A high performance bench scale process for isolation from inclusion bodies, refolding and dimerisation of a thiol-engineered recombinant therapeutic protein.

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Abstract

The use of laboratory procedures is often inefficient for materialisation of recombinant therapeutic proteins in *Escherichia coli* (*E. coli*) for pre-clinical evaluation. Approaches such as scaling out shake flask cultivation can be laborious, inefficient and expensive. These inefficiencies can be compounded if the protein requires post-translational modification such as multimerisation. We previously used laboratory methods to produce the <60kDa, recombinant biotherapeutic, RB1. We were aware, a priori, that dimeristion of RB1 could double the molecular weight of the protein and increase its systemic retention in the human body by avoiding renal filtration. Here we modified RB1 by substituting a native residue for an unpaired cysteine, generating eRB1, in order to favour its dimerisation. Laboratory methods failed to achieve >20% disulphide-bridged homodimerisation or monomer of sufficient purity to enable chemi-dimerisation. As such we established a set of high performance, bench-scale, unit operations for cultivation of *E. coli* cells expressing eRB1, the isolation of eRB1 inclusion bodies, refolding and disulphide-based dimerisation of ≥40% of total eRB1 and finally successful chemi-dimerisation of remaining monomeric eRB1. The establishment of scalable procedures can now enable future investigations of eRB1 and other <60kDa biologics for which significant bench-scale production is required for pre-clinical evaluation.

Keywords *Escherichia coli*, inclusion body refolding, thiol engineering, recombinant biotherapeutic

1. Introduction

We previously investigated a recombinant biotherapeutic protein, RB1, which posed challenges characteristic of early-stage biologics development i.e. the need for high-performance but still relatively small-scale production and for protein engineering steps to increase efficacy. In this study we propose and test a bench-scale process for RB1 production that offers a high level of performance, is scalable and in a rapid and efficient manner provides sufficient material for efficacy evaluation. We demonstrate the process using a variant of RB1 that has undergone protein-engineering to yield eRB1 in order to address the possibility of low systemic retention. We suggest the principle value in thie work is in mapping out a process for production and isolation of dimerised therapeutics at bench scale that is also scalable. We hope to provide a useful production starting point for researchers wishing to dimerise a given therapeutic protein for reasons of efficacy in addition to the goal of producing sufficient material for pre-clinical investigation.

Our previous investigation of RB1 showed that it forms homoaggregate inclusion bodies (IBs) when expressed in the *E. coli* cytosol. IB formation can be desirable for high performance production of biologics, due to the high degree of purity of the constituent monomers (1), resistance to proteolysis (2) and ease of isolation from other *E. coli* host cell material (3). However developing such steps is often a challenge in a research setting geared toward clinical investigation as opposed to product materialisation. As such we were eager in this study to establish a set of procedures for addressing IB production that ideally were compatible with both bench scale equipment and any future pilot-scale unit operations. These steps would enable isolation of IBs then solubilisation and refolding of recombinant protein in a

way that is rapid and yields a sufficient quantity of therapeutically active protein for efficacy studies.

The efficacy of a given biologic is frequently determined by its ability to bind to a specific target. This target binding often must be robust to a wide range of microenvironments, levels of target expression, and genetic variation across patient populations (4,5). In this study our strategy was to construct a variant, eRB1, in which a residue was substituted for an unpaired cysteine able to form a disulphide bridge between monomers (6,7). This was based on the *a priori* prediction that such dimerisation would double the effective molecular weight (MW) of the protein to take it above the so-called 'renal threshold'. Upon administration to patients, RB1 is predicted to have a high propensity to loss by excretion due to its having a molecular weight (MW) below the renal threshold. Proteins of MW less than 30-60 kDa are likely to be excreted rapidly from patients during glomerular filtration (14). This is often problematic for treating disease states with recombinant proteins, as therapeutic target sites are typically scarce. As such, the longer a biologic can be retained in the circulation the more likely are encounters with therapeutic targets. Successful dimerisation of eRB1 will in effect double its MW and take it above the renal threshold. We assembled DNA encoding eRB1 by site-directed mutagenesis of the RB1 open reading frame (ORF) and subcloning the resultant RB1 ORF into an *E. coli* expression plasmid.

In their native state many proteins use multiple intramolecular disulphide bonds between cysteine residue thiol groups to stabilise tertiary structure. Disulphide bonds cannot form in the *E. coli* cytoplasm as it is a reducing environment in which glutaredoxin and thioredoxin pathways act to reduce any thiol bridges via oxidation (8) of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to cationic

$NADP^+$. As such, our strategy would require IB formation ideally to persist in the eRB1 variant and would involve dimerisation of eRB1 only after its isolation from IBs. We investigated approaches to catalyse disulphide bridge formation between eRB1 monomers *in vitro* by testing refolding conditions that favour disulphide bond formation and shuffling.

We were also keen to develop alternative linking chemistries that might render the dimer bond stable to *in vivo* encounters with reducing microenvironments. To do this we investigated chemical crosslinking of monomeric eRB1 using bis-maleimidohexane (BMH) to form a thioether bridge. BMH is commonly used for crosslinking cysteine residues to form thioether bonds. Maleimides are preferred for this process due to their high reactivity in aqueous solution, specificity for thiol groups and reactivity at neutral pH (9). Maleimide crosslinking is now a well-established bioprocess option for dimerisarion of many licensed biotherapeutics (10–12), including Rentuximab vedotin and Trastuzumab emtansine, both of which are approved by the United States Food and Drug Administration (13). A scalable process for BMH-mediated crosslinking could significantly shorten the timescale for materialisation of dimerised therapeutic proteins of which eRB1 serves as an example.

2. Materials and Methods

2.1 Chemicals, enzymes, and materials

All chemicals were purchased from Sigma (UK) and were of analytical grade unless stated otherwise. Enzymes were purchased from New England Biolabs (UK) unless stated otherwise.

2.2 Construction of therapeutic eRB1 expression strain

The *E. coli* TOP10 strain (Invitrogen, Paisley, UK) was used for cloning steps and the BL21 DE3 pLysS strain (Stratagene, Linford Wood, UK) for eRB1 expression. The RB1 ORF, encoded by a plasmid based on a pET29a vector (Novagen, UK) backbone, was modified to encode a cysteine substitution by site directed mutagenesis performed using the QuikChange® Site-Directed Mutagenesis Kit (Agilent, UK) and following the manufacturer's instructions. The mutation was achieved with a single base substitution that converted the wild type codon to a cysteine codon, resulting in the eRB1 ORF. The eRB-29a vector now encoding eRB1 was transformed into BL21 DE3 pLysS *E. coli* using standard molecular biology techniques to give the eRB-BL21 strain (15).

2.3 High cell density *E. coli* cultivation

7.5 L New Brunswick BioFlo 110 bioreactors, following a protocol described previously (16), were used to cultivate cells expressing eRB1. Harvested process stream was divided into 500 mL centrifuge tubes (Beckmann) and centrifuged at 6000 x g for 20 minutes at 4°C before storage overnight at -20ºC.

2.4 Refolding and purification of eRB1

After overnight storage at -20°C cells were resuspended to 20% w/v solids in 50 mM Tris/50 mM NaCl, pH 7.4 and subjected to 5 passes at 500 Bar in a Lab60 homogenizer to release the intracellular product (APV Invensys, London, UK). The homogenate was then divided into Beckmann 500 mL centrifuge tubes, and the insoluble cell debris pelleted by centrifugation at 6000 x g for 1 hour at 4ºC. The inclusion body fraction was purified using methods previously described (16) and solubilised in 8 M urea / 0.1 M Tris / 2 mM dithiothreitol (DTT) / 440 mM reduced glutathione (GSH) / 1 mM ethylenediaminetetraacetic (EDTA) pH 9.3. The protein concentration of this mixture was determined by Bradford Assay before being added

at a rate of 1 mL / minute to a refolding buffer of 0.1 M Tris/0.5 M L-Arginine/2 mM EDTA/1 mM GSSG pH 8.5, at 4°C using a Model EP-1 Econo Pump (Bio-Rad Laboratories Ltd., Hertfordshire, UK) with stirring at 200 RPM. The volume of refolding buffer was adjusted to ensure a final protein concentration of 100 µg/mL and the solution was left to stand for 72 hours for refolding to take place. The solution was then dialysed against 50 mM sodium acetate pH 5 buffer for 24 hours with two buffer changes before loading onto a HiTrap SP FF Cation Exchange Column (GE Healthcare Sciences, Buckinghamshire, UK) and elution with a gradient of 0-1 M NaCl. Size Exclusion chromatography was performed with a 500 mL Fast Flow Sepharose column (GE Healthcare Sciences). Fractions were analysed by non-reducing sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) for the presence of monomeric and dimeric eRB1.

2.5 Chemical Cross-linking

SEC fractions of eRB1 confirmed by SDS PAGE to contain no dimer were pooled and diluted to 5 mg/mL. As a precaution to remove any residual dimer not visible by SDS PAGE we added 5 mM tris (2-carboxyethyl) phosphine (TCEP) to the solution for a 30 minute incubation. This incubation with reducing agent should convert the remaining dimer to monomer. The TCEP was then removed, by desalting with a PD10 column (GE Healthcare Sciences) and 10 µL of 5.5 mg/mL bismaleimidohexane (BMH), from Thermo Fisher Scientific, Rockford, USA, in dimethylsulfoxide (DMSO) was added per mL of protein solution. This corresponds to 1:2 molar ratio of protein:BMH. The mixture was incubated for 1 hour at room temperature, and then the reaction was quenched by desalting with a PD10 column. The crosslinked protein was then separated from monomer and other reactants by cation exchange chromatography.

2.6 Expression and isolation of inclusions bodies formed by eRB1 in *E. coli*.

We sought to establish a high performance bench scale production and recovery strategy in which the host strain was cultivated to high cell density using chemically defined media and a bioreactor to grow cells to high density (16). A triton/urea-based washing protocol would be used to obtain purified inclusion bodies (17) and a final two-step chromatographic process used to separate and purify monomeric and dimeric eRB1 (Figure 2).

E. coli strain BL21 DE3 pLysS was used as host for the expression of eRB1. A seed culture of 200 mL LB with 35 µg/mL kanamycin was inoculated with a glycerol stock and grown at 37°C, 250 RPM until OD_{600} = 1.0. This seed culture was then used as a 10% inoculum for a 400 mL defined media culture grown at 30°C, 200 RPM until OD_{600} = 5.0. This defined culture was used as inoculum for a 7.5 L New Brunswick BioFlo 110 bioreactor containing 3.6 L defined media, to provide a starting OD_{600} of 0.5. This bioreactor culture was grown following the Matos et al. protocol (16), induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at mid-exponential phase, OD_{600} = 56. 48 hours after initial inoculation (Figure 3) the entire process stream was transferred to 0.5 L centrifuge bottle for centrifugation at 6000 x *g* in a J2-M1 device (Beckman-Coulter, Fullerton, CA, USA) with a JA10 rotor. Supernatant was decanted from the resultant pellets which were then frozen by storage at -20ºC overnight. Frozen cell pellets were resuspended to 20% w/v in homogenisation buffer (50 mM Tris-HCl, 50 mM NaCl, pH 7.4) and subjected to 5 passes at 500 Bar in a Lab 60 homogenizer (APV Invensys, London, UK) to achieve complete lysis. The crude extract was centrifuged for 1 hour at 6,000 x g, 4°C, and the IB pellet washed with urea and Triton X-100 containing buffers to give a purified pellet (17).

2.7 One step refolding and dimerisation of eRB1 from inclusion bodies

Recombinant biotherapeutics are often highly disulphide-linked, and so require extensive refolding when expressed within cytosolic IBs in *E. coli* cells. Altering the reducing conditions within the cytosol through gene knock outs (23) and co-expression of folding chaperones (24, 25) has been shown to allow the recovery of active, correctly folded disulphide bonded proteins. Targeting recombinant therapeutic proteins to the oxidative periplasm has also successfully yielded active protein. However, IB-based refolding methods can enable a high yield of pure protein to be obtained due to the ease of separation from other cellular debris, the resistance of the IB to proteolysis, and the high yield obtainable due to the cytosolic compartment forming the majority of the volume of an *E. coli* cell (26).

In this procedure the IB pellet was resuspended in solubilisation buffer (8 M Urea/0.1 M Tris/2 mM DTT/440 mM GSH/1 mM EDTA pH 9.3). SDS-PAGE (Figure 4) was then used to estimate eRB1 yield and the protein solution was then added drop-wise to a pH 8.5 refolding buffer of 0.1 M Tris-HCl/0.5 M L-Arginine/2 mM EDTA/1 mM oxidised glutathione (GSSG) and incubated at 200 RPM, 4°C for 72 hours.

3. Results and Discussion

3.1 Thiol engineering of the novel recombinant biotherapeutic eRB1.

Thiol groups and the disulphide bonding they direct are known to be key determinants of the biological function (18) and malfunction (19) of human proteins. Thiol engineering has previously been shown by others to facilitate the anchorage of protein units onto polymer capsules (20) and other immobilisation matrices (21). We also previously used protein engineering for removal of unpaired cysteine residues to reverse disulphide-driven multimerisation (22).

RB1 is a multidomain soluble protein with an even number of cysteine residues all of which are understood to form intramolecular disulphide bonds (Figure 1). The codon for a residue lying within an exposed loop of RB1 was converted to a cysteine codon using site directed mutagenesis to effect a single base pair substitution. The mutation was confirmed by sequencing and the engineered RB1 (eRB11) ORF was subcloned into pET29a in frame with a carboxy terminal hexahistidine tag to simplify purification.

3.2 Purification of eRB1

Poor results were observed with nickel affinity chromatography (data not shown) so a two-step purification procedure was developed using cation exchange followed by size exclusion chromatography. Cation exchange (CEX) was operated as a bind/elute process to remove impurities that had carried over from the previous stage and concentrate the protein solution down from 1-2L refolding buffer to 10-20mL total in elution buffer. For CEX, the refolded protein solution was dialysed into binding buffer (50 mM sodium acetate, pH 5.0), loaded onto a pre-equilibrated HiTrap SP FF Cation Exchange (GE Healthcare Sciences) column and eluted with a linear gradient of 0-1 M NaCl at a flow rate of 1 mL/min (Figure 5A). The fractions containing eRB1 in monomeric and dimeric forms (Figure 5B) were then pooled, dialysed into Dulbecco's phosphate buffered saline, concentrated to a volume of 5 mL and then separated on a Superdex 500 Size Exclusion Chromatography (GE Healthcare Sciences) column (Figure 6A). Purity of the eluted fractions was estimated at 95% by SDS-PAGE analysis (Figure 6B) and the yield of purified protein determined by absorbance at 280 nM (Table 1). In total, 108 mg of purified eRB1 was obtained, which correlates to a yield from fermentation of 27 mg/L. This was divided

approximately 50:50 between monomer and dimer forms. The monomer fraction was then pooled to use for further chemical crosslinking.

3.3 Chemi-dimerisation of remaining eRB1 monomer

Monomeric fractions from the size exclusion chromatography were pooled and reduced by incubation with tris(2-carboxyethyl)phosphine (TCEP) to remove any co-eluted dimer then chemically crosslinked with BMH to form a thioether-bridged dimer. Once the crosslinking reaction was complete, cation exchange chromatography was used to separate eRB1 from reactants (Figure 7A). Thioether bonds are far more resistant to reduction-based scission than disulphide bridges. As such the persistence of dimer bands during reducing SDS-PAGE is evidence of thioether-based eRB1 dimerisation (Figure 7B).

4. Conclusions

We were motivated to establish a high performance process for timely materialisation of the relatively low MW (<60kDa) biotherapeutic eRB1 for extensive pre-clinical characterisation. We intended that scaling up of each step will be straightforward and the process should be applicable to a range of different biotherapeutics that possess similar amino acid sequence properties as those of eRB1, depicted in Figure 1.

Figure 2 sets out our proposed 14-step production process with which we achieved greater than 95% purity in the final product. All steps of the process were scalable but the final SEC step is relatively slow so would need to be replaced with approaches such as anion exchange or hydrophobic interaction chromatography in order to achieve levels of purity mandated by regulators within acceptable timescales.

We successfully dimerised RB1 by point mutation to bring about a single amino acid substitution, generating eRB1, then subjecting the protein to oxidising conditions during an IB refolding step. We were also able to convert any remaining undimerised eRB1 protein to homodimers by thioether bridging of thiol groups, a method that has been applied to a variety of recombinant protein therapeutics, such as IgG1 monoclonal antibodies (27), somatostatin (28) and other complex heterodimers (29). Previously, when using laboratory methods to materialise RB1 variants we achieved ≤20% disulphide-bridged homodimerisation and the remaining monomer was of insufficient purity for successful chemi-dimerisation.

In conclusion, we present a largely scalable bench process for production of a homodimeric biotherapeutic protein that achieves high levels of performance with respect to the level of disulphide-based dimerisation (≥40%) and the level of purity, which is sufficient to enable chemi-dimerisation of remaining monomer. We anticipate this work will inform future laboratory-scale pre-clinical investigation of putative biotherapeutics of comparable MW and sequence.

Acknowledgements

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Figures

Figure 1. Schematic of therapeutic recombinant protein RB1. Hopps-Woods hydropathy analysis and sequence characteristics of RB1, hydrophilic residues are shown above central line, hydrophobic below.

Figure 2. High performenace bench scale process for eRB1 production. A) Process flow sheet of eRB1 production and isolation. B) Process diagram for which the process flow sheet provides a key.

Figure 3. Growth profile of *E. coli* cells harbouring eRB1 expression plasmid. Cell growth performance in a 7.5 L bioreactor. IPTG was added to 1mM concentration at OD_{600} = 56 to induce eRB1 expression. Data is representative of n=3 biological repeats.

Figure 4. **eRB1 IB isolation.** SDS PAGE analysis of inclusion body washing strategy. Lane 1: resuspended insoluble fraction post homogenisation (step 5 in Figure 2). Lane 2: purified inclusion body fraction post washing; Lane 3: purified RB1 monomer as marker.

Figure 5. **Isolation of refolded eRB1.** (A) Cation exchange chromatography of refolded eRB. Samples were eluted with a linear gradient of 0-1 M NaCl and a flow rate of 1 mL/min. (B) A selection of 1 mL fractions (fraction number indicated above lanes) were taken and analysed by non-reducing SDS PAGE to visualised monomer and dimer forms of eRB1. Red arrow indicates dimer. Purifided, monomeric RB1 was run in lane labelled ‘RB1’ for comaprison.

Figure 6. Purification of eRB1 monomer and dimer solutions. (A) Size exclusion chromatography of eRB1 containing fractions containing predominantly dimer or

monomer. (B) 5 mL fractions analysed by non-reducing SDS-PAGE to visualise monomer (lanes 240, 250 and 260) and dimer (lanes 270, 280 and 290). Black triangle indicates dimer MW, grey triangle indicates monomer MW.

Figure 7. Chemi-dimerisation of remaining eRB1 monomer. Crosslinking of eRB1 monomers with BMH renders them resistant to dimer-bond scission by reducing conditions. (A) Cation exchange chromatography was used to separate eRB1 from BMH reactants. (B) Photographs of reducing SDS PAGE performed on samples that contained disulphide-linked eRB1 dimers (eRB1 -SS-) and eRB1 monomer treated with BMH to drive thiother-linked dimerisation (eRB1 -S-). Black triangle indicates dimer MW, grey triangle indicates monomer MW.

Purification stage	Total protein (mg)	Purity of eRB1(%)	Dimer Percentage (%)
Insoluble fraction	ND	<10	ND
Washed inclusion bodies	211	80	NA
Cation exchange peaks	151	90	40
Size Exclusion fractions 271-300	53.2	>95	>95

Table 1 Protein concentration and purity obtained at different points in recovery.

Figure 1

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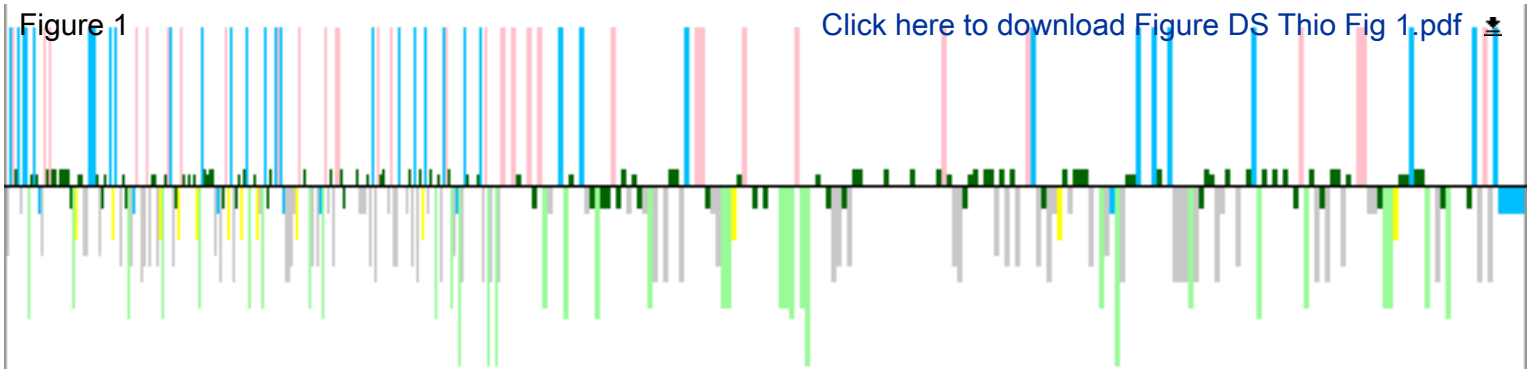

Acidic Aromatic Basic Aliphatic Polar Cysteine

Figure 2

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A)

1 7.5 L eRB-BL21 Fermentation

2 Harvested eRB-BL21

Resuspended in 50 mM Tris/50 mM NaCl, pH 7.4

3 Homogenisation (5 x 500 Bar)

4 Centrifugation (4°C, 6000 g, 1 hr)

5 Inclusion body fraction

6 Washing

7 8 M Urea/0.1 M Tris/2 mM DTT/440 mM GSH/1 mM EDTA pH 9.3 solubilisation

8 Refolding in 0.1 M Tris/0.5 M L-Arginine/2 mM EDTA/1 mM GSSG pH 8.5 4°C, 200 RPM, 72 hrs

9 Dialysis 2x against 50 mM sodium acetate pH 5

10 Microfiltration

11 Cation Exchange Chromatography

12 Dialysis 2x against DPBS

13 Microfiltration

14 Size Exclusion Chromatography

B)

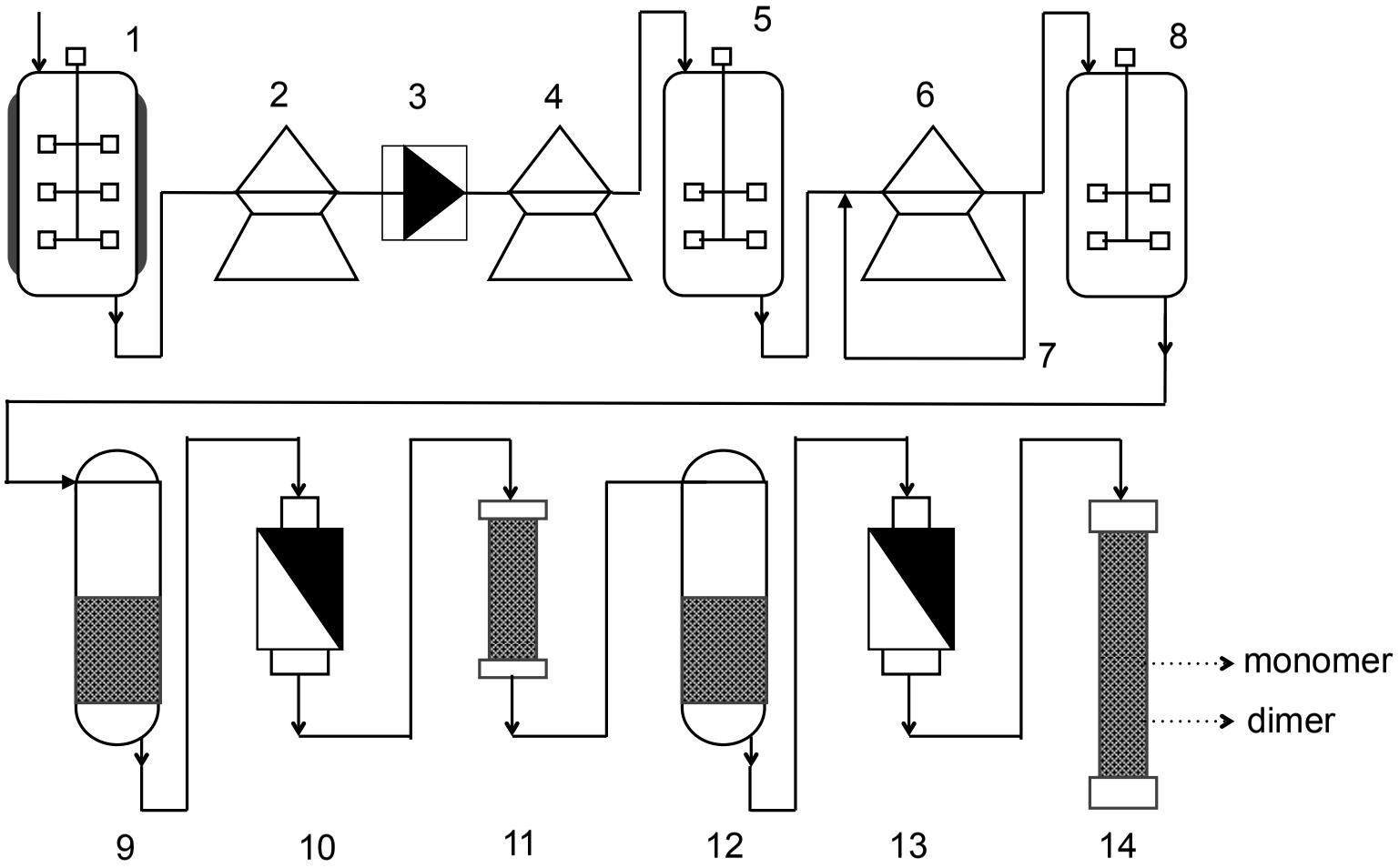

Figure 3

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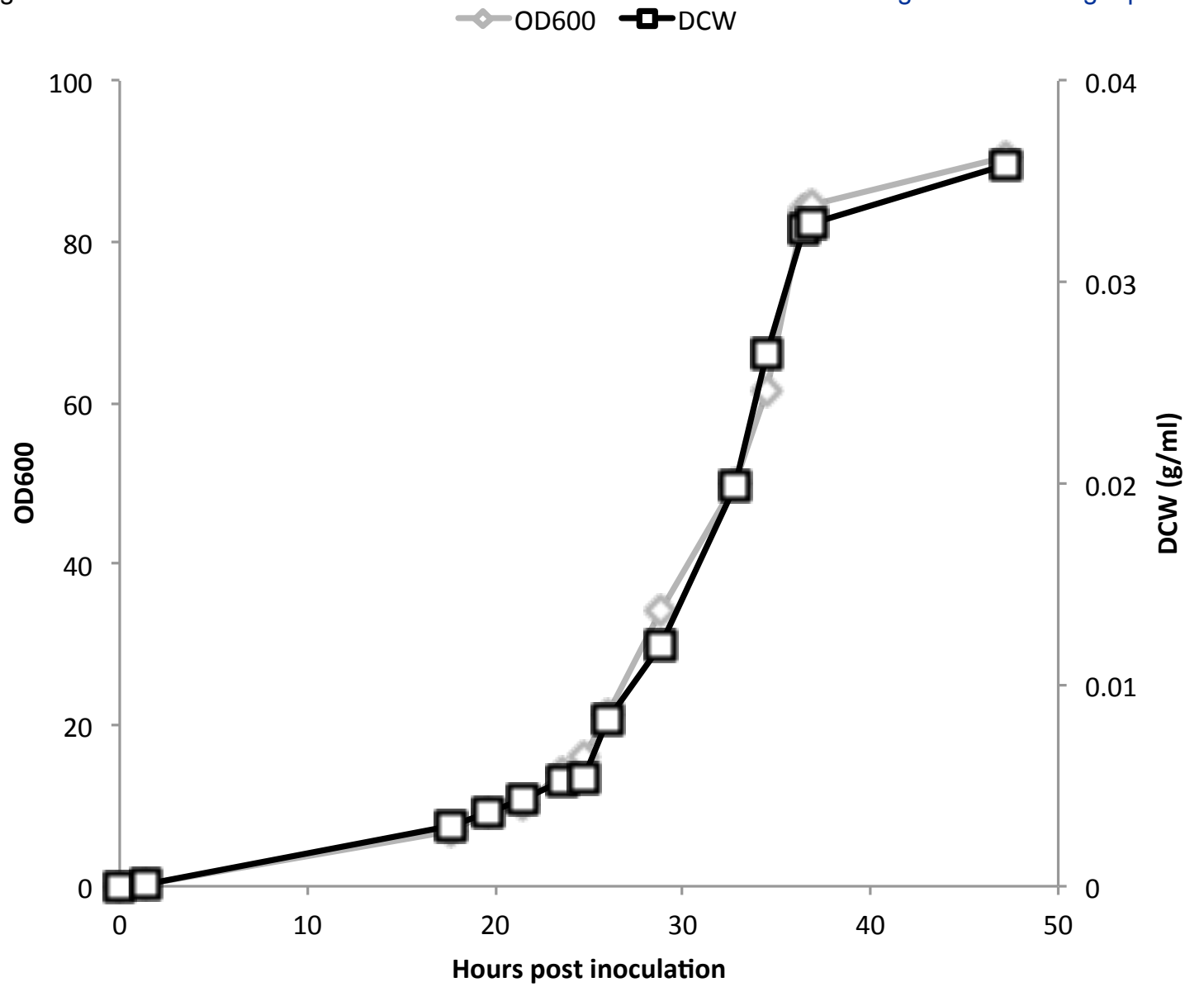

Figure 4

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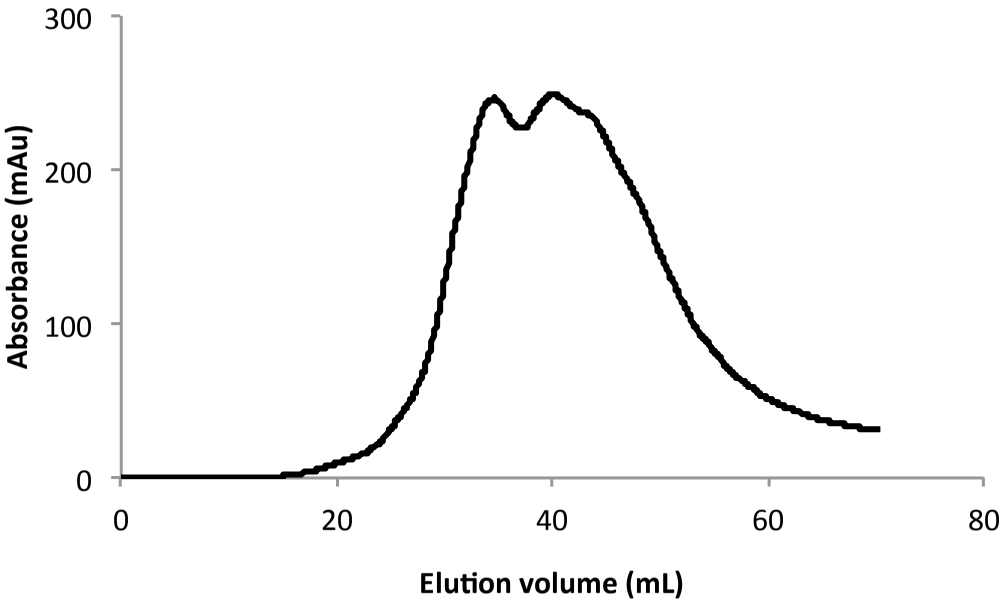
Figure 5

(A)



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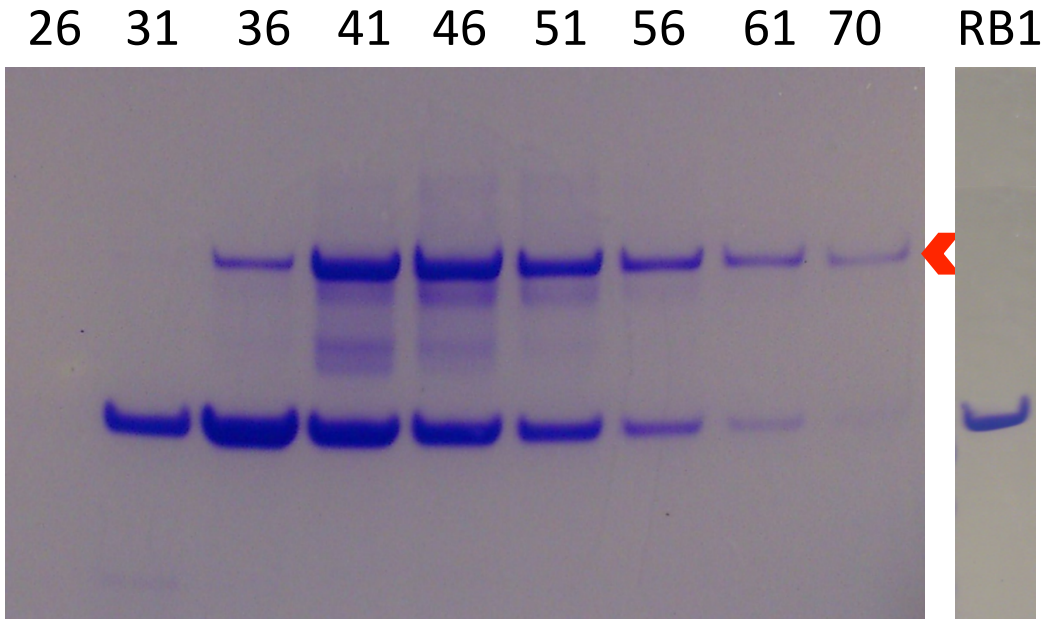

Figure 6

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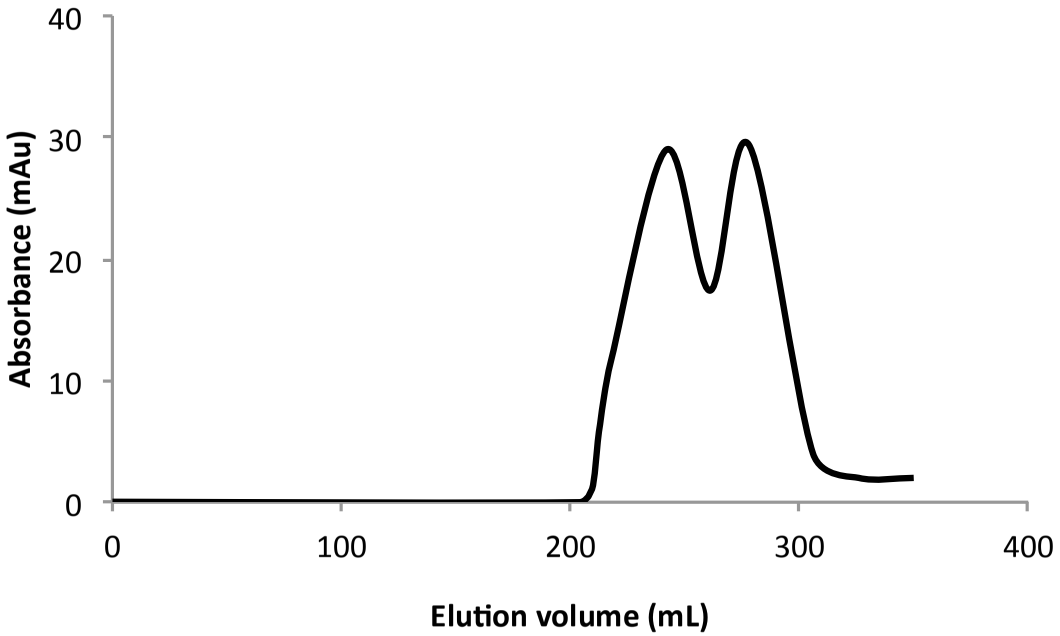

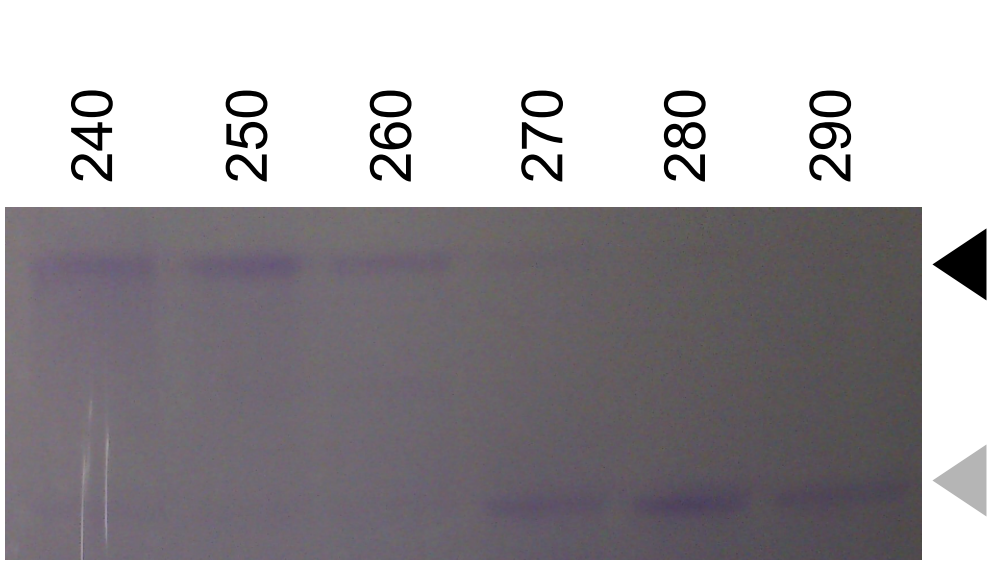

Figure 7

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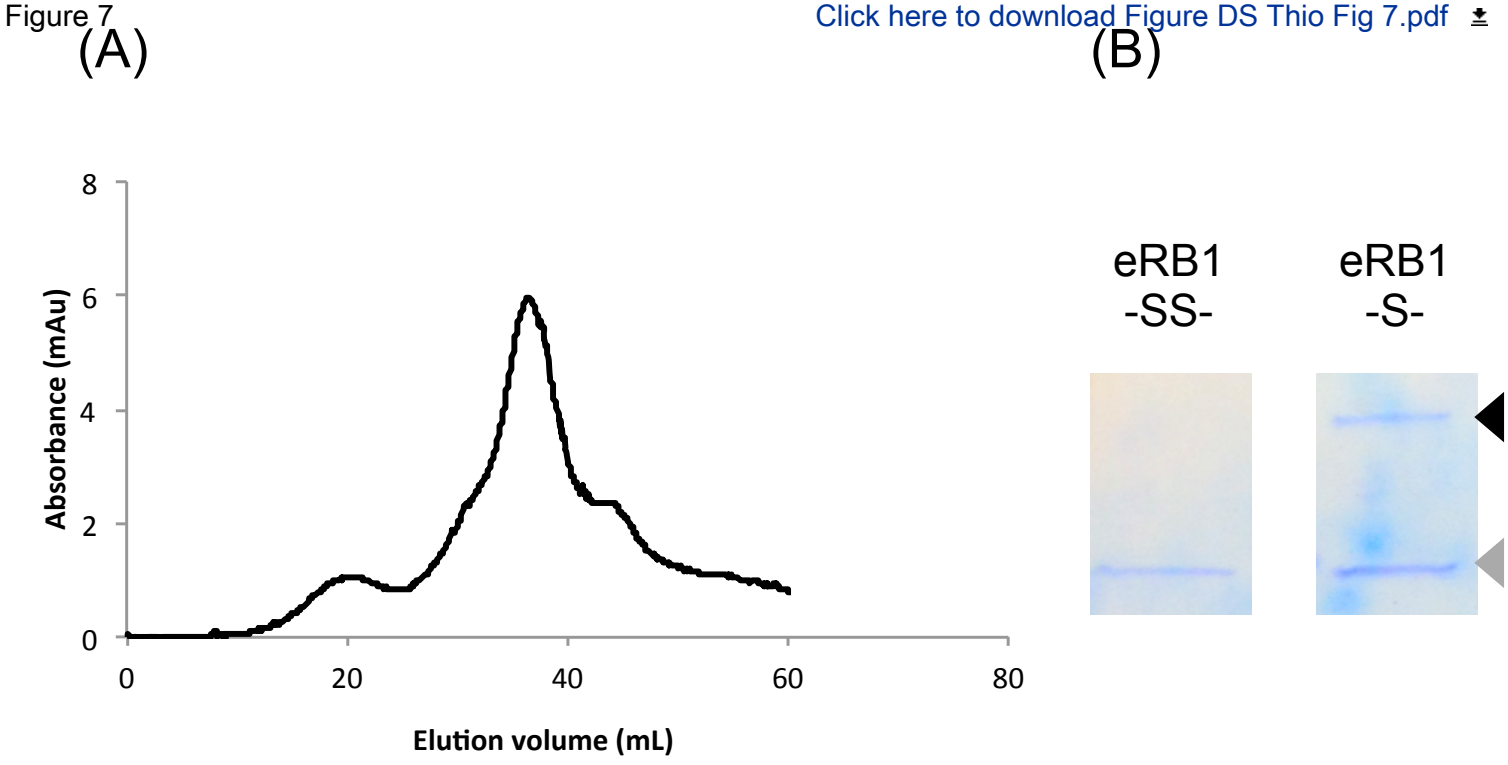

Cover Letter and Responses to Reviewers