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A microscale method of protein extraction from bacteria: Interaction of *Escherichia coli* with cationic microparticles

Alexandru Trefilov[a], Moritz Imendoerffer[a], Gerhard Sekot[a], Florian Strobl[a], Alois Jungbauer[a,b], Rainer Hahn[a,b,*]

[a] Austrian Centre of Industrial Biotechnology, Muthgasse 18, 1190 Vienna, Austria
[b] Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna (BOKU), Muthgasse 18, 1190 Vienna, Austria

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ABSTRACT

We developed a simple, highly selective, efficient method for extracting recombinant proteins from *Escherichia coli*. Our recombinant protein yield was equivalent to those obtained with high pressure homogenization, and did not require exposure to harsh thermal, chemical, or other potentially denaturing factors. We first ground conventional resin, designed for the exchange of small anions, into microparticles about 1 μm in size. Then, these cationic microparticles were brought convectively into close contact with bacteria, and cell membranes were rapidly perforated, but solid cell structures were not disrupted. The released soluble components were adsorbed onto the cell wall associated microparticles or diffused directly into the supernatant. Consequently, the selective adsorption and desorption of acidic molecules is built into our extraction method, and replaces the equally effective subsequent capture on anion exchange media. Simultaneously to cell perforation flocculation was induced by the microparticles facilitating separation of cells yet after desorption of proteins with NaCl. Relative to high pressure homogenization, endogenous component release was reduced by up to three orders of magnitude, including DNA, endotoxins, and host cell proteins, particularly outer membrane protein, which indicates the presence of cell debris.

1. Introduction

Extraction of proteins from bacteria without disrupting the cells is a key simplification of conventional purification processes and isolations of labile proteins. Typically, isolation of recombinant proteins from bacteria involves a cascade of operations, including cell harvest, cell disruption, and homogenate clarification to remove cell debris, followed by a combination of chromatographic methods to obtain highly pure protein (Demain and Vaishnav, 2009). Currently, a variety of methods are available for cell disruption, including enzymatic digestion, chemical treatments, and ultrasonication. For preparative scale protein extractions, high pressure homogenization and milling techniques are the preferred methods (Middelberg, 1995). Also, osmotic and thermal shock can be applied as supplementary forces to enhance the performance of other disruption techniques (Harrison, 1991). In all cases, and irrespective of the scale, the removal of generated cell debris is required before further processing the obtained cell homogenate (Balasundaram et al., 2009). Membrane filtration or chromatographic techniques, which commonly follow the disintegration step in a downstream processing routine, are susceptible to blocking and fouling when small particles are present in the process solution. Thus, efficient removal of cell debris or other solid components is difficult. Centrifugation, depth filtration, or a combination thereof is typically applied, but again, difficulties arise with small particle extractions. High product yields can be achieved by increasing the number of disintegration cycles to further fragment the cell debris, but eventually, this leads to an increase in viscosity. Consequently, very high centrifugal forces or large filter units are required, but efficient clarification results in low throughput yields. When working at the laboratory scale, these problems can be solved with high performance equipment; but at preparative, or even more industrial

* Corresponding author at: Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna (BOKU), Muthgasse 18, 1190 Vienna, Austria. Tel.: +43 1476546671.
E-mail address: Rainer.Hahn@boku.ac.at (R. Hahn).

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scales, this processing step represents a severe bottle-neck. Thus, alternative techniques are required with higher selectivity for extracted components.

Typically, the bacterial cell envelope consists of a plasma membrane and a peptidoglycan cell wall. Gram negative strains, such as *E. coli*, contain a thin peptidoglycan layer and an outer membrane composed of a lipopolysaccharide complex, also known as endotoxin. Endotoxin removal below a critical, nontoxic limit during downstream processing is crucial for any biopharmaceutical product. In general, the integrity and functionality of a cellular system is maintained by the selective molecular sieving properties of its membranes. The bacterial peptidoglycan layer, which provides the shape of the cell, typically allows diffusion of relatively large molecules, due to its loose meshwork (Demchick and Koch, 1996; Vázquez-Laslop et al., 2001); in contrast, cell membranes are generally impermeable to passive transport of macromolecules, like proteins. Chemical or physical methods for increasing cell membrane permeability, e.g., organic solvent and osmotic shock treatments, have been shown to be more selective for the release of intracellular proteins compared to cell disruption methods (Harrison, 2011).

Membranes are composed of amphiphilic molecules that form hydrophobic, introversive domains and hydrophilic chains that are exposed to the aqueous surfaces. The structural stiffness of these so called bilayers is primarily limited, due to the interfacial tension of water, which is excluded from the hydrophobic regions (Hancock et al., 1994; Nikaido, 2003). Such a structure is susceptible to internal and external pressures, depending on the enclosed volume. Moreover, weak interactions between membrane moieties allow lateral movement and transverse rearrangements, which makes the bilayer vulnerable to disaggregation when subjected to surface tension-reducing agents. However, membranes of unicellular specimens which are extensively exposed to environments contain divalent cations, like Ca^{2+} and Mg^{2+}, which increase structural stiffness by minimizing the repulsive forces between the predominantly negatively charged hydrophilic residues (van Loosdrecht et al., 1990). The negative charges on microbial surfaces also promote the electrostatic interaction of these cells with positive charges on anion exchange resin (Terada et al., 2006). The attractive or repulsive force intensities between charges in an aqueous medium depend on the buffer conditions, such as pH and ionic strength, and on the interfacial distance. Previously, it was shown that some electrostatic interactions can perturb membrane integrity and lead to cell inactivation (Gottenbos et al., 2001; Leroueil et al., 2008; Palermo et al., 2011). As a possible perturbation mechanism, it has been proposed that divalent cations, which are crucial for membrane integrity, could be exchanged with a high density of positive charges or neutralized, due to complexation with chelating groups (Poortinga et al., 2002).

Our protein extraction method comprises five steps. First, we prepared cationic microparticles with an average diameter of one micron, by grinding up a strong basic anion exchange resin; second, we homogenously mixed a bacterial cell suspension with the microparticles; third, we incubated the mixture without further agitation; fourth, we resuspended the flocs in an elution buffer; and fifth, we separated the liquid from the solid fraction.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma Aldrich (St. Louis, Missouri, US), Merck (Darmstadt, Germany), or Invitrogen (Carlsbad, CA, US).

2.2. Preparation of microparticles

Ion exchange resins were purchased from Sigma Aldrich. Dowex Marathon A2 is a gel-type, strong basic, anion exchange resin, supported on a polystyrene divinylbenzene polymeric matrix, and functionalized with dimethyl ethanol ammonium. Dowex Marathon MSC is a macroporous, strong acidic, cation exchange resin, functionalized with sulfonic groups. Amberlite IRA458 is a gel-type, strong basic, anion exchange resin supported on a divinylbenzene crosslinked acrylic matrix, and functionalized with trimethyl ammonium. Resins were washed by mixing 20:1 (v/v) with 50% ethanol and 20:1 (v/v) double deionized water (ddH_2O), preconditioned to their sodium or chloride form with 10:1 (v/v) 2 M NaCl for cation exchangers and anion exchangers, respectively. Then, beads were washed 5 times by mixing 20:1 (v/v) with ddH_2O until the supernatant conductivity was below 1 mS/cm and the pH was neutral. Resin beads were then wet-ground with a pestle and mortar, until the main solid fraction had an average diameter of about 1 μm. Grinding of 40 g resin in 120 g ddH_2O was performed with a motor-driven pestle and mortar for 24 h. Particle size was measured by optical microscopy with the Live Cell system (Leica Microsystems, Wetzlar, Germany). Particle size distributions were estimated statistically from an equivalent circular diameter by counting 5000–50,000 discrete optical projections using the image analysis software JMicroVision (Roduit, N., University of Geneva, Switzerland Version 1.2.7). The shapes of ground particles were considered irregular, based on optical projections. Microparticle concentrations were determined by weighing the wet pellet and relating it to the total suspension volume. Suspensions were adjusted by centrifugation on a Heraeus Multifuge X3R with a TX-750 Swinging Bucket Rotor (from Thermo Fisher Scientific, Vienna, Austria) in 50 mL tubes, for 2 h, at 4000 × *g* and 23 °C; wet pellets were resuspended in ddH_2O.

2.3. Cells

E. coli HMS174 cells were batch cultivated in a programmable, logic-controlled reactor with semisynthetic media. The media was supplemented with 2.5 g NH_4Cl and 2.1 g $(NH_4)_2SO_4$ per liter to provide a nitrogen source. Yeast extract was added (0.15 g/g dry cell mass) to accelerate the initial growth. After media sterilization in the bioreactor, the pH was adjusted to 7 with 12.5% ammonia solution. The synthetic media was used for the subsequent fed batch culture. A buffer (3 g KH_2PO_4 and 4.58 g K_2HPO_4 per liter media) was added to provide buffering capacity and to serve as a phosphate and potassium source. The other media components were added in sufficient quantity to produce 1 g of biomass: 0.25 g $C_6H_5Na_3O_7 \times 7H_2O$, 0.10 g $MgSO_4 \times 7H_2O$, 0.02 g $CaCl_2 \times 2H_2O$, 50 μL trace element solution, and 3.3 g $C_6H_{12}O_6 \times 1H_2O$. For cultivations of *E. coli* that expressed recombinant superoxide dismutase (SOD), 4 mg $CuCl_2 \times 2H_2O$ and 3.2 mg $ZnSO_4 \times 7H_2O$ per g dry cell mass were added. The recombinant proteins, green fluorescent protein mutant 3.1 (GFPmut3.1) and SOD, were induced to express with the addition of isopropylthio-β-galactoside (IPTG) at 20 μM/g dry cell mass. Approximately 10 L of cell broth was collected from the fed-batch culture and stored at 4 °C. Extraction and homogenization experiments for GFP and for SOD were started within 1–20 h after the cell broth was collected from the reactor. In that interval, cell suspensions and pellets were stored at 4 °C.

2.4. High pressure homogenization

Cells were collected from fermentation broth by centrifugation (50 mL at 4000 × *g* for 10 min) at 23 °C. Cells were suspended in buffer containing 50 mM TRIS, pH 8.0, and 100 mM NaCl at a concentration of 25% (v/v) cells; then, 100 mL batches of cells were