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Comparison of histidine-tag capture chemistries for purification following chemical extraction

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Abstract

The purification of a 6x-histidine tagged viral coat protein (L1) in expanded mode directly following chemical extraction from the cytoplasm of *Escherichia coli* HMS174(DE3) is investigated. Chelating adsorbents based on the ligands iminodiacetic acid (IDA) and nitrilotriacetic acid, using chelated metal ions Ni^{2+} and Cu^{2+}, were compared. The use of Ni^{2+}–IDA resulted in a high purification factor (9.7) and moderate recovery yield (58%). However, the eluted fractions had an overall L1 purity less than 50% and were therefore significantly contaminated with other host proteins. In batch tests, Cu^{2+}–IDA was found to be superior to all other combinations as it was characterised by higher binding capacities and faster adsorption kinetics. A subsequent immobilised metal affinity chromatography-expanded bed adsorption experiment using Cu^{2+}–IDA resulted in a higher L1 purification factor (20), recovery yield (71%) and purity (89%). The process presented here combines direct chemical extraction with expanded bed recovery. It is simpler than traditional methods, and should find more widespread application in the recovery of inclusion body proteins. Robust pseudo-affinity ligands such as metal chelates show potential for selective primary recovery of unfolded proteins, and could be used for further processing such as on-column refolding.

Keywords: Expanded bed adsorption; Adsorption; Immobilized metal affinity chromatography; *Escherichia coli*; Inclusion bodies; Proteins; Metal complexes; Histidine

1. Introduction

The over-expression of recombinant proteins in *Escherichia coli* often leads to their intracellular accumulation in solid granules known as inclusion bodies (IBs). Conventional recovery processes for IBs consist of a highly conserved set of steps [1]: cell disruption [2]; centrifugation [3]; dissolution and column purification prior to refolding [1]. These traditional methods are largely evolved from laboratory-scale approaches based upon inclusion body release followed by in vitro solubilisation. A major intensification of the process can be achieved by using in situ solubilisation of the inclusion bodies without mechanical cell disruption [4] followed by an adsorption-based primary recovery operation, thereby also eliminating the need for a clarification step. This new flowsheet was recently implemented using a combination of chemical extraction and subsequent immobilised metal affinity chromatography (IMAC)-based expanded bed adsorption (EBA) for dramatically simplified IB processing [5].

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EBA is a unit operation for the direct recovery of bio-products from particulate containing feeds [6–8]. It has been used successfully with a number of different feedstocks such as bacterial [9,10], yeast [11,12] and mammalian [13,14] cell fermentation broths. In comparison to these feedstocks, the increased viscosity of the lysate and different physical structure of the unfolded protein following chemical extraction, is expected to lead to very different mass transfer characteristics and significantly altered process performance. The influence of process parameters on the adsorption chemistry and kinetics on the separation are therefore investigated here to try to understand the process in greater detail.

The direct chemical extraction of IB protein from *E. coli* using 8 *M* urea, and the use of spermine to compact the released DNA enabling the coupling of extraction and expanded bed recovery, has been shown to be effective [5]. However, the yield and purity following expanded bed recovery remained lower than anticipated. Further work is therefore required to investigate the effectiveness of direct recovery following chemical extraction. This work necessarily entails investigation of capture chemistries to improve expanded bed performance.

The most widely used metal ions in IMAC are the first row transition metal ions: Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} [15]. Their affinity towards electron donating groups varies in the order $Cu^{2+} > Ni^{2+} > Zn^{2+} \approx Co^{2+}$, whereas their selectivity follows the order $Cu^{2+} < Ni^{2+} < Zn^{2+} \approx Co^{2+}$ [16,17]. These metal ions can also be immobilised using various chelating ligands, of which only iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) derivatised adsorbents are available commercially. IDA forms tridentates with metal ions whereas NTA forms tetradentates. This means that, for six coordination number metal ions, three and two orbitals, respectively, remain free to interact with side chains of residues. The choice of metal ion and chelator is often a function of the particular separation required and whether productivity or purity is desired. We therefore compare the dynamic capacity of the resin for denatured protein using Ni^{2+} and Cu^{2+} as the immobilised metal, and IDA or NTA as the chelating group.

The model protein in this study is the major capsid protein (L1) of human papillomavirus type 16 (HPV 16). HPV16 has been linked with the development of cervical cancer [18,19]. Recombinant L1 is expressed in *E. coli* as a fusion protein with a C-terminal 6x-histidine tag thereby facilitating its recovery by IMAC. The objectives of this work are to assess the use of chemical extraction to release insoluble L1 from the whole broth following fermentation, and to optimise subsequent IMAC-EBA purification. The use of Cu^{2+}- or Ni^{2+}-charged IDA- or NTA-Streamline for the binding of L1 is then compared using equilibrium binding and batch uptake kinetics. These data are then compared with breakthrough curves generated in expanded mode on the same adsorbents. The characteristics of the recovery of the unfolded protein from the chemical extraction mixture using fluidised adsorbent particles are central to the performance of the integrated extraction–recovery process.

2. Materials and methods

All experiments were performed at room temperature unless otherwise stated.

2.1. Fermentation

Recombinant *E. coli* strain HMS174(DE3) containing vector pET16-L1 expressing the L1 major coat protein from human papillomavirus 16 as an inclusion body [20] was kindly provided by the Imperial Cancer Research Fund (London, UK). The recombinant L1 protein was expressed in the form of an inclusion body using fermentation conditions described previously [21].

2.2. Chemical extraction and preparation of feedstock

2.2.1. Feedstock A: Extraction from cells resuspended in buffer

The cell suspension, following induction [21], was centrifuged at 8000 *g* and 4 °C for 15 min and then resuspended in HEPES–EDTA buffer [0.16 *M* 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), 4.89 m*M* EDTA, pH 8.7] to give a cell density of $A_{600\ nm}$=130. 61.5 ml of this cell suspension was then mixed with 48 g of urea and 1.2 g of spermine-4 HCl to give 100 ml of final extraction

mixture (0.1 *M* HEPES, 3 m*M* EDTA, 8 *M* urea, 34.5 m*M* spermine, pH 9.0 at $A_{600\ nm}$=80). Following the addition of urea and spermine, extractions were performed overnight (12 h) at 37 °C and 200 rpm. This extended extraction time was dictated by practical constraints and the observation that endothermic urea dissolution, rather than extraction, was rate limiting. 6 m*M* $CaCl_2$ was then added to the extraction mixture to chelate EDTA and the mixture incubated for a further 30 min. The DNA–spermine complex was then removed by low-speed centrifugation (5000 *g* for 15 min) and the pH of the supernatant adjusted to 8.0 immediately before column loading.

2.2.2. Feedstock B: Extraction from cells resuspended in fermentation supernatant

The freshly harvested cell pellets (prepared as above) were suspended using cell-free supernatant of fermentation broth (following centrifugation as above) to give a cell density of $A_{600\ nm}$=130. The powdered forms of HEPES, urea, EDTA and spermine-4 HCl were then added to 61.5 ml of cell suspension to mimic a large-scale in situ extraction. The final composition of the extraction mixture was therefore the same as above except for the (unidentified) additional components introduced by the fermentation supernatant. All subsequent procedures were the same as in the preparation of Feedstock A.

2.3. EBA operation

IMAC-EBA was conducted using a custom-designed 65 cm height×1.0 cm diameter glass column (Soham Scientific, Ely, UK). The adsorbent used was either: Streamline Chelating functionalised with IDA (Pharmacia, UK); or Streamline base matrix functionalised with NTA (donated by Qiagen, Germany). The details of the IMAC-EBA operation were essentially the same as described previously [5]. The specific differences were: (a) each adsorbent was charged with two settled volumes (SVs) of either 50 m*M* $NiSO_4$ or $CuCl_2$ in distilled water in packed bed mode to test the effect of different metal ions on L1 protein capture; (b) in the case of the Cu^{2+}–IDA combination, 5 m*M* imidazole was included in the wash buffer; (c) for the NTA based adsorbent, the column was first washed with 5 SVs of regeneration buffer as the adsorbent was supplied in a Ni^{2+} pre-charged form.

2.4. Measurement of adsorption isotherms and binding kinetics

The two metal ions, Ni^{2+} and Cu^{2+}, and two functional groups, IDA and NTA, were tested to investigate the L1 binding isotherm and kinetics.

2.4.1. Adsorbent preparation

The adsorbent (10 ml SV) was first stripped of any chelated metal ions by incubation with regeneration buffer (100 m*M* EDTA, 200 m*M* phosphate, pH 4.5) (2×10 ml). The resin was then: washed with distilled water (5×40 ml); charged with 100 m*M* $NiSO_4$ or $CuCl_2$ (10 ml); and finally, equilibrated with loading buffer (5×40 ml). The pH of the decanted supernatant was then measured at 8.0.

2.4.2. Batch adsorption isotherm

Extraction was conducted at $A_{600\ nm}$=80 as above. Following extraction, the pH of the extraction mixture was adjusted to 8.0 and diluted with IMAC-EBA loading buffer to give feedstock of the following cell densities ($A_{600\ nm}$=80, 72, 64, 56, 48, 40, 32, 24, 16, 8). 1.8 ml of feedstock at each cell density was then mixed with 0.2 ml of a 50% (v/v) resin slurry and incubated at 100 rpm for 12 h. The adsorbent was then removed by centrifugation (1000 *g*, for 5 min) and the collected supernatant assayed for residual L1 protein.

2.4.3. L1 binding kinetics

Twelve tubes of identical composition were prepared containing: 1.5 ml of fresh extraction mixture at $A_{600\ nm}$=80 (adjusted to pH 8.0 following extraction); and 0.15 ml of a 50% (v/v) slurry of adsorbent. These were then incubated as described above. Each tube was removed at a pre-determined time, immediately centrifuged (as above), and then assayed for L1 protein.

2.5. Analytical methods

All samples were clarified by centrifugation at 18 000 *g* for 15 min before measuring the con-

centration of total protein, DNA and recombinant L1 protein, unless otherwise mentioned.

2.5.1. Measurement of total protein and recombinant L1 protein

Total protein in each sample was measured using a Coomassie Plus Protein Assay Kit (Pierce, 23236T) using bovine serum albumin (BSA) as the standard. L1 protein concentration was determined using an enzyme-linked immunosorbent assay (ELISA) method based on the binding of the 6x-His fusion tag to an antibody conjugated with Ni^{2+} as well as horseradish peroxidase (India HisProbe Ab, 15165, Pierce). The details of each assay are described elsewhere [5].

2.5.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein samples were diluted threefold in gel loading buffer [2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 50% (w/v) glycerol, 62.5 m*M* Tris–HCl at pH 6.8, 0.01% (w/v) bromophenol blue] and then boiled for 3–5 min. A 5-μl volume of sample was loaded into each well of a pre-cast polyacrylamide gel (Bio-Rad, 161-1106) and electrophoresis conducted using a Bio-Rad Protean 3 Cell system at a voltage of 80 V. The gel running buffer used was 0.025 *M* Tris, 0.192 *M* glycine, 0.1% (w/v) SDS at pH 6.8. Protein bands were detected by Coomassie G-250 staining using GelCode Blue Stain reagent (Pierce, 24590).

3. Results and discussion

Feedstock composition can often limit the application of expanded-bed chromatography due to the interaction of cell surfaces, DNA and other substances with adsorbent particles, causing their aggregation and potentially leading to bed instability and channelling [22,23]. Despite these difficulties, EBA has found widespread application in the large-scale purification of proteins from mammalian and microbial feedstocks in industrial bioprocessing [24,25]. However, where intracellular proteins are the target, feedstocks are often prepared by high-pressure homogenisation prior to EBA capture and the physical and chemical properties of such a feedstock are very different from those produced by chemical extraction [4,21]. The latter contains high-molecular-mass DNA and 8 *M* urea, which make it a highly viscous feed and therefore likely to cause problems related to aggregation, clogging and fouling of the adsorbent. Nuclease treatment of the released DNA [26,27] cannot be used due to the presence of denaturant. Therefore, the highly negative charges of the phosphate backbone of the host DNA were neutralised by the multiple positive charges on spermine to form an insoluble DNA–spermine complex [28]. This selective precipitation of the DNA by spermine has been employed to render the chemical extraction mixture compatible with the subsequent expanded bed stage [5].

In addition to the increased viscosity, several of the chemicals employed in the chemical extraction process [4], specifically the Tris buffer and EDTA, interfere significantly with the binding of L1 protein to Ni^{2+}–IDA. The Tris buffer was therefore replaced with HEPES, and $CaCl_2$ was added to chelate the remaining EDTA without affecting the extraction efficiency of L1 protein [29]. The work presented here optimises ligand capture chemistry with respect to final L1 recovery and purity.

3.1. IMAC-EBA purification of L1 protein following chemical extraction

More than 60% of the applied L1 protein was recovered by Ni^{2+}–IDA EBA using Feedstock A (cells centrifuged and re-suspended in buffer before extraction, see Materials and methods), with a purification factor of approximately 10 (chromatogram not shown). Fed-batch culture is frequently employed for the large-scale production of recombinant protein at high-cell density. Following fermentation, direct addition of powdered extraction chemicals to the cell suspension would be far simpler than centrifugation and resuspension. This direct extraction using fermentation broth is termed in situ extraction. Our unoptimised laboratory-scale fermentation typically yielded low cell concentrations ($A_{600\ nm}$ of 22–23), which were considered to present too little of a challenge for in situ extraction, especially as the efficiency of L1 extraction is relatively insensitive to cell concentration [21]. We therefore chose, in this study, to concentrate the fermentation broth by

centrifugation and resuspension in supernatant. This gave a simulated fermentation suspension at a high cell concentration, close to the maximum typically expected from fed-batch fermentation ($A_{600\ nm}$ of 130, Feedstock B, Materials and methods). Application of this feedstock to the IMAC-EBA column, following chemical extraction, gave the chromatogram shown in Fig. 1. The recovery of L1 (58%), degree of purification (9.7) and purity (37%) were virtually the same as those using Feedstock A, confirming that removal of fermentation media prior to extraction is unnecessary. The protein composition at each stage of the purification using Feedstock B was shown by SDS–PAGE (Fig. 2) to be virtually indistinguishable from that for Feedstock A (data not shown), further confirming that cell washing is not necessary. In both cases, however, there is considerable scope to improve product purity and recovery.

3.2. L1 binding to different combinations of metal ion and chelating group

The model protein L1 is tagged with six histidines at its C-terminus [20], but, unlike other His-tagged proteins, L1 was found in preliminary studies to have very weak binding to Ni^{2+}–NTA. This prevented the use of imidazole or a down-shift in pH for the selective removal of contaminating proteins during the expanded bed wash stage. Of the rules established in IMAC, copper is known to exhibit the strongest binding to histidine among borderline metal ions [15]. However, there are several instances where, for specific proteins, the usual rules cannot be applied. It is also probable that the functional groups employed to chelate the metal ion may play a role in the overall binding of L1 protein.

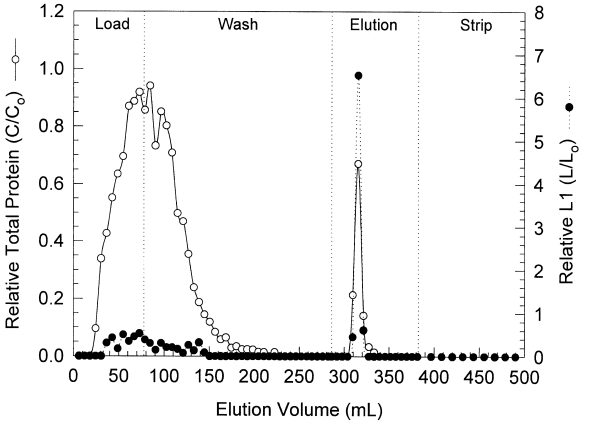


Fig. 1. Chromatogram for the IMAC-EBA purification (Ni^{2+}–IDA) of L1 protein using Feedstock B (see Materials and methods).

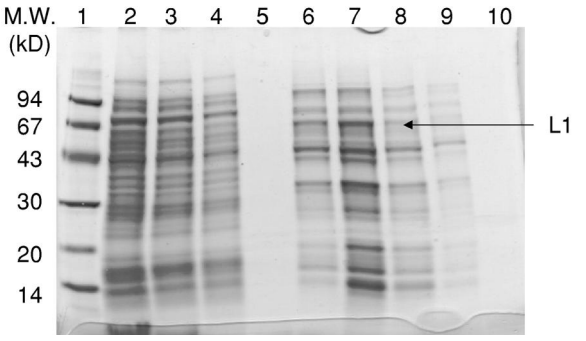


Fig. 2. SDS–PAGE of selected fractions from the IMAC-EBA experiment plotted in Fig. 1. Lanes: 1 = molecular mass marker; 2 = feedstock; 3 = flow-through (61.0 ml); 4 = wash (121.1 ml); 5 = wash (240.3 ml); 6 = elution (309.5 ml); 7 = elution (315.6 ml); 8 = elution (321.5 ml); 9 = elution (327.4 ml); 10 = stripping (443.7 ml).

To improve the recovery yield and purity of L1 protein during IMAC-EBA, we investigated the binding characteristics of L1 protein to different combinations of metal ions, Ni^{2+} and Cu^{2+}, and functional groups, IDA and NTA. The batch adsorption isotherms obtained using different combinations of metal ion-functional group are shown in Fig. 3. The Langmuir isotherm expressed as:

$$q = \frac{Q_m c}{K_d + c} \tag{1}$$

was fitted to the data, where c (mg/l) and q (mg L1 protein/ml adsorbent) denote the L1 concentration in bulk solution and adsorbed at equilibrium, respectively. Q_m is the maximum binding capacity (mg L1 protein/ml adsorbent) and K_d the dissociation constant (mg/ml). Copper showed the highest maximum binding capacity and tightest association with L1 protein, regardless of functional group (see Table 1). In the case of IDA, copper showed almost 10-fold tighter binding to L1 than nickel. The Cu^{2+}–IDA combination also showed higher (approximately 20%) maximum binding capacity than Cu^{2+}–NTA,

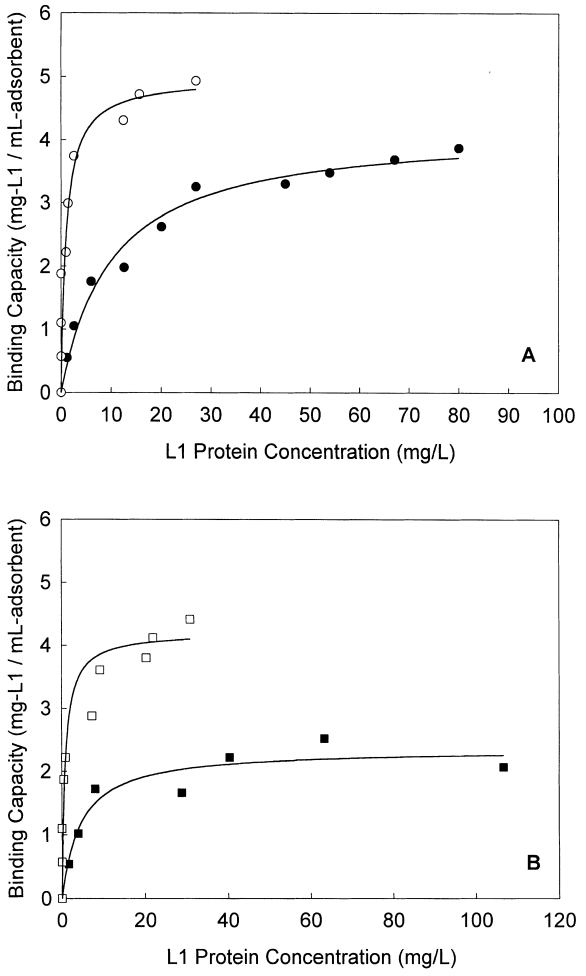


Fig. 3. Batch adsorption isotherm for L1 protein to different binding partners. L1 protein binding to IDA (A): Ni^{2+}–IDA (●) and Cu^{2+}–IDA (○). L1 protein binding to NTA (B): Ni^{2+}–NTA (●) and Cu^{2+}–NTA (○). The solids lines were fitted using the Langmuir model (Eq. (1)).

while exhibiting almost the same binding strength for L1 protein.

The binding kinetics of L1 protein to different metal ion-functional group combinations was also investigated using crude extraction mixture (see Fig. 4 and Table 2). The data were fitted to first-order kinetics expressed as:

$$q = Q_m \, e^{-t/\lambda} \quad (2)$$

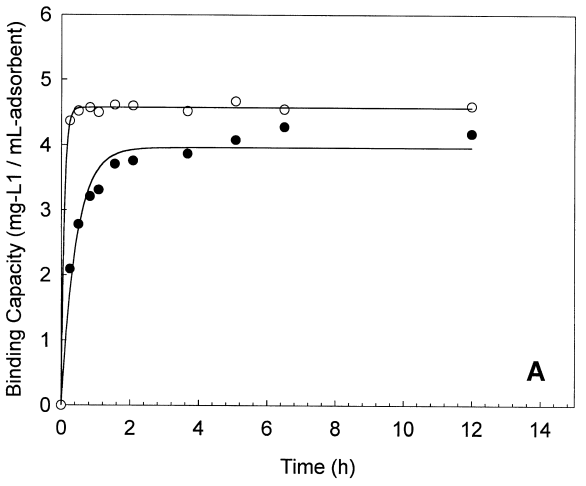
where t is the incubation time (h), Q_m the equilibrium binding capacity (mg L1 protein/ml adsorbent), and λ the time constant (h), respectively. First-order kinetics were selected in order to make qualitative comparisons between the lumped forward time constants rather than perform a more complex analysis yielding little further insight into the gross capture process. The estimated maximum binding capacities for each combination were in good agreement with those from the batch adsorption study. For IDA, L1 protein showed approximately five times faster binding to Cu^{2+} than to Ni^{2+} as was shown by the difference in the estimated time constant (see Table 2). The binding kinetics of L1 to Cu^{2+}–IDA and Cu^{2+}–NTA were quite similar, exhibiting almost equal time constants. When Ni^{2+} was used as a ligand, L1 showed approximately twofold faster binding to Ni^{2+}–NTA than to Ni^{2+}–IDA while the maximum binding capacity of Ni^{2+}–NTA was much lower than that of Ni^{2+}–IDA.

The binding studies for L1 protein to Ni^{2+} show the importance of selection of the right combination of ligand and functional group. For instance, where maximum binding capacity is required, Ni^{2+} should be coupled with IDA. However, when binding strength rather than capacity is important, as is the

Table 1
Binding parameters of L1 protein to different binding partners comprising metal ions and functional groups as indicated

Binding parameter	Binding partners			
	Ni^{2+}–IDA	Cu^{2+}–IDA	Ni^{2+}–NTA	Cu^{2+}–NTA
Q_m (mg L1 protein/ml adsorbent)	4.19	4.98	2.35	4.21
K_d (mg/ml)	10.09	1.04	4.53	0.84

The Langmuir adsorption isotherm (Eq. (1)) was used for parameter estimation.



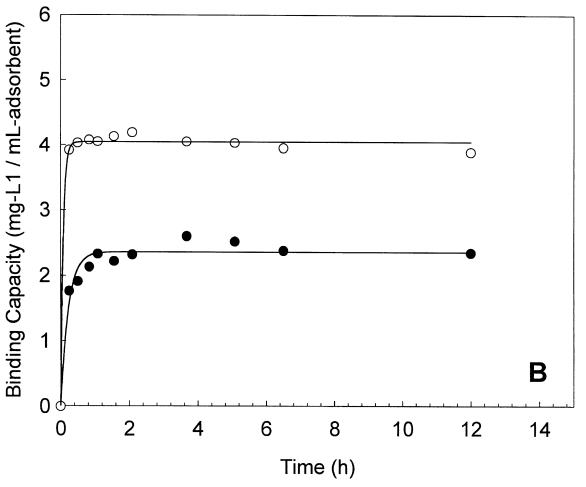


Fig. 4. Kinetics of L1 protein binding to different binding partners. L1 protein binding to IDA (A): Ni^{2+}–IDA (●) and Cu^{2+}–IDA (○). L1 protein binding to NTA (B): Ni^{2+}–NTA (l) and Cu^{2+}–NTA (○). The solid lines were fitted using first-order kinetics (Eq. (2)).

case when maximising the purity of target protein by employing a harsh washing condition, it is advantageous to couple Ni^{2+} to NTA. During expanded bed adsorption, the height of the bed is directly related to feed flow-rate and these determine the contact time of a target protein with adsorbent. Usually 2–3-fold bed expansion is employed [30], restricting the adjustable range of feed flow-rate available to control the contact time of the protein with the ligand. Therefore the kinetics of protein binding to the chosen ligand are critical to the effective retention of target protein during sample loading. In this situation, the Ni^{2+}–NTA combination would be the better choice for the effective capture of L1 protein as it shows faster binding to L1

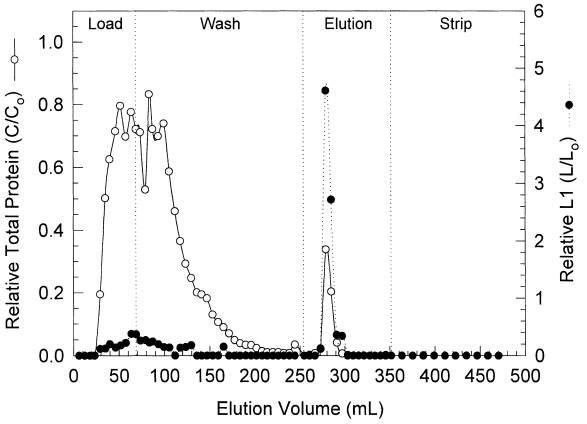


Fig. 5. Chromatogram for the IMAC-EBA purification (Ni^{2+}–NTA) of L1 protein using extraction mixture as a feedstock. Extraction was conducted at $A_{600\ nm}=80$ using 100 m*M* HEPES, 8 *M* urea, 3 m*M* EDTA, 34.5 m*M* spermine-4 HCl at pH 9.0. Following the spiking of 6 m*M* $CaCl_2$ into the post-extraction mixture, the DNA–spermine complex was removed by low-speed centrifugation (5000 *g* for 15 min) prior to column loading.

Table 2
Parameters estimated from the study of binding kinetics of L1 protein to different binding partners comprising metal ion and functional group as indicated

Binding parameter	Binding partners			
	Ni^{2+}–IDA	Cu^{2+}–IDA	Ni^{2+}–NTA	Cu^{2+}–NTA
Q_m (mg L1 protein/ml adsorbent)	3.96	4.57	2.36	4.06
Time constant, λ (h)	0.43	0.08	0.22	0.07

Parameters are defined by Eq. (2).

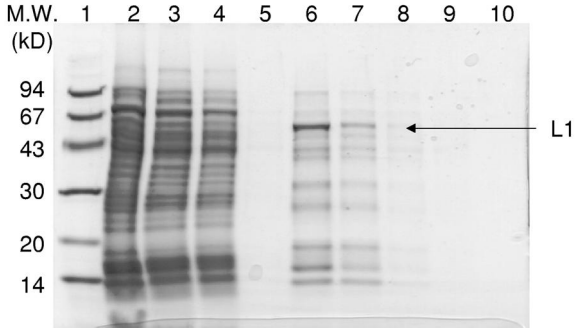


Fig. 6. SDS–PAGE of selected fractions from the IMAC-EBA experiment reported in Fig. 5. Lanes: 1 = molecular mass marker; 2 = feedstock; 3 = flow-through (57.4 ml, collected after 57.4 ml of cumulative effluent volume); 4 = wash (111.7 ml); 5 = wash (232.2 ml); 6 = elution (279.1 ml); 7 = elution (285.0 ml); 8 = elution (291.0 ml); 9 = elution (297.2 ml); 10 = stripping (422.8 ml).

protein than Ni^{2+}–IDA (Table 2 and Fig. 5). Alternative combinations of ligand and functional group would cause undesirable leakage of target protein due to binding kinetics rather than binding capacity limitation, which cannot be rectified easily due to the restricted range of operating flow-rates in EBA.

NTA is often described as being more selective than IDA for the capture of histidine tagged proteins and here we can compare purifications using Ni^{2+}–IDA (Figs. 1 and 2) and Ni^{2+}–NTA (Figs. 5 and 6). The batch adsorption and binding kinetics results reveal that Ni^{2+}–IDA has a much higher binding capacity for L1, while binding kinetics are slower and binding strength weaker. However, the recovery yields for L1 in each case using IMAC-EBA were almost the same (66% for Ni^{2+}–IDA and 71% for Ni^{2+}–NTA) while protein compositions (as shown by SDS–PAGE) and purity of L1 (47% for Ni^{2+}–IDA and 62% for Ni^{2+}–NTA) in the eluted fractions were different. The working capacities (L1 protein recovered in the elution divided by adsorbent volume) [31] were calculated as 1.89 and 2.08 mg L1/ml for Ni^{2+}–IDA and Ni^{2+}–NTA, respectively. Comparison with data in Table 1 indicates that available binding sites in the Ni^{2+}–IDA combination are massively under-utilised due to its slow and weak binding with L1. Conversely, nearly 90% of maximum binding capacity assessed by batch binding study (see Table 1) is used in the Ni^{2+}–NTA combination. The Ni^{2+}–NTA combination also gave higher purity of L1 in the eluted fractions, possibly due to its tighter association with L1 protein than with contaminating proteins. However, despite these binding advantages, the density distribution of the NTA adsorbent was unfavourable for our feedstock as the bed front was severely dispersed and continuous overflow of the fine adsorbent particles was monitored during the sample loading, resulting in approximately 10% loss of adsorbent. This continuous elutriation did not occur when the IDA based adsorbent was used. This observation re-emphasizes the importance of selecting the best combination of ligand and functional group for a given protein, and also the necessity for practical testing of the chosen candidate in an operational configuration.

3.3. Application in EBA

The batch adsorption and kinetics studies for L1 protein suggested that either Cu^{2+}–IDA or Cu^{2+}–NTA would be most effective for the capture of L1 during IMAC-EBA. Of these, we selected Cu^{2+}–IDA for the following reasons. Firstly, it has the highest binding capacity for L1 whilst demonstrating virtually the same binding kinetics and similar dissociation constant (see Tables 1 and 2). Secondly, the density distribution of NTA-based adsorbent is unfavourable for our feedstock as discussed above.

The chromatogram in Fig. 7 summarises the IMAC-EBA purification using Cu^{2+}–IDA. The feedstock used was produced by chemical extraction

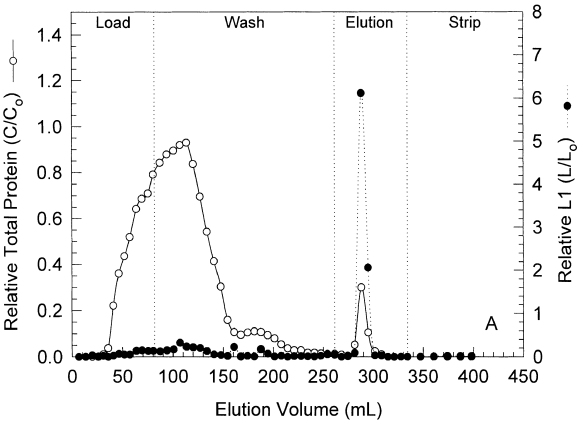


Fig. 7. Chromatogram for the IMAC-EBA purification (Cu^{2+}–IDA) of L1 protein using extraction mixture containing fermentation supernatant as a feedstock (see Materials and methods).

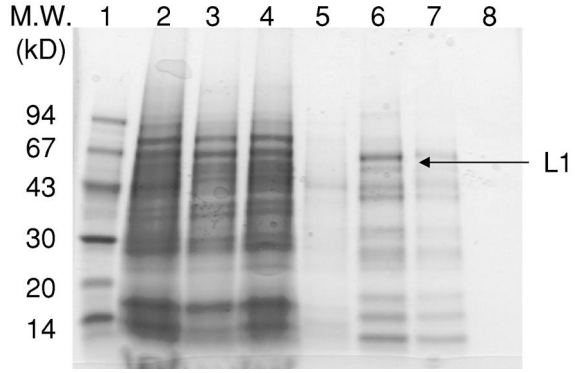


Fig. 8. SDS–PAGE of selected fractions from the IMAC-EBA experiment reported in Fig. 7. Lanes: 1=molecular mass marker; 2=feedstock; 3=flow-through (63.7 ml, collected after 63.7 ml of cumulative effluent volume); 4=wash (120.2 ml); 5=wash (187.7 ml); 6=elution (288.3 ml); 7=elution (295.2 ml); 8=stripping (374.2 ml).

from concentrated fermentation broth ($A_{600\ nm}$= 130). Unlike the previous run using Ni^{2+} as the ligand, 5 m*M* imidazole was included in the washing step to enhance the removal of contaminating proteins. L1 protein loss during the loading and washing periods was smaller than in previous cases (shown in Fig. 1), resulting in higher recovery yield of 71% and a purification factor of 20. The relatively low leakage of L1 is due to the faster and tighter binding to Cu^{2+} than to Ni^{2+}. The shoulder observed in the total protein data at the end of the washing period (elution volume between 160 and 200 ml) was mainly due to the elution of contaminating proteins as no significant L1 protein was detected in this region. Imidazole selectively removed the contaminating proteins. The purity of eluted L1 measured by ELISA was 89%. The SDS–PAGE (Fig. 8) did not reflect this improvement as L1 exists as a mixture of full-length L1 (approximately at M_r 60 000) and smaller L1 fragments (approximately at M_r 43 000, 32 000 and 14 000). These smaller fragments were found to be reactive towards anti-His antibody and were present from the beginning of L1 protein expression after isopropyl β-D-thio-galactopyranoside (IPTG) induction, but not in uninduced cells (data not shown). Either N-terminal proteolytic degradation of full-length L1 [20], or the presence of internal start codons in the open reading frame of HPV 16 L1 [32], is believed to be responsible for these fragments.

An overall summary of the IMAC-EBA experiments performed is presented in Table 3. Overall, the copper and IDA combination was found to be the most effective for L1 capture, resulting in better recovery yield, purification factor and purity. This confirms the results of the batch binding studies.

4. Conclusions

The integration of chemical extraction and IMAC-

Table 3
Summary of IMAC-EBA experiments conducted in this study

	Experiment number according to the order of appearance in the text			
	Run 1	Run 2	Run 3	Run 4
Related chromatogram	Not shown	Fig. 1	Fig. 5	Fig. 7
Binding partner	Ni^{2+}–IDA	Ni^{2+}–IDA	Ni^{2+}–NTA	Cu^{2+}–IDA
Feedstock				
Origin[a]	Feedstock A	Feedstock B	Feedstock A	Feedstock B
Volume (ml)	65.5	78.8	68.8	80.4
Total protein (mg/ml)	14.8	15.4	14.7	16.7
L1 protein (mg/ml)	0.691	0.591	0.673	0.729
Purification efficiency				
Working capacity [31] (mg L1/ml adsorbent)	1.89	1.71	2.08	2.64
Recovery of L1 protein (%)	65.6	57.7	70.5	70.5
Purity of L1 (%)	46.5	37.4	62.4	88.9
Purification factor	9.9	9.7	13.6	20.4

[a] See Materials and methods for details of Feedstock A and Feedstock B.

EBA has significant potential for improving process economics in the recovery of inclusion body protein. Although the major problems associated with the direct coupling of these two techniques have been identified and solved previously [5], some aspects required further optimisation.

Firstly, the large-scale applicability of the new technique employing direct addition of powdered chemicals to fermentation broth was evaluated. The feedstock simulating the in situ extraction (Feedstock B) was found to give almost the same purification performance as was achieved using extraction buffer (Feedstock A), indicating that the developed technique could be integrated directly with the fermentation step without affecting process efficiency. Secondly, the causes of the gradual breakthrough of L1 during loading and low purity during elution in the IMAC-EBA were investigated. Batch adsorption isotherm and kinetic binding studies for L1 to four different combinations of metal ion and functional groups (i.e., Ni^{2+}–IDA, Cu^{2+}–IDA, Ni^{2+}–NTA, Cu^{2+}–NTA) revealed that L1 has very different binding behaviour depending on the chelating group and metal ion. Furthermore, this difference was significant enough to control the purification performance. It was shown that binding kinetics rather than binding capacity are critical to the efficiency of the IMAC-EBA purification of L1. Cu^{2+}–IDA was found to be the best binding partner for L1 using batch binding studies. This was confirmed to be the case in IMAC-EBA, resulting in improved L1 recovery yield (71%), purification factor (20) and purity (89%).

In this paper, a radically new process re-defining the traditional inclusion body recovery process is presented and optimised. The simple combination of chemical extraction and IMAC-EBA eliminates several unit operations in the conventional flowsheet, thereby achieving process simplification and intensification. Although the process was demonstrated using the L1 protein from the HPV 16 viral capsid, the developed process is generic, simple and scalable, and deserves to find more widespread application.

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