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Two-step method to isolate target recombinant protein from co-purified bacterial contaminant SlyD after immobilised metal affinity chromatography

Céline B. Parsy [a,*], Caroline J. Chapman [b], Antony C. Barnes [a], John F. Robertson [b], Andrea Murray [a]

[a] *OncImmune Limited, Clinical Sciences Building, Nottingham City Hospital, Hucknall Road, Nottingham NG5 1PB, UK*
[b] *Tumor Immunology Group, University of Nottingham, Division of Breast Surgery, Nottingham City Hospital, Hucknall Road, Nottingham NG5 1PB, UK*

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Abstract

As part of a study to purify the internal domain of HER2 (ICD) from recombinant expression, through metal immobilised affinity chromatography (IMAC), we encountered a contaminant, SlyD, a 29 kDa native *E. coli* protein. SlyD is a recurrent contaminant, with a histidine rich domain enabling binding to IMAC columns and thus co-elution with the target protein. Research has been carried out on this protein and its purification, however, no work mentions how to treat it as a true contaminant or describe procedures to isolate it from target proteins. In this report, we described a two-step chromatographic method for the purification of ICD, including IMAC as a capture step and size exclusion chromatography (SEC) as a polishing step. IMAC allowed us to purify ICD from bacterial crude with SlyD co-eluting. SEC then allowed us to resolve ICD from SlyD and achieve a purity greater than 95% for ICD. However, this method has been developed to accommodate any protein whose molecular weight is different enough from SlyD to be separated by SEC.
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1. Introduction

The past 20 years have seen the development of techniques and tools for the expression and purification of recombinant proteins which have transformed the way scientists approach protein production and research. Vector constructs allow over-expression of an increasing amount of protein-encoding DNA sequences. Naturally low-abundant protein can then be expressed in gram quantity through a suitable expression vector and host combination. Some vectors can also insert extra DNA sequences leading to the production of tagged proteins, which incorporate extra amino-acid sequences like hexa-histidine [1], GST [2] or streptavidin [3]. These tags can then be used to purify the recombinant protein by single-step affinity chromatography through corresponding matrix and ligand [4,5]. Immobilised metal affinity chromatography (IMAC) is one such example and generally involves the fusion of a six-histidine tag to the target protein, though the tag can vary in length (4–10 histidines). This tag interacts with a chromatography matrix charged with metal ions (mainly nickel and cobalt) due to the affinity of histidine for these ions. As a result, recombinant fusion protein can be purified to homogeneity from a bacterial lysate in only one step.

However, the progress in recombinant protein expression and purification has raised new challenges. The over-expression of a target protein by the host bacteria has been linked to the over-expression of native bacterial proteins, as a response to stress conditions following the high production of foreign material [6–8]. Some of these native proteins have properties that cause them to co-purify with the target protein when IMAC is being used, as they show an affinity to divalent cations [9]. The most studied recurrent contaminant of IMAC purification is SlyD, a 196 amino acids protein, known to be present in *E. coli* during lysis when infected by bacteriophage ΦX174 [10]. SlyD

* Corresponding author. Tel.: +44 115 823 1871; fax: +44 115 823 1881.
E-mail address: celine.parsy@nottingham.ac.uk (C.B. Parsy).

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also acts as a nucleotide binding-protein but its major activity is thought to be a peptidoylproline cis–trans isomerase, showing three domains [11]. The first domain (residues 1–95) is similar to the FK binding proteins superfamily, the second domain (residues 148–179) is rich in histidines while the third one (residues 149–196) corresponds to the metal-binding region. Due to the histidine rich domain, SlyD can therefore bind to metal matrices in native or denatured conditions [12] as would histidine-tagged fusion proteins.

Most studies of SlyD have focused on how to purify it on its own for specific studies on SlyD activity and properties [7,10,13–15]. However, no publications have reported on the problems SlyD can generate if present in a recombinant protein preparation. If SlyD cannot be removed from a sample, accurate quantitation of the target protein becomes impossible. Moreover, SlyD is expressing isomerase and chaperone activities, which could lead to unwanted interaction or reaction with the target protein, hence there is an urgent need to find a method that facilitates full removal of SlyD from the protein preparation which is preferably applicable to a wide range of proteins.

Here, we describe a method to purify the internal domain of HER2 (ICD) from bacterial expression through affinity chromatography. As SlyD co-eluted with the target protein, we then developed a way to remove the contaminant by size exclusion chromatography (SEC). The significance of this work is based on the fact that the purification process can be applied to a broad spectrum of proteins, as no specific characteristic is required to carry out SEC. By applying this process to the purification of ICD, we reached a high purity protein, free of SlyD.

2. Materials and methods

2.1. Cloning and expression

Specific cDNA for the internal domain of HER2 (ICD) was subcloned, with addition of a small tag, into the pET21b expression vector (Novagen, EMD Biosciences, Madison, Wisconsin, USA). The recombinant proteins were expressed in BL21 (DE3) (Novagen), grown in 20 l of overnight express media (Novagen). The cells were harvested by centrifugation for 10 min at 3000 × *g* at 4 °C. These steps were performed by Davies Fermentation, (Athens, Georgia, USA). After centrifugation, the pellets were stored at −80 °C and shipped to our laboratory.

2.2. Lysis

One pellet of BL21(DE3)(ICD) (5 g) was thawed on ice and resuspended in 20 ml lysis buffer (6 M guanidine chloride (GuHCl), 100 mM Tris–HCl, 100 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol, 1% Triton, pH 8.0). The lysate was sonicated three times using a 1 × 10 s pulse program at 25% power using a Sonopuls Ultrasonic Homogeniser (Progen Scientific Ltd, Mexborough, UK) and left rolling for at least 1 h at room temperature. A centrifugation step at 15000 × *g* for 30 min was then performed to pellet cellular debris. The supernatant containing soluble proteins was then recovered, diluted to 50 ml with solubilisation buffer (6 M GuHCl, 100 mM Tris–HCl, 100 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, pH 8.0), and filtered sequentially through 1.2 μm, 0.8 μm, 0.45 μm and 0.2 μm cellulose ester filters (Whatman, Brentford, UK).

2.3. Immobilised affinity chromatography

The filtered lysate was loaded at 2.5 ml/min onto a 5 ml HisTrap FF crude column (GE Healthcare Bio-Sciences, Uppsala, Sweden), using an AKTA Prime Plus purification system (GE Healthcare Bio-Sciences, Uppsala, Sweden) previously equilibrated with 5 column volumes (CV) of solubilisation buffer, at a flow-rate of 5 ml/min. The flow-through was collected in 10 ml fractions. The column was then washed with 5 CV of the same solubilisation buffer at a flow rate of 5 ml/min and then 5 CV of washing buffer (8 M urea, 100 mM Tris–HCl, 100 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, pH 8.0) at the same flow-rate and 10 ml fractions were collected during the washes. Elution was performed using a gradient from 0 to 100% of elution buffer (8 M urea, 100 mM Tris–HCl, 100 mM NaH_2PO_4, 0.5 M NaCl, 500 mM imidazole, pH 8.0) over 10 CV and a final 5 CV wash was performed with 100% elution buffer, 5 ml fractions were collected throughout the elution step. The entire purification was monitored using the PrimeView software (GE Healthcare Bio-Sciences). All relevant fractions were selected from the chromatogram and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions free of SlyD were pooled together and dialysed for 24 h at 4 °C in refolding buffer (1 M urea, 50 mM Tris–HCl, 2 mM reduced glutathione, 0.02 mM oxidised glutathione, 0.005% Tween, pH 8.0). Fractions containing SlyD were pooled together and processed further through SEC.

2.4. SEC and refolding

Fractions containing SlyD were saturated with 60% ammonium sulphate, by adding 0.39 g of ammonium sulphate per ml of contaminated sample. After the ammonium sulphate has dissolved, the sample was allowed to roll overnight at 4 °C and then centrifuged at 15000 × *g* for 30 min. The supernatant was discarded and the pellet was resuspended in 5 ml of elution buffer and filtered through a 0.2 μm Minisart syringe filter (Sartorius, Epsom, UK).

The sample was then injected into an AKTA Prime Plus 5 ml injection loop (GE Healthcare Bio-Sciences) connected to a HiPrep 16/60 Sephacryl S-200 HR (GE Healthcare Bio-Sciences), previously equilibrated with 150 ml of refolding buffer and run at 0.5 ml/min with the same buffer. The chromatography was monitored using PrimeView software (GE Healthcare Bio-Sciences). All relevant fractions were analysed by SDS-PAGE.

2.5. Storage

Both samples, refolded after affinity chromatography or SEC, were then pooled together and dialysed for 24 h at 4 °C in dialysis

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buffer (50 mM Tris–HCl, 200 mM NaCl, pH 8.0). A 1 ml aliquot was removed for analysis by SDS-PAGE and Western blotting. The rest of the sample was stored at −80 °C in 50% glycerol, 1 mM DTT and 0.1 mM EDTA final concentration.

2.6. Analytical techniques

2.6.1. SDS-PAGE

Electrophoresis was performed for samples throughout the purification process, using the Bio-Rad Protean 3 gel electrophoresis system (BioRad, Hercules, California, USA Bio-Rad). The samples were diluted 1:1 in SDS-loading buffer (6% SDS, 10% β-mercaptoethanol, 4% glycerol, 1.25 M Uridine 5′-triphosphate Tris salt, bromophenol blue), and boiled for 10 min. 10 μl of sample, along with 5 μl of a Wide Molecular Weight Range protein marker (Sigma, Gilligham, UK) for silver-staining analysis or 5 μl of Precision Plus Protein all blue marker (BioRad) for Western blot analysis were loaded and run on a 10% Tris–HCl precast gel (Bio-Rad) according to the manufacturer's instructions, at 150 V for 50 min.

2.6.2. Silver-staining

After completion of electrophoresis, gels to be analysed by silver-staining were fixed for 30 min into 10% acetic acid, 40% methanol. The gels were transferred into sensitiser solution (30% ethanol, 7% sodium acetate trihydrate, 0.5% gluteraldehyde, 0.2% sodium thiosulphate) for a minimum of 2 h, followed by three washes in distilled water for 5 min. Staining was performed by adding a silver nitrate solution (0.1% silver nitrate, 0.014% formaldehyde) to the gels for 45 min in a recipient cover with foil to avoid light interference, followed by a single wash in distilled water for 30 s. The gels were then transferred in the development solution (2.5% sodium carbonate decahydrate, 0.008% formaldehyde) twice and stopped by transferring the gels into 5% Tris, 2.2% acetic acid, pH 7.0.

2.6.3. Western blot analysis

The proteins were transferred after SDS-PAGE onto a Trans-blot Transfer Medium membrane (BioRad) using blotting buffer (1× Tris-Glycine buffer (Biorad), 20% methanol) and a Trans-Blot SD Semi-dry Transfer Cell (Biorad) at 15 V for 45 min. The membrane was then blocked for 2 h in 3% bovine serum albumin (BSA) in phosphate buffer saline solution (PBS), and washed three times for 5 min in 10 ml of 0.1% Tween 20 in PBS. It was followed by membrane incubation for 2 h with 10 ml of PBS supplemented with 1:1000 dilution of mouse anti His-Tag monoclonal IgG1 antibody (Novagen) or mouse ErbB 2 IgG1 antibody [CB11] (anti-ICD) (Abcam, Cambridge, UK), followed by three washes as before. A second incubation was performed for 1 h with 10 ml of PBS supplemented with 1:1000 dilution of polyclonal rabbit anti-mouse immunoglobulins horse radish peroxidise (HRP) conjugated antibody (DakoCytomation, Glostrup, Denmark). A further wash step was performed as before followed by three washes for 5 min in 10 ml distilled water. Detection was performed by adding 1 ml of 3,3′,5,5′-tetramethylbenzidine (TMB) (Promega, Madison, Wisconsin, USA) and incubation for 3 min. The development reaction was stopped by adding 10 ml of distilled water.

2.6.4. Quantitation

Crude protein concentrations were measured using the BCA Protein Assay kit (Pierce, Rockford, Illinois, USA) following the manufacturer's instructions. The purified protein was quantified using a Cary 50 spectrophotometer (Varian, Mulgrave, Australia) and measuring the absorbance at 280 nm. The concentration in mg/ml, was then calculated using the specific extinction coefficient of ICD and its molecular weight according to the Beer-Lambert law.

2.6.5. Protein sequencing of contaminant protein

After purification by IMAC, the elution fraction containing ICD and a contaminant protein was analysed by gel electrophoresis and silver stained. The gel was then transferred to The University of Nottingham Biopolymer Synthesis and Analysis Unit [16], where the contaminant protein was excised from the gel for sequencing by LC–MS/MS electrospray. Results were analysed on Swiss-Prot database.

3. Results and discussion

3.1. Extraction and solubilisation of recombinant ICD

Previous experiments showed ICD is expressed in inclusion bodies (data not shown). In order to achieve the purification of this protein, the cells had to be lysed and disrupted. The pellet treatment with ultrasonic homogenisation allowed the cell lysis and the inclusion bodies to be broken and also helped to reduce the viscosity of the lysate. The addition of Guanidine-chloride in the lysis buffer also helped in the solubilisation of the proteins even expressed as inclusion bodies. Guanidine chloride was chosen over urea as it is a stronger denaturing agent [17] and it allowed an easier lysis in only two steps.

Filtration steps performed prior to IMAC enhanced the clarity of the lysate and allowed the loading of a non viscous sample on the column.

3.2. Immobilised affinity chromatography

The use of a vector, providing a sequence for the expression of a poly-Histidine tag, and the possibility of using pre-packed column of Nickel coupled with sepharose, allowed the whole purification to be automated with the use of a chromatography purification system. The use of columns specifically designed for the loading of crude proteins also allowed the reduction of back pressure that we observed when using HisTrap HP (GE Healthcare Bio-Sciences) columns.

IMAC allowed a fast and efficient purification from crude bacterial preparation with only two proteins being captured from the original lysate (Fig. 1A). However, even if the protein was loaded at half the speed compared to the elution, ICD remained in the flow-through and four cycles of purification were needed to be performed to exhaust the lysate of target protein. This may be

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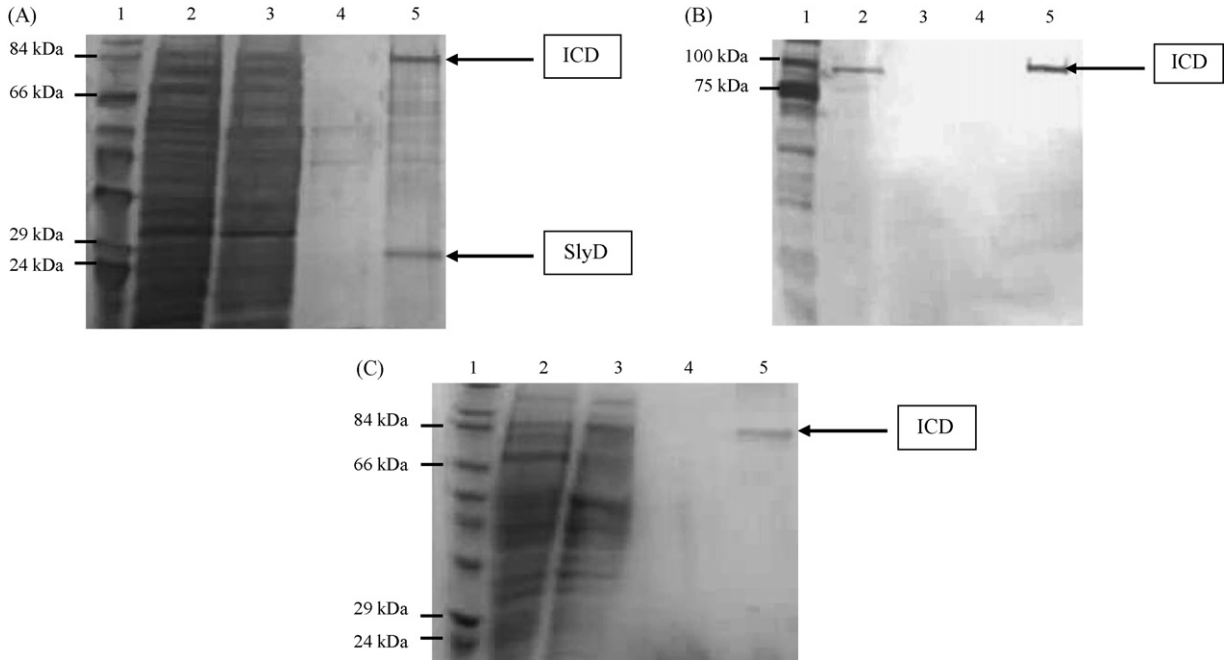

Fig. 1. SDS-PAGE and Western-blot analysis of the different ICD IMAC purification steps. Lane 1: molecular weight marker; lane 2: induced cell homogenate, lane 3: non-binding proteins (flow-through), lane 4: 20 mM imidazole wash; lane 5: elution pooled fractions. (A) SDS-PAGE developed by silver-staining. (B) Western blot analysis using anti-ICD antibody. (C) SDS-PAGE developed by Coomassie staining.

explained by the amount of soluble proteins present in the bacterial crude acting as a screen between the His-tag on the ICD and the Nickel ions present on the columns. However, when using this protocol for the purification of other antigens, we could observe the presence of SlyD only in the first flow-through. Further runs of the lysate showed no more SlyD co-eluting with the target protein (data not shown). SlyD may therefore be competing with a stronger affinity for binding on the Nickel matrix than the original His-tagged protein due to its Histidine rich domain. This affinity may also be dependent on the actual SlyD concentration in the lysate, the size of the target proteins as well as the expression yield. Fig. 1A shows the SDS-PAGE analysis of the different steps in the affinity chromatography from capture to elution by imidazole gradient. From this analysis and a Western blot using anti-ICD antibody (Fig. 1B), it was possible to confirm the presence of ICD, at a molecular weight of 83 kDa (67 kDa plus 16 kDa for the small tag). The contaminant protein, whose apparent molecular weight was 29 kDa was assumed as being SlyD, based on previous references to a protein co-eluting during IMAC and being the principal contaminant with a molecular weight of 29.6 kDa [7,2,8,11,13]. In order to confirm this result, protein sequencing was performed by electrospray mass spectrometry. Two peptides sequences were identified: FNVEVVAIR and DVFMGVDELQVGMR. A Blast search performed from the Swiss-Prot database returned SlyD from *E. coli* as the highest matching sequence, which confirms the previous assumption as SlyD being the contaminant protein present in the purification process.

Silver staining was favoured over Coomassie staining as we observed that SlyD could not always be visualised using Coomassie even when present as a high proportion of the total protein. Fig. 1C shows exactly the same samples and loading order as that shown in Fig. 1A but the SDS-PAGE gel was Coomassie stained. Lane 5 from both figures shows that SlyD is visible when silver stained and represents about 40% of the total protein loaded but does not appear with Coomassie staining at all. However, examples of poor protein staining with silver nitrates compared to Coomassie have been reported before [18]. This emphasizes that the method of visualisation for contaminants is of prime importance in the purification process. Therefore optimisation steps for analysis need to be performed in order to identify the best purification analysis methods, in this case silver staining.

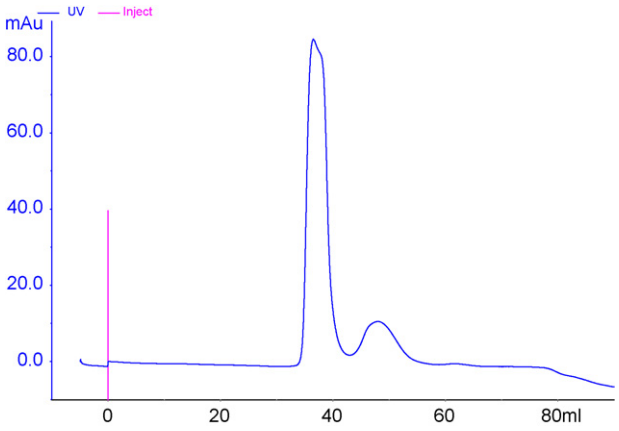

Fig. 2. SEC performed with HiPrep 16/60 Sephacryl S-200 HR (120 ml of gel). Sample concentrated up to 5 ml. Elution buffer: 1 M urea, 50 mM Tris–HCl, 2 mM reduced glutathione, 0.02 mM oxidised glutathione, 0.005% Tween, pH 8.0. Flow-rate: 0.5 ml/min. UV detection at 280 nm (mAu).

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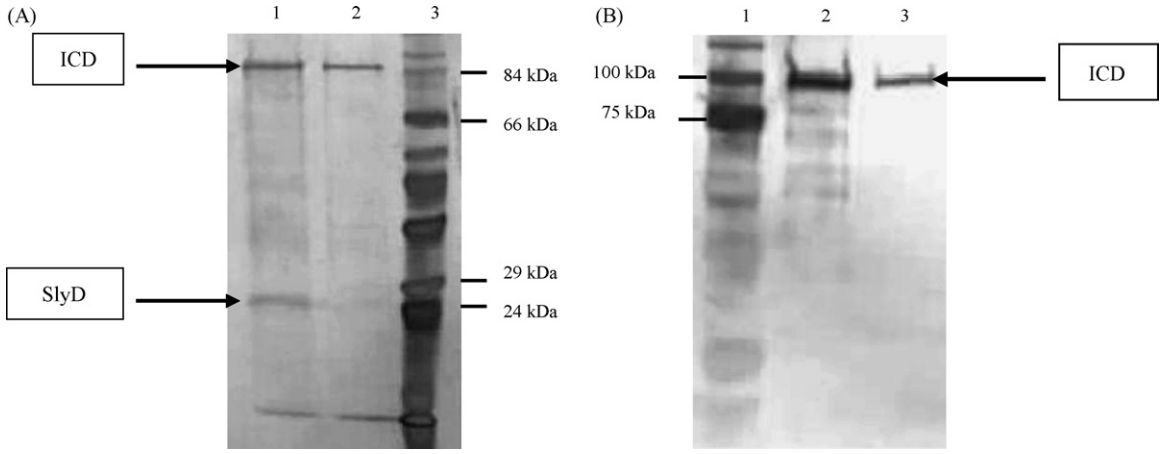

Fig. 3. SDS-PAGE and Western-blot analysis of the different ICD SEC samples. (A) SDS-PAGE developed by silver-staining. Lane 1: loaded sample (IMAC elution fractions), lane 2: S200 elution (pooled fractions), lane 3: molecular weight marker. (B) Western blot analysis using anti-ICD antibody. Lane 1: molecular weight marker; lane 2: loaded sample (IMAC elution fractions), lane 3: S200 elution (pooled fractions).

3.3. SEC analysis

As ICD purified from the IMAC showed SlyD contamination, another step was necessary to remove it. Other chromatography steps, like anion exchange and cation exchange chromatography could be potential candidates to achieve this target. However, with a theoretical p*I* of 4.86 for SlyD and 5.30 for ICD, anion exchange experiments would allow the binding of the two proteins but they would potentially co-elute together as well, while cation exchange experiments, on the other hand, could have led to sample precipitation or non binding. These techniques can be time-consuming to optimise and need to be specific for every target protein. Our objective was to be able to use a method which could be applicable to the large variety of proteins we purify in our laboratory that have different characteristics to ICD. With these considerations, the use of SEC appeared to be a suitable way to remove SlyD for a protein whose size is different from 29 kDa. In this study, we used a Sephacryl S-200 HR column. In other studies we have achieved complete resolution from SlyD with a protein of 32 kDa, a difference of only 3 kDa from SlyD molecular weight, but with approximately 80% loss of the target protein (data not shown). However, if other columns or other media are used, like a Superdex 75 column, the resolution could be greatly increased [19]. Optimisation of the experimental conditions is then essential to achieve the degree of resolution required. However, optimisation for SEC is more straightforward than for other chromatography methods as less parameters are involved. Careful consideration needs to be given to column size, column packing and flow rate, but these variables are more easily controlled than those that govern the success of ion exchange chromatography for example. SEC is also a gentle technique, which can be used in native conditions as well as denaturing ones with a broad range of buffer compositions, the only consideration being the stability of the target protein in the running buffer.

The ammonium sulphate precipitation, a standard method of protein concentration, allowed the elution sample volume to be reduced in order to perform SEC. No dialysis was required as the gel filtration column was compatible with the elution buffer composition. This method also allowed refolding to be performed on the column as the SEC can act as a dialysis step. After injection onto the Sephacryl S-200 column and subsequent chromatographic separation, the sample was resolved (Fig. 2), ICD eluted between 34 and 43 ml of elution volume and SlyD eluted from 44 to 55 ml. The corresponding fractions were pooled and the ICD sample was dialysed in storage buffer.

Table 1
Purification of ICD from a 5 g cell pellet

Sample	Total protein[a] (mg)	Yield (%)	Protein purity[b] (%)
Soluble faction	132	N/A	N/A
Ni2+ elution fractions	8	6	50
SEC	4	3	95

[a] Protein quantitation by BCA assay.
[b] Protein quantitation by $OD_{280\,nm}$

3.4. Characterisation of the purified recombinant ICD

Analysis of the initial and final samples of the SEC were made by SDS-PAGE and Western blot (Fig. 3A and B), showing a molecular weight of about 83 kDa for ICD and a purity reaching 95% by silver staining.

The total amount of ICD being purified from a 5 g pellet is 4 mg (Table 1). This relatively low result can be explained by the relative poor expression of ICD due to its high molecular weight. However, when we used this process for the purification of antigens, for which over-expression is more efficient, we observed a much higher yield of pure protein (data not shown).

4. Conclusion

We have reported the development of a two-step process for the purification of recombinant protein overexpressed in *E. coli*, showing SlyD contamination. After an immobilised metal affinity chromatography step, which led to SlyD being the principal contaminant, as commonly experienced when using this

technique, SEC of the concentrated eluted sample allowed the resolution of the target recombinant protein, here ICD, from SlyD. The purity of the target protein was in excess of 95%. The only physical characteristics employed in this process are the presence of a His-tag on the recombinant protein and separation according to the target protein size which must be different from the SlyD molecular weight. This method can therefore be easily applied to any recombinant protein system exhibiting a His-tag and whose molecular weight is different enough from 29 kDa to be resolve by gel filtration.

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References

[1] A. Hoffmann, R.G. Roeder, Nucleic Acids Res. 19 (1991) 6337.
[2] R.Y. Poon, T. Hunt, Anal. Biochem. 218 (1994) 26.
[3] J.C. Bonnafous, R. Seyer, M. Tence, J. Marie, M. Kabbaj, A. Aumelas, J. Recept. Res. 8 (1988) 295.
[4] E. Hochuli, H. Dobeli, A. Schacher, J. Chromatogr. 411 (1987) 177.
[5] M.C. Smith, T.C. Furman, T.D. Ingolia, C. Pidgeon, J. Biol. Chem. 263 (1988) 7211.
[6] F.H. Arnold, Biotechnology (NY) 9 (1991) 151.
[7] C. Wulfing, J. Lombardero, A. Pluckthun, J. Biol. Chem. 269 (1994) 2895.
[8] J. Porath, Trac-Trends AnalChem 7 (1988) 254.
[9] C. Mateo, G. Fernandez-Lorente, B.C. Pessela, A. Vian, A.V. Carrascosa, J.L. Garcia, R. Fernandez-Lafuente, J.M. Guisan, J. Chromatogr. A. 915 (2001) 97.
[10] T. Mitterauer, C. Nanoff, H. Ahorn, M. Freissmuth, M. Hohenegger, Biochem. J. 342 (Pt 1) (1999) 33.
[11] V.M. Bolanos-Garcia, O.R. Davies, Biochim. Biophys. Acta 1760 (2006) 1304.
[12] S. Mukherjee, A. Shukla, P. Guptasarma, Biotechnol. Appl. Biochem. 37 (2003) 183.
[13] S. Hottenrott, T. Schumann, A. Pluckthun, G. Fischer, J.U. Rahfeld, J. Biol. Chem. 272 (1997) 15697.
[14] W.D. Roof, S.M. Horne, K.D. Young, R. Young, J. Biol. Chem. 269 (1994) 2902.
[15] C. Scholz, B. Eckert, F. Hagn, P. Schaarschmidt, J. Balbach, F.X. Schmid, Biochemistry 45 (2006) 20.
[16] The University of Nottingham, School of Biomedical Sciences, Biopolymer Synthesis and Analysis Unit, http://www.nottingham.ac.uk/biomedsci/template.php?page=corefacilities&subpage=biopolymer.
[17] G.S. Chhatwal, C. Lammler, H. Blobel, Med. Microbiol. Immunol. (Berl.) 173 (1984) 19.
[18] M. Schleicher, D.M. Watterson, Anal. Biochem. 131 (1983) 312.
[19] Amersham-Biosciences, Gel filtration, Principles and Methods Handbook, Amersham Biosciences (2002).