Generation of novel trimeric fragments of human SP-A and SP-D after recombinant soluble expression in E. coli

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ABSTRACT

Surfactant treatment for neonatal respiratory distress syndrome has dramatically improved survival of preterm infants. However, this has resulted in a markedly increased incidence of sequelae such as neonatal chronic inflammatory lung disease. The current surfactant preparations in clinical use lack the natural lung defence proteins surfactant proteins (SP)-A and D. These are known to have anti-inflammatory and anti-infective properties essential for maintaining healthy non-inflamed lungs. Supplementation of currently available animal derived surfactant therapeutics with these anti-inflammatory proteins in the first few days of life could prevent the development of inflammatory lung disease in premature babies. However, current systems for production of recombinant versions of SP-A and SP-D require a complex solubilisation and refolding protocol limiting expression at scale for drug development.

Using a novel solubility tag, we describe the expression and purification of recombinant fragments of human (rfh) SP-A and SP-D using Escherichia coli without the need for refolding. We obtained a mean (± SD) of 23.3 (± 5.4) mg and 86mg (± 3.5) per litre yield of rfhSP-A and rfhSP-D, respectively. rfhSP-D was trimeric and 68% bound to a ManNAc-affinity column, giving a final yield of 57.5mg/litre of highly pure protein, substantially higher than the 3.3mg/litre obtained through the standard refolding protocol. Further optimisation of this novel lab based method could potentially make rfhSP-A and rfhSP-D production more commercially feasible to enable development of novel therapeutics for the treatment of lung infection and inflammation.

1. Introduction

Treatment of neonatal respiratory distress syndrome (RDS) with exogenous surfactant has dramatically increased survival of preterm infants (Horbar et al., 1993; Schoendorf and Kiely, 1997; Malloy and Freeman, 2000). However, the corollary to this is an increase in survivors after preterm birth living with ventilator related lung damage and oxygen toxicity. As a result, between 50–70% of extremely preterm neonates may go on to develop inflammatory and emphysematous-like lung damage. This can result in neonatal chronic lung disease and a prolonged requirement for oxygen throughout infancy and early childhood.

Natural lung surfactant is composed of a variety of phospholipids as well as surfactant proteins A (SP-A), SP-B, SP-C and SP-D (Perez-Gil, 2008). The main function of lipid surfactant and SP-B and SP-C is to lower the surface tension of the alveolar air-liquid interface at end expiration to prevent alveolar collapse and facilitate breathing. Contrastingly, SP-A and SP-D act mainly as essential innate immune defence proteins which have key roles in keeping the lung in a non-infected, hypo-responsive and non-inflamed state (Wright, 2005; Fakih et al., 2015).

There is a wealth of literature demonstrating that SP-A and SP-D are implicated in the innate clearance of viruses, fungi and gram negative and gram positive bacteria (Pastva et al., 2007; Watson et al., 2019; Ujma et al., 2019). Moreover, SP-A and SP-D have been shown to be involved in the clearance of dead and dying apoptotic cells and to have key immunomodulatory effects on dendritic cells, macrophages and T-cells (Borron et al., 1996; Lin et al., 2010). These functions are essential in maintaining the lung in a hypo-responsive state to prevent inflammatory damage of the thin alveolar-capillary membrane and minimise the
Natural-derived surfactant preparations available commercially currently are manufactured by organic solvent extracts from animal lungs and thus they do not contain the water-soluble SP-A and SP-D (Sato and Ikegami, 2012; Baroutis et al., 2003). Similarly, recombinant versions of SP-A and SP-D are not included in the new synthetic surfactant therapeutics (Sato and Ikegami, 2012; Salgado et al., 2014).

SP-A and SP-D are collectins, composed of a N-terminal region, a collagen-like tail, a neck and a globular ligand-binding head domain, also known as the carbohydrate recognition domain (CRD). These proteins form functional trimeric units which bind to carbohydrates in a calcium-dependent manner, alongside protein receptors (Hoppe and Reid, 1994; Zhang et al., 2001; Jakel et al., 2013). SP-A and SP-D trimers can further oligomerise into octadecamers or oligomers containing up to 32 trimeric units, respectively (Strang et al., 1986). Due to their large size and complex quaternary structure, the production of full-length recombinant SP-A and SP-D for therapeutic purposes has been problematic. This is due to obstacles such as low expression yields, requirement of eukaryotic expression systems, difficulties in handling and obtaining a defined oligomeric state, as well as a requirement for administration in EDTA to prevent agglomeration and preserve solubility (Salgado et al., 2014; Haagsman et al., 1990; Brown-Augsburger et al., 1996; Sato et al., 2010).

Smaller recombinant fragments of human (rh) SP-A1 and SP-D which lack the majority of the collagen-like domain have been produced and shown to form functional trimeric units. These fragments consist of the carbohydrate recognition domain (CRD), the α-helical neck domain and a short segment of eight G-X-Y repeats from the collagen domain. rfhSP-D has been well characterized structurally and functionally and demonstrated to maintain many of the functions of the full length protein (Watson et al., 2017; Clark, 2010; Madan et al., 2001). rfhSP-D has been produced and shown to be effective at neutralizing respiratory syncytial virus

Sub-cloned into a pT7 expression vector as a fusion gene with His6-NTwt or His6-NT*, with a 3C protease cleavage site between the two genes on the N-terminal side of rfhSP-A or rfhSP-D (Fig. 1). Standard cleavage and ligation procedures were then used to make the fusion gene constructs (Watson et al., 2017). Constructs were then used to transform chemically competent E. coli BL21 (DE3).

2.2. Protein expression

Glycerol stocks of transformed E. coli BL21 (DE3) were grown in LB medium containing 70 mg/L kanamycin. Initial growth was overnight at 37 °C with shaking (180 rpm). 5 mL of this culture was then used to inoculate a fresh 500 mL of LB medium, containing 70 mg/L kanamycin which was grown at 30 °C with shaking (180 rpm) until the OD600 was ~1. Expression was induced by addition of isopropyl-β-d-1-thiogalactopyranoside (IPTG) to
a concentration of 0.5 mM, expression was undertaken overnight at 20 °C with shaking (180 rpm). After harvesting the cells through centrifugation at 4,000 x g for 20 min, the pellet was resuspended in 30 mL of 20 mM Tris–HCl, pH 8.0 and the cell solution was stored at -20 °C for at least 24 h.

2.3. Purification of rfhSP-A and rfhSP-D

Cell suspensions were defrosted and inclusion bodies were harvested through centrifugation at 7,200 x g for 40 min (4 °C). The pellet was subsequently suspended in 20 mM Tris–HCl, 1 mM CaCl₂, 2 M urea, pH 8.0 and sonicated for 1 s pulses at 80% amplitude (for a total of 2 min) at 4 °C. Insoluble fractions were then removed through centrifugation at 30,000 x g for 30 min. To purify the protein, the supernatant was applied to a Ni-sepharose column (GE Healthcare) which had been equilibrated in 20 mM Tris–HCl, 1 mM CaCl₂, 2 M Urea, pH 8.0. Washing of the column was undertaken using 20 mM Tris–HCl, 5 mM imidazole at pH 4 with a decreasing amount of urea (2 M, 1 M, 0.5 M and no urea). The bound fusion protein was then eluted using 20 mM Tris–HCl with 300 mM imidazole, pH 8.0. Imidazole was removed through dialysis at 4 °C overnight using 20 mM Tris–HCl, pH 8. After concentration of the protein, it was cleaved in 20 mM Tris–HCl, pH 8 with 1 mM DTT using 3C protease at a 1:10 ratio (w/w) for 6 h, at 4 °C. rfhSP-A and rfhSP-D were then purified through reapplying to the Ni-sepharose to remove the His-tagged NT protein. rfhSP-D was purified as above but without the presence of urea.

2.4. Size-exclusion chromatography

Size exclusion chromatography was undertaken as previously described (Sorensen et al., 2009). Briefly, 200 μL of purified rfhSP-A or rfhSP-D was loaded onto a Superdex 200 h 10/30 column equilibrated in TBS with 5 mM EDTA, pH 7.4. The samples were run through at 0.3 mL/min and protein elution was detected using optical absorbance at = 280 nm, this was compared to molecular weight standards including 12.4 kDa cytochrome c, 29 kDa carbonic anhydrase, 66 kDa BSA, 150 kDa alcohol dehydrogenase and 443 kDa apoferritin.

2.5. Affinity chromatography

ManNAc and mannan-sepharose columns (15 mL) were produced in Southampton, as previously described (Sorensen et al., 2009). rfhSP-A or rfhSP-D were applied to the column in 20 mM Tris, 150 mM NaCl (TBS) with 5 mM CaCl₂, pH 7.4 using an Äkta 900 system, as previously described (Watson et al., 2017). Columns were then washed in 20 mM Tris, 1 M NaCl, 5 mM CaCl₂, pH 7.4. Protein was eluted in TBS with 5 mM EDTA, pH 7.4.

2.6. Western blot

Western blot analysis was undertaken as previously described (Watson et al., 2017) using a monoclonal mouse α-native human (nh) SP-A primary HYB 238 – 04 (1 mg/mL), diluted 1:1,000 or a polyclonal rabbit α-rfhSP-D antibody (1.6 mg/mL), diluted 1:1,000 (Duvoix et al., 2011), to identify rfhSP-A and rfhSP-D, respectively. Detection was undertaken using a HRP-conjugated Goat α-Mouse IgG (H + L) antibody (Life Technologies, UK) (62–6520), diluted 1:10,000.

3. Results

3.1. Soluble expression of rfhSP-A and rfhSP-D using NT*

We previously used NTₜₜ as an expression tag to successfully over-express rfhSP-A in E. coli cells. However, this tag resulted in the protein residing within the inclusion body containing fraction, thus it required a subsequent solubilisation and refolding step using 8 M urea (Watson et al., 2017). Using removable fusion proteins NT* and NT* cloned to rfhSP-A and rfhSP-D (Fig. 1), we attempted to express these fragments as soluble proteins.

NTₜₜ-rfhSP-A was expressed as a predominantly insoluble protein (Fig. 2A). However, through sonication using non-denaturing amounts of urea (2 M), almost 50% of the NTₜₜ-rfhSP-A1 fusion protein could be obtained in the soluble fraction (Fig. 2B). NT* in fusion with rfhSP-A allowed for similarly high levels of protein expression, (Fig. 2A). However, NT* allowed for nearly all of the fusion protein to be expressed in the soluble fraction (Fig. 2B). Both NT* and NTₜₜ allowed for high levels of rfhSP-D protein expression, the majority of which was expressed in the soluble fraction (Fig. 2C and D). Other solubility tags including Trx, OmpA and PelB were also used for comparison. However, these gave only low levels of expression of insoluble protein (data not shown).

3.2. Purification of rfhSP-A and rfhSP-D

The NT*–rfhSP-A and NT*–rfhSP-D fusion constructs were cloned to contain an N-terminal His₉ tag. This allowed purification of the fusion proteins by Nickel affinity purification. The purified NT*–rfhSP-A and NT*–rfhSP-D fusion proteins were subsequently cleaved with 3C protease to remove the NT* solubility tag (Fig. 1). After cleavage, rfhSP-A and rfhSP-D were then purified using a second round of nickel affinity purification. This removed the His₉ tagged NT* and 3C enzyme using negative selection (Fig. 3).

Purification by nickel affinity purification resulted in a highly pure rfhSP-A, with no observable contamination from NT* or any other protein, as determined by SDS-PAGE (Fig. 3 A and C). rfhSP-A was confirmed using western blot analysis for detection of rfhSP-A using a monoclonal antibody against native human SP-A (Fig. 3 B). Upon cleavage of NT*–rfhSP-D, an additional higher order contaminating band was also seen (Fig. 3 C). However, this was not recognized by the rfhSP-D western blot and was removed with subsequent purification by affinity chromatography (Fig. 3D and 5C). The identity of rfhSP-A and rfhSP-D were confirmed using mass spectrometry (data not shown).

This streamline purification technique of solubly expressed protein yielded a mean (± SD) of 23.3 (± 5.4) mg (n = 4) of highly pure rfhSP-A and 86 mg (± 3.5) of rfhSP-D (n = 3) per litre of bacteria. This compared with the expression of rfhSP-D using the standard protocol which required time consuming solubilisation and refolding steps and yielded only 15.75 (± 1.06) (n = 2) of total protein, which was highly contaminated with bacterial proteins (Fig. 3E).
3.3. rfhSP-A and rfhSP-D produced using NT* are trimeric

The formation of trimeric units is essential for the biological activities of SP-A and SP-D. Thus to determine the trimeric structure of purified rfhSP-A and rfhSP-D produced using NT*, they were analysed using size-exclusion chromatography. Size-exclusion chromatography demonstrated that a mean (± SD) proportion of 24 (± 4.3)% (n=4) of rfhSP-A produced using NT* eluted at the expected volume for trimeric rfhSP-A. This aligned with trimeric rfhSP-A produced using the previously used solubilisation and refolding protocol (Fig. 4A). However, a large proportion of rfhSP-A also had a higher apparent molecular weight of >443 kDa.

Comparatively 88.5 (± 4.0, n = 4)% of rfhSP-D expressed and purified using NT* eluted at the expected volume for trimeric rfhSP-D aligning with the elution volume of ManNAc purified rfhSP-D produced by our previous solubilisation and refolding protocol (Fig. 4B).

3.4. rfhSP-D produced using NT* is functional in binding to ManNAc

To demonstrate the functionality of rfhSP-A and rfhSP-D in binding to carbohydrates in a calcium-dependent manner, they were further purified using ManNAc-affinity chromatography. rfhSP-A produced using NT* did not bind to ManNAc-coupled sepharose columns or other carbohydrate columns including mannan and maltose (Fig. 5A). However, 68% of the rfhSP-D purified using NT* did bind to a ManNAc column in a calcium-dependent manner; this was able to be eluted specifically in the presence of EDTA (Fig. 5B).

Elution of rfhSP-D from the ManNAc column yielded a highly pure rfhSP-D preparation, as determined by SDS-PAGE (Fig. 5C). Manufacture using NT* with subsequent ManNAc affinity chromatography resulted in 57.5 mg/Litre of functional trimeric rfhSP-D. This is substantially higher than the 3.3 mg of ManNAc purified rfhSP-D produced using the standard solubilisation and refolding protocol.
4. Discussion

Functional trimeric SP-A and SP-D fragments could have therapeutic potential in limiting ventilator and oxygen induced lung inflammation in preterm infants to help reduce the development of neonatal chronic lung disease. However, the current expression and purification systems of rfhSP-A and rfhSP-D require a solubilisation and refolding process which is time-consuming and difficult to undertake on an industrial scale (Kaur et al., 2018). Here we demonstrate the over expression and purification of rfhSP-A and rfhSP-D in a streamline process using a novel solubility tag, NT*. Furthermore we demonstrate that rfhSP-D produced by NT* is trimeric and functional in binding to ManNAc in a calcium-dependent manner.

We have demonstrated a novel expression system for the streamline production of pure, trimeric and functional rfhSP-D which gave a final yield of 57.5 mg/litre after ManNAc affinity purification. This is
substantially higher than the 3.3 mg/litre obtained using the standard rhfSP-D process where 80–90% of solubilised protein is lost during refolding due to precipitation (Knudsen et al., 2007). This system also allowed the over expression and purification of rhfSP-A as a soluble protein, giving a yield of 23.3 (± 5.4) mg/litre, higher than the ~ 12 mg/litre of yields than previously obtained during expression with NT\text{wt} and refolding (Watson et al., 2017).

The majority of rhfSP-D expressed using this novel solubility tag was trimeric and functional in binding to ManNAC. However, only a quarter of rhfSP-A was of trimeric structure and no rhfSP-A bound to ManNAC, mannan or maltose affinity columns. This contrasts to rhfSP-A previously expressed as an insoluble protein using NT\text{wt} where a fraction of refolded rhfSP-A did bind to carbohydrate affinity columns. This difference in functionality could be due to the different expression environment in the soluble fraction of the bacterial cell compared to that during the refolding step (Heath et al., 2015; Schlegel et al., 2013). Furthermore, variation of expression and purification parameters could have an impact on the functionality of the end product and this remains to be understood (Schlegel et al., 2013). As compared with rhfSP-D, rhfSP-A required sonication in 2 M urea to allow dissociation from the inclusion bodies. Although this is unlikely to denature the protein, this could lead to slight alterations in the rhfSP-A structure which could impact on functionality. Requirement for 2 M urea is likely required due to the rhfSP-A interacting with the insoluble Escherichia coli cell fraction. SP-A is known to be inherently more lipophilic than SP-D, hence SP-A is frequently purified by butanol extraction (Watson et al., 2017). Purification of rhfSP-A expressed as a soluble protein using 2 M is different to the previously used refolding process in the presence of glycerol, which may stabilise the CRD. Further development purifying rhfSP-A in different solutions with various additives could be tested to try to obtain functional rhfSP-A using this novel approach. Furthermore, modification of expression conditions as well as the bacterial strains used could be trialled to optimise the soluble protein expression.

Notably, although this allows the soluble expression of higher yields of rhfSP-A and rhfSP-D than previously possible, this is still the first iteration of a lab scale system. Preliminary work expressing NT\textsuperscript{+}·rhfSP-D with a different enzyme cleavage site allowed for a >70% higher initial yield (Watson, 2016). However this construct could not be used due to non-specific enzymatic cleavage. This alternative construct which results in higher expression yields demonstrates that there is likely scope to further optimise constructs and expression conditions to obtain improved yields. Through further optimization, process development and use of industrial fermenters, it is likely that a yield of rhfSP-D in the range of grams per litre could be obtained, as has been done for other heterologous proteins (Fordjour et al., 2019; Lu et al., 2015). There are clear advantages of expressing rhfSP-A and rhfSP-D in E. coli due to cost and yield (Bill, 2014; Kaur et al., 2018). Other expression systems such as the yeast expression system using Pichia pastoris have previously been used to express rhfSP-D relatively cheaply and in high yields and may merit further investigation as to scalability (Hakansson et al., 1999). In this present soluble bacterial expression system, NT\textsuperscript{+} is cleaved from rhfSP-D through addition of an enzyme. This is removed through facilitated purification and incorporation of a His\textsubscript{6} tag. However, the impact of adding multiple steps on the scalability of this laboratory based process also merits consideration.

5. Conclusions

We have used a novel soluble expression system to allow over-expression of high levels of soluble rhfSP-A and rhfSP-D. This advance increases the feasibility of further detailed investigations on the structure function relationships of recombinant fragments of SP-A and SP-D compared to the native proteins. Furthermore, it represents a significant step forward to scalable development of rhfSP-D and rhfSP-A as novel therapeutics for the treatment of lung infection and inflammation.
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