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# Use of the amicyanin signal sequence for efficient periplasmic expression in *E. coli* of a human antibody light chain variable domain

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## Abstract

Periplasmic localization of recombinant proteins offers advantages over cytoplasmic protein expression. In this study signal sequence of amicyanin, which is encoded by the *mauC* gene of *Paracoccus denitrificans*, was used to express the light chain variable domain of the human κIO8/O18 germline antibody in the periplasm of *E. coli*. The expressed protein was purified in good yield (70 mg/L of culture) in one step from the periplasmic fraction by affinity chromatography using an engineered hexahistidine tag. Circular dichroism spectroscopy was used to determine if the secondary and tertiary structures of the protein and its thermal stability corresponded to those of the native folded protein. The expressed and purified protein was indeed properly folded and exhibited a reasonable thermal transition temperature of 53°C. These results indicate that the amicyanin signal sequence may be particularly useful for prokaryotic expression of proteins which are prone to mis-folding, aggregation or formation of inclusion bodies, all of which were circumvented in this study.

## Introduction

*Escherichia coli* is widely used in the production of recombinant proteins, including approximately 30% of therapeutic proteins that have been approved by the FDA [1]. Problems associated with protein expression in *E. coli* include degradation by intracellular proteases and the correct formation of disulfide bonds in the cytoplasm. The correct formation of disulfide bonds is especially important in biological therapeutics, where inefficient disulfide formation has been shown to limit the yield of the expressed protein [2]. High levels of cytoplasmic expression of recombinant proteins can also lead to formation of inclusion bodies and mis-folding of the proteins. This is especially prevalent during the



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expression of recombinant immunoglobulin domains [3–6]. These problems can be circumvented by translocation of the recombinant proteins into the periplasm of *E. coli*. The periplasm is an oxidizing environment compared to the cytoplasm. This facilitates formation of disulfide bonds that are often required for correct protein folding. The periplasm has a lower concentration of proteolytic enzymes and host cell proteins in general [7]. Thus, the periplasm is enriched in the relative concentration of the recombinant protein which facilitates its purification. For the above reasons the formation of inclusion bodies in the periplasm is much less likely than in the cytoplasm. Recombinant proteins are translocated to the periplasm via membrane-associated secretion systems that recognize an N-terminal signal sequence. The cleavage of the signal peptide during export of the recombinant protein to the periplasm yields a protein with its correct N-terminal residue. A variety of signal peptides have been used for this purpose. Some of the most widely used signal peptides are pelB from *E. carotovora* and ompA, DsbA, and TolB from E. *coli* [8–10]

Amicyanin [11] is a periplasmic type I copper protein, referred to as a cupredoxin [12], which is encoded by the *mauC* gene [13] of *Paracoccus denitrificans*. Recombinant amicyanin was previously expressed in *E. coli* at high levels [14]. In this study, we describe the use of the N-terminal signal sequence of amicyanin to express the light chain variable domain ($V_L$) of a human antibody in the periplasm of *E. coli*. The κI O8/O18 germline antibody $V_L$ was chosen for expression as it is a well characterized member of this family [15]. Correct folding of this protein requires formation of a disulfide bond and as with many antibodies and single chain fragments, aggregation and incorrect folding of these recombinant proteins has been problematic [16]. The folding and stability of this $V_L$ has previously been characterized by circular dichroism (CD) and thermal stability studies [15]. Thus, it was possible to assess the integrity of the recombinant protein that was expressed in this study by comparison to those results. The results of this study demonstrate the utility of the amicyanin signal sequence as an alternative for recombinant protein expression in *E. coli*. It should also be noted that an increasing number of recombinant human single chain fragments are being developed for therapeutics and so there may be additional applications of the results which are presented.

## Materials and Methods

### Protein expression

DNA encoding the κI O8/O18 germline $V_L$ with the N-terminal signal sequence of *mauC* from *P. denitrificans* and a C-terminal hexahistidine tag was codon-optimized for expression in *E. coli*, and synthesized by GenScript (Piscataway, NJ). The sequence of the DNA and the amino acid sequence of the protein that it encodes are shown in Table 1. The synthetic gene was cloned into a pET11a expression plasmid at the Nde1 and BamH1 restriction sites. *E. coli* BL21(DE3) cells were transformed with this plasmid, and cells were cultured in LB media at 37° C in the presence of 100 μg/ml ampicillin. When the $A_{600}$ of the culture reached 0.6, 0.4 mM IPTG was added to the culture to induce expression and incubation was continued at 30° C for 12 hours.

## Protein purification

The recombinant κI O8/O18 $V_L$ was purified from the periplasmic fraction of the *E. coli* cells. The periplasmic fraction was obtained using a lysozyme/osmotic shock method [11, 17]. The harvested cells were resuspended in 10 mM Tris-HCl buffer, pH 8.0, at a ratio of 6 mL/g of wet cell weight. The buffer also contained 20% w/v sucrose, 0.7 mM EDTA, 2 mg/mL of lysozyme, 1 mM $MgCl_2$, 0.01 mg/mL of DNase and 200 μM phenylmethylsulfonyl fluoride. After incubation for 20 min at 30° C with shaking, an equal volume of $H_2O$ was added and incubation continued for a total of one hour. The spheroplasts were then removed by centrifugation and the periplasmic fraction was in the supernatant. The His-tagged κI O8/O18 $V_L$ was purified from the periplasmic fraction by affinity chromatography using a Ni-NTA column (Qiagen). Protein concentration was determined from the absorbance of the pure protein at 280 nm using an extinction coefficient of 16,055 $M^{-1}$ $cm^{-1}$ that was calculated from the amino acid composition of the protein using the Expasy ProtParam tool (http://web.expasy.org/protparam/) [18].

## Protein characterization

Size exclusion chromatography was performed with a HiPrep 16/60 Sephacryl S-300 HR column (GE Healthcare). The equilibration and elution buffer was 10 mM potassium phosphate, pH 7.5, with 150 mM NaCl. The flow rate was 1.0 mL/min. Methylamine dehydrogenase, cytochrome *c*-553, cytochrome *c*-551i, and amicyanin with molecular weights of 124, 30, 22, and 11.5 kDa, respectively, were used as standards.

CD spectra were recorded using a J-810 spectropolarimeter equipped with a Peltier temperature controller (Jasco corp., Tokyo, Japan). Samples contained 3 μM protein in 10 mM potassium phosphate buffer, pH 7.5. For temperature-dependence studies, samples were equilibrated at each temperature for 2 min before CD measurements were recorded. The measured ellipticity, $\theta_{meas}$, was converted to mean residue molar ellipticity [θ], through the formula $[\theta] = \theta_{meas}/nlc$, where $n = 113$ is the number of amino acid residues in the protein, $l$ is the optical pathlength in mm, and $c$ is the molar concentration of the protein. Thermal unfolding of the protein secondary structure was determined from the increase in the negative CD signal at 205 nm which results from an increase in the fraction of unordered structure [19]. The value of the thermal transition temperature ($T_m$) was determined as the inflection point of the sigmoidal temperature-dependence curves of the corresponding normalized signal intensity, which was identified as the extremum of the first derivative with respect to the temperature. The fraction of folded protein at a given temperature $T$ was determined using eq 1 where [θ] is the mean residue molar ellipticity at 205 nm and the subscript indicates the temperature. Values of [θ] at 40° C and 70° C were deemed to correspond to 100% and 0% folded state, respectively.

$$Fraction\,folded = \frac{[\theta]_T - [\theta]_{70}}{[\theta]_{40} - [\theta]_{70}} \quad (1)$$

## Results

### Protein expression and purification

The recombinant κI O18/O8 germline $V_L$ was successfully expressed in *E. coli* and purified. The yield of pure protein was 70 mg/L of cell culture. The purified protein ran as a single band on SDS-PAGE (Figure 1A). When the protein was subjected to size exclusion chromatography it eluted as a single peak consistent with it being present exclusively as a monomer (Figure 1B). Comparison of the elution volume of the $V_L$ to other protein standards indicated that it eluted at a position corresponding to a molecular mass of about 9 kDa which is lower than that determined by SDS-PAGE and calculated from the amino acid sequence of 12.8 kDa. However, this observation is consistent with anomalously lower values for molecular mass determined by size exclusion chromatography that have been previously observed with $V_L$ domains [20–22]. Since the expressed protein is an antibody domain and not an enzyme, it does not have an activity to assay. Evidence for the structural and functional integrity of the expressed protein was provided by the demonstration that it binds to protein L resin, which is specific for antibody kappa light chains, and is often used for affinity purification of immunoglobulins [23]. In this study we chose to add a His-tag for purification because the Ni-NTA superflow resin used for purification has a much higher capacity for bound protein than the protein L resin. It was also confirmed by Western blot that the purified protein reacted with an anti-His-tag antibody. These results show that use of the amicyanin signal sequence allowed for expression of high levels of monomeric $V_L$ in the periplasm, which could be easily purified in a single step with no evidence of aggregation or inclusion bodies.

### Protein structure and stability

The secondary structure of the κI O18/O8 $V_L$ has previously been characterized by CD spectroscopy [15]. As such, the CD spectrum of the purified recombinant protein was obtained and compared with the published data. The spectrum shown in Figure 2A (solid line) is essentially identical to that previously reported for κIO18/O8 $V_L$. It has a β-sheet secondary structure as indicated by the minimum around 216 nm. The spectrum also exhibits a second minimum at 232 nm, which can be assigned to type I β-turn structure [24]. The minimum between 200–205 nm that appears at higher temperatures (Figure 2A, dashed line) is generated by unordered structure [19]. These features of the CD spectrum are essentially identical to those reported for the native protein and are consistent with the x-ray crystal structure of the germline Vk1 O18/O8 light chain variable domain [15] (PDB entry 2Q20).

These results indicate that the recombinant $V_L$ protein that was expressed in the periplasm using the amicyanin signal sequence is properly folded with regard to secondary and tertiary structures. It is also important to assess the thermal stability of this recombinant protein. CD spectra were recorded at temperatures from 40° C to 70° C. In Figure 2A the spectra of the native protein at 40° C, completely denatured protein at 70° C, and the protein at the $T_m$ are shown. The $T_m$ of the protein, as determined from the temperature-dependent perturbations of the CD signal at 205 nm was 53° C (Figure 2B). This value is in agreement with the previously reported value of 56° C for κI O18/O8 $V_L$ 15].

## Discussion

As stated earlier, there are certain advantages to expressing recombinant proteins in the periplasm of *E. coli* rather than in the cytoplasm. The amicyanin signal sequence is shown to be an efficient tool for periplasmic recombinant protein expression. It may also be particularly useful for expression of proteins which are prone to mis-folding or formation of inclusion bodies. The *mauC* gene encodes amicyanin, a Type I copper protein that is localized in the periplasm [11]. It was shown that incorporation of the very tightly bound copper occurs during the folding of amicyanin in the periplasm [25]. In cases such as this, the timing of the translocation of the protein is important to avoid mis-folding and aggregation that can result from unavailability of copper at the critical point in protein folding. Recombinant expression of $V_L$ proteins and immunoglobulins has also been problematic due to the tendency to form inclusion bodies. For expression of some proteins such amicyanin and κI O18/O8 $V_L$, as well as many others, the level of recombinant protein expression may not necessarily be as important as the timing and rate at which the protein is being expressed and co-translationally translocated into the periplasm. The amicyanin signal sequence may be a useful alternative to other commonly used signal sequences for expression of proteins in *E. coli* and other prokaryotes.

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## Abbreviations

| | |
|---|---|
| **$V_L$** | light chain variable domain |
| **CD** | circular dichroism |
| **$T_m$** | thermal transition temperature |

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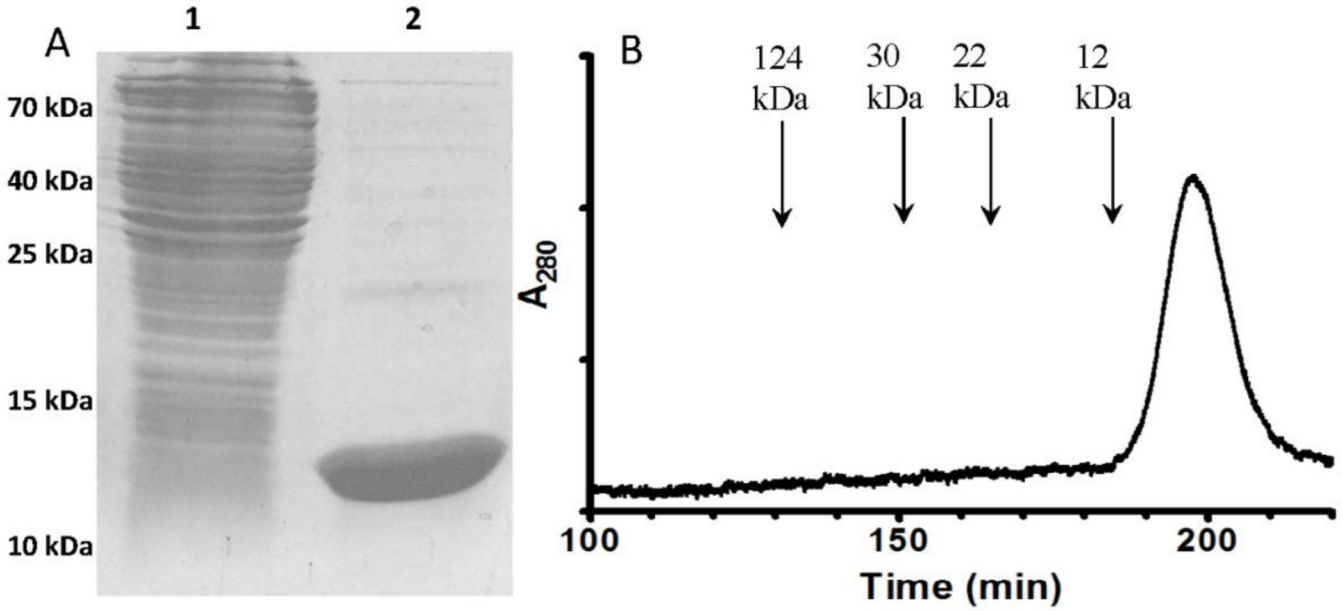


**Figure 1.**

SDS-PAGE and size exclusion chromatography of the expressed and purified κIO18/O8 $V_L$. **A.** SDS-PAGE was performed using a 12.5% gel. Lane 1 is a whole cell extract of the *E. coli* cells from which the recombinant $V_L$ was purified. Lane 2 is the protein that was purified from the periplasmic fraction of the cells. The positions of MW markers which were also run on the gel are indicated. **B.** The elution profile obtained when the purified recombinant $V_L$ was subjected to size exclusion chromatography is shown with the positions of elution of other proteins of known molecular weight indicated.

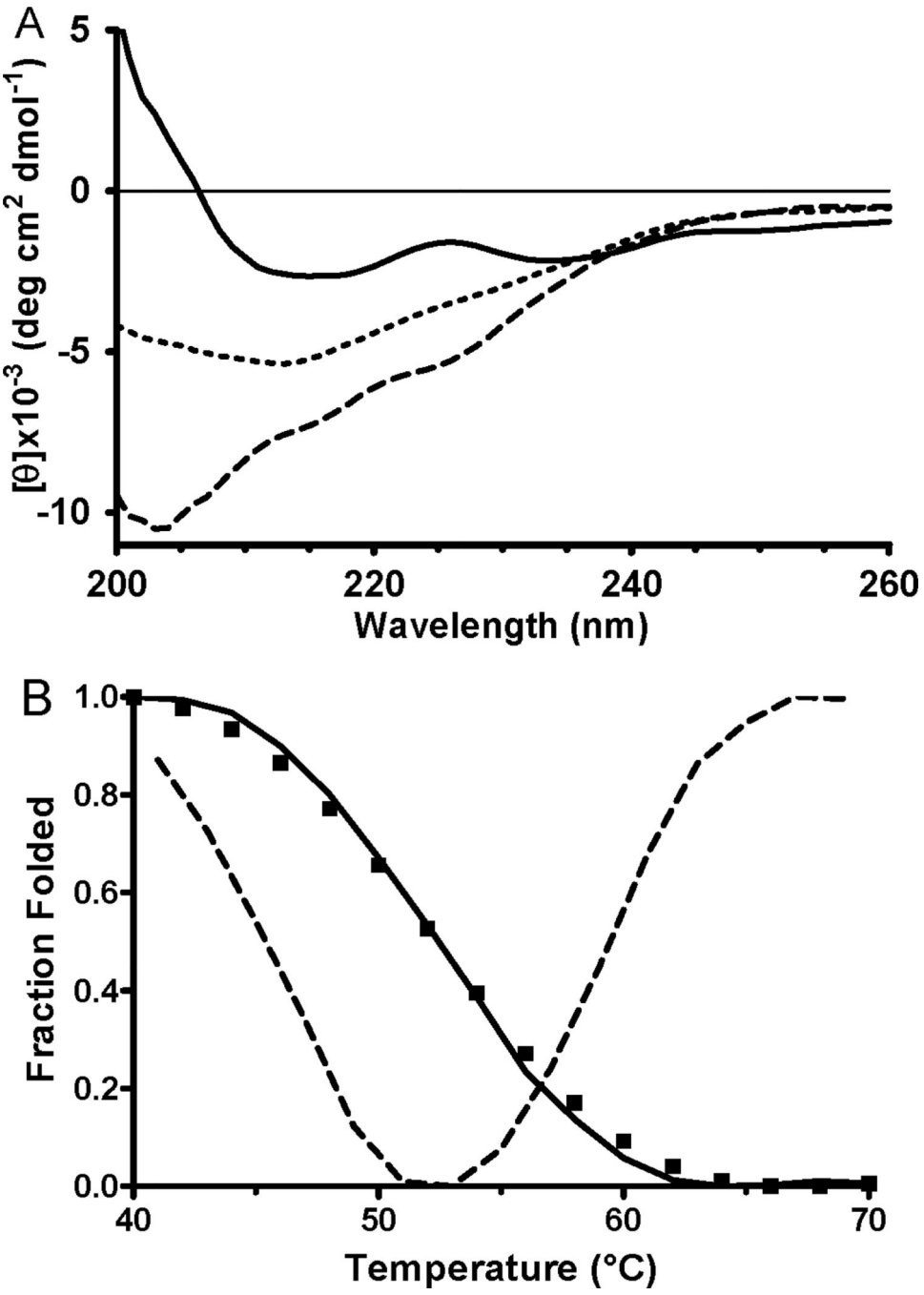


**Figure 2.**

CD spectra of purified recombinant κI O18/O8 $V_L$ and determination of the $T_m$ for the protein. CD spectra of the protein were recorded at a range of temperatures up to 70°C. **A.** For clarity only three spectra are shown which were recorded at 40°C (solid line), 53°C (short dashed line) and 70°C (long dashed line). **B.** The values of normalized mean residue molar ellipticity at 205 nm as a function of temperature are shown (solid line) as well as the 1st derivative of that line which was used to determine the $T_m$ (dashed line).

## Table 1

Sequences of the synthetic gene used to express κI O18/O8 $V_L$ and the protein that is encoded by the gene.

**ATGATTTCCGCTACCAAAATCCGCTCATGCCTCGCGGCCTGTGTCTTGGCTGCCT**
**TTGGAGCCACCGGAGCCCTTGCCTC**GACATTCAAATGACTCAATCCCCGTCATCC
CTGTCAGCGAGTGTCGGTGATCGCGTCACGATCACGTGCCAGGCGTCTCAAGACA
TTAGCAACTACCTGAATTGGTACCAGCAGAAACCAGGTAAGGCCCCGAAACTCTTG
ATCTACGACGCGTCCAATTTGGAAACAGGCGTGCCGAGTCGCTTTAGCGGTAGCG
GAAGCGGCACCGATTTCACCTTCACCATCAGTTCCCTTCAGCCGGAAGACATCGC
CACCTACTATTGTCAACAGTATGACAATCTGCCATATACGTTTGGCCAGGGCACCA
AACTGGAAATCAAG**CACCATCATCATCATCAT**TAG

**M I S A T K I R S C L A A C V L A A F G A T G A L A** D I Q M T Q S P S S L S A S V
G D R V T I T C Q A S Q D I S N Y L N W Y Q Q K P G K A P K L L I Y D A S N L E T
G V P S R F S G S G S G T D F T F T I S S L Q P E D I A T Y Y C Q Q Y D N L P Y T
F G Q G T K L E I K **H H H H H H**

(Top) The DNA sequence of the gene encoding κIO18/O8 $V_L$ with the N-terminal *mauC* signal sequence and the sequence encoding the C-terminal hexahistidine tag underlined.

(Bottom) The amino acid sequence encoded from this gene is shown. The N-terminal signal sequence which is cleaved during expression and hexahisidine tag that is used for affinity purification are underlined.