

RESEARCH ARTICLE

Extraction of recombinant periplasmic proteins under industrially relevant process conditions: Selectivity and yield strongly depend on protein titer and methodology

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Abstract

In this work, we attempted to identify a method for the selective extraction of periplasmic endogenously expressed proteins, which is applicable at an industrial scale. For this purpose, we used an expression model that allows coexpression of two fluorescent proteins, each of which is specifically targeted to either the cytoplasm or periplasm. We assessed a number of scalable lysis methods (high-pressure homogenization, osmotic shock procedures, extraction with ethylenediaminetetraacetic acid, and extraction with deoxycholate) for the ability to selectively extract periplasmic proteins rather than cytoplasmic proteins. Our main conclusion was that although we identified industrially scalable lysis conditions that significantly increased the starting purity for further purification, none of the tested conditions were selective for periplasmic protein over cytoplasmic protein. Furthermore, we demonstrated that efficient extraction of the expressed recombinant proteins was largely dependent on the overall protein concentration in the cell.

KEYWORDS

chromoproteins, cytoplasmic protein extraction, detergent extraction, *Escherichia coli*, high-pressure homogenization, osmotic shock

1 | INTRODUCTION

Much scientific research has been performed to learn how to drive periplasmic expression of complex recombinant proteins in gram-negative bacteria.¹⁻⁴ The oxidative environment of the periplasm favors disulfide bridge formation,^{5,6} and the presence of specific chaperones can enable correct protein folding.^{7,8} Notably, of the 25 known cellular proteases in *Escherichia coli*, only seven are present in the periplasm.⁹

Proteins destined for the periplasm are synthesized in the cytoplasm with an N-terminal signal peptide targeting them to the inner membrane (IM). Translocation into the periplasm can occur via the general secretory

(Sec) or twin-arginine-translocation (Tat) pathway.^{6,10,11} Within the Sec pathway, unfolded proteins are posttranslationally (SecA/SecB-dependent) or cotranslationally (SRP pathway) translocated via the Sec translocase that transports proteins across the IM. In contrast, the Tat-protein transport system forms a complex that enables translocation of cargo proteins in their folded state. Upon translocation, the transported protein's signal peptide is cleaved off by a signal peptidase, producing the authentic N-terminus of the mature protein of interest (POI).^{12,13}

From the perspective of downstream processing, periplasmic expression potentially offers a big advantage. Recombinant protein extraction is facilitated by the fact that only the outer membrane (OM) must be disintegrated to release the protein from the cell.

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Ideally, periplasmic extracts contain only minimal amounts of cell wall and cytoplasmic components, facilitating the subsequent downstream processing steps.^{9,14}

However, selective OM perforation without damaging the IM is not an easy task.⁹ A variety of methods has been proposed, all of which involve destabilizing the OM using either physical forces (shear, heat, or osmotic pressure) or through the chemical removal of membrane components. For example, periplasmic proteins (e.g., antibody fragments) have been extracted using ethylenediaminetetraacetic acid (EDTA) combined with heat, successfully yielding relatively pure extracts.¹⁵ EDTA is a metal-chelating agent that promotes the binding of divalent cations present in the membrane (mainly Mg^{2+}), consequently leading to a rapid change in permeability as the asymmetry of the OM is broken down.¹⁶⁻¹⁸ Such methods involve the loss of 30–50% of the lipopolysaccharide component, but the morphological cell structure remains intact, and even cell viability can be restored to some extent following extraction.^{16,19}

Osmotic shock (OS) procedures rely on the rapid transfer of cells into solutions having a very low solute concentration. This causes the osmotic transmembrane pressure to drastically increase, leading to cell bursting and fragmentation. OS methods are widely used on the laboratory scale, and are reportedly selective for periplasmic proteins.²⁰⁻²² Freeze-and-thaw methods have also been successfully applied, either as the sole cell disintegration method, or to enhance the performance of another method.²³

Periplasmic release can also be induced by chemicals other than chelating agents, for example, detergents, amino acids, polyols, chaotropic salts, and solvents.^{18,21,24} One comparison of extraction methods using low concentrations of chemicals revealed that extraction with sodium deoxycholate (DOC) at a concentration of 0.1% was highly efficient and selective for some periplasmic proteins, and produced an extract containing low levels of host-cell protein (HCP).²¹ Many other approaches for periplasmic protein extraction are described in literature, including glycol ether treatment and surfactant treatment,²⁵ and the application of chemicals other than DOC, such as glycine for permeabilization²⁵ or the use of chloroform.²⁶

In an industrial setting, in addition to selectivity (e.g., the exclusive release of periplasmic proteins), the large-scale applicability of a method is very important. The current industrial standard is protein release by high-pressure homogenization (HPH),²⁷ which is efficient, easy to develop, and straightforward to scale-up. Operating conditions for selective periplasmic release have not yet been reported. When using a simplified and adapted method, OS is partly applicable on a large scale.²⁸ The addition of lysozyme increases efficiency, but the lysozyme must be removed during downstream processing. EDTA/heat methods are also potentially applicable on a large scale. However, the use of other chemicals may be restricted due to toxicity and environmental issues, and applications must be evaluated on a case-by-case basis.

The previously published data have several weaknesses. In most studies, cells have been grown in shake flasks rather than in bioreactors, and thus do not reflect the same physiological state of cells in a production scenario. Moreover, due to feasibility constraints, many

studies have been performed using frozen cells, and the cell disruption from freezing and thawing raises questions regarding the specificity of the methods.^{21,29} Additionally, the expression levels and protein titers have either been very low or scarcely mentioned in the published works, making comparability difficult or even impossible. Data are also scarce regarding DNA and endotoxin release when using different extraction methods,²⁵ and these impurities strongly influence the requirements for subsequent downstream processing, particularly the chromatographic steps.³⁰ Further characterization of cell fragmentation (e.g., debris size) with HPH has only been published in a few studies.³¹ Understanding debris size and distribution is particularly important in terms of the clarification performance of homogenates and extracts when applying centrifugation and filtration.

In the present study, our main goal was to investigate the potential of various methods for periplasmic protein release under industrial process conditions, using fed-batch fermentations at medium-to-high cell densities, and thereby producing high protein titers. We selected three widely used methods for periplasmic protein release: OS, EDTA/heat extraction, and DOC extraction. We also investigated HPH at low pressure and repeated passages for this purpose. As a workhorse for this study, we developed a model production system based on two chromoproteins: a monomeric red fluorescent protein (mCherry) that is expressed in the cytoplasm, and a superfolder green fluorescent protein (sfGFP) that is targeted to the periplasm. This system allows investigation of the selectivity of protein extraction methods by fluorescence measurements. To uncouple the effects of coexpression of the mCherry and sfGFP proteins, we also comparatively studied two single-protein systems. For this analysis, GFPmut3.1 was expressed in the cytoplasm, and sfGFP in the periplasm. To assess applicability on an industrial scale, we also comprehensively analyzed the extracts with regard to DNA and endotoxin levels, and measured cell fragmentation by analytical centrifugation including laser monitoring.

2 | MATERIALS AND METHODS

2.1 | Construction of expression systems

2.1.1 | Strains, plasmids, and primers

All enzymes and kits were purchased from New England Biolabs (NEB, Ipswich, MA). For cloning purposes, we purchased chemically competent *E. coli* K-12 NEB-5 α cells from NEB. Expression vectors were transformed into the *E. coli* strains BL21(DE3) (NEB) and HMS174(DE3) (Novagen[®], Madison, WI). Cells were cultured and processed following the manufacturer's protocols. We cloned the gene-of-interest sequences into the expression vector pET30a via the NdeI and EcoRI restriction sites. The primers were purchased from Sigma-Aldrich (St. Louis, MO). All constructs were confirmed by sequencing (Microsynth, Vienna, Austria).

2.1.2 | Cytoplasmic and periplasmic fluorescent protein reporter system (cyto-peri-FP system)

The reporter system is based on two fluorescent proteins: mCherry, which is expressed in the cytoplasm (cyto-mCherry), and sfGFP, expressed in the periplasm (peri-sfGFP). Figure 1a presents a schematic overview of the construct design. We amplified mCherry from the vector pCD256ΔEc_P11_mCherry using Q5 PCR.³² The forward primer contained an XbaI restriction site and the 6xHis tag, and the reverse primer contained a BsaI restriction site and a spacer. We also used Q5 PCR to amplify sfGFP.³³ The forward primer contained the BsaI restriction site, the DsbA leader sequence, and a FLAG tag, and the reverse primer contained a BamHI restriction site. The PCR fragments were digested using the corresponding restriction enzymes (XbaI, BsaI, and BamHI), and the pET30a vector was digested using XbaI and BamHI. All three fragments were simultaneously ligated using T4 Ligase, and the ligation product was transformed into *E. coli* NEB5α (NEB). After plasmid preparation, the plasmid was transformed into the expression strains BL21(DE3) and HMS174(DE3).

sfGFP reporter system (peri-sfGFP system)

We amplified sfGFP and introduced a myc tag (included in the overhang of the reverse primer). The PCR product sfGFP, having an N-terminal FLAG tag, DsbA leader, and C-terminal myc tag, was cloned into a pET30a vector using XbaI and BamHI, and then the generated plasmid was transformed into BL21(DE3) and HMS174(DE3).

GFPmut3.1 reporter system (cyto-GFPmut3.1 system)

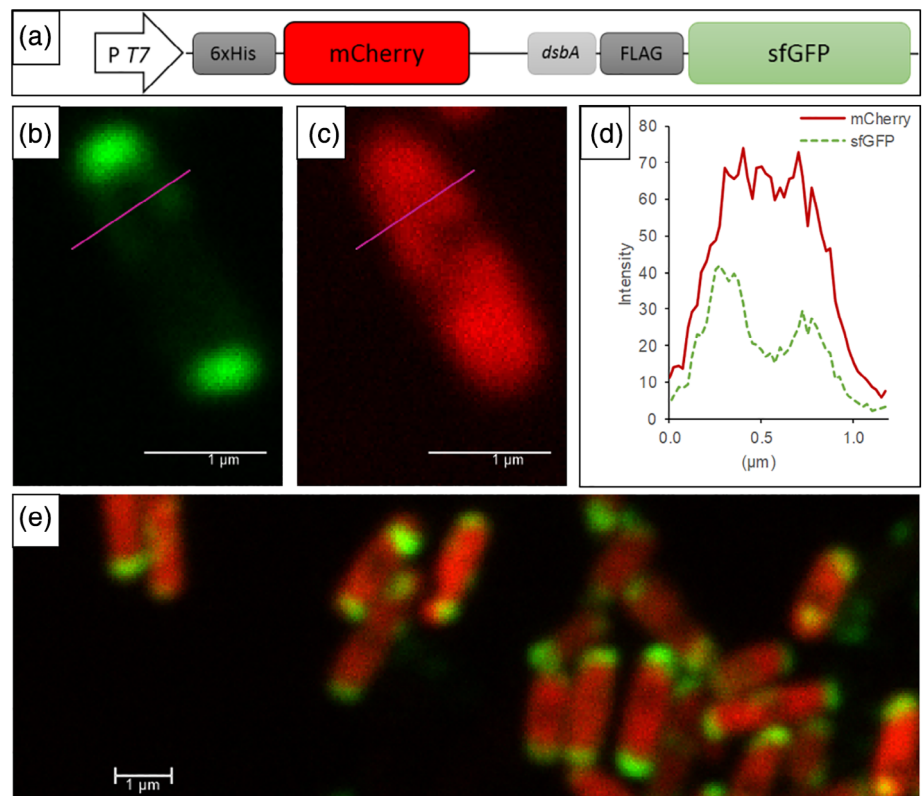
The pET30a vector containing the sequence for GFPmut3.1 was transformed into BL21(DE3) and HMS174(DE3).

2.2 | Culture media and expression conditions

Cells were cultivated in a fed-batch process using 1.5 L DASGIP[®] benchtop glass bioreactors (Eppendorf, Hamburg, Germany). The batch and fed-batch cultivation media contained glucose as the carbon source, NH₄Cl and (NH₄)₂SO₄ as the nitrogen source, and KH₂PO₄ and K₂HPO₄ as the phosphate source and as the buffer system. The media was also supplemented with MgSO₄, CaCl₂, trace elements, and Na-citrate. Yeast extract was added to only the batch medium, to boost growth at the start. Details regarding medium composition and preparation have been described by Striedner et al.³⁴ The pH was maintained at 7 ± 0.05 throughout the cultivation using 12.5% ammonia solution. The temperature was maintained at 37°C during the batch phase and 30°C during the feed phase. A cell concentration of 3 g/L was reached in batch mode, and cultivation was continued in fed-batch mode for two further generations without recombinant protein expression (14 hr, μ = 0.1/hr).

We started expression of the recombinant model proteins (either sfGFP and mCherry together, or sfGFP or GFPmut3.1 alone) via induction with 5 μmol isopropyl-β-D-thiogalactopyranosid/g cell dry mass (CDM). Then cultivation and recombinant protein expression was continued for two generations (14 hr, μ = 0.1/hr). Approximately, 1.2 L fermentation broth was harvested, and centrifuged for 10 min at

FIGURE 1 Expression system including cytoplasmic mCherry and periplasmic sfGFP (cyto-peri-FP system). (a) Schematic overview of the construct design, containing the gene of interest (sfGFP and mCherry), the dsbA leader sequence (for cotranslational export of sfGFP to the periplasm), and the fused tags (N-terminal 6xHis tag on mCherry to facilitate purification, and N-terminal FLAG tag on sfGFP to monitor DsbA leader cleavage). (b) CLSM picture, with the green fluorescence channel, of one *Escherichia coli* HMS174(DE3) cell exhibiting sfGFP located at the two extremities of the cell. (c) CLSM picture, with the red fluorescence channel, showing mCherry evenly distributed over the cell. (d) Fluorescence intensity along the purple line drawn in panels (b) and (c), showing higher intensities of green fluorescence at the edges and even distribution of red fluorescence. (e) CLSM of BL21(DE3) cells with this cyto-peri-FP system, with an overlay of the green and red channels. CLSM, confocal laser scanning microscopy



8,000g at 10°C. Then the cell pellet was resuspended in buffer containing 100 mM Tris, pH 8, to a concentration of 25% wet cell pellet (approximately 50 g CDM/L).

2.3 | Protein extraction methods

All extraction methods were evaluated at an industrially relevant cell density of approximately 40 g CDM/L.

2.3.1 | High-pressure homogenization (HPH)

Cells were resuspended in 100 mM Tris/HCl buffer, pH 8 to obtain a concentration of 40 g CDM/L. Then this suspension was homogenized for multiple passages at various pressures ranging from 5/50 to 70/700 bar (first stage/second stage) using a PandaPLUS 2000 homogenizer (GEA, Düsseldorf, Germany). The minimal working volume was 150 ml. Samples were centrifuged for 10 min at 16,000g, and the supernatants were used for analysis.

2.3.2 | DOC extraction

A cell suspension of 50 g CDM/L was supplemented with 100 mM Tris/HCl, 0.75% DOC stock solution to obtain a suspension of 40 g/L CDM and 0.15% DOC. We then incubated 1-ml aliquots of this suspension at RT for up to 7 hrs on a rotator. The extraction was stopped by centrifugation for 10 min at 16,000g, and the supernatants were used for analysis.

2.3.3 | EDTA and heat extraction

To the 50 g/L CDM cell stock in 100 mM Tris/HCl buffer, pH 8, we added 50 mM EDTA in the same buffer to generate a 40 g/L CDM suspension with 10 mM EDTA. We then incubated 1-ml aliquots of this suspension at 60°C for 2 hrs using an Eppendorf thermoblock. Extraction was stopped by centrifugation for 10 min at 16,000g, and the supernatants were used for analysis.

2.3.4 | Osmotic shock (OS)

Cells were resuspended at 40 g/L CDM in 100 mM Tris/HCl buffer, pH 8. This suspension was then centrifuged for 5 min at 5,000g, and the supernatant was analyzed. The cells were resuspended in hypertonic solution (100 mM Tris/HCl, 10 mM EDTA, and 20% sucrose, pH 8) and incubated on ice for 10 min. After centrifugation (10 min, 7,000g), the supernatant was saved for analysis, and the cells were resuspended in hypotonic solution (100 mM Tris/HCl, and 10 mM EDTA, pH 8) and incubated on ice for 10 min. After centrifugation (10 min, 16,000g), the supernatant was analyzed.

2.4 | Analytical methods

2.4.1 | Model protein quantification using fluorescence

Fluorescence measurements were performed using a Tecan analyzer infinite 200Pro (Tecan Group Ltd, Männedorf, Switzerland). For mCherry, the excitation wavelength was 587 nm and the emission wavelength was 610 nm. For sfGFP and GFPmut3.1, the excitation wavelength was 485 nm and the emission wavelength was 520 nm. For quantification, calibration was performed using in-house purified target proteins. We purified mCherry with immobilized metal affinity chromatography using His-Tag-specific binding on an Ni-Sepharose HP column (GE Healthcare Bio-Sciences, Uppsala, Sweden). GFP and sfGFP were purified through anion exchange chromatography using a Capto Q column (GE Healthcare Bio-Sciences, Uppsala, Sweden), followed by hydrophobic interaction chromatography using a Butyl Sepharose HP column (GE Healthcare Bio-Sciences, Uppsala, Sweden), and finally size exclusion chromatography using a Superdex 75 column (GE Healthcare Bio-Sciences, Uppsala, Sweden). We applied the Beer-Lambert law to determine the concentrations of the standards using the absorbance at 280 nm and the corresponding excitation coefficients.

2.4.2 | Confocal laser scanning microscopy (CLSM)

Cells were fixed with 4% paraformaldehyde solution for 30 min. We used a confocal microscope (TCS SP8 Leica Microsystems, Mannheim, Germany) equipped with a pulsed white light laser (WLL 2), that was tunable between 470 and 670 nm. Fluorescence emissions were collected using hybrid spectral detectors. For mCherry, the excitation wavelength was 585 nm and the emission wavelength was 594–656 nm. For sfGFP, the excitation wavelength was 490 nm and the emission wavelength was 497–549 nm. Images were acquired using a HC PL APO 100×/1.4 Oil CS2 objective (TCS SP8 Leica Microsystems, Mannheim, Germany). We performed deconvolution of the pictures using Huygens Deconvolution Software (Scientific Volume Imaging, Hilversum, Netherlands).

2.4.3 | Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

We mixed 13 µl of each sample with 5 µl ×4 LDS sample buffer (Invitrogen, Waltham, MA) and 2 µl reducing agent (×10). After heating these mixtures at 70°C for 10 min, we loaded 15 µl on an SDS-polyacrylamide NuPAGE™ 4–12% Bis-Tris Protein Gel, 1.0 mm (Invitrogen, Waltham, MA). Electrophoretic separations were performed for 45–50 min at 200 V (400 mA) on gels in MES-SDS running buffer, and then the gels were stained with Coomassie.

2.4.4 | Total protein quantification

Total protein content was determined using the Qubit™ Protein Assay Kit (Invitrogen, Waltham, MA). The results were not influenced by the intrinsic fluorescence of the utilized chromoproteins.

2.4.5 | DNA quantification

We performed DNA quantification using a DNA Quantitation Kit (Bio Rad, Hercules, CA), with Hoechst 33258 as dye. Depending on the expected concentration, we added 2–5 μ l of sample to 200 μ l fluorochrome solution (2 μ g dye per ml). The calibration curve was generated using Calf Thymus DNA standard (Sigma–Aldrich). We measured fluorescence (excitation at 346 nm and emission at 460 nm) using a Tecan analyzer infinite 200Pro (Tecan Group Ltd, Männedorf, Switzerland).

2.4.6 | Endotoxin quantification

We performed endotoxin quantification using the EndoZyme® II Recombinant Factor C Assay (Hyglos, Bernried am Starnberger See, Germany).

2.4.7 | Particle size analysis

To approximate the postextraction particle size distribution of cells and cell debris, we used the LumiSizer (LUM GmbH, Berlin, Germany), an analytical photo centrifuge based on light transmission profiles. Centrifugation was performed at 2,000 rpm, acquiring a profile at 865 nm every 10 s for a total of 50 min. Due to the density differences between intact cells and disrupted cells/debris, we used sedimentation velocity to compare the different samples. We calculated approximate size ranges, assuming a density of 1.090 kg/m³ for intact cells and 1.300 kg/m³ for cell debris.

3 | RESULTS AND DISCUSSION

3.1 | Novel reporter system for investigating selective periplasmic extraction

To selectively determine the extraction efficiency for periplasmic proteins in a simple and accurate manner, we developed the cyto-peri-FP expression system based on two chromoproteins. The construct is shown in Figure 1a. While mCherry resides in the cytoplasm, the DsbA leader sequence upstream of the sequence encoding sfGFP enables its cotranslational transport across the IM. Both mCherry and sfGFP were engineered with an N-terminal tag: 6xHis and FLAG, respectively. This tagging approach enabled purification and detection of both leader peptide expression and cleavage.

We assessed the system's functionality using CLSM. The examined cells exhibited a faint green film at their outer surface (Figure 1b), as well as large intense green spots at the cell extremities, representing inclusion bodies of active sfGFP. Cytoplasmic mCherry was evenly distributed in the cell (Figure 1c). The fluorescence intensity along the drawn purple lines in Figure 1b,c is plotted in Figure 1d, representing the approximate distribution of the two proteins within the cell. In the harvested material used for the extraction experiments, the mCherry concentration was approximately 0.56–0.70 mg/ml, and the sfGFP concentration was approximately 0.25–0.32 mg/ml. Chromoprotein concentrations were determined by measuring the fluorescence of samples after full extraction and removal of cell debris and inclusion bodies. Correct cleavage of the DsbA leader was confirmed by western blot analysis with anti-FLAG antibody, which is specific only for N-terminal FLAG. Anti-6xHis antibodies were used to confirm mCherry expression (Figure S1). The data shown in Figure 1b–d were produced using strain HMS174(DE3), and similar results were obtained with the strain BL21(DE3) (Figure 1e and Figure S2).

3.2 | Method optimization

Using four different methods, we performed a comparative study of the selective extraction of periplasmic proteins. Figure 2 outlines the action principles of these methods, together with a scheme of selective periplasmic release exemplified with our model system. For each method, we applied a cell concentration of 40 g CDM/L, representing industrially relevant conditions. We also investigated two frequently used industrially relevant host strains of *E. coli*: the B-strain BL21 (DE3) and the K-strain HMS174(DE3).^{35,36} The initial screening experiments with HPH were performed using the HMS174(DE3) strain, and confirmed using the BL21(DE3) strain. The comprehensive comparative approach was conducted only using the BL21(DE3) strain.

HPH is usually performed at a pressure range of 700–1,000 bar, over several passages, to achieve full disruption and complete release

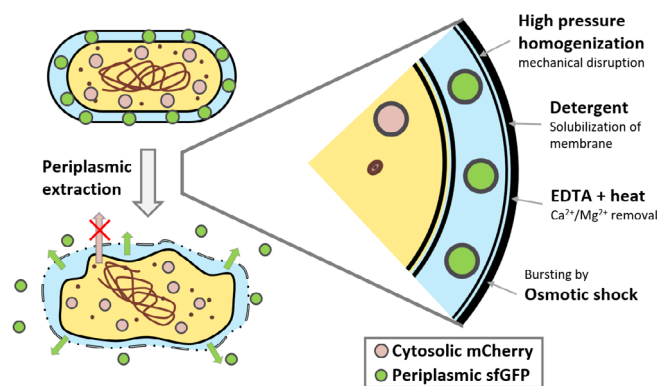


FIGURE 2 Schematic representation of methods for potential selective periplasmic protein extraction, including their action principles

of the POI.³⁷ In this study, our approach was to explore process conditions in the low-pressure range. We hypothesized that the OM would be disintegrated, and that only sfGFP would be released while mCherry would remain within the weakly fragmented cell (debris) assembly. We screened the pressures ranging from 50 to 200 bar over several passages, with the second pressure stage always being 10% of the first stage. We defined a reference for full release using two passages at 700 bar. Figure 3 shows the normalized protein release data based on fluorescence measurements, indicating that we did not identify any operating range enabling selective release. Under the conditions tested, we observed no discrimination between release of cytoplasmic mCherry and periplasmic sfGFP. Within the very-low-pressure range of 50–100 bar, the cells seemed highly resistant, with only a minor fraction of proteins released even after a high number of passages. Microscopic images of treated cells revealed that the cells were either fully disrupted or completely intact, with no images showing slight damage of cells that could eventually lead to selective periplasmic release (Figure S3). A data-set with strain BL21 yielded the same qualitative results (Figure S4), although BL21 is slightly more resistant to pressure due to its more spherical and compact shape.³⁸

Preliminary experiments involving protein extraction with EDTA demonstrated that this method was ineffective at temperatures below 50°C (data not shown). On the other hand, using a temperature that was too high could be detrimental to proteins. Since the sfGFP, GFP, and mCherry proteins are relatively heat stable, we used temperatures of 50 and 60°C for our studies. Control experiments without EDTA showed that the detergent was essential for effective protein release (Figure S5).

Extraction using DOC was initially screened at DOC concentrations ranging from 0.01 to 0.2%, since these concentrations have reportedly yielded successful extraction of periplasmic proteins.²¹ However, in our

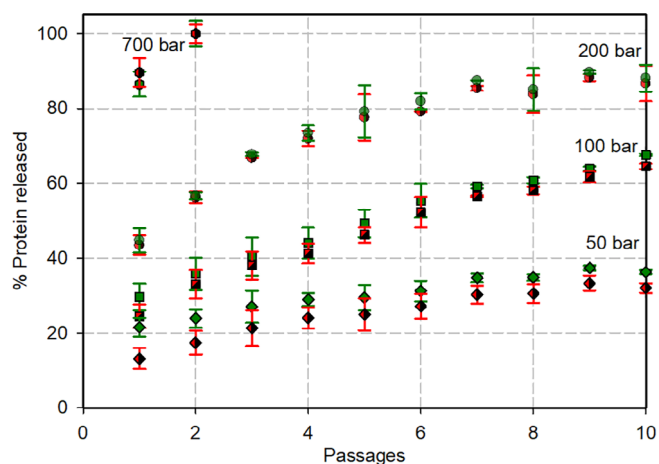


FIGURE 3 The release of sfGFP and mCherry during HPH for up to 10 passages, with pressures ranging from 50 to 700 bar in the first stage and from 5 to 70 bar in the second stage (with the second stage always 10% of the first stage pressure), relative to the reference process of 700/70 bar for two passages. Fully green symbols indicate released sfGFP, red semi-filled symbols indicate released mCherry, hexagons indicate 700/70 bar, circles indicate 200/20 bar, squares indicate 100/10 bar, and diamonds indicate 50/5 bar

expression system, low concentrations were not effective. A 0.2% DOC concentration resulted in high fragmentation of the cells and partial solubilization of the membrane fraction, such that only a very small debris fraction remained after centrifugation. We found that a DOC concentration of around 0.15% was applicable, and was thus included in the comparative analysis. We performed protein extraction by OS using a two-step procedure: the cells were first resuspended in a hypertonic solution, and then transferred into a hypotonic solution.

3.3 | Cyto-peri-FP system

Figure 4 shows the extraction efficiency and HCP impurities from all methods with the cyto-peri-FP system. Clearly, EDTA extraction yielded the relatively best results, obtaining complete extraction and moderate purity. These results were promoted by the heat precipitation of HCPs, combined with cell disintegration promoted by heat. However, we could not achieve selective release of sfGFP. HPH and DOC extractions also yielded full recovery of both POIs. As expected, purity was very low with these methods. The OS procedure produced extracts with a comparable purity to EDTA extraction, and an overall cumulative yield of nearly 100%. However, OS extraction achieved no discrimination between mCherry and GFP, and both POIs were found in all three process fractions—namely, the cell wash, hypertonic, and hypotonic steps.

3.4 | Peri-sfGFP system

The same extraction methods and experimental conditions applied in the cyto-peri-FP system were also applied to another model expression system that only produced periplasmic sfGFP (peri-sfGFP system; Figure 5). In this system, the sfGFP concentration was 0.6 mg/ml. The results with the peri-sfGFP system followed essentially the same trends in terms of purity; however, OS and EDTA extractions produced much lower yields (approximately 40%). HPH and DOC extractions showed the same results—high extraction efficiency and low purity—as obtained with the cyto-peri-FP system. The lower yields could be explained by more stable cell membranes due to comparably less metabolic burden by expressing only one POI in moderate amounts and less inclusion body formation.

3.5 | Cyto-GFPmut3.1 system

We also used a third model system, the cyto-GFPmut3.1 system, to investigate the effect of high levels of recombinant protein in the cytoplasm. With this system, we focused on the performance of the methods postulated to favor selective periplasmic extraction—namely, OS, EDTA, and DOC extractions. GFPmut3.1 was expressed in the cytoplasm with a titer of 7 mg/ml, corresponding to 175 mg/g CDM. All extracts appeared relatively pure, which was due to the high titer of the recombinant protein (Figure 6). In contrast to the other expression systems, with the cyto-GFPmut3.1 system, the OS method

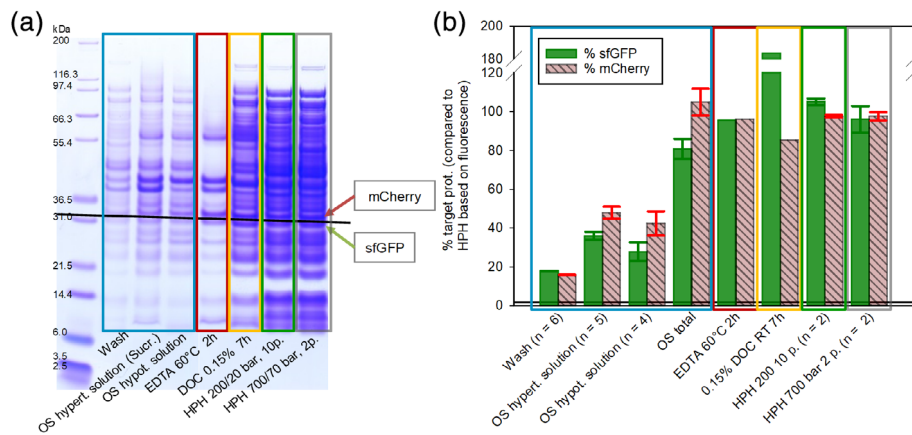


FIGURE 4 Analytical results of different extraction methods using the cyto-peri-FP system. (a) SDS-PAGE showing POI and host-cell protein impurity levels. (b) Relative quantities of POI in extracts based on fluorescence measurements. Blue frame indicates extraction by OS, red frame indicates extraction by EDTA combined with heat, yellow frame indicates extraction by DOC, green frame indicates extraction by HPH for 10 passages at 200/20 bar (first/second stage), and gray frame indicates extraction by HPH for 10 passages at 700/70 bar (first/second stage). DOC, deoxycholate; EDTA, ethylenediaminetetraacetic acid; HPH, high-pressure homogenization; OS, osmotic shock; POI, protein of interest; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

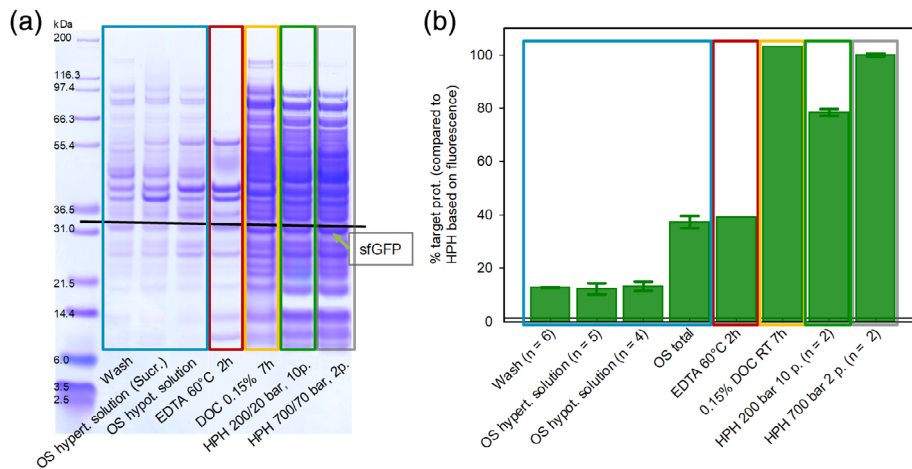


FIGURE 5 Analytical results of different extractions with the peri-sfGFP system. (a) SDS-PAGE showing POI and host-cell protein impurity levels. (b) Relative quantities of POI in extracts based on fluorescence measurements. Blue frame indicates extraction by OS, red frame indicates extraction by EDTA combined with heat, yellow frame indicates extraction by DOC, green frame indicates extraction by HPH for 10 passages at 200/20 bar (first/second stage), and gray frame indicates extraction by HPH for 10 passages at 700/70 bar (first/second stage). DOC, deoxycholate; EDTA, ethylenediaminetetraacetic acid; HPH, high-pressure homogenization; OS, osmotic shock; POI, protein of interest; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

yielded full extraction and high purity. EDTA extraction yielded full recovery, but the fluorescence signal significantly decreased after 30 min of incubation at 60°C, suggesting increased heat sensitivity of GFPmut3.1 under these conditions (Figure S5).

3.6 | Influences of protein concentration and molecular weight

Our results suggested that the common classification of whole-cell versus periplasmic protein extraction may be somewhat ambiguous. Traditionally β -galactosidase (520 kDa) and glucose-6-phosphate-

dehydrogenase (128 kDa) are regarded as cytoplasmic marker proteins, while β -lactamase (42 kDa) and alkaline phosphatase (86 kDa) are used as periplasmic markers.^{2,24} However, Vazquez et al³⁹ performed a study using OS procedures and proposed that there was no discernable selectivity according to protein location, but that extraction efficiency was instead determined by protein size, with more efficient release of smaller proteins. Indeed, rather than the IM or OM, it appears that the crucial barrier is the peptidoglycan mesh, which is not destroyed during OS and thus functions as a sieve with a cutoff of approximately 100 kDa. The release efficacy is 90% for proteins with a molecular weight below 14 kDa, approximately 70% for a 25-kDa protein, and only 40% for proteins of between 66 and 97 kDa in size.

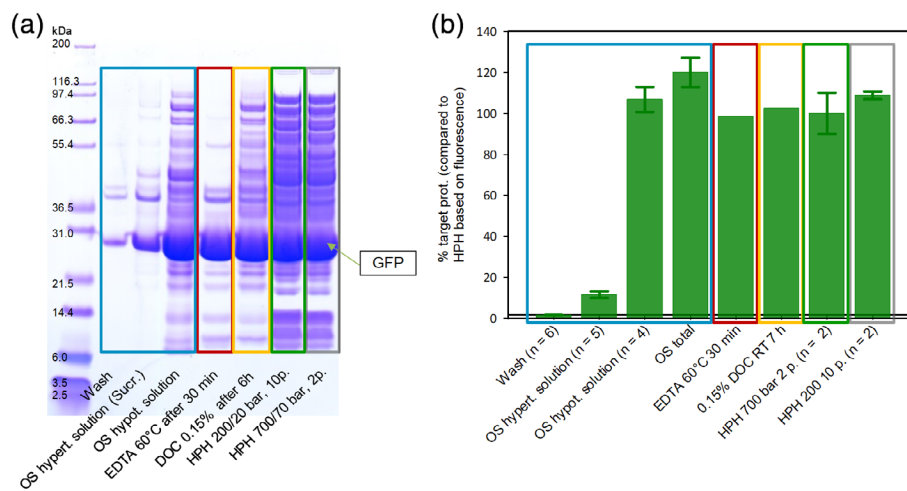


FIGURE 6 Analytical results of different extractions with the cyto-GFPmut3.1 system. (a) SDS-PAGE showing POI and host-cell protein impurity levels. (b) Relative quantities of POI in extracts based on fluorescence measurements. Blue frame indicates extraction by OS, red frame indicates extraction by EDTA combined with heat, yellow frame indicates extraction by DOC, green frame indicates extraction by HPH for 10 passages at 200/20 bar (first/second stage), and gray frame indicates extraction by HPH for 10 passages at 700/70 bar (first/second stage). DOC, deoxycholate; EDTA, ethylenediaminetetraacetic acid; HPH, high-pressure homogenization; OS, osmotic shock; POI, protein of interest; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

Based on the results with different model proteins, Vasquez et al excluded protein concentration as an influencing factor. However, our present data contradict this hypothesis. GFPmut 3.1 has a molecular weight of 26.4 kDa, and we found that its extraction efficiency was nearly 100% with the OS procedure, as well as for all other methods. These findings strongly indicate that the protein concentration was a major factor. Additionally, in this context, the relative low extraction efficiency for sfGFP can be explained by the reduced driving force caused by the lower protein titer of 0.6 mg/ml.

Based on our comparison of four extraction methods using three different model protein systems, we propose that a truly suitable method for periplasmic extraction at the preparative industrial scale has not yet been established. Nevertheless, the established extraction methods can be more or less disruptive, allowing strategies to obtain periplasmic protein at high purity. The various cell disintegration methods are based on different action principles, and thus produce different impurity profiles. Specifically, a high expression level combined with a gentle extraction method will generally yield recombinant protein of high purity, and if the POI is expressed in the periplasm and a gentle extraction method is used, the results will appear to be “selective periplasmic extraction.”

3.7 | DNA, endotoxins, and protein impurities

To complement our SDS-PAGE analysis, we also determined the total protein concentrations in all extracts (Table 1). As expected, HPH yielded the highest protein release, with concentrations of up to 20 mg/ml depending on the expression level of the recombinant target protein. By considering the target protein concentration, we determined that the HCP impurity level was 10–15 mg/ml, which is in the same range as reported by Nesbeth et al⁴⁰ OS, EDTA, and DOC

extractions yielded much lower protein impurity levels. Notably, the heat treatment involved in the EDTA method further contributed to the low HCP concentration. Extraction of GFPmut3.1 with EDTA yielded an extremely low value of only 0.5 mg HCP/ml, which corresponds to approximately 95% purity with respect to protein impurities.

Aside from purity in terms of HCP, contaminating levels of DNA and endotoxins constitute an important issue for downstream processing and purification, as these molecules must be reduced to very low levels for pharmaceutical applications.^{9,41} Table 1 summarizes the DNA and endotoxin levels of our extracts. DNA concentrations in the HPH extracts were consistently around 0.9 mg/ml with all three expression systems. Voulgaris et al reported the same DNA concentration when using the same host.⁴² For the other methods, the DNA concentrations were all below 0.2 mg/ml, indicating that the OS, EDTA, and DOC extraction methods are less disruptive and do not cause the release of large DNA molecules. The cyto-GFPmut3.1 system generally exhibited slightly lower DNA content in all extracts.

Endotoxin concentrations were all in the range of 10^6 – 10^7 EU/ml, with the exception of DOC extractions, which were lower by one order of magnitude. Trevilov et al⁴³ reported endotoxin values in the same range using HPH. The generally high endotoxin levels with all methods can be explained by the location of endotoxins in the OM, such that even methods that do not cause complete cell disruption can lead to high endotoxin release. With regard to the lower values with DOC, it is possible that DOC methods may partially mask endotoxins, resulting in an apparent lower value in the assay.

3.8 | Cell fragmentation

Cell fragmentation, and the resulting distribution of debris size, is a highly relevant process parameter for extract clarification.³¹

TABLE 1 DNA, endotoxin, total protein content, and purity of extracts

Extraction method	Cyto-Peri-FP system (mCherry/sfGFP)					Cyto-GFPmut3.1 system					Peri-sfGFP system						
	DNA (mg/ml)	Endotoxin (EU/ml)	Tot. Prot. (mg/ml)	Purity mCherry (%)	Purity sfGFP (%)	DNA (mg/ml)	Endotoxin (EU/ml)	Tot. Prot. (mg/ml)	Purity (%)	DNA (mg/ml)	Endotoxin (EU/ml)	Tot. Prot. (mg/ml)	Purity (%)	DNA (mg/ml)	Endotoxin (EU/ml)	Tot. Prot. (mg/ml)	Purity (%)
HPH 700/70 bar, two passages	0.92	5.9E+06	14.6	5	3	0.69	8.4E+06	17.6	39	0.74	3.6E+06	14.2	6	0.74	3.6E+06	14.2	6
HPH 200/20 bar, 10 passages	0.93	5.3E+06	15.2	5	3	0.79	1.1E+07	20.3	37	0.80	4.4E+06	11.0	6	0.80	4.4E+06	11.0	6
OS wash	0.14	1.4E+06	1.4	8	6	0.04	2.4E+06	1.2	10	0.13	1.3E+06	2.0	6	0.13	1.3E+06	2.0	6
OS hypertonic solution	0.05	7.1E+06	4.0	9	4	≤0.03	6.9E+06	2.8	28	0.05	4.0E+06	2.4	5	0.05	4.0E+06	2.4	5
OS hypotonic solution	≤0.03	6.4E+05	2.9	11	4	0.05	3.8E+06	12.2	65	≤0.03	8.7E+05	2.4	5	≤0.03	8.7E+05	2.4	5
10 mM EDTA, 60°C, 2 hr	0.18	2.9E+06	3.6	20	10	0.08	8.4E+06	7.6	61 ^a	0.23	1.7E+06	2.8	12	0.23	1.7E+06	2.8	12
0.15% DOC, 7 hr	0.17	1.4E+05	7.2	9	11 ^b	0.05	5.5E+05	9.6	73	0.08	1.8E+05	7.7	12	0.08	1.8E+05	7.7	12

Note: Purity is calculated based on the ratio of protein of interest and total protein.

Abbreviations: DOC, deoxycholate; EDTA, ethylenediaminetetraacetic acid; HPH, high-pressure homogenization; OS, osmotic shock.

^aAfter 30 min, we observed full extraction with a purity of 95%; however, after 2 hr of EDTA/heat treatment, the fluorescence signal decreased.

^bDue to solubilization of sfGFP from IB, the sfGFP content according to fluorescence was 180%, resulting in a relatively high purity value.

We investigated this aspect using a dispersion analyzer—an analytical centrifuge that can instantaneously measure the extinction of transmitted light across the entire length of the settling sample. Figure 7 shows the results of these measurements. Density distribution was plotted against the sedimentation velocity, which is a function of particle size and viscosity and, thus, centrifugability. By considering the viscosity and density, the particle size can be calculated. Since exact fragment densities are difficult to determine, we entered a density of 1.09 kg/m³ for cells,⁴⁴ and assumed a density of 1.3 kg/m³ for fragments and a viscosity of 1.3 mPa s for our approximate estimation of size. The low viscosity was a result of our dilution of the samples before measurement.

Using the above-described assumptions, we determined that the cell size was around 1 μm, which is in agreement with previous data.^{38,45} For the cyto-GFPmut3.1 system, the cell size was slightly bigger than measured for the other systems. With OS methods, the produced debris size showed different appearances in different systems. For the cyto-GFPmut3.1 system, which yielded full extraction, the debris size distribution exhibited unimodal distribution within the range of 240 nm. For the peri-sfGFP system, the debris size distribution rather suggested shrinking of the cells and only minor disintegration, which was also reflected by the low extraction efficiency. For the system involving mCherry and sfGFP, we observed a bimodal distribution of shrunk and disintegrated cells, which is also in accordance with the measured extraction efficiency of approximately 70%.

EDTA extraction resulted in a relatively narrow distribution at around 700–800 nm, indicating that cells were not completely fragmented despite complete extraction of target proteins. This was further confirmed by CLSM measurements revealing intact cell structures, but no remaining fluorescence signal of the soluble target proteins within the cells (Figure S6). Leive et al demonstrated that such cells are still somewhat viable and can continue growing after EDTA treatment, although they used a temperature of only 37°C in their work.¹⁶

DOC extraction resulted in a wide particle size distribution. Notably, previous experiments have revealed that DOC concentrations higher than 0.15% result in only small particles with sizes of <240 nm, indicating full membrane disruption. Apparently, when using a DOC concentration of 0.15%, larger membrane structures were still present. All model systems responded to DOC treatment in essentially the same way.

HPH almost completely disrupted the cells, resulting in cell debris size of 100–300 nm. After HPH at 200 bar, we observed slightly larger particle sizes compared to HPH at 700 bar.

Our cell fragmentation results were reproducible after independent fermentations and extractions (Figure S7). The results presented in Figure 7, represent the sedimentation behavior in a centrifugal force field under dilute conditions. Preparative centrifugation at process scale will most likely perform similarly. Our results suggest that the majority of particles can be separated using standard equipment. In terms of separability, EDTA extraction appeared to be the best option, as it produced no small debris particles. For a full evaluation of the methods, centrifugation trials must be performed on larger scales

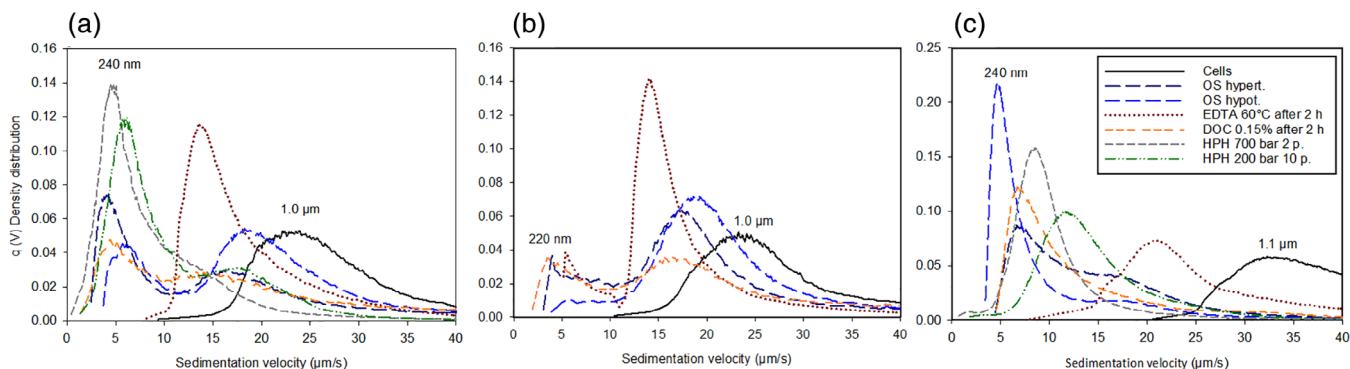


FIGURE 7 Sedimentation velocity of the remaining cells and cell debris after extractions performed with the three investigated systems in BL21(DE3) host cells expressing periplasmic sfGFP and cytoplasmic mCherry (a), periplasmic sfGFP (b), and cytoplasmic GFPmut3.1 (c)

and followed by filter screening; however, this was beyond the scope of our present study.

4 | CONCLUSIONS

Here we compared the performance of four methods for selective extraction of periplasmic proteins. As a monitoring tool, we used chromoproteins as cytoplasmic and periplasmic markers. In contrast to other reports, we did not achieve true selectivity. We observed apparent selectivity for periplasmic extraction when applying nondestructive extraction methods with a relatively high target protein concentration. However, the same results were achieved with a cytoplasmic protein, as demonstrated with the cyto-GFPmut3.1 system. Our findings are in agreement with those of Vasquez et al, who suggested that OS procedures are selective only in terms of protein size, and independent of a protein's cytoplasmic or periplasmic location.³⁹ In addition to different action principles that lead to extraction, we clearly demonstrated that the target protein concentration was a major driving force of a method's efficiency. Besides extraction efficiency and purity of extracts, we also examined aspects relevant to the subsequent downstream processing.

HPH caused full mechanical disruption, with consequently high levels of HCP, DNA, and endotoxins. The residual debris size was small, and applying HPH at lower pressure and with more passages did not yield relevant improvement in terms of impurity levels. EDTA treatment combined with heat resulted in large cell-like residual structures, thus simplifying centrifugation. The extracted target protein showed high purity, supported by the fact that heat treatment induced concurrent protein precipitation. However, this method is difficult to apply for temperature-sensitive proteins. Combination with other chemicals, such as urea,⁴⁶ may be an option to circumvent heating, but can also induce target protein denaturation. Extraction with the detergent DOC was efficient, and is easy to apply and implement. Since no temperature increase is required, this method is also applicable for more sensitive proteins. Membrane solubilization resulted in relatively high protein impurity contents and smaller particle sizes. The OS method resulted in pure extracts; however, the POI was contained in two or three different fractions. Moreover, efficient

extraction was associated with rather small particle sizes, and scaling up to an industrial scale seems challenging due to the stepwise procedure. Overall, depending on the target protein, our findings indicate that DOC extraction and EDTA/heat extraction are valuable alternatives to HPH as a release method for both cytoplasmic and periplasmic proteins.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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