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Non-chromatographic preparative purification of enhanced green fluorescent protein

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ABSTRACT

Recombinant enhanced green fluorescent protein (eGFP) is used as a marker in numerous applications in biomedical research and diagnostics. For these applications, the macromolecule needs to be provided in a highly purified form. The conventional purification process of eGFP usually consists of multiple subsequent preparative chromatography steps. Since this procedure is costly and time-consuming, an alternative chromatography-free purification process was investigated. This process was a combination of three-phase partitioning (TPP) and preparative crystallization including an ultrafiltration/diafiltration (UF/DF) intermediate step. After the TPP step, eGFP with a purity level suitable for preparative crystallization of 82.5–85.0% and a yield of 84–92% was obtained depending on the scale. After cross-flow UF/DF, the crystallization was performed in parallelized mL-scale stirred tanks. A favorable robust crystal morphology was obtained combined with fast crystallization kinetics when two polyethylenglycols and ethanol were used simultaneously as crystallization additives. The crystallization process can easily be scaled-up to obtain large amounts of highly purified, concentrated eGFP with a purity >99% after a crystal wash step and resolubilization. The proposed chromatography-free purification procedure gives reason to expect significant reductions of costs and required process time compared to conventional preparative chromatography. Q2

1. Introduction

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Green fluorescent protein (GFP) was originally discovered in 1961 while purifying the bioluminescent protein aquorin from the jellyfish *Aequorea* (Shimomura et al., 1962). GFP has proven to be a valuable tool for widespread applications in biomedical research. GFP can be passively applied as e.g. a reporter gene, cell marker, or fusion tag, and can be actively applied as an indicator for e.g. protease action, transcription factor dimerization, or Ca^{2+}-sensitivity (Tsien, 1998). GFP is particularly suited as a marker for gene expression and localization of proteins in living organisms since its fluorescence intensity is not influenced by exogenous substrates and cofactors (Chalfie et al., 1994; Misteli and Spector, 1997). Purified GFP was characterized based on X-ray diffraction analyses of its crystals. The purification procedure prior to crystallization consisted typically of multiple chromatography steps (Deschamps et al., 1995). Purified GFP was first crystallized by Morise et al. (1974). Perozzo et al. (1988) crystallized GFP for X-ray diffraction analysis using vapor diffusion techniques (McPherson, 1982). A recombinant GFP (S65T) mutant expressed in *Escherichia coli* was reported by Heim et al. (1995) which showed a four- to six-fold increase in amplitudes of single excitation peaks compared to the wild type. This protein and other mutants were later called enhanced green fluorescent protein (eGFP).

Due to the use of eGFP in above mentioned biomedical applications, the protein needs to be available in a highly purified form, preferably >99% purity, in larger amounts. In order to achieve the high level of purity, several sequential preparative packed-bed chromatography procedures are usually applied after homogenization and clarification of the fermentation broth. However, the chromatography procedures are known to have low purification capacities, to be time-consuming, and costly. Therefore, non-chromatographic alternatives were sought or combinations of preparative chromatography and non-chromatographic methods

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were investigated in order to reduce the number of chromatography steps. In this context, purification by aqueous two-phase extraction with polyethylene glycol (PEG)/salt followed by precipitation by Zn^{2+} (Jain et al., 2004a) and purification by aqueous two-phase extraction with PEG/Na-polyacrylate was reported (Johansson et al., 2008). Another non-chromatographic purification alternative is three-phase partitioning (TPP) which has been successfully applied to numerous proteins (Pike and Dennison, 1989; Dennison and Lovrien, 1997; Przybycien et al., 2004; Ward, 2009; Ward and Swiatek, 2009). This procedure is typically applied as an early step in downstream processing after broth homogenization and clarification. Due to the extraordinary stability of GFP, this method involving salting-out using ammonium sulfate in combination with solvent precipitation using *t*-butanol is applicable without difficulty as reported in literature (Jain et al., 2004b). The combination of TPP followed by hydrophobic interaction chromatography was described by Vessoni Penna et al. (2003, 2004, 2005).

However, the aim of obtaining a high final purity >99% was not met in the above mentioned literature. On the other hand, it was shown that preparative crystallization in combination with a simple appropriate preceding purification step can lead to proteins with the desired high purity in short processing times at high yields (Hebel et al., 2013a; Smejkal et al., 2013a). It was further shown previously that the preparative crystallization step in stirred-tank crystallizers was easily scalable from the mL-scale to the L-scale (Hebel et al., 2013b; Smejkal et al., 2013b). As to the knowledge of the authors, preparative crystallization as an alternative purification step of eGFP in stirred tanks was not reported in literature so far. Hence, the aims of the present work were: (i) apply TPP as preceding step to purify an eGFP mutant to a level which is expected to be appropriate for crystallization and perform an intermediate ultrafiltration/diafiltration (UF/DF) step, (ii) perform non-agitated µL-scale vapor diffusion and micro-batch crystallization experiments of the eGFP mutant based on the conditions from the above described literature, and (iii) transfer the obtained experience to the stirred mL-scale as described by Hekmat et al. (2007) and investigate the preparative crystallization step in these scalable mL-scale stirred-tank crystallizers. This work describes the development of a novel purification process. It was not the aim to demonstrate the production of purified eGFP as a stand-alone product.

2. Materials and methods

2.1. Batch production of eGFP and protein assays

The eGFP used had two point mutations (F64S, S65T) with a 6xHis-tag attached directly to the N-terminus without spacer. The total number of amino acids was 244, the molecular weight was 27.66 kDa, and the theoretical pI was 6.0. Data on the solubility and other thermodynamic properties were not available. This protein was overexpressed in *E. coli* BL21(DE3) (pQE vector backbone, T5 promoter) which was cultivated in a 50 L stirred-tank bioreactor (Bioengineering AG, Wald, Switzerland) using an auto-induction medium containing 5 g L^{-1} lactose (Riesenberg et al., 1991). Batch cultivation was at $T = 37\,^{\circ}C$ and the pH was controlled at 6.8 using a 25% NH_4OH-solution. The fermentation broth was homogenized directly by three passages with intermediate cooling using a high pressure homogenizer type Ariete NS3015 (GEA Niro Soavi, Parma Italy) at 800 bar. The flow rate was 150 L h^{-1} and the temperature was controlled in order not to exceed 30 °C. The homogenized solution was then clarified batch-wise at room temperature using a floor-standing centrifuge type Rotixa 50 RS with a nominal capacity of 4 L × 1 L at 4000 × g for 10 min (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The total protein concentration of the solutions was measured by Bradford assay (Bradford, 1976). The concentration of the eGFP was measured by fluorescence assay (Elsliger et al., 1999). The excitation wavelength was 485 nm, the emission wavelength was 515 nm. The pH dependency of the fluorescence assay was accounted for in the data analysis by calibrating at two different pH levels of pH 6.5 and pH 8.0. Both assays were performed in a 96-well microplate reader type Tecan Infinite M200 (Tecan Group AG, Männedorf, Switzerland).

2.2. Purification of eGFP by immobilized metal affinity chromatography, anion exchange chromatography, and size exclusion chromatography

Purification of eGFP to >99% purity by SEC-HPLC for calibration of protein assays and sitting drop crystallization screening experiments was performed at room temperature by four consecutive chromatography steps using an ÄKTA explorer 100 chromatography system (GE Healthcare, Munich, Germany): (1) immobilized metal affinity chromatography (IMAC), (2) first size exclusion chromatography (SEC) step, (3) anion exchange chromatography (AEX), and (4) second SEC step. The initial recovery of eGFP from the clarified cell homogenate was performed by IMAC using a 5 mL prepacked HisTrap FF crude column (GE Healthcare, Munich, Germany) which was equilibrated with binding buffer (50 mM NaH_2PO_4, pH 7.9, 500 mM NaCl) in advance. Impurities were removed using a step gradient to 4% elution buffer (50 mM NaH_2PO_4, pH 7.9, 500 mM NaCl, 500 mM imidazole) and the target protein was eluted by a step gradient to 100% elution buffer. Target protein containing fractions were pooled and the buffer was subsequently exchanged for AEX binding buffer (50 mM Tris pH 8.5) in a first SEC step using a HiPrep 26/10 desalting column (GE Healthcare, Munich, Germany). The desalted fractions containing eGFP were then loaded onto a self-packed Unosphere Q column (1 cm × 13 cm, column volume 10 mL, Bio-Rad Laboratories, Munich, Germany) at a flow rate of 2 mL min^{-1}. eGFP was eluted with help of a linear gradient from 0% to 50% AEX elution buffer (50 mM Tris, pH 8.5, 1 M NaCl) over five column volumes. Aggregates and further impurities were removed by a second SEC step by injecting 2 mL of the AEX-purified eGFP solution onto a HiLoad 16/600 Superdex 75 pg column (GE Healthcare, Munich, Germany) and eluting isocratically with a continuous flow of crystallization buffer (50 mM Tris, pH 8.0) at a flow rate of 1 mL min^{-1}.

2.3. Purification of eGFP by three-phase partitioning and hydrophobic interaction chromatography

The three-phase partitioning (TPP) method consisted of two steps (Dennison and Lovrien, 1997). In the first step, a protein solution saturated with ammonium sulfate (30% saturation) was thoroughly mixed in a vortexer for 10 min with pure *t*-butanol (volume ratio 1:1). After centrifugation at room temperature at 4000 × g for 10 min using a laboratory centrifuge type 5415 R (Eppendorf Zentrifugen GmbH, Leipzig, Germany), a three-phase system was formed with an upper organic phase, a semi-solid interphase, and a lower aqueous phase. The upper phase contained host cell proteins (HCP), lipids, and other hydrophobic substances. The interphase consisted mainly of precipitated HCP. The lower phase contained mainly dissolved eGFP and some hydrophilic HCP. In the second step, the lower phase which was separated from the upper phase and the interphase was again thoroughly mixed with pure *t*-butanol in a vortexer for 10 min (volume ratio 1:2). Again, a three-phase system was formed where the upper phase consisted predominantly of *t*-butanol, the interphase was mainly precipitated eGFP, and the lower phase contained hydrophilic HCP. After centrifugation at 4000 × g for 10 min, the upper and lower phases were discarded and the precipitated eGFP was dissolved in

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