamic capacity of the Streamline matrix was found to be 235 U(ADH)/ml matrix. The total capacity of the matrix at 50% ADH breakthrough was found to be 1075 U(ADH)/ml matrix. This compares favourably with values for Streamline Phenyl low-sub (a hydrophobic interaction matrix) of 260 U(ADH)/ml dynamic capacity and 750 U(ADH)/ml total capacity [20].

3.3. Expanded bed experiments

The results of the residence time distribution curves were used to determine the stability of the bed on expansion. From these traces the number of theoretical plates in the system was found to be in the range 32–39. The values obtained show a variation of 18% across the range of results obtained. This is within the maximum variation of 20% for bed stability recommended by Pharmacia [24] and it is reasonable to assume that the bed is stable upon expansion and the flow through it is approximately plug.

The results of the packed bed trials predicted that the ADH peak would occur within the buffer C (buffer+6 m*M* EDTA) step. Protein and ADH assays were carried out in the load, wash and elution at 3-min intervals. Values for protein concentration and ADH activity were calculated and plotted using Microcal Origin.

The expanded bed experiment described above was carried out on three occasions in order to check for reproducible results. A typical experimental trace is shown in Fig. 3, along with data for ADH and protein concentrations and yield values in Table 2.

The initial run on the expanded bed showed an ADH recovery on elution of 99% and a purification factor with respect to contaminating protein of 11. Although these are very positive results, subsequent experiments showed a reduced recovery on elution to a steady state of approximately 60% yield. This was believed to be due to initial bed contamination not being completely removed and hence a more rigorous CIP protocol was adopted (see Section 2.4). In addition, it was noted that the ADH peak was crossing over the 6 m*M* EDTA to 50 m*M* EDTA step boundary, and this was likely to effect recovery

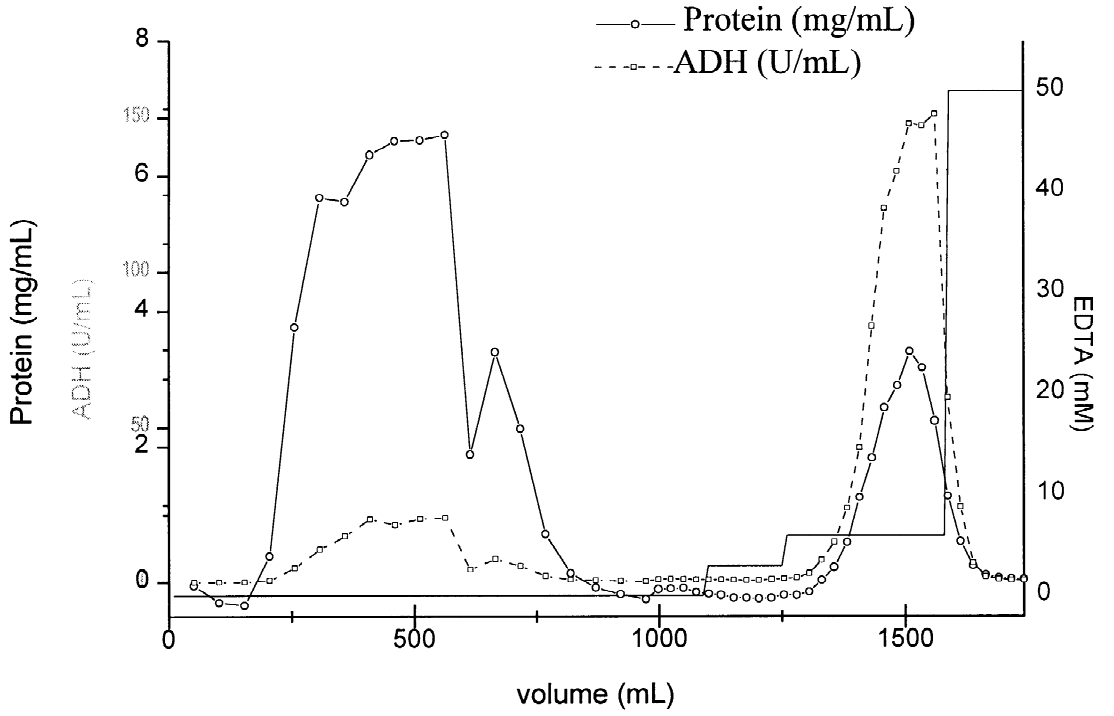

Fig. 3. An example of ADH (left-hand scale, values 0, 50, 100, 150) and protein concentrations (left-hand scale, values 0, 2, 4, 6, 8) in column outflow during expanded bed experiment, along with buffer step changes as shown by the EDTA trace (line without symbols).

values since ADH in 50 m*M* EDTA will be inactive due to subunit separation and therefore will not register on rate assays. Consequently the EDTA concentration employed in the elution was gradually increased to attempt to contain the ADH peak entirely in the third step. It was noted that, although 6 m*M* EDTA eluted the ADH, increasing the EDTA concentration resulted in earlier elution of the ADH peak. The EDTA concentration was increased to 20 m*M* before the peak was clearly contained in the third step. After these changes the recovery was stable at 80% or greater over multiple runs, and the improved CIP appeared effective at removing contaminants. In addition further stability work showed that ADH maintained 90–95% initial activity in 20 m*M* EDTA solution for at least 2 h at room temperature, longer if kept at 4°C.

These results compare reasonably favorably with more common, less specific chromatographic methods such as ion-exchange or hydrophobic interaction chromatography, although the purification factor is quite low for an affinity separation. This is thought

Table 2
Load, wash and elution sample pool data from a sample expanded bed run showing yield and purification factor for each stage

	Volume (ml)	ADH (units)	Protein (mg)	Specific activity (U/mg)	Purification factor	Yield (ADH, %)
Load	360	25 300	3070	8.24	1	79.9
Wash	973	6690	2490	2.69	0.3	21.1
Elution (buffer A)	70	65	0	N/A	N/A	0.2
(Buffer B)	154	97	0	N/A	N/A	0.3
(Buffer C)	323	24 748	366	67.6	8.2	78.2
(Buffer D)	222	98	7	14	1.7	0.3

to be due in part to the use of EDTA as an elution mechanism. Since EDTA in high concentrations (such as the 50 m*M* in the final elution step) removes the metal ions from the matrix, the system becomes more irreversible in nature and prevents re-binding of contaminating proteins and the development of a chromatographic profile. As a result the purification suffers because all proteins bound to the matrix will tend to co-elute. This can be improved with an accurate step elution protocol but the nature of the EDTA elution mechanism is always likely to cause problems in this area.

In addition the binding and elution of ADH was found to be extremely susceptible to small variations of pH since the choice of pH 7.2 for the buffers is extremely close to the pK_a of histidine, affecting the charge on the imidazole ring and hence the ability to bind. Consequently extreme care has to be taken in selecting the pH of buffers to ensure consistent results. Further work is considering the possibility of modifying buffer pH to ensure more consistent binding without affecting enzyme activity.

The major concern with the work lies in the purification factor with respect to contaminating proteins, which although acceptable for a less specific method such as ion exchange is quite low for what is essentially an affinity separation. The nature of the EDTA elution is likely to contribute to lower purification factors, since EDTA strips off the metal ions from the matrix thus preventing further binding to the column. Consequently anything bound to the column runs the risk of being eluted in one step if the metal ions are detached in this step. This can be minimised by ensuring that the ADH is eluted at as low an EDTA concentration as possible.

4. Conclusions

The work performed here shows that yeast ADH can be effectively purified from unclarified homogenates of pressed baker's yeast using immobilised metal-ion affinity chromatography in an expanded bed. The recovery of ADH on elution from the expanded bed compares favorably with other results shown in literature, although concerns about the long term stability of the matrix must be addressed before more detailed conclusions can be drawn. The introduction of a more rigorous CIP protocol into the system should increase the life of the matrix. The concern over the relatively low purification factor achieved indicates that careful control to narrow the elution peak or lower the amount of unwanted protein in the elution through the use of a tight EDTA step elution is necessary for such systems.

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