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Journal of Chromatography A, 874 (2000) 27–43

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Immobilised metal affinity chromatography of β-galactosidase from unclarified *Escherichia coli* homogenates using expanded bed adsorption

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Received 3 August 1999; received in revised form 20 December 1999; accepted 11 January 2000

Abstract

The development of an expanded bed process for the direct extraction and partial purification of β-galactosidase from unclarified *Escherichia coli* homogenates using its natural affinity for metal loaded STREAMLINE Chelating is described. Small packed beds were used to determine the effect of chelated metal ion (Cu^{2+}, Ni^{2+}, Co^{2+} or Zn^{2+}), loading pH and ionic strength on the selective binding capacity, and recovery of β-galactosidase from clarified homogenates. An elution protocol was developed using the competitive displacer, imidazole, to recover β-galactosidase in 87% yield and 3.4-fold purification. These results were then used to develop a separation for the recovery of β-galactosidase from unclarified homogenates in a 2.5-cm diameter expanded bed. Although Ni^{2+} loaded STREAMLINE Chelating had a 5% dynamic capacity for β-galactosidase of just 118 U ml^{-1} (0.39 mg ml^{-1}), the low capacity was thought to be due to the large size of the target (464 000) relative to the exclusion limit of the macroporous adsorbent. Despite this low capacity, Ni^{2} STREAMLINE Chelating was used successfully to recover β-galactosidase from an unclarified homogenate in 86.4% yield and at 5.95-fold purification. The degree of purification relative to a commercial standard, as assessed using the purification factor and sodium dodecyl sulphate–polyacrylamide gel electrophoresis was high suggesting that this pseudo-affinity procedure compared favourably with alternative methods.

Keywords: Expanded bed adsorption; Fluidised bed; Extraction methods; *Escherichia coli*; Streamline chelating; β-Galactosidase; Enzymes

1. Introduction

Many of the bioproducts of the biotechnology industry are proteins which are of interest because of their enzymatic activities, specific recognition interactions or other therapeutic actions [1]. These may be formed in, or obtained from a variety of sources such as fermentation broths of bacterial, yeast or mammalian cells or preparations of naturally occurring tissues or fluids. Direct extraction of the product from the crude process solution by adsorption to a chromatography medium would both make the product less susceptible to proteolytic degradation leading to higher yields and lead to better process economics through a reduction in the overall number of stages [1,2]. Expanded bed adsorption (EBA) eliminates the need for filtration and/or centrifugation and concentration steps and has the advantage over other

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0021-9673/00/$ – see front matter
PII: S0021-9673(00)00087-X

contacting methods of near plug flow of liquid resulting in adsorption performance similar to packed beds. The development of suitable adsorbents, in terms of both base matrix and ligand is central to the successful implementation of this technology [1]. Several matrices have been developed and tested [3,4], which have established selection criteria for size and density range and physical and chemical resistance. However the range of ligand receptor interactions tested has been identified as still requiring research [4].

The choice of ligand for expanded bed adsorption is a balance between high selectivity and the stability required to operate in crude feedstreams [1]. Robust ligands such as ion-exchange [5–8] or hydrophobic groups [9,10] have been used effectively in EBA. Although such adsorbents can be used in the purification of a large number of products, their lack of specificity may lead to saturation of the adsorbent with other components from the feedstock. In addition, the relatively high ionic strength of some fermentation broths can detrimentally reduce the performance of ion-exchange ligands [1,4,5,11,12].

Affinity ligands are considered to be the most specific of all the ligands employed in downstream processing, since their interaction is based on biological recognition rather than physico–chemical properties [13]. However, the high expense and proneness to inactivation of fragile proteinaceous affinity ligands, such as Protein A, antibodies or lectins, makes them unsuitable for use in the harsh environments encountered early on in downstream processing. Recent developments have seen the replacement of delicate high molecular mass biological ligands with smaller, cheaper synthetic ligands [14], such as reactive dyes [15], mixed mode and custom designed ligands [16]. The applications of such ligands in direct contacting and specifically in expanded bed operations have been described [14,17–21]. It has been suggested that general affinity ligands such as those which form metal chelates may also represent the appropriate balance between selectivity and robustness required for EBA [1,5,11].

Immobilised metal affinity chromatography (IMAC) exploits the affinities for metal ions that are exhibited by functional groups on the surfaces of proteins [22,23]. The advantages of IMAC include the stability of the metal chelates over a wide range of solvent conditions and temperatures, the high metal loadings that result in high protein loading capacities and the ease of product elution and ligand regeneration [24]. These small, inexpensive ligands can complex a variety of metal ions making it possible to bind a range of different proteins. The high ionic strengths often encountered in biological feedstocks actually serves to reduce non-specific electrostatic interactions and the presence of detergents or denaturants (such as 8 *M* urea or 6 *M* guanidine hydrochloride) does not affect the interaction [25]. Since the introduction of IMAC many applications of the principle have been developed. Schemes have been developed for the purification of both proteins exhibiting a natural affinity for metals such as human interferon-γ [26] and catalase [27], and also some which have a genetically engineered affinity, for example human atrial natriuretic peptide [28], lipoxygenase [29] and β-galactosidase [30]. Such engineering enables the one step isolation of a 95% pure protein from mixtures containing less than 1% of target protein [31].

In this paper we describe the use of STREAMLINE Chelating in expanded bed mode for the purification of the intracellular enzyme β-galactosidase from unclarified *Escherichia coli* homogenates. This is an appropriate model system, as *E. coli* is often used as an expression system for recombinant proteins [32,33] and β-galactosidase may be used as an affinity handle [34] and to hydrolyse milk products, a process of importance because of lactose intolerance in many people [35]. Various schemes for the expression, release and purification of β-galactosidase are also available in the literature for comparison [35–40]. The aim of the work presented here was to develop an optimised process, capable of delivering β-galactosidase at high purity and yield, to investigate the effect of suspended solids such as cell debris on the adsorption process, and finally, to establish a protocol for the application of immobilised metal ligands in the expanded bed adsorption of naturally occurring metal binding proteins.

2. Experimental

All experiments were performed at room temperature (20°C) unless otherwise noted.

2.1. Materials

STREAMLINE Chelating (100–300 μm, 1.2 g ml^{-1}, 40 μmol Cu^{2+} ml^{-1} gel) used in this work was a gift from Amersham Pharmacia Biotech (Uppsala, Sweden). This has the metal chelating ligand iminodiacetic acid (IDA) attached to it. *E. coli* 10221 (ATCC 23725) was purchased from the NCIMB (Aberdeen, UK). This bacterium is a constitutive mutant for β-galactosidase expression, a K12 derivative of genotype *lac*- (i−z+y+). Polypropylene glycol (average M_r 1025) was purchased from BDH (Poole, UK). All other chemicals used were obtained from Sigma–Aldrich (Poole, UK).

2.2. Fermentation

E. coli 10221 were revived and maintained using nutrient agar plates (13 g l^{-1} nutrient broth, 15 g l^{-1} technical agar), which were stored after growth at 4°C. Fresh plates were streaked each fortnight. An overnight McCartney Bottle culture (10 ml, nutrient broth, 13 g l^{-1}) was used as a pre-inoculum for the main inoculum, a shake flask culture (100 ml, defined media, as below – except 5 ml l^{-1} glycerol) which was also grown overnight at 37°C, 300 rpm in an LH orbital incubator [Inceltech (UK), Berkshire, UK].

All fermentations were performed in an LH 2000 fermentor [Inceltech (UK)] with a working volume of 5 l. The growth medium was a modified defined media originally developed for the high-cell-density fermentation of *E. coli*, the main change was glycerol replacing glucose as carbon source [41]. The pH was maintained at 6.8 by addition of 33% (v/v) ammonia or 2 *M* nitric acid. The fermentation temperature was controlled at 37°C, whilst air was sparged at a rate of 4 l min^{-1}. The agitation speed was 800 rpm throughout the fermentation. Foaming was controlled as necessary through the manual addition of further polypropylene glycol 1025. Samples (20 ml) were collected during the fermentation for determination of the turbidity at 600 nm, dry mass and β-galactosidase activity. After 12–14 h, the resulting fermentation broth was removed and split into 400-ml fractions. These were stored at 4°C until used.

2.3. Homogenisation

The cells were harvested from the stock fermentation broth (400-ml fractions) by centrifugation (10 000 rpm, 4°C for 15 min). The pellet was then resuspended in buffer (100 m*M* sodium phosphate pH 7.5) to give a final volume of 200 ml. The cells were then disrupted in an ice bath using a sonicator (Heat Systems, NY, USA). Disruption was carried out for a total of 14 min, using 30 s pulses with 1 min in between to allow the disruptate to cool. The cell extract was then treated with an endonuclease (Sigma–Aldrich, E-1014), active on all forms of DNA and RNA. Specifically, magnesium chloride was added to 2 m*M*, together with 10 μl of endonuclease (340 U μl^{-1}). The homogenate was then incubated with agitation for 2.5 h at 34°C. Sodium chloride was then added to give the desired molarity (which varied with experiment) and the pH adjusted using 100 m*M* sodium phosphate (with the same molarity sodium chloride) to give a final volume of 400 ml. For experiments involving cell-free extracts, this homogenate was centrifuged (20 000 rpm, 4°C for 20 min) and the pellets discarded.

2.4. Packed bed optimisation experiments

STREAMLINE Chelating was loaded with metal ions batchwise over a sintered funnel. The adsorbent (approx. 20 ml) was first washed with distilled water (200 ml), then loaded using a 50 m*M* solution of the sulphate salt of the required metal ion (Cu^{2+}, Ni^{2+}, Co^{2+}, or Zn^{2+}), in distilled water (100 ml). This was then washed with 100 m*M* sodium acetate, 0.5 *M* sodium chloride, pH 4.0 (200 ml), to remove loosely bound metal ions, and distilled water (100 ml). The small packed bed scouting experiments were carried out using a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech). STREAMLINE Chelating (pre-loaded with metals), was packed into either a HR 5/5 or a HR 5/10 column giving bed volumes of 1 or 2 ml, respectively. Clarified *E. coli* homogenate was applied to the column from either a 500-μl or a 50-ml sample loop, and fractions collected using a Frac-100.

These columns were then used to test variations in binding selectivity and capacity for β-galactosidase with the metal ions (Cu^{2+}, Ni^{2+}, Co^{2+}, or Zn^{2+}). A

1-ml bed of metal loaded adsorbent was first washed with 100 m*M* sodium phosphate, 0.5 *M* NaCl, pH 7.5 (10 ml) at a flow-rate of 0.5 ml min^{-1} (150 cm h^{-1}). Clarified *E. coli* homogenate (16 ml, 3.97 mg ml^{-1} protein, 106 U ml^{-1} β-galactosidase) was then pumped through at the same flow-rate. The column was then washed (5 ml), followed by elution with 60 m*M* imidazole (5 ml), all in the above buffer. The load, wash and elution volumes were collected and assayed for protein concentration and β-galactosidase activity.

An investigation of the optimum pH and ionic strength for selective adsorption of β-galactosidase was performed. The pH was varied from 6.5 to 8, whilst the ionic strength was varied by adding sodium chloride to a final concentration between 0 and 1.0 *M*. Clarified *E. coli* homogenate (12 ml, 3.20–3.56 mg ml^{-1} protein, 104–112 U ml^{-1} β-galactosidase) was loaded onto a 1 ml bed of Ni^{2+} STREAMLINE Chelating at a flow-rate of 0.5 ml min^{-1} (150 cm h^{-1}). The column was then washed (5 ml) and eluted with 60 m*M* imidazole (5 ml) in the appropriate buffer. Detailed methodologies are included in the appropriate figure legend.

The elution behaviour of β-galactosidase from Ni^{2+} STREAMLINE Chelating was studied using imidazole. Elution was performed using both gradients and steps, details being found in the appropriate legend. Typically, 500 μl of a clarified *E. coli* homogenate (3.67–4.32 mg ml^{-1} protein, 119–131 U ml^{-1} β-galactosidase) would be applied to a 2 ml bed of adsorbent at 0.5 ml min^{-1}. Under these conditions all the β-galactosidase activity would be bound. Fractions (1 or 0.5 ml) were collected for analysis of the protein concentration and β-galactosidase activity.

2.5. Expanded bed chromatography of β-galactosidase

Laboratory-scale expanded bed adsorption was carried out in a STREAMLINE 25 column (1 m×2.5 cm diameter) with valving and tubing enabling upflow and downflow through the column [42], together with hydraulic operation of the upper adapter using a second peristaltic pump. The settled bed height of STREAMLINE Chelating was 20.4 cm (100 ml adsorbent). The adsorbent was loaded with Ni^{2+} ions at the start of each experiment and regenerated using EDTA at the end of each run, prior to clean-in-place (CIP). This was to ensure the total removal of strongly bound proteins at the end of elution and to ensure any metal ions lost during sample application were subsequently fully replaced. The packed adsorbent was first washed with distilled water, then 50 m*M* nickel sulphate in distilled water (200 ml) was applied at 10.5 ml min^{-1} (130 cm h^{-1}). Weakly bound and free nickel ions were then washed from the column using 100 m*M* sodium acetate, 0.5 *M* NaCl pH 4.0 (500 ml) at the same flow-rate.

The column was then equilibrated with 100 m*M* sodium phosphate, 1.0 *M* sodium chloride, 6 m*M* imidazole pH 8.0 at 10.5 ml min^{-1} (130 cm h^{-1}). During this process the bed was expanded to a height of 40 cm, the adapter being positioned just above the top of the expanded bed. At least 30 min was allowed for bed stabilisation prior to feed application. *E. coli* homogenates were then applied at room temperature, the flow-rate being decreased to maintain a height of 40 cm or 2% expansion [43]. After sample application, weakly adsorbed contaminating proteins and particulates in the bed voids were washed out using the equilibration buffer, whilst steadily increasing the flow-rate to the value before loading. The bed was then allowed to settle and the upper adapter lowered to the top of the packed adsorbent. Elution was performed in a downflow direction at 10.5 ml min^{-1} (130 cm h^{-1}). Fractions were collected throughout loading, washing and elution and stored on ice. These were then analysed for turbidity (unclarified feedstocks only), total protein concentration and β-galactosidase activity.

The adsorbent was regenerated in the expanded mode at a flow-rate of 4.1 ml min^{-1} (50 cm h^{-1}) using 500 ml of each of the following 1 *M* NaOH, 1 *M* NaCl and 70% ethanol. Between each of these treatments the bed was washed using 300 ml distilled water at the same flow-rate. The adsorbent was used five times in the expanded mode with unclarified homogenates of dry mass as above and volumes between 200 and 2000 ml. Following each cycle of loading and regeneration, the adsorbent was monitored for changes in its fluidisation properties and

binding to β-galactosidase. The adsorbent was stored in the column between experiments in 20% ethanol.

2.6. Protein determination and enzyme assays

Protein determination was carried out using the Pierce Coomassie reagent with bovine serum albumin (BSA) being used as standard, according to standard protocols (for example see Ref. [21]). All measurements were made using clarified samples (8800 *g* for 8 min) to determine soluble protein concentration.

β-Galactosidase activity was determined (essentially according to Ref. [44]) by adding clarified enzyme solution (33 μl) to a 1-ml cuvette containing the following solutions: 0.3 *M* sodium phosphate, 0.003 *M* magnesium chloride, pH 7.5 (333 μl); 1.0 *M* β-mercaptoethanol (100 μl); 0.01 *M* Tris–acetate, 0.01 *M* magnesium chloride, 0.014 *M* *o*-nitrophenyl, β-D-galactopyranoside (ONPG), pH 7.5 (167 μl); and distilled water (367 μl). Enzyme activity was then measured spectrophotometrically at 405 nm by monitoring the hydrolysis of ONPG. Results are expressed in terms of enzyme activity where 1 unit (U) is defined as the amount of β-galactosidase required to hydrolyse 1 μmol of ONPG to *o*-nitrophenyl (ONP) and galactose per minute at 20°C. The extinction coefficient of ONP used was 3.1 mM^{-1} cm^{-1} [45].

The amount of activity contained within unbroken bacterial suspensions was assessed by diluting the sample to give 1 ml, of an optical density (O.D.) at 600 nm of <0.5, followed by incubation with mixing with 30 μl toluene at 37°C for 30 min [37,46,47]. The sample was then allowed to cool to room temperature before assay using the method above except without the clarification step.

2.7. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis analysis of expanded bed fractions

An Amersham Pharmacia PhastSystem horizontal gel-electrophoresis unit was used to carry out sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analyses of the protein fractions, essentially according to standard protocols [21,48]. Gels used were PhastGel Gradient 10–15 in conjunction with PhastGel SDS buffer strips. The gels were stained using PhastGel Blue R (Coomassie R350).

3. Results and discussion

3.1. Fermentation of E. coli 10221

E. coli 10221 is a constitutive mutant for the expression of β-galactosidase, which using these fermentation conditions, gave a final level of approximately 8.6% total protein. After 12 to 14 h fermentation, a cell suspension of O.D. 600 nm up to 30, corresponding to a dry mass of 11.4 mg ml^{-1} (1.14%, w/w) and containing β-galactosidase activities between 100 and 120 U ml^{-1} was harvested. The conductivity at the end of the fermentation was approximately 20 mS cm^{-1}. The fermentation broth was stored at 4°C rather than −20 or −70°C, as frozen samples have been reported to be more ''sticky'', which can result in blockage of the distributor of the expanded bed [6].

3.2. Homogenisation

Sonication resulted in cell homogenates (after dilution to a 400 ml volume) of total protein content 3 to 5 mg ml^{-1}, β-galactosidase activity 100 to 130 U ml^{-1} and O.D. at 600 nm of approximately 5.5.

In initial expanded bed experiments to determine whether β-galactosidase could be purified using IMAC in an EBA format, serious hydrodynamic problems were encountered. Soon after the start of feed application, the adsorbent particles formed aggregates leading to channelling within the bed and a degree of bed collapse. This lead to a decreased capacity for β-galactosidase, and a lengthy wash period. This aggregation was thought to be due to nucleic acids released during homogenisation associating with cell debris and adsorbent particles, forming large networks. Treatment of the homogenate with an endonuclease, active on all forms of DNA and RNA eliminated this problem. All the homogenates used in this work were treated with the nuclease, whether they were used in a packed or an expanded bed. The nuclease treatment was found not to alter adsorption performance in packed bed scouting experiments [49].

3.3. Choice of metal ion for binding β-galactosidase to STREAMLINE Chelating

The metal ions most commonly used in IMAC are the first row transition metal ions (Cu^{2+}, Ni^{2+}, Co^{2+} and Zn^{2+}). The IDA ligands immobilised on the STREAMLINE matrix, can co-ordinately bond to these metal ions through their nitrogen and two carboxylate oxygens. The remaining sites in the co-ordination sphere of the ions are occupied by water or buffer molecules, and may be displaced by protein functional groups. The groups responsible for retention on these immobilised metals have been suggested to be histidine, cysteine and tryptophan, and terminal amines [24,50], but due to the chemistry and surface availability of these groups, protein retention is dominated by histidine residues [51].

Table 1 gives the amounts of total protein and β-galactosidase eluted from the adsorbent in packed bed experiments with different chelated metal ions and clarified homogenates. Flow-rates were maintained at approximately 150 cm h^{-1} throughout the following optimisation experiments for approximate consistency with the determined fluidisation characteristics of STREAMLINE Chelating.

Previous studies have shown that variations in the affinity of model proteins can be rationalised in terms of the presence, multiplicity and micro-environments of histidine residues [52,53], leading to the following rules. Any protein displaying a single histidine residue on its surface is bound by chelated Cu^{2+}, whilst those displaying at least two histidines in their surface are retained by Ni^{2+}. Anything binding to either Co^{2+} or Zn^{2+} is due to the presence of two adjacent surface histidine residues. This condition may be met by two residues on an α-helix separated by two or three amino acids or simply as a result of the foldings of the polypeptide backbone [54]. The target enzyme, β-galactosidase is a large enzyme of M_r 464 000 [55,56] made up of four subunits, each of 1023 amino acids, of which 33 are histidines [35]. It is retained on Cu^{2+} STREAMLINE Chelating, however a large number of contaminating proteins are also bound reducing the purification achievable (Table 1). The Ni^{2+} loaded adsorbent also binds the enzyme, but with a lower amount of general *E. coli* protein making higher purifications possible. The chelated Zn^{2+} retains a small amount of the applied activity, but in an expanded bed protocol being applied as the first stage of a purification, the first requirement is for a large capacity for the target protein. Ni^{2+} loaded STREAMLINE Chelating was therefore chosen for further experimentation.

3.4. Choice of pH and ionic strength for loading

The selectivity exhibited by a metal–IDA complex can also depend on the composition of the mobile phase. The conditions usually employed for adsorption are aqueous buffers of pH between 7 and 8, including an intermediate concentration of a salt to suppress non-specific electrostatic interactions.

The influence of ionic strength (varied through the addition of various concentrations of sodium or potassium chloride) on the binding equilibrium between hen egg white lysozyme (HEWL) and Cu^{2+}–IDA Sepharose CL-4B, has recently been studied [57]. At concentrations of added salt ≤ 0.2 *M*, the adsorption data correlated with Langmuir predic-

Table 1
Choice of metal ion for retention of β-galactosidase on STREAMLINE Chelating

	Total protein (mg)	β-Galactosidase activity (U)	β-Galactosidase yield (eluted peak) (%)	β-Galactosidase specific activity (U mg^{-1})	Purification factor
Applied feed	63.5	1696	(100)	26.7	(1.00)
Elution peak					
Cu^{2+}	14.2	427	25.2	30.2	1.13
Ni^{2+}	7.5	560	33.0	75.0	2.81
Zn^{2+}	2.5	196	11.6	79.4	2.97
Co^{2+}	0.2	3	0.2	20	0.82

tions. However at concentrations ≥0.5 *M* the data followed a Freundlich–Langmuir model, indicating positive co-operativity. A range of added sodium chloride concentrations of 0, 0.25, 0.5, 0.75, 1 *M* and of pH 6.5, 7, 7.5, and 8 were tested in this work, in order to assess their effect on a multi-component preparative separation. Table 2 shows that at a constant pH, the amount of β-galactosidase retained increased with increasing sodium chloride concentration, as did the level of retained protein. The purification factor also increased indicating a higher affinity of β-galactosidase for the adsorption sites than general protein. These results were consistent with observations of the capacity of Cu^{2+}–IDA Sepharose CL-4B for HEWL [57] which increased to a maximum at 1 *M* NaCl and then decreased with further increases in salt concentration. The effect of pH on adsorption was not as pronounced as expected, there still being retention at pH 6.5, below the pK_a of the histidine residues (surface histidines residues have pK_a values between 6 and 7 [24]) although purification factors were higher at 7.5 and 8. A pH of 8 and 1.0 *M* sodium chloride were chosen for expanded bed adsorption, as salt concentrations any higher than this may result in protein precipitation and concomitant losses of target protein.

These results also indicate that β-galactosidase may be recovered directly from the fermenter, as its conductivity of 20 mS cm^{-1} lies approximately midway between 0 and 0.25 *M* NaCl, although the capture of β-galactosidase would be lower than using the above buffered preparation. It is interesting to note here that a conductivity of greater than 5 mS cm^{-1} would be expected to seriously reduce the efficiency and capacity of an ion-exchange separation due to the saturation of binding sites with small charged species [5,58].

3.5. Elution using imidazole in packed beds

Retention on immobilised metals is due mainly to the presence of surface accessible histidine residues, however additional secondary interactions involving ionic or hydrophobic forces may exist [24]. It is not unreasonable, therefore to expect that, amongst the various proteins of the *E. coli* lysate that bound to the chelated metal, a variety of strengths of interaction may exist. The use of selective elution strategies might therefore be useful in obtaining a product of increased purity. Elution of bound proteins may be achieved using either a decreasing pH gradient or by introducing a competitive agent. A decrease in pH to below 6 is usually sufficient to elute bound proteins, although this can lead to a degree of denaturation and loss of yield and/or a requirement for rapid quenching of the eluted fractions. Competitive agents such as imidazole, histidine or amine groups such as glycine are also effective at displacing proteins. Addition of strong chelators such as EDTA is usually reserved to strip the column of metal ion in the regeneration stage of operation. This is because it can lead to contamination of eluted fractions with metal ions or loss of metal ions from metallo-proteins and subsequent loss of activity or shape. We therefore, chose to investigate the use of imidazole as an eluent as it is inexpensive, does not effect subsequent purification steps, as would a

Table 2
Influence of (a) ionic strength, and (b) pH on the binding of β-galactosidase to Ni^{2+} STREAMLINE Chelating

	NaCl (*M*)	Ionic strength (mS cm^{-1})	pH	Total protein bound (%)	β-Galactosidase bound (%)	Purification factor
(a)	0	11.2	7.5	11.1	22.8	2.06
	0.25	30.2	7.5	14.1	39.7	2.82
	0.5	48.9	7.5	14.6	42.8	2.92
	0.75	62.5	7.5	14.9	45.9	3.08
	1.0	74.8	7.5	15.6	50.8	3.25
(b)	0.5	48.9	6.5	13.3	33.9	2.56
	0.5	48.9	7	14.9	38.3	2.57
	0.5	48.9	7.5	14.6	42.8	2.92
	0.5	48.9	8	13.7	41.0	3.00

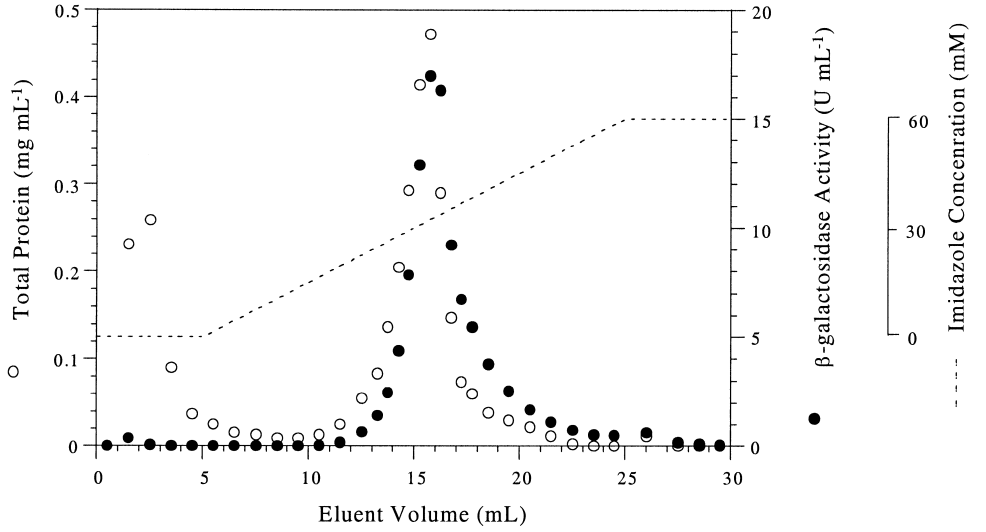

Fig. 1. Gradient elution of β-galactosidase from Ni^{2+} STREAMLINE Chelating.

change in pH and does not scavenge metals from the bed as other competitive eluents may.

Gradient elution (0 to 60 m*M* imidazole) of a small sample of clarified extract gave just a single peak, as shown in Fig. 1. This peak contained all of the applied activity at a purification factor of 1.43 (Table 3). This peak could not be further separated using different steps of imidazole around 30 m*M*, however it does contain a whole number of different proteins as just 29% of the loaded protein passed

Table 3
Development of gradient and stepwise elution strategies for the recovery of β-galactosidase in higher purity[a]

Elution conditions (imidazole)	Purification stage	Volume of step (ml)	Total protein (%)	β-Galactosidase activity (%)	β-Galactosidase specific activity (U mg^{-1})	Purification factor
1. Gradient	Flowthrough	5	29.0	0.7	0.67	0.02
(0 to 60 m*M*)	Gradient	20	65.0	94.1	39.9	1.43
2. Stepwise	Flowthrough	5	26.1	1.3	1.72	0.05
(30 m*M*, 60 m*M*)	30 m*M*	10	62.3	71.7	40	1.15
	60 m*M*	10	1.1	13.6	442	12.7
3. Stepwise	Flowthrough	5	25.9	1.3	1.74	0.05
(18 m*M*, 60 m*M*)	18 m*M*	15	52.8	31.1	20	0.59
	60 m*M*	10	8.9	56.6	216	6.38
4. Stepwise	Flowthrough	5	27.5	1.4	1.82	0.05
(12 m*M*, 60 m*M*)	12 m*M*	15	48.8	10.7	7.9	0.22
	60 m*M*	10	15.0	77.9	187	5.20
5. Stepwise	Flowthrough	5	27.4	1.1	1.17	0.04
(6 m*M*. 60 m*M*)	6 m*M*	20	38.3	1.8	1.34	0.05
	60 m*M*	10	25.7	86.8	99	3.38

[a] The running buffer was 100 m*M* Na Phosphate, 1.0 *M* NaCl, pH 8.0.

through the column unbound. The reason for the coelution of all the bound protein in the single peak is unknown at present.

Despite this finding, alternative elution strategies involving stepwise changes in the level of imidazole were also investigated, as earlier work had suggested that the use of a low concentration of imidazole may be used to elute some contaminating proteins without elution of β-galactosidase (see Table 3). A step to 30 m*M* imidazole may be used to elute the majority of the bound protein and some β-galactosidase, however 13.6% of the enzyme was recovered in the 60 m*M* step, at a purification factor of 12.7. This indicates that the target enzyme has a higher affinity for the chelated Ni^{2+} than does the majority of the general protein. Although this material is very pure, it is not very useful as an elution step as it represents a low fraction of the β-galactosidase in the load. The magnitude of the first step of imidazole concentration was then reduced in order to try to elute the contaminating proteins, before using the 60 m*M* step to elute the product. As the level of imidazole present in the first step was decreased, so the levels of protein and β-galactosidase were reduced. By using a two-step elution (Table 3) of 6 m*M* imidazole to elute the contaminating protein, the following 60 m*M* step may be used to release 86.8% of the applied β-galactosidase at a purification factor of 3.38.

We also tested the inclusion of a level of 6 m*M* imidazole in all the running buffers, in order to prevent binding of the protein eluted in the 6 m*M* step above, during the load. This was expected to enable better capture of β-galactosidase during the load, and also, through a reduction in the number of elution steps, an increase in productivity. For these reasons, two further experiments were carried out using 2-ml beds of Ni^{2+} STREAMLINE Chelating challenged with 5 column volumes of clarified *E. coli* homogenate, in order to compare the inclusion of 6 m*M* imidazole in the running buffer with the above two step scheme (6 m*M*, then 60 m*M* imidazole) (Table 4). There did appear to be some improvement in the purification achieved when the 6 m*M* imidazole was included in the running buffer, possibly through prevention of the binding of other host proteins. This elution scheme was therefore selected for the expanded bed adsorption of this enzyme from unclarified homogenates.

3.6. Comparison of the adsorption performance in different operating modes

Following the optimisation of adsorption and elution conditions for β-galactosidase, the metal loaded adsorbent was deployed in expanded mode for the preparative recovery of the enzyme. Initial tests were directed at investigating any problems in contacting this feedstream with the adsorbent and any differences between the packed and expanded mode of operation. Clarified and unclarified cell homogenates were therefore applied to 100 ml of Ni^{2+} STREAMLINE Chelating in either packed or expanded mode, initially at a constant superficial velocity of 130 cm h^{-1} (10.5 ml min^{-1}). Application was continued until the level of β-galactosidase in the effluent reached up to 95%, as results show in Fig. 2. The shapes of the breakthrough curves are

Table 4
Development of stepwise elution strategies for the recovery of β-galactosidase in higher purity[a]

Elution conditions (Imidazole)	Purification stage	Volume of step (ml)	Total protein (%)	β-Galactosidase activity (%)	β-Galactosidase specific activity (U mg^{-1})	Purification factor
Two-step elution (6 m*M*, then 60 m*M*)	Flowthrough	10	52.0	17.6	9.4	0.34
	Wash	10	23.4	8.7	10.4	0.37
	6 m*M*	15	4.1	3.4	23.4	0.84
	60 m*M*	10	16.5	58.2	98.2	3.53
6 m*M* Imidazole included in running buffers	Flowthrough	10	59.3	17.8	8.6	0.30
	Wash	15	23.2	9.5	11.7	0.41
	60 m*M*	10	12.9	61.7	137	4.79

[a] The running buffer was 100 m*M* Na Phosphate, 1.0 *M* NaCl, pH 8.0.

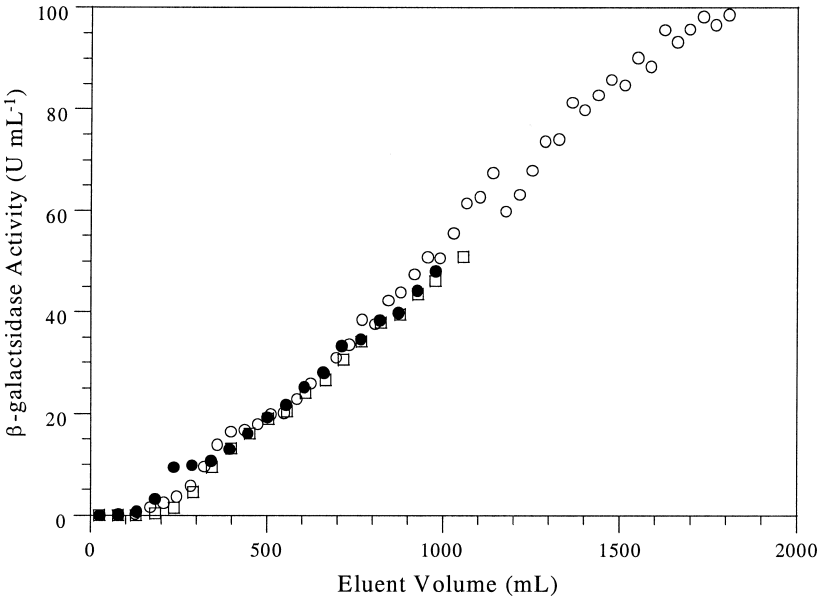

Fig. 2. Comparison of breakthrough curves in the packed and expanded bed adsorption of β-galactosidase onto Ni^{2+} STREAMLINE Chelating. Clarified homogenate onto a packed bed (●), clarified homogenate onto an expanded bed (□) and unclarified homogenate onto an expanded bed (○). A 100-ml bed (settled height of 20.4 cm) of STREAMLINE Chelating was packed into a STREAMLINE 25 column. The columns were charged with Ni^{2+} as described in Experimental and then equilibrated with 100 m*M* sodium phosphate, 1 *M* NaCl, 6 m*M* imidazole, pH 8.0 as appropriate in the packed or expanded mode.

very similar, irrespective of mode of operation or the presence or absence of cell debris. The curves also overlap, indicating the good run-to-run reproducibility achieved with the unclarified *E. coli* feed. The breakthrough curves for the clarified feed in packed and expanded mode suggest that the expanded mode of operation causes a slight reduction in the capacity of the adsorbent for β-galactosidase (as the curves overlap and the voidage and hence the amount of target protein present but not adsorbed in the voids of the bed is greater for the expanded bed). By comparing the expanded contacting with clarified and unclarified feeds, the presence of cells and cell debris appears to cause no change in the position or shape of the breakthrough curve in the expanded mode.

3.7. Frontal analysis of β-galactosidase on Ni^{2+} STREAMLINE Chelating

Frontal analysis was employed to determine the maximum quantity of unclarified *E. coli* homogenate that can be applied to the bed of Ni^{2+} STREAMLINE Chelating before significant β-galactosidase breakthrough occurs. The data also provides quantitative information on the dynamic and total capacity of the resin for the target, enabling comparison with other methods and modes of separation. The effluent concentrations of turbidity, total protein and β-galactosidase during the application of 2000 ml of unclarified homogenate to the bed are shown in Fig. 3. The turbidity and total protein are seen to breakthrough simultaneously after approximately 120 ml (approximately the interstitial voidage of the bed). The turbidity breakthrough curve was sharp indicating no hold-up of solids within the column whilst the total protein trace was slightly shallower due to its smaller size and possibly suggesting some slight interaction with the adsorbent. In contrast, the applied β-galactosidase activity was removed from the feedstream due to the much higher selectivity of the column for the enzyme under these conditions. Complete breakthrough of β-galactosidase occurred after approximately 2000 ml had been applied. The optimum load was determined as between 200 and 300 ml.

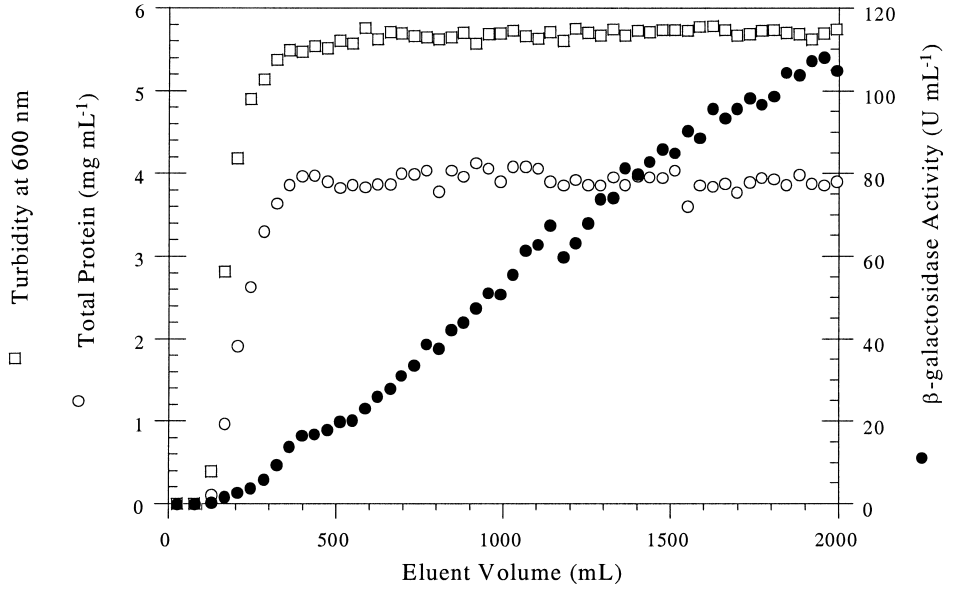

Fig. 3. Determination of the dynamic binding capacity of Ni^{2+} STREAMLINE Chelating for β-galactosidase using frontal analysis.

The dynamic capacity of Ni^{2+} STREAMLINE Chelating for β-galactosidase under these conditions was calculated at 5% as 118 U ml^{-1} and the total capacity as 881 U ml^{-1}. These capacities can be converted to mg β-galactosidase per ml Ni^{2+} STREAMLINE Chelating using the specific activity of pure β-galactosidase. A sample of grade VIII purity β-galactosidase (Sigma–Aldrich) was therefore assayed using the same protocol and in the same buffer, giving a specific activity of 302 U mg^{-1}. This value was similar to existing literature values (at varying temperatures and slightly different conditions) of 650 U mg^{-1} (Sigma–Aldrich; at 37°C), 600 U mg^{-1} ([46]; at 25°C) and 570 U mg^{-1} ([37]; at 37°C).

This would indicate a 5% dynamic capacity of 0.39 mg ml^{-1} and a total capacity of 2.92 mg ml^{-1}. The reason for these low capacities and possibly for the shallow breakthrough curve is the size of the target enzyme 464 000 relative to the exclusion limit of the macroporous adsorbent. The fractionation range for this material is up to M_r $4 \cdot 10^6$. A crude comparison can be made between the Stokes radius [59] of β-galactosidase (physicochemical molecular properties approximated using data of Wallenfels and Weil [47]) and a hypothetical protein of M_r $4 \cdot 10^6$, which are 67 and 125 Å, respectively. This demonstrates the level of steric hindrance to the diffusion of the large β-galactosidase molecule which might be expected from the pores of this matrix. Adsorption would probably be limited to an external layer of the adsorbent, depending on the exact nature of the pore size distribution. Such problems have been observed in the purification of other large proteins such as IgM (M_r 950 000) using porous adsorbents, where it was shown that a more open pore structure is beneficial [60].

3.8. Metal affinity expanded bed adsorption of β-galactosidase from unclarified E. coli homogenate

Using the optimised conditions developed in the packed beds and a volume of unclarified *E. coli* homogenate (200 ml) such that there was no significant loss during the load or wash phase, an experiment was performed to clarify, concentrate and partially purify β-galactosidase using Ni^{2+} STREAMLINE Chelating. The resulting chromatogram and purification table are shown in Fig. 4 and Table 5, respectively. Fig. 4 shows how with the load of just two settled column volumes, the wash begins just as the solid debris and general protein are

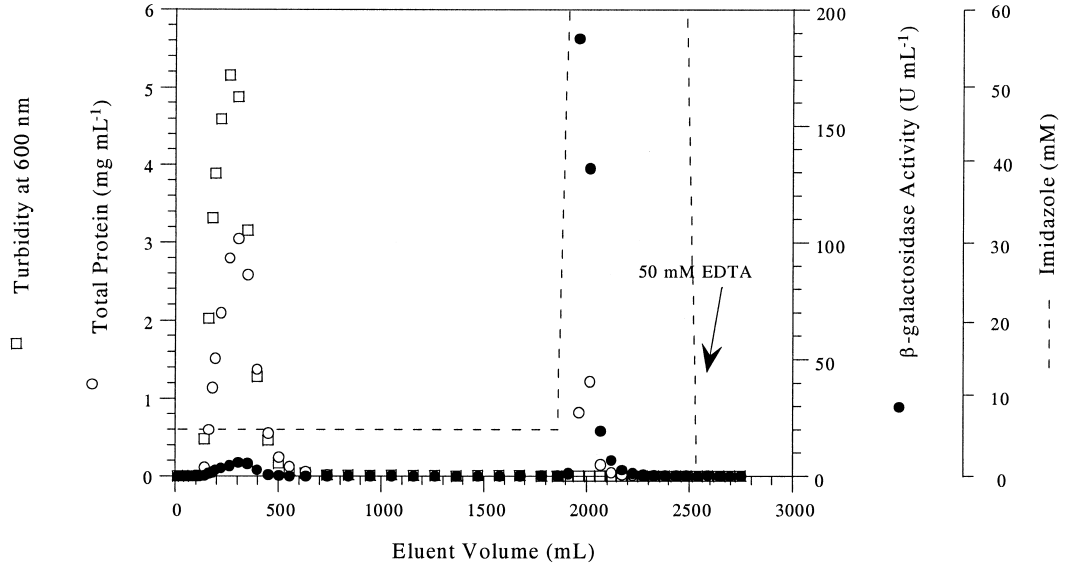

Fig. 4. Metal affinity expanded bed adsorption of β-galactosidase from an unclarified *E. coli* homogenate using Ni^{2+} STREAMLINE Chelating. A 100-ml bed (settled height of 20.4 cm) was expanded to 40 cm (200 ml) with 100 m*M* sodium phosphate, 1 *M* NaCl, 6 m*M* imidazole, pH 8.0 at 10.5 ml min^{-1} (130 cm h^{-1}). Unclarified *E. coli* homogenate (200 ml, turbidity at 600 nm of 5.46, 4.12 mg ml^{-1} protein, 107.3 U ml^{-1} β-galactosidase) was then injected onto the bed, which was subsequently washed with the fluidising buffer until the absorbance at 280 nm returned to the baseline. The pump was then switched off and the adsorbent allowed to settle, the upper adapter lowered and the flow direction reversed. Elution was carried out first with 60 m*M* imidazole in 100 m*M* sodium phosphate, 1 *M* NaCl, pH 8.0, and then with 50 m*M* EDTA in distilled water.

beginning to breakthrough. The level of β-galactosidase activity in the effluent rose to 5.4% of the inlet value, and just 0.5% of the applied enzyme was lost in the flowthrough. During the wash, a further 5.3% of the target was lost in the column effluent, however a total of 77% of the general protein passed through the column unretarded. The elution protocol used was as devised in the small packed beds. The bound material was eluted in a sharp peak of approximately 210 ml. This peak contained 86.4% of the applied β-galactosidase activity and only 14.6% of the general protein loaded. There was no evidence of any fouling of the adsorbent or of any cellular debris being bound or released in the eluate. The high level of activity recovered in the eluted fractions indicates that the enzyme is not denatured during its extraction using this technique. The purification factor of 5.95 is high for what is usually

Table 5
Metal affinity expanded bed adsorption of β-galactosidase using Ni^{2+} STREAMLINE Chelating

Purification stage	Volume (ml)	Total protein (mg)	β-Galactosidase activity (U)	β-Galactosidase yield (%)	β-Galactosidase specific activity (U mg^{-1})	Purification factor
Unclarified homogenate	200	824	21 462	(100)	26	(1.00)
Flowthrough	200	52	97	0.5	1.86	0.07
Wash	1632	584	1139	5.3	1.95	0.08
Elution 1 (60 m*M* imidazole)	629	120	18 552	86.4	154.6	5.95
Elution 2 (50 m*M* EDTA)	314	0.34	34	0.2	100	3.85
Yield (%)		91.8	92.4			

considered a pseudo-affinity interaction, and is a useful addition to the clarification and concentration usually achieved in this type of capture step.

The time taken for the metal affinity expanded bed to go through one cycle of the purification process (equilibration [30 min], Ni^{2+} loading [20 min], removing loosely bound Ni^{2+} [40 min], equilibration and expansion [60 min], loading [22 min], washing [90 min], elution [90 min]) was about 412 min. The productivity of the process for β-galactosidase was therefore $2.9 \cdot 10^{-1}$ U ml^{-1} min^{-1} (based on the dynamic capacity to 5% breakthrough of 140 U ml^{-1} in this experiment, and the yield). However, if the CIP procedure is included in the calculation, the productivity dropped to $1.3 \cdot 10^{-1}$ U ml^{-1} min^{-1} (as the CIP took an additional 512 min). The extra time required for charging the column with Ni^{2+} ions and regeneration with EDTA has a negative effect on the productivity of IMAC protocols compared with simpler ion-exchange methods. On balance though, the adoption of such stages could improve adsorbent life and result in a decreased number of stages through improved purity.

A further step designed to elute any remaining tightly bound proteins together with the chelated metal ions using 50 m*M* EDTA released little further protein. Analysis of the level of Ni^{2+} ions in the eluted β-galactosidase pool was not attempted, but this data may prove useful for the large scale adoption of the technique as any contamination may complicate subsequent procedures. Indeed, a potential problem with the use of Ni^{2+} in the manufacture of therapeutics is the fact that this metal ion is a sensitising agent. This problem could be tackled in the future either by careful selection of subsequent polishing steps to remove the contaminating metal ions or by using a slightly different metal chelating ligand thereby retaining the metal ion more strongly or enabling a different, less toxic, metal to be used.

3.9. SDS–PAGE analysis

A further semi-quantitative analysis of the metal affinity expanded bed adsorption of β-galactosidase was made using SDS–PAGE. Fig. 5 shows a Coomassie Blue stained gel of the important fractions. As indicated previously β-galactosidase has a molecular mass of 464 000 and is made up of four identical monomers of M_r 116 000. A whole range of proteins are present in the *E. coli* lysate (lane 3),

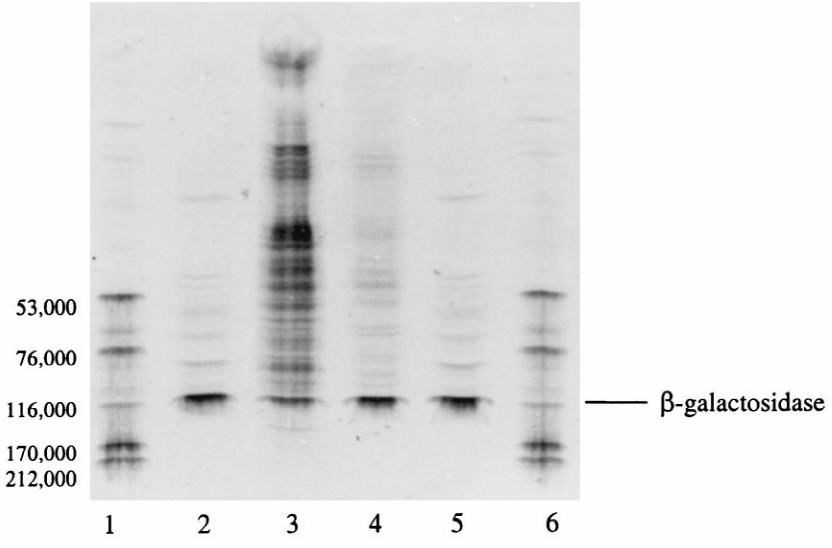

Fig. 5. SDS–PAGE (Coomassie Blue stained, reducing conditions) of the purification of β-galactosidase from an unclarified *E. coli* homogenate using Ni^{2+} STREAMLINE Chelating. Where: lane 1, high-molecular-mass standards (myosin, 212 000, α-macroglobulin, 172 000, β-galactosidase, 116 000, transferrin, 76 000 and glutamic dehydrogenase, 53 000); lane 2, commercially available β-galactosidase (Sigma–Aldrich, purity grade VIII); lane 3, *E. coli* homogenate feedstream; lane 4, elution stream (60 m*M* imidazole); lane 5, as lane 2; lane 6, as lane 1.

β-galactosidase being one of the major bands present. The degree of purification achieved is clear when comparing lanes 3 and 4, the β-galactosidase being the only protein concentrated, although there are a large number of weakly binding co-eluting proteins. Lanes 2 and 5 contain a sample of commercially available β-galactosidase (grade VIII purity, Sigma–Aldrich) which also contains a number of impurities, some of which may be degradation products. This SDS–PAGE analysis appears to be consistent with the values of the purification factors above and the fact that the specific activity in the eluate pool of 155 U mg^{-1} is not far short of that in the sample of purified enzyme (302 U mg^{-1}).

3.10. Cleaning-in-place procedures

In direct extraction operations unclarified broths containing nucleic acids, lipids and other components normally removed prior to column chromatography are contacted with adsorbents. Special care has to be taken to thoroughly clean the adsorbent between runs to maintain performance [4]. Indeed as in conventional packed chromatography, stationary phase costs can be significant unless they can withstand a certain number of purification cycles [61]. In expanded bed adsorption CIP procedures are usually particularly rigorous due to the crude nature of the feedstocks. A slightly modified CIP procedure, originally used to clean the anion exchanger STREAMLINE DEAE [5] was employed in this work. The protocol used was a sequence of solutions containing 1 *M* NaOH, distilled water, 1 *M* NaCl, distilled water and then 70% ethanol followed by a 20% ethanol preservative solution. This was designed to sanitise the adsorbent and then remove residual compounds bound via strong electrostatic and hydrophobic interactions, respectively. Table 6 and the β-galactosidase breakthrough curves for the unclarified homogenate shown in Fig. 3 demonstrate how little change in the properties of the adsorbent or the resulting chromatogram occur over the initial five uses. This adsorbent can therefore be used and cleaned repetitively using this simple CIP procedure, which might be expected to damage many proteinaceous affinity ligands.

3.11. Comparison of metal affinity expanded bed adsorption with alternative purification methods for β-galactosidase and other model systems

A number of purification schemes for the enzyme β-galactosidase are compared in Table 7. The schemes are varied as there are a number of properties of the target enzyme that may be exploited for purification. Amongst others, the enzyme has a low isoelectric point (estimated as 5.5 [62]) and there is also an affinity ligand available, the substrate analogue *p*-aminophenyl-β,D-thiogalactopyranoside. Several of the isolation procedures included [37,39,40] are complete pilot or process scale methods. In addition, two recent studies have reported the expanded bed adsorption of β-galactosidase using anion exchangers. The IMAC technique developed here compares favourably with the alternative methods presented and resulted in one of the highest yields (86.4%). This is probably due to the reduction in the number of separate operations required to purify the product, which can improve process yields by a reduction in losses due to individual stage losses and due to time-dependent proteolytic damage or other modifications. It should be noted that the data needs to be analysed carefully, as the end product of each scheme has a different degree of purity which can be difficult to compare due to differences in assay procedure. Despite this, the purification achieved using Ni^{2+} STREAMLINE Chelating compares favourably with the multi-step procedures. It

Table 6
Effect of clean-in-place (CIP) on the performance of STREAMLINE Chelating

Number of CIP cycles	Superficial velocity (cm h^{-1}) for 2% bed expansion in running buffer at 20°C	Dynamic capacity (to 5%) for β-galactosidase
0	135	121
1	131	118
5	130	140

Table 7
Comparison of various purification schemes for the recovery of β-galactosidase

Number of steps	Purification procedure: Unit operation (matix/ligand)	Capacity (U ml^{-1})	Yield (%)	Purification fold	β-Galactosidase specific activity (U mg^{-1})	Productivity (U ml^{-1} min^{-1})	Ref.
Single	Expanded bed metal affinity chromatography (Ni^{2+} loaded STREAMLINE Chelating)	118–140	86.4	5.95	155	$2.9 \cdot 10^{-1}$	This work
Single	Expanded bed anion-exchange chromatography (Accell Plus QMA)		83.0	2.2	37.3		[38]
Single	Expanded bed (STREAMLINE DEAE)	84	63.0	6.0	28.0		[36]
Multiple	1. Aqueous two-phase extraction		78	–	79		[35]
	2. Ion-exchange chromatography		68	6.33	500		
	3. Immobilised metal affinity chromatography (Ni^{2+} IDA agarose)		65	8.1	640		
Multiple	1. Centrifugation						[37]
	2. Heat treatment						
	3. Centrifugation						
	4. Precipitation						
	5. Centrifugation		62.5	4.33	132		
Multiple	1. Ammonium sulphate precipitation						[39]
	2. Centrifugation						
	3. Affinity chromatography		87.2	9			
Single	1. Aqueous two-phase extraction						[40]
	2. Centrifugation		75	12	260		

also achieved a reasonable level of purification for a front line process and therefore could ultimately lead to a reduction in the size and expense of subsequent steps. The dynamic capacity of this adsorbent is similar to that reported for the binding of this enzyme to STREAMLINE DEAE in expanded bed procedures of 84 U ml^{-1} [36], although the process described here is also successful in feeds of higher ionic strength and is therefore a more versatile technique.

4. Conclusions

A procedure for the metal affinity expanded bed adsorption and partial purification of β-galactosidase from unclarified *E. coli* homogenates has been developed. Ni^{2+} loaded STREAMLINE Chelating was used to isolate β-galactosidase at 5.95-fold purification with an enzyme activity yield of 86.4%. The process indicates that IMAC techniques are a useful addition to other interaction types already applied successfully in EBA. The ability to achieve adsorption under conditions of high ionic strength or in solutions containing detergents or denaturants together with the robust yet selective nature of the solid phase might improve the range of products that can be separated using the EBA technique or provide an alternative to existing processes.

Group specific (or pseudo-affinity) methods for protein purification have been shown in this and other studies to give, depending on the protein, a useful degree of purification. A drawback in the design of a purification scheme for a given novel protein is the current requirement for exhaustive screening of existing interaction types. In order for IMAC to be successful, such a protein would need to have a number of metal chelating sites displayed in its surface. The greater the number, the higher the capacity and degree of purification one could hope to

achieve. Future studies will be aimed at demonstrating how, by introducing a tail of chelating amino acids one can facilitate such a proteins downstream processing using IMAC giving one step purifications in expanded beds.

5. Nomenclature

ADH	Means Alcohol dehydrogenase
BSA	Means Bovine serum albumin
EBA	Means Expanded bed adsorption
HEWL	Means Hen egg white lysozyme
IDA	Means Iminodiacetic acid
IMAC	Means Immobilised metal affinity chromatography
ONP	Means *o*-Nitrophenyl
ONPG	Means *o*-Nitrophenyl, β-D-galactopyranoside

Acknowledgements

The authors acknowledge support for this work from the Biotechnology and Biological Sciences Research Council (BBSRC), UK and also thank Amersham Pharmacia Biotech (Uppsala, Sweden) for the provision of chromatographic materials and equipment.

References

[1] H.A. Chase, Trends Biotechnol. 12 (1994) 296–303.
[2] F.P. Gailliot, C. Gleason, J.J. Wilson, J. Zwarick, Biotechnol. Prog. 6 (1990) 370–375.
[3] R. Hjorth, Trends Biotechnol. 15 (1997) 230–235.
[4] J. Thommes, Adv. Biochem. Eng. Biotechnol. 58 (1997) 185–230.
[5] Y.K. Chang, H.A. Chase, Biotechnol. Bioeng. 49 (1996) 204–216.
[6] A.-K. Barnfield-Frej, R. Hjorth, A. Hammarstrom, Biotechnol. Bioeng. 44 (1994) 922–929.
[7] M. Hansson, S. Stahl, R. Hjorth, M. Uhlen, T. Moks, Bio/Technology 12 (1994) 285–288.
[8] W. Noppe, I. Hanssens, M. De Cuyper, J. Chromatogr. A 719 (1990) 327–331.
[9] M.P. Smith, M. Bulmer, R. Hjorth, N.J. Tichener-Hooker, Proceedings of the 5th World Congress of Chemical Engineering, Vol. 2, July 1996, pp. 565–570.
[10] M.P. Smith, Ph.D. Thesis, University College London, London, 1997.
[11] H.A. Chase, J. Mol. Recognit. 11 (1998) 1–5.
[12] J. Thommes, A. Bader, M. Halfar, A. Karau, M.-R. Kula, J. Chromatogr. A 752 (1996) 111–122.
[13] S.R. Narayanan, J. Chromatogr. A 658 (1994) 237–258.
[14] G.E. McCreath, H.A. Chase, R.O. Owen, C.R. Lowe, Biotechnol. Bioeng. 48 (1995) 341–354.
[15] S.B. McCloughlin, C.R. Lowe, Rev. Prog. Coloration 18 (1988) 16–28.
[16] C.R. Lowe, S.J. Burton, N.P. Burton, W.K. Alderton, J.M. Pitts, J.A. Thomas, Trends Biotechnol. 10 (1992) 442–448.
[17] M. Bjorklund, M.T.W. Hearn, J. Chromatogr. A 743 (1996) 145–162.
[18] Y.K. Chang, G.E. McCreath, H.A. Chase, Biotechnol. Bioeng. 48 (1995) 355–366.
[19] N. Garg, I. Yu, G. Mattiasson, B. Mattiasson, Bioseparation 6 (1996) 193–199.
[20] P.E. Morgan, O.R.T. Thomas, P. Dunnill, A.J. Sheppard, N.K.H. Slater, J. Mol. Recognit. 9 (1996) 394–400.
[21] R.O. Owen, G.E. McCreath, H.A. Chase, Biotechnol. Bioeng. 53 (1997) 427–441.
[22] J. Porath, J. Carlsson, I. Olsson, G. Belfrage, Nature 258 (1975) 598–599.
[23] J. Porath, Prot. Exp. Purif. 3 (1992) 263–281.
[24] F.H. Arnold, Bio/Technology 9 (1991) 151–156.
[25] J. Porath, B. Olin, Biochemistry 22 (1983) 1621–1630.
[26] Z. Zhang, K.-T. Tong, M. Belew, T. Pettersson, J.-C. Janson, J. Chromatogr. 604 (1992) 143–155.
[27] G.S. Chaga, A.S. Medin, S.G. Chaga, J. Porath, J. Chromatogr. 604 (1992) 177–183.
[28] D.L. Wilkinson, N.-T. Ma, C. Haught, R.G. Harrison, Biotechnol. Prog. 1 (1995) 265–269.
[29] X.S. Chen, A.R. Brash, C.D. Funk, Eur. J. Biochem. 214 (1993) 845–852.
[30] S. Piesecki, W.-Y. Teng, E. Hochuli, Biotechnol. Bioeng. 42 (1993) 178–184.
[31] S.A. Lopatin, V.P. Varlamov, Appl. Biochem. Microbiol. 31 (1995) 221–227.
[32] R.C. Hockney, Trends Biotechnol. 12 (1994) 456–463.
[33] A.P.J. Middelberg, Biotechnol. Adv. 13 (1995) 491–551.
[34] E. Flaschel, K. Freihs, Adv. Biotechnol. 11 (1993) 31–78.
[35] B.M. Brena, L.G. Ryden, J. Porath, Biotechnol. Appl. Biochem. 19 (1994) 217–231.
[36] M.J. Atolozaga, R. Jonas, A.L. Schneider, S.A. Furlan, M.D. Carvalho-Jonas, Bioseparation 7 (1998) 137–143.
[37] J.J. Higgins, D.J. Lewis, W.H. Daly, F.G. Mosqueira, P. Dunnill, M.D. Lilly, Biotechnol. Bioeng. 20 (1978) 159–182.
[38] J.A.M. Pereira, P.D.T. Viera, E. Rosa, G.M. Pastore, C. Santana, Appl. Biochem. Biotechnol. 70–72 (1998) 779–787.
[39] P.J. Robinson, M.A. Wheatley, J.-C. Janson, P. Dunnill, M.D. Lilly, Biotechnol. Bioeng. 16 (1974) 1103–1112.

[40] A. Veide, A.-L. Smeds, S.-O. Enfors, Biotechnol. Bioeng. 25 (1983) 1789–1800.
[41] D. Riesenberg, K. Menzel, V. Schultz, K. Schumann, G. Veith, G. Zuber, W.A. Knorre, Appl. Microb. Biotechnol. 14 (1990) 77–82.
[42] A.-K. Barnfield-Frej, H.J. Johansson, S. Johansson, P. Leijon, Bioproc. Eng. 16 (1997) 57–63.
[43] Y.K. Chang, H.A. Chase, Biotechnol. Bioeng. 49 (1996) 512–526.
[44] G.R. Craven, E. Steers, C.B. Anfinsen, J. Biol. Chem. 240 (1965) 2468–2477.
[45] K. Wallenfels, Methods Enzymol. 5 (1962) 212–219.
[46] P.P. Gray, P. Dunnill, M.D.J. Lilly, Ferment. Technol. 50 (1971) 381–387.
[47] K. Wallenfels, R. Weil, in: P.D. Boyer (Ed.), The Enzymes, Academic Press, London, 1972, pp. 617–663.
[48] U.K. Laemmli, Nature 227 (1970) 680–685.
[49] R.H. Clemmitt, H.A. Chase, unpublished observations.
[50] J. Porath, Trends Anal. Chem. 7 (1988) 254–259.
[51] T.W. Hutchens, T.T. Yip, J. Chromatogr. 500 (1990) 531–542.
[52] E.S. Hemdan, Y.-J. Zhao, E. Sulkowski, J. Porath, Proc. Natl. Acad. Sci. 86 (1989) 1811–1815.
[53] Y.-J. Zhao, E. Sulkowski, J. Porath, Eur. J. Biochem. 202 (1991) 1115–1119.
[54] E. Sulkowski, Bioessays 10 (1989) 170–175.
[55] A. Kalnins, K. Otto, U. Ruther, B. Muller-Hill, EMBO J. 2 (1983) 593–597.
[56] A. Veide, T. Lindbach, S.O. Enfors, Enzyme Microb. Technol. 11 (1989) 744–751.
[57] W. Jiang, M.T.W. Hearn, Anal. Biochem. 242 (1996) 45–54.
[58] G. Sofer, L. Hagel, Handbook of Process Chromatography: A Guide To Optimisation, Scale-up and Validation, Academic Press, London, 1997.
[59] H.A. Chase, in: A. Mizrahi (Ed.), Downstream Processes: Equipment and Techniques, Alan R. Liss, 1988, pp. 159–204.
[60] H.A. Chase, B. Machielse, D. Naveh, Bioseparation 7 (1997) 47–55.
[61] J.E. Porter, M.R. Ladisch, Biotechnol. Bioeng. 39 (1992) 717–724.
[62] M.H. Heng, C.E. Glatz, Biotechnol. Bioeng. 42 (1993) 333–338.