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Continuous processing of fusion protein expressed as an *Escherichia coli* inclusion body

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Abstract

In this study we develop the components of an integrated process for the continuous extraction and purification of a histidine-tagged fusion protein expressed as an inclusion body in *Escherichia coli*. Lac21 was selected as a model peptide and was expressed as a fusion to ketosteroid isomerase. A purification strategy was developed on a 1-ml batch column before successful scale-up and transfer to a continuous purification system, having a bed volume of 240 ml. Preliminary experiments proved cleavage of the fusion protein. The use of chemical extraction and continuous chromatography gives a flowsheet far superior to the traditional methods for inclusion body processing.
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Keywords: *Escherichia coli*; Fusion protein

1. Introduction

A growing interest in peptides has developed as a result of favourable research into their function and properties. A small number of peptides are commercially available, predominantly serving roles in the biopharmaceuticals market as, for example, hormones and antibiotics. The self-assembling properties of peptides are intriguing, and this has the potential to bring about their use in fields such as tissue engineering and the development of biocompatible implants. In 1999 the global peptide pharmaceutical market was estimated at US$350 million, with 15% growth annually up to 2005 [1]. Traditionally, peptide production is by means of chemical synthesis using techniques based on that devised by Merrifield [2]. This methodology has major drawbacks in that it is expensive, costing US$50 to US$42 000 per mol of a single, protected amino acid residue [3], plus only relatively small quantities can be produced per synthesis. To capitalise on this new, rapidly growing market, technology must be developed that both satisfies the increasing demand and provides product at low cost.

So-called ''genetic modification'' of cells has been routinely used for protein-production since the 1980s, predominantly using the bacterium *Escherichia coli* [4,5]. This technology can be extended to peptide expression to dramatically decrease costs compared with those from chemical synthesis. By definition, peptides are small, short sequences, which are either poorly expressed in *E. coli*, or are rapidly degraded. It is for this reason that they are produced as part of a much larger fusion protein, with the target peptide only released during downstream processing after cleavage at a strategically located position in the amino acid sequence. The fusion partner can also be engineered to facilitate purifica-

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tion [6], detection of the fusion protein from a complex mixture [6], or to avoid degradation by endogenous host proteases [5,7]. Inclusion bodies are commonly produced when recombinant protein is over-expressed in *E. coli*. These dense, refractile particles comprise mainly inactive recombinant protein, with other bacterial contaminants. Denaturation is a lesser issue when the end product is peptide rather than protein since complex tertiary structure formation generally cannot occur in these short sequences.

Efficient and cost-effective downstream processing techniques are essential for handling the large volumes produced by fermentation. The traditional route for recombinant protein recovery involves a large number of costly unit operations. Cells are disrupted by high-pressure homogenisation and the insoluble inclusion bodies are collected by centrifugation. Inclusion bodies are then solubilised using strong denaturant prior to refolding. Multiple cell disruption passes are required to decrease cell debris size [8]. Elimination of cell disruption and inclusion body centrifugation has recently been achieved by Choe and co-workers [9,10] based on a novel chemical extraction technique originally developed by Falconer et al. [11–13]. The technique solubilises cytoplasmic inclusion bodies by exploiting the synergistic effects of chaotrope (urea or guanidine hydrochloride) and EDTA, directly on intact cells suspended in fermentation media at high cell density.

Subsequent purification is achieved by batch column, ion-exchange, hydrophobic, covalent, or metal-chelate chromatography [14]. The nature of the purification is often intimately linked with the selected fusion partner. Batch column processing is not ideal for numerous reasons; the volume that can be processed per run is limited by the capacity of the column used, and the vessel must be cleaned after each use leading to dead-time when the column is not being used for its designed purpose. At process scale these points lead to an increase in production cost which may render the production route uneconomic, especially for commodity products. A move to continuous processing is thus desirable; however, limited technological advancements in this area have discouraged this practice. One notable limitation to continuous protein purification is obtaining sufficient protein-containing cells. Since most fermentation is conducted in batch, numerous fermentations in parallel may be required to enable purification to be conducted in a truly continuous manner.

Annular chromatography is a continuous process that has been known for over 40 years [15–17]. This technique has only recently been extended to bioseparations [18–20], and the only commercial unit available employing annular chromatography is the Preparative Continuous Annular Chromatograph (P-CAC) (Prior Technologies, Götzis, Austria). The equipment is based upon two rotating cylinders, with the resultant annulus filled with chromatography medium (Fig. 1). A stationary distribution head is located at the top of the column, which has eight inlets, spaced 45° apart for the introduction of different mobile phases to the column. Ninety exit ports spaced 4° apart are found at the bottom of the column for fraction collection. As the components move downwards through the chromatography medium the bed is continuously rotating at a constant speed, and this leads to two-dimensional separation. The flow-rate of the sample onto the column, Q and the length of the column, L determine the retention time of the sample components. The rotation speed, Ω, only governs the angle of elution. Typically the P-CAC is run between 60 and 600° per hour, with higher rotational speeds used during column packing [21].

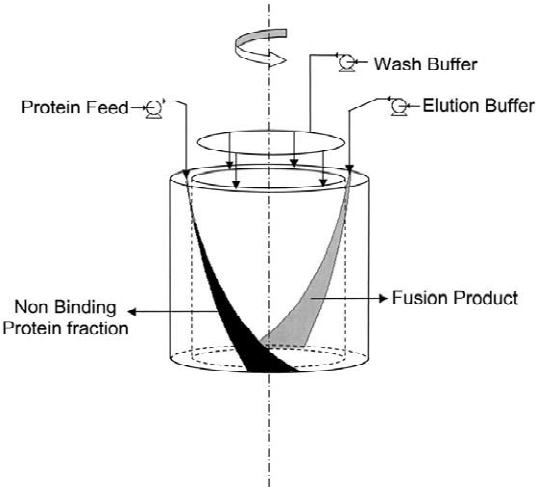


Fig. 1. Schematic representation of fusion protein purification using continuous annular chromatography.

In this paper we present a study of continuous peptide production and purification using recombinant DNA technology, combined with novel chemical extraction and fusion protein purification using the P-CAC. The peptide Lac21 was chosen as the model peptide for this study. This 21-amino acid peptide forms α-helices and its sequence based upon that of the C-terminal domain from the Lac repressor [22]. Lac21 peptide can form four-chain coiled-coils, and in bulk solution is in dynamic equilibrium between monomer and tetramer states [22]. Lac21 is particularly active at oil–water interfaces and causes an interfacial pressure greater than most proteins once adsorbed [23]. The peptide is expressed as a fusion to ketosteroid isomerase (KSI) and a 6× histidine tag, engineered-in to facilitate immunological detection and purification by immobilised metal affinity chromatography (IMAC). Novel chemical extraction is employed to release the cellular contents with simultaneous inclusion body solubilisation, eliminating the requirements for conventional cell disruption and centrifugation. After preliminary batch column validation, IMAC is conducted using the P-CAC and its successful use demonstrates that processing can potentially be carried out in a continuous mode, leading to a reduction in the costs associated with industrial protein and peptide production. Preliminary batch column studies of fusion-protein cleavage using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) plus ammonia are presented, confirming that the peptide can be cleaved from the fusion partner following IMAC purification.

2. Experimental

2.1. General

Oligonucleotides were purchased from The Protein and Nucleic Acid Facility, University of Cambridge (Cambridge, UK). DNA sequencing to verify the plasmid construct was done by The DNA Sequencing Facility, University of Cambridge (Cambridge, UK). All DNA restriction and modifying enzymes were purchased from New England Biolabs (Hitchin, UK). All work was conducted at room temperature unless noted otherwise.

2.2. Bacterial strains and plasmid

Escherichia coli strain DH5α (Invitrogen, Paisley, UK) was used as the host for initial subcloning work. Protein expression was conducted with *E. coli* strain BLR(DE3)pLysS (Novagen, Madison, WI, USA). Plasmid pET-31(b) (Novagen) was used as the expression vector.

2.3. Construction of recombinant plasmid

pET-31(b) was digested with *Alw*NI to linearise the plasmid. A site susceptible to 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) cleavage following translation, and a DNA recognition site for *Bsp*MI, were then introduced into the plasmid. This was done by annealing two complementary oligonucleotides with sticky ends compatible with those generated by *Alw*NI digestion and ligating this with the linearised plasmid. DNA encoding Lac21 was introduced by digestion of the newly modified vector with *Bsp*MI, and ligating this with a further two annealed oligonucleotides encoding the peptide (carrying *Bsp*MI compatible sticky ends). The DNA sequence of the modified plasmid was verified by sequencing.

2.4. Recombinant protein expression

Following initial 200-ml shake flask trials, fermentation was conducted using a 5-l fermentor (BioFlo 3000, New Brunswick Scientific, Edison, UK) with a 4-l working volume. Terrific broth (Sigma, Poole, UK) supplemented with 10 g/l glycerol (Sigma) and 0.3 ml/l polypropylene glycol (PPG) (BDH, Lutterworth, UK) was used as the growth medium. Immediately prior to inoculation, filter (Millex-GS 0.22 μm, Millipore, Bedford, MA, USA) sterilised carbenicillin (Sigma) was added to a final concentration of 50 μg/ml, and chloramphenicol (Sigma) to a final concentration of 30 μg/ml. The fermentor was operated at 37 °C without pH control (pH varied from 7.1 to 6.0). Foaming was controlled by automated addition of PPG as required. Induction of protein expression was at OD_{600} 7–8 by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Melford Laboratories, Ipswich, UK) to a final concentration of 1 m*M*. The period of induction was

4 h and bacterial cells were stored as pellets at −20 °C following centrifugation at 8000 *g* for 15 min.

2.5. Chemical extraction

Chemical extraction buffer comprised 8 *M* urea (Fisher Scientific, Loughborough, UK), 0.1 *M* Hepes (Fisher), 3 m*M* EDTA (Sigma) and 12 g/l spermine–4HCl (Fluka, Gillingham, UK), at a final equivalent cell OD_{600} of 80. The protein content of the supernatant was determined via a Bradford assay using Coomassie Plus Protein Assay Reagent (Perbio Science UK, UK), following centrifugation (8000 *g* for 15 min). This supernatant was stored at 4 °C until required. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with Coomassie staining was conducted using the mini Protean 3 electrophoresis system (Bio-Rad Labs., Hemel Hempstead, UK) to assess protein production and the success of chemical extraction. Tris–HCl and Tris–tricine Ready Gels (Bio-Rad Labs.) were used as per the manufacturer's protocols, and run at 100 and 200 V, respectively. To prepare the chemical extraction mixture for IMAC purification, the composition was adjusted to 10 m*M* imidazole (Acros, Loughborough, UK) 8 *M* urea, 50 m*M* Hepes (Fisher). To remove the EDTA from the extraction mixture, $CaCl_2$ (Acros) was added to a concentration of 6 m*M*.

2.6. Batch column chromatography

A 1-ml HiTrap affinity column associated with a FPLC system (LCC500 Plus, Pharmacia, with two P500 pumps (Amersham Biosciences, Chalfont, UK) was charged with two column volumes (CV) of 200 m*M* $NiSO_4 \cdot 6H_2O$ (Sigma), followed by washing with 5 CV of distilled water. Flushing with 7.2 CV of washing buffer (15, 30 or 45 m*M* imidazole, 8 *M* urea, 50 m*M* Hepes) equilibrated the column for loading of the protein feed (1.5 CV of 10 m*M* imidazole, 8 *M* urea, 50 m*M* Hepes, 4.5 mg/ml protein). A total of 7.2 CV of washing buffer, followed by 4.8 CV of elution buffer (150 m*M* imidazole, 8 *M* urea, 50 m*M* Hepes) were pumped through the column. Successively, 1-ml fractions were collected and Bradford assays conducted using Coomassie Plus Protein Assay Reagent to determine protein concentrations. Further protein analysis was done using SDS–PAGE as described above.

2.7. Continuous chromatography: P-CAC

The annulus of a P-CAC system (outer radius 7.5 cm, inner radius 6.5 cm) (Prior Separation Technologies, Götzis, Austria) was filled with 240 ml Sepharose Fast Flow (Amersham Biosciences) to give a bed height of 5.5 cm. A layer of glass beads (Prior Separation Technologies) was applied directly above the bed such that inlet ports were immersed. The system was packed by pumping distilled water through it at a flow-rate of 95 ml/min for 20 min using a Kronlab VP120 pump (Sinsheim, Germany), at a system rotational speed of 10 rpm. The resin was charged for 1 h by pumping 1.3 CV of 200 m*M* $NiSO_4 \cdot 6H_2O$ through the bed at a flow-rate of 5 ml/min, and 15 CV of distilled water at 60 ml/min. The rotational speed was 3 rph (1080°/h) during this procedure. The resin was washed with a further 10 CV of distilled water applied at 60 ml/min, with rotation of 3 rph. Equilibration was conducted over 15 min at a rotational speed of 1 rph. Washing buffer (45 m*M* imidazole, 8 *M* urea, 50 m*M* Hepes) was pumped through the main inlet into the headspace at a flow-rate of 30 ml/min. Dummy feed (10 m*M* imidazole, 8 *M* urea, 50 m*M* Hepes) was applied through a separate inlet at 3.2 ml/min, and at 180° to this elution buffer (150 m*M* imidazole, 8 *M* urea, 50 m*M* Hepes) was introduced at 10 ml/min. For IMAC purification, the dummy feed was substituted for real feed, with all other conditions remaining identical. Fractions were collected from each of the 90 exit ports at 1, 1.5 and 2 h after feed commencement. Protein concentrations were determined by Bradford assays using Coomassie Plus Protein Assay Reagent. Samples with significant protein content were further analysed by SDS–PAGE as detailed in Section 2.5.

2.8. Chemical cleavage

The pH of the eluted protein solution resulting from batch column purification was adjusted to 3 with concentrated HCl (Sigma). 100× molar excess of Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) buffer (0.1 *M* TCEP (CN Biosciences, UK), 8 *M* urea, 50 m*M* Hepes, pH 3) was added to 8.5

nmol of KSI::Lac21 fusion protein, based on the number of cysteine residues in the fusion protein. The reduction reaction was allowed to proceed for 30 min, at which time 1000× molar excess of 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) buffer (0.1 *M* CDAP, 8 *M* urea, 50 m*M* Hepes, pH 3) was added to cyanylate the sulfhydryl groups. After 15 min, ammonia was added to a final concentration of 1 *M* and this final cleavage stage was allowed to proceed for 3 h. The cleavage product was precipitated by adding an equal volume of trichloroacetic acid (Sigma) and incubating on ice for 30 min. This was then centrifuged at 14 000 *g* for 15 min. The supernatant was discarded and the pellet washed with 500 μl absolute ethanol (Fisher). Finally the pellet was resuspended in 100 μl elution buffer.

2.9. Western blotting

The pET-31(b) plasmid incorporates a coding sequence for a 6× histidine tag that can be used for detection as well as purification. Proteins were separated by SDS–PAGE, and transferred to a nitrocellulose membrane using the Mini-Trans-Blot Electrophoretic Transfer cell (Bio-Rad Labs.). Detection was conducted using INDIA HisProbe-HRP and CN/DAB Substrate Kit (both Perbio Science UK).

3. Results and discussion

The modifications made to pET-31(b) allow multiple copies of DNA encoding a peptide to be inserted into the vector while retaining potential for cleavage using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) and ammonia. This gives a simple strategy for peptide release following purification. Lac21 was selected as the model peptide for this study since its sequence lacks cysteine, and it was expected to be expressed well as it derives from the naturally occurring Lac repressor protein [22,24]. In this study only a single copy of the DNA encoding Lac21 was inserted into the plasmid.

3.1. Expression

Initial tests of protein expression were conducted in 200-ml shake-flask cultures. Chemical extraction was used to release proteins for analysis. A comparison of protein expression in induced and non-induced cells harbouring the unmodified ketosteroid isomerase (KSI) plasmid is shown in Fig. 2a, lanes 1 and 2. Histidine-tagged KSI has a molecular mass of approximately 15.8 kDa, and the absence of a band of this position for non-induced cells indicates strict repression prior to induction. A distinct band corresponding to this weight is shown in lane 2, indicating successful expression of KSI. Fermentation, including IPTG induction, was conducted using bacteria transformed with the modified plasmid engineered for KSI::Lac21 expression. Crude, unpurified bacterial protein following total chemical extraction, as described in Section 2, is shown in lane 3. A dominant band is apparent at approximately 18 kDa, which is 2.5 kDa (the molecular mass of Lac21) greater than KSI.

An antibody to Lac21 is not available, and unique

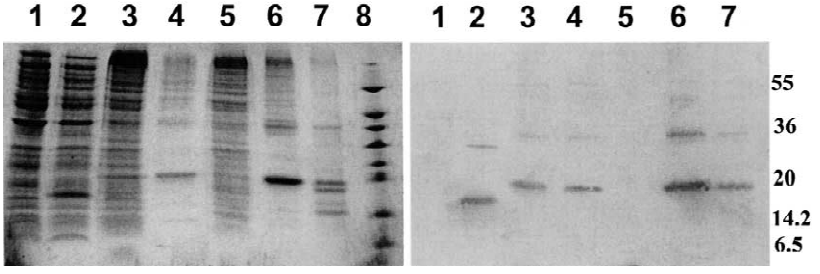


Fig. 2. (a) SDS–PAGE analysis of samples. Lanes: (1) unpurified non-induced unmodified (KSI) plasmid; (2) unpurified, induced unmodified (KSI) plasmid; (3) unpurified, induced KSI::Lac21; (4) P-CAC purified KSI::Lac21; (5) Flow-through after P-CAC washing; (6) purified KSI::Lac21 used for cleavage; (7) fragments produced by CDAP plus ammonia cleavage; (8) molecular mass marker. (b) Western blot of (a) for detection of 6× histidine sequence. Number on right hand side corresponds to molecular mass (10^3).

identification of Lac21 by Western blot analysis was not possible. However, the fusion-protein incorporates a 6× histidine tag C-terminal to Lac21, to which several detection systems are commercially available. Western blotting was conducted on an identically loaded gel run in parallel with that shown in Fig. 2a, the results of which are shown in Fig. 2b. No histidine tag is detected in lane 1, which confirms tight repression. Product bands are identified as expected based on molecular mass, but a low-intensity band of double the molecular mass is also apparent. This is thought to be a product dimer.

3.2. Batch column chromatography

The strategy employed for purification was immobilised metal affinity chromatography (IMAC), exploiting the affinity of the 6× histidine tag for nickel. Initial purification was conducted using a 1-ml column to quickly establish a valid purification protocol for subsequent transfer onto the continuous P-CAC system. Three washing conditions were examined to reduce non-specific contaminant binding: 15, 30 and 45 m*M* imidazole. Fractions were collected throughout purification and protein content assessed by Bradford assay (Fig. 3). A large and broad protein peak appears, for all three batches, when washing is commenced. This was attributed to contaminating protein having low affinity for the chelating resin. Elution was conducted with 150 m*M* imidazole, giving a distinct product peak. Peak area decreases with increasing imidazole concentration in the wash buffer. SDS–PAGE confirms the product following washing with 15 m*M* is heavily contaminated with bacterial protein (Fig. 4, lane 2). Clearly, there is a trade off between product purity and product recovery, as is often the case. It was decided that 45 m*M* imidazole gave the best results, as a pure product was required for subsequent cleavage. This was the condition selected for transfer to the P-CAC preparative system.

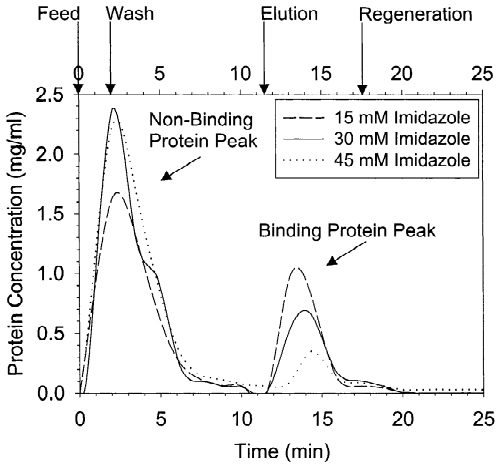


Fig. 3. Influence of imidazole concentration in the washing buffer on protein retention.

3.3. Continuous chromatography: P-CAC

The successful small-scale batch column purification strategy was transferred to the P-CAC system, which has a bed volume of 240 ml and can be operated continuously. After equilibration of the system the feed was applied. Theoretical elution angles and times were derived using calculations based upon column dimension and flow-rates [25]. Samples were taken from each outlet 1, 1.5 and 2 h after feed application and protein content quantitated by Bradford assay. As for the batch column purification (Fig. 3), two major protein peaks occur for all

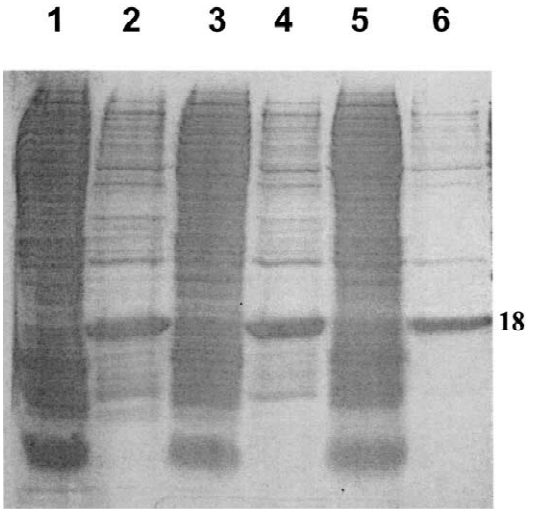


Fig. 4. SDS–PAGE analysis of batch column purifications. Lanes: (1,3,5) flow-through after washing with buffer containing 15, 30, or 45 m*M* imidazole; (2,4,6) eluate. Number on right hand side corresponds to molecular mass ($\times 10^3$).

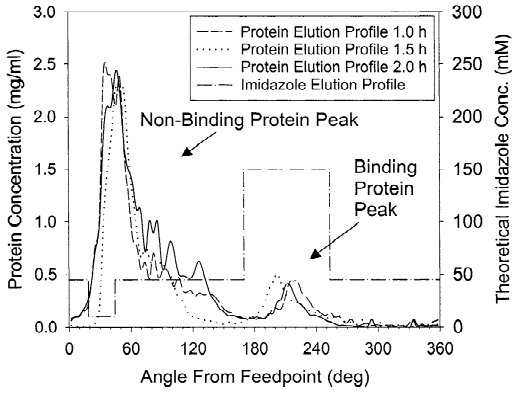


Fig. 5. Comparison of protein flow-through profiles at sampling times 1.0, 1.5, and 2.0 h.

time points (Fig. 5), and these were at the expected angles for contaminating material and Lac21 fusion product. Fig. 6 shows the SDS–PAGE results for the two major peaks identified in Fig. 5. Lanes 3, 5 and 7 show a dominant band at the molecular mass corresponding to KSI::Lac21 fusion protein. Lane 8 shows the feed material, confirming that the continuous purification strategy is successful and that it provides a product of similar purity to the batch column. This finding emphasises two major outcomes. Firstly, we have shown that development of purification strategies on small batch columns for transfer to P-CAC is legitimate (this will be discussed in more detail in the next section). Secondly we have demonstrated the use of the P-CAC preparative system for continuous purification of an affinity-tagged fusion protein. The protein profiles for the three sampling time points are all approximately superimposed on each other (Fig. 5), which illustrates that the angle of elution for any protein remains approximately constant and does not drift with time. Fig. 6 confirms that the protein compositions remain constant with time. Product purity is maintained as shown by lanes 3, 5 and 7.

The product sample purified by P-CAC is shown in both Lane 4 of Fig. 2a and lane 7 of Fig. 6. The identity of the putative product band was confirmed by detecting the histidine tag through Western blot analysis (Fig. 2b). The Western blot analysis is negative in Lane 5 of Fig. 2b, indicating no detectable product in the wash stage.

3.4. Comparison of batch column and P-CAC purification

As demonstrated in Section 3.3, protocols developed using a batch column can be directly transferred to the P-CAC and used for protein purification. The total protein profiles analysed using Bradford assays for the batch column and P-CAC are directly compared in Fig. 7. Batch column purification results using 45 m*M* imidazole in the washing buffer were selected for comparison with the P-CAC since this gave the best balance between loss and purity of fusion-protein. Based on the observed elution angle, α, the retention time, t_r, was calculated. In order to compare P-CAC with batch column results, the retention times for bed length, L and

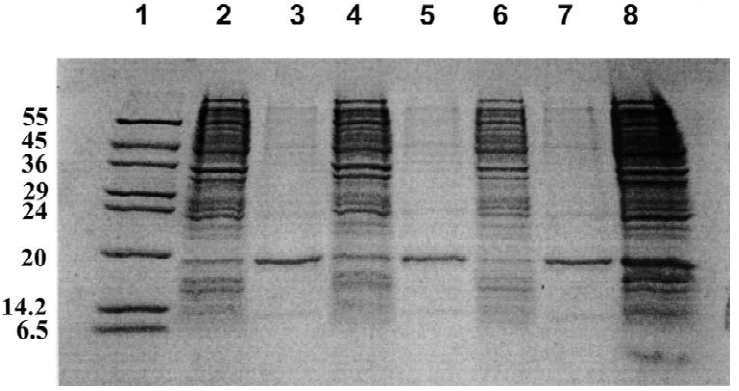


Fig. 6. SDS–PAGE analysis of proteins from P-CAC purification. Lanes: (1) molecular mass marker; (2,4,6) washing flow-through; (3,5,7) eluate; (8) protein feed. Numbers on the left hand side correspond to molecular masses ($\times 10^3$).

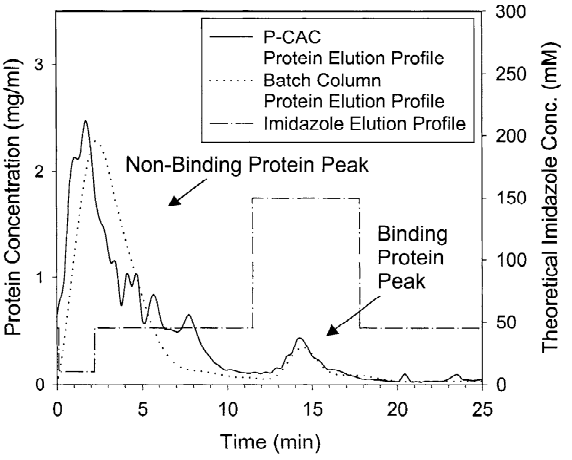


Fig. 7. Direct comparison of protein flow-through profiles of batch column and P-CAC purification strategies.

linear velocity of the mobile phase, u_m, were corrected according to Eq. (1), where k' is the capacity coefficient.

$$t_r = \frac{L}{u_m} \times (1 + k') \quad (1)$$

It is clear that the two routes of purification achieve highly comparable results. A quantitative comparison is given in Table 1. Complete protein recovery is achieved for both situations, with the percentage of protein in the elution peak almost identical for the batch column and P-CAC runs with a 45 m*M* imidazole wash. This assertion is confirmed by visual inspection of Fig. 7. The purity of the protein in the elution peak is considered very high for a single chromatographic step, and very comparable for both batch column and P-CAC purification techniques.

3.5. Cleavage

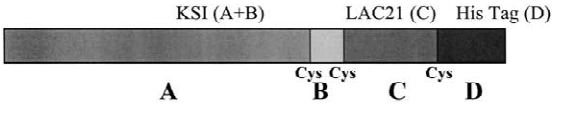
Our ultimate aim is to use the new developments presented in this work for the production of peptides using scaleable and economic technology (direct chemical extraction and continuous purification). Following the successes highlighted above, we wished to finally prove that the fusion protein could be cleaved to release the target peptide, lac21. The reagent CDAP is not commonly used for fusion protein cleavage; however, Nakagawa et al. [26] first suggested its potential for to assist cleavage at cysteine residues. A standard protocol for optimal use of the chemical is not available. Taking the amino acid sequence of Lac21 into account, CDAP was selected as the cleavage reagent, since Lac21 is devoid of cysteine moieties.

After preliminary experiments (data not shown), a basic protocol for conducting the cleavage reaction was established in batch (see Section 2). Protein purified by batch column chromatography was used, as this was slightly more concentrated and only a small amount of protein was required for this initial study. An illustration of the cleavage products expected, and their sizes, is shown in Fig. 8.

Protein analysis by SDS–PAGE, both before and after CDAP cleavage, is shown in Fig. 2a. Three new bands of approximate sizes 16.5, 14 and 13 kDa are evident on lane 7 of the gel following CDAP cleavage. The band at approximately 18 kDa is the initial fusion product, and this is significantly depleted following cleavage (protein at 18 kDa in Lane 7 is less intense than in lane 6). Referring to Fig. 8, the cleavage products in Lane 7 are identified as ABC, AB, and A in Fig. 8, respectively. The presence of uncleaved fusion protein in the reaction mixture indicates that the reaction is incomplete and

Table 1
A comparison of protein recovery and purification using batch column and continuous purification

	Protein in. feed (mg/ml)	Imidazole in wash (m*M*)	Protein recovered		Protein in elution peak		Purity of elution peak
			(mg)	(%)	(mg)	(%)	
Batch column	4.5	15	6.86	102	1.95	28	−
		30	7.17	107	1.37	21	+
		45	7.27	109	0.58	9	++
P-CAC	3.9	45	14.0 mg/min	112	1.37 mg/min	11	++



Fragment	Molecular Weight (kDa)	pI
ABCD	18.2	8.07
ABC	16.6	5.92
AB	14.3	5.66
A	13.2	5.48
BCD	4.9	7.21
CD	3.9	7.18
D	1.5	6.62
BC	3.4	8.07
C	2.3	8.22
B	1.1	6.73

Fig. 8. Hypothetical, partial fragmentation pattern of KSI::Lac21 fusion protein following CDAP plus ammonia cleavage. Associated theoretical isoelectric points (p*I*s) of fragments are also detailed.

must be further optimised. The other fragments expected to result from cleavage are smaller, ranging between 4.9 and 2 kDa. The smallest fragment that could be detected in Fig. 2a is approximately 6.5 kDa, and so visualisation of the smaller fragments was not expected. Only histidine-tagged fragments larger than 6.5 kDa were expected to be detected on the corresponding Western blot (Fig. 2b, lane 7). The only species in Fig. 8 that is bigger than this size and has a his tag is the uncleaved fusion protein, and it can be seen that this is indeed the only species detected by Western blot (Fig. 2b). This provides further confirmation that the cleavage reaction has been successful, and that we would expect to be able to recover Lac21 peptide from the post-cleavage reaction mixture. This will be the focus of a subsequent study.

4. Conclusions

We have studied the production and purification of a fusion protein, followed by its cleavage to release a desired target peptide. Once expression of KSI::Lac21 fusion protein had been successfully established, chemical extraction was conducted and the fusion protein was purified by IMAC. The strategy was first developed using a 1-ml batch column, before its successful transfer to the P-CAC, which had a column volume of 240 ml. Thus we have demonstrated scale-up by a factor of 240, and in doing so have established a continuous affinity purification process. To our knowledge, this is first time that continuous affinity chromatography using P-CAC has been reported in the literature.

Fig. 9 shows a fully integrated process flowsheet that is achievable based on the proof-of-concept and optimisation work presented in this study. Following fermentation, the fermentation broth is added to a tank, and extraction chemicals are added. Recent work by Choe [27] has demonstrated that this chemical extraction can be conducted directly on intact cells in fermentation broth, at high cell density ($OD_{600} = 130$), removing the need for the cell-washing stage employed in this work. Following extraction, residual particulate and precipitated DNA [28] can be removed by centrifugation or filtration, prior to P-CAC recovery. Chemical cleavage releases the target peptide, which might be recoverable by continuous ion-exchange chromatography, based on differences in theoretical isoelectric points (see Fig. 8). We are in the process of altering the KSI sequence to provide improved separation of fragments based on their p*I*.

The process in Fig. 9 can be compared with the traditional inclusion body process flowsheet [10]. This flowsheet would involve cell washing, repeated cell disruption by homogenisation, repeated centrifugal recovery and washing of the inclusion bodies, and batch-wise solubilisation prior to chromatographic purification of the denatured fusion protein. This process is difficult to operate continuously, due

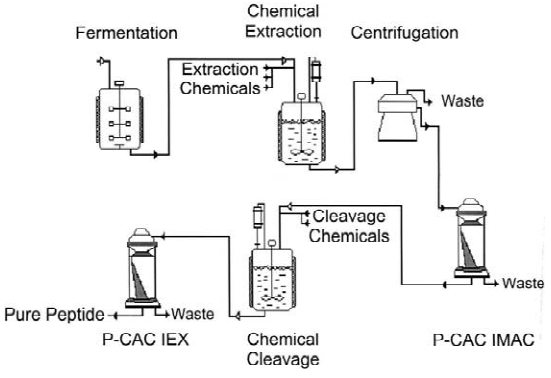


Fig. 9. The proposed integrated process for continuous peptide purification. Flowsheet drawn with the assistance of SuperPro Designer 4.9 (Intelligen Inc., Scotch Plains, NJ, USA).

to the need for batch-wise and repetitive homogenisation and centrifugation. Chemical extraction enables continuous processing to be achieved, and the use of P-CAC makes this a reality.

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