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N^{pro} fusion technology: On-column complementation to improve efficiency in biopharmaceutical production

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ABSTRACT

N^{pro} fusion technology, a highly efficient system for overexpression of proteins and peptides in *Escherichia coli*, was further developed by splitting the autoprotease N^{pro} into two fragments to generate a functional complementation system. The size of the expression tag is thus reduced from 168 to 58 amino acids, so by 66%. Upon complementation of the fragments auto-proteolytic activity is restored. This process has been shown for three model proteins of different size, a short 16 aa-peptide, MCP-1, and lysozyme. Moreover, the complementation was still functional after immobilization of the N-terminal fragment to a solid support which enables recycling of the immobilized fragment. This strategy enhances overall productivity of N^{pro} Fusion Technology and thus allows more efficient production of recombinant proteins with reduced costs and in higher yields. Overall, the N^{pro} complementation system has, depending on the size of the target molecule, potential to increase the productivity up to 4 fold for batch refolding and even more for on-column refolding strategies by the proven possibility of regeneration of the immobilized fragment.

1. Introduction

N^{pro} Fusion Technology has been widely used to produce proteins with an authentic N-terminus [1]. This technology for overexpression of peptides and proteins in *Escherichia coli* utilizes the autoprotease N^{pro} derived from *Pestiviruses* as a self-cleaving expression tag. Upon folding N^{pro} releases the target with an authentic N-terminus. The target therefore must be expressed as a C-terminal fusion to the autoprotease. Deposition of such N^{pro} fusion proteins in inclusion bodies enables the production of heterologous protein of high yields and purity [2], facilitated downstream processing [3] and expression of small and toxic peptides [4,5]. However, deposition in inclusion bodies requires solubilization and refolding steps for recovery of functional products. Solubilization of inclusion bodies is a crucial step, which needs proper optimization of conditions to reach the highest possible yields [6]. Refolding by rapid dilution into kosmotropic buffers finally renders autoprotease N^{pro} active to release the target protein by one precise cut in the C-terminal at residue C168. Current N^{pro} Fusion Technology using the mutant EDDIE of the wild type autoprotease designed by exchange of 11 amino acids (aa) exerts improved cleavage. This improvement has been demonstrated for pharmaceutically relevant targets [1,7] as well as for antimicrobial peptides [8]. The major shortcoming of this expression tool is overexpression of a tag 19 kDa in size. Reducing the size of the expression tag by a complementation strategy will concomitantly reduce production costs and increase overall expression performance of N^{pro} Fusion Technology. To reach this goal, we undertook the design of N^{pro} Fusion Technology as a complementation system.

The ability of protein fragments to reassemble through non-covalent interactions into functional enzymes is possible and has been shown for fragment-complementation of bovine pancreatic ribonuclease [9], T-nuclease from *Staphylococcus aureus* [10], β-galactosidase [11], dihydrofolate reductase [12], *Renilla* luciferase [13], and proteins such as green fluorescence protein and variants thereof [14].

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We used a mutated N^{pro} variant of classical swine fever virus strain Alfort, termed Δ21N^{pro} 37. In this mutant the first 21 residues of the wild type sequence are deleted. Deletion of these 21 N-terminal amino acids had no impact on proteolytic activity, but inhibited interaction with the cellular immune system via interferon-regulatory factor 3 which is important for production of biopharmaceuticals [15,16]. Based on structural knowledge [17], we split Δ21N^{pro}37 into two parts: The N-terminal fragment was designed to harbor the protease domain containing all catalytically important residues, while the C-terminal fragment was fused to the target molecule. We used three different targets ranging from 16 to 157 amino acids in length. All fragments as well as the full-length variant were overexpressed in *E. coli* and mostly deposited as inclusion bodies. Inclusion bodies were solubilized under chaotropic conditions and subsequently refolded by rapid dilution. Reassembly of the active autoprotease N^{pro} structure led to release of the targets.

The proof of principle for the complementation approach was shown by utilizing three different targets: (1) pep6His, 16 amino acids of randomized order with a C-terminal 6His-tag; (2) monocyte chemoattractant protein MCP-1, a chemokine of therapeutic interest, 79 amino acids in length; and (3) lysozyme with a C-terminal 6His-tag and a molecular weight of 17.8 kDa. All subsequent experiments to investigate the expression yield, ratio of soluble to insoluble fractions of overexpressed fusion protein, solubilization yield, and different refolding conditions and strategies during complementation were carried out with pep6His as target, which had shown the highest cleavage yield in the preliminary experiments. All experiments were carried out in direct comparison to results obtained for the full-length fusion protein of the same target. In Fig. 1 the target release is schematically depicted comparing a full-length fusion molecule (A) to the complementation set up (B).

Schmoeger et al. previously showed that contact with the chromatographic matrix enhanced the shift from aggregates to the intermediate or monomeric state and therefore promoted refolding [18]. Therefore, our goal was to prove the applicability of our developed complementation system for on-column refolding (for schematic depiction of target release see Fig. 1C). This methodology holds the potential for significant further improvement since the immobilization of one fragment to a stationary phase will enable re-usage for several cycles of cleavage. Therefore, we investigated the immobilization of the GST-tagged N-terminal fragment by binding to Glutathione-Sepharose affinity resin, the cleavage yield, and the potential for recycling of the immobilized fragment were investigated.

2. Material and methods

2.1. Cloning of constructs

2.1.1. Construct Δ21N^{pro}37 was used as a template

Two N-terminal Δ21N^{pro}37 Δ107-168 and three C-terminal fragments Δ112N^{pro}37 were generated using corresponding primers including appropriate restriction sites. For exchange of the N^{pro} sequence, restriction sites NdeI (5′) and SpeI (3′) were used. After PCR amplification DNA fragments were digested and one N-terminal and three C-terminal inserts were subcloned into pET30a-c(+), an IPTG-inducible expression vector from Novagen based on the pET expression. For the second N-terminal fragment, its sequence was amplified by PCR and subcloned into pGEX-4T-2 (GE Healthcare, Uppsala, Sweden) using restriction sites BamHI and XhoI. pGEX-4T-2 provides the fusion protein with an N-terminal GST-tag. After transformation of *E.-coli* NovaBlue (Novagen, Merck Millipore, Billerica, MA, USA), all sequences were verified by sequencing (MWG Eurofins). See Table 1 for all generated variants.

2.2. Protein expression and process analysis

Protein expression was performed using *E. coli* BL21(DE3) (Novagen, Merck Millipore, Billerica, MA, USA) carrying the T7 polymerase gene for protein expression using the T7 promoter [19,20]. An overview of the Δ21N^{pro}37-pep6His reference full-length fusion protein and the corresponding protein fragments thereof used for the complementation system is listed in Table 1. For the proof of concept experiments expression is performed in laboratory scale: After transformation, overnight cultures were diluted 1:100 and cells were grown in up to 400 mL of medium (10 g peptone, 7 g yeast extract and 2.5 g NaCl per liter) at 37 °C and 220 rpm. When an OD_{600} between 0.5 and 1.0 was reached, expression was induced with 0.1 mM IPTG. After 2–4 h, cells were collected by centrifugation and stored at −20 °C. Samples before (-I, 250 μL) and after induction (+I, 125 μL) were collected by centrifugation for SDS-PAGE analysis. For all further experiments and investigation of optimal solubilization and refolding conditions, expressions were performed in larger scale in a 10 L (5 L working volume) computer-controlled bioreactor (MBR, Wetzikon, CH) as described previously [20]. Optical density (OD) is measured at 600 nm. Bacterial dry matter was determined by centrifugation of 10 mL of the cell suspension, re-suspension in distilled water followed by centrifugation, and re-suspension for transfer to a pre-weighed beaker, which is then dried at 105 °C for 24 h and re-weighed. The progress of bacterial growth was determined by calculating the total amount of biomass (total bacterial dry matter BDM; also termed cell dry weight CDW). The evaluation of the expressed fusion protein was performed with SDS-PAGE by means of a linear regression curve of a purified EDDIE (autoprotease) standard. Aliquots of 1 mg CDW of each fermentation broth were harvested by centrifugation at 14,000 rpm for 10 min (Centrifuge 5415R, Eppendorf, Germany) and the supernatants were decanted. Each pellet was resuspended in 200 μL lysis buffer (27 mM Tris/HCl, pH = 8.2, 25 mM EDTA, 10 mM $MgCl_2$ x $6H_20$, 28.6 mM 2-mercaptoethanol) and after rigid mixing 50 μL of a aqueous 2 mg/mL lysozyme stock solution and 50 μL of a benzonase stock

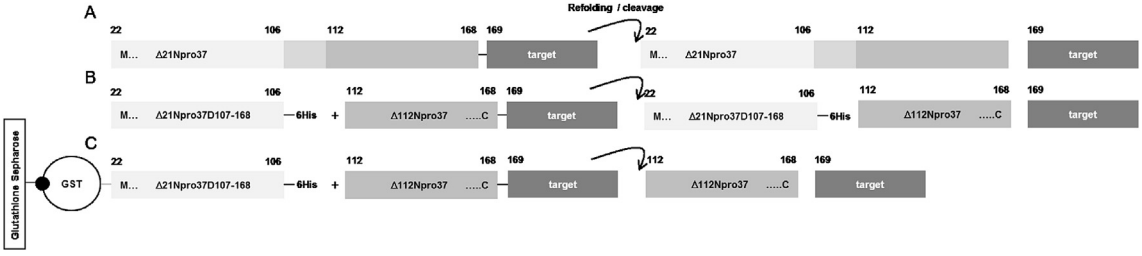

Fig. 1. Schematic depiction of N^{pro} target liberation and the Δ21N^{pro}37 complementation system. (A): Δ21N^{pro}37 – full-length autoprotease as fusion tag; (B): N^{pro}37 complementation system with the N- and C-terminal fragment used for off-column complementation; (C): N^{pro}37 complementation system with the GST-tagged N-terminal fragment used for on-column complementation by affinity binding and corresponding C-terminal fragment fused to the target molecule. Left: Refolding set up; Right: Fragments in solution after successful refolding/cleavage.