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Mild and cost-effective green fluorescent protein purification employing small synthetic ligands

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ABSTRACT

The green fluorescent protein (GFP) is a useful indicator in a broad range of applications including cell biology, gene expression and biosensing. However, its full potential is hampered by the lack of a selective, mild and low-cost purification scheme. In order to address this demand, a novel adsorbent was developed as a generic platform for the purification of GFP or GFP fusion proteins, giving GFP a dual function as reporter and purification tag. After screening a solid-phase combinatorial library of small synthetic ligands based on the Ugi-reaction, the lead ligand (A4C7) selectively recovered GFP with 94% yield and 94% purity under mild conditions and directly from *Escherichia coli* extracts. Adsorbents containing the ligand A4C7 maintained the selectivity to recover other proteins fused to GFP. The performance of A4C7 adsorbents was compared with two commercially available methods (immunoprecipitation and hydrophobic interaction chromatography), confirming the new adsorbent as a low-cost viable alternative for GFP purification.

1. Introduction

The green fluorescent protein (GFP) from jellyfish *Aequorea victoria* is the most extensively studied reporter protein and is a well-developed marker in bioprocess control and analysis, applied to monitor dynamic processes in cells, organisms and organs (e.g. gene expression, intracellular localization and protein–protein interaction studies) [1–3]. GFP-like proteins do not require cofactors, enzymes or substrates other than molecular oxygen for the formation of the chromophore [4,5]. This property makes the formation of the chromophore easier in live organisms, tissues and cells. Other advantages of these proteins include the low-toxicity and high stability in a wide range of pH and solvents, the easy detection in a bulk cell suspension without cell disruption, and relatively small molecular weight (25–30 kDa) which contributes to a low burden to host cells [5]. Due to the attractive properties of GFP, its use can be extended as an alternative protein tag for the purification of GFP fusion proteins through chromatography. However, this application requires the existence of a binding ligand immobilized onto a chromatographic matrix. This would represent a highly attractive protein purification technique allowing for high recovery yields of the target protein [6,7]. However there is a lack of suitable ligands targeting a wide range of proteins, including GFP.

To date, there is no purification process that combines selective GFP recovery at affordable costs [8,9]. Purification processes for GFP fusion proteins require multiple steps of chromatography or immunoaffinity separations [10–16]. Multi-step purification protocols involve the use of different techniques, including hydrophobic-interaction chromatography (HIC) [13], immobilized metal affinity chromatography (IMAC)[11], and aqueous two-phase extraction (ATPE) [12]. The common feature between these techniques is the employment of structural ligands (e.g. metal chelators, hydrophobic ligands), which are known to present low selectivity and operate in harsh conditions (e.g. 4 M ammonium sulphate in HIC). Moreover, the use of metal chelator ligands can lead to metal leaching and contamination of the target protein [17–19]. Immunoaffinity separation methods involve the use of monoclonal antibodies as biological ligands, which despite of exhibiting high selectivity, also present poor stability during the purification

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process with higher associated costs [20–22]. Synthetic ligands emerged to address the disadvantages of biological and structural ligands as they can combine high-selectivity, robustness and stability with cost-effectiveness. Moreover, they can be easily produced by chemical synthesis, using unique chemistries, such as the triazine chemistry and multicomponent reactions (MCRs) as the Ugi reaction. The Ugi reaction is simpler and less time consuming; furthermore, it offers the possibility to create a greater diversity of combinatorial ligand libraries based on peptoidal scaffolds [23,24]. The Ugi reaction combines four main components: an amine (primary or secondary), and oxo-compound (aldehyde or ketone), a carboxylic acid and an isocyanide [25]. This MCR presents unique selectivity as only one product is obtained from different starting materials. Synthetic ligands based on the Ugi reaction have been so far used for the purification of highly relevant therapeutic proteins such as immunoglobulins [26,27] and erythropoietin [28]. Recently, a ligand based on the Ugi reaction was also developed for binding to a novel affinity tag, with the aim of creating a universal strategy for the purification of recombinant proteins [25].

In this work, a generic platform for the purification of GFP and GFP fusion proteins was explored and benchmarked against the current methodologies in place. A synthetic affinity ligand based on the multicomponent Ugi Reaction was selected from a solid-phase combinatorial library due to its ability to capture and selectively elute GFP, and employed to create a simple and cost-effective purification scheme for GFP fusion proteins.

2. Materials and methods

2.1. Materials

All chemicals used were at least 98% pure and the solvents were pro-analysis grade. The plasmid pET21c was kindly provided by Prof. Alice S. Pereira (Requimte, Portugal). The competent cells *Escherichia coli* BL21(DE3) and NZY5α were purchased from NZYTech. DNase I was obtained from Roche (Lisboa, Portugal). The chromatographic materials cross-linked agarose (Sepharose™ CL-6B), PD-10 Columns – Sephadex™ G – 25M and HiScale16/20 columns were obtained from GE Healthcare (Uppsala, Sweden). Captiva 96-well filtration block and empty columns (0.8 cm × 6.5 cm) were purchased from Varian (Lisboa, Portugal), Brand Black immunograde 96-well microplates and 96-well UV half area (Greiner) were supplied from VWR International (Lisbon, Portugal). The 96-well transparent microplates were acquired from Sarstedt (Lisbon, Portugal). Rabbit anti-GFP polyclonal antibody ab290 and goat anti-GFP polyclonal antibody ab97051 were acquired from Abcam (Cambridge, UK). The Novex HRP Chemiluminescent substrate reagent kit was purchased from Life Technologies, Paisley, UK. Novex® Sharp protein standard was obtained from Invitrogen, UK and low molecular weight protein marker was purchased from Nzytech (Lisboa, Portugal).

2.2. Synthesis of the combinatorial library

Solid-phase synthesis of the ligands library was based on the multicomponent Ugi reaction and performed in a 96-well filtration block. Aldehyde-activated agarose beads were used as the starting solid support following previously reported methodologies [26,29]. The combinatorial library comprised a total of 64 affinity ligands and each ligand was obtained from the reaction between the aldehyde-functionalized agarose, an amine, a carboxylic acid and isopropyl isocyanide. Each ligand presented a different combination of amine and carboxylic acid functionalities as shown in Fig. 1a.

2.3. Combinatorial Library screening with crude extracts containing GFP

The screening of the combinatorial libraries was initiated with the regeneration and equilibration of the resins (250 mg/well of the 96-well filtration block) functionalized with different synthetic ligands (total of 64 ligands). In the regeneration step, 250 μl of regeneration buffer (0.1 M NaOH, 30% (v/v) isopropanol) were added to each well alternated with 250 μl of distilled water for a total of three times. The equilibration was then conducted by adding 12 × 250 μl of the equilibration buffer (PBS), the fractions (250 μl) were collected on a 96-well microplate UV-half area and the absorbance was measured at 280 nm until $A_{280\,nm}$ was ≤0.005. Both steps were carried out under gravity. Once equilibrated, 250 μl of the crude extract containing GFP (0.66 mg/ml; see SI for details on GFP expression and extract preparation) was loaded on each well and incubated at 4 °C for 1 h under manual shaking to guarantee a good mixing between the resin and the target protein. After incubation, the flow-through was collected in a black 96-well microplate (VWR) by placing it below the 96-well block and then centrifuging (170 × *g*, 1 min). Afterwards, each well was washed with binding buffer (PBS 12 × 250 μL); between each wash, samples were collected in a black 96-well microplate by centrifugation (170 × *g*, 1 min). After the screening, the resins in all 96-well blocks were regenerated and stored at 0–4 °C in 20% (v/v) ethanol. Total protein concentration in the collected samples was quantified by BCA. GFP fluorescence was also measured. The selectivity and binding percentage was determined according to Eqs. (1) and (2):

$$\text{Selectivity} = \frac{R_1}{R_2} = \frac{\text{GFP bound/total protein bound}}{\text{GFP loaded/total protein loaded}} \quad (1)$$

$$\%\,\text{Binding (w/w)} = \frac{\text{GFP bound}}{\text{GFP loaded}} \times 100 \quad (2)$$

2.4. Re-screening of lead ligands with crude extracts containing GFP

The screening of the promising lead ligands with GFP was performed as described above with an additional step of elution after washing, using two different elution buffers: 0.1 M glycine–HCl, pH 3 and 0.1 M glycine–NaOH, pH 11, respectively. The bound target was eluted by adding the indicated elution buffer (5 × 250 μl). Total protein concentrations and GFP concentration in the samples were quantified by BCA and GFP fluorescence assays. The same evaluation was carried out with the blank (unmodified agarose) with GFP. The recovery yield was determined according to Eq. (3):

$$\%\,\text{Recovery yield (w/w)} = \frac{\text{GFP eluted}}{\text{GFP bound}} \times 100 \quad (3)$$

The selectivity of the ligands for GFP and the purity of the samples were evaluated by densitometry analysis of SDS-PAGE gels (12.5% acrylamide gels) using Image J software.

2.5. On-column purification of GFP from crude cell extract with A4C7 adsorbents

The purification of GFP from the crude extract using ligand A4C7-Sepharose™ column was performed as described above. About 250 mg moist gel (500 μl of 50% (w/v) slurry) of immobilized lead ligand was packed in 4 ml columns (0.8 cm × 6 cm), the assay ran under gravitational flow, and the fractions were collected in 1.5 ml microcentrifuge tubes covered with aluminum foil to protect from light and kept at 4 °C. A blank assay was also performed on unmodified agarose by loading a sample of the crude extract containing GFP. The fractions obtained were analysed by BCA assay, GFP

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