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Rapid and efficient purification of RNA-binding proteins: Application to HIV-1 Rev

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ABSTRACT

Non-specifically bound nucleic acid contaminants are an unwanted feature of recombinant RNA-binding proteins purified from *Escherichia coli* (*E. coli*). Removal of these contaminants represents an important step for the proteins' application in several biological assays and structural studies. The method described in this paper is a one-step protocol which is effective at removing tightly bound nucleic acids from over-expressed tagged HIV-1 Rev in *E. coli*. We combined affinity chromatography under denaturing conditions with subsequent on-column refolding, to prevent self-association of Rev while removing the nucleic acid contaminants from the end product. We compare this purification method with an established, multi-step protocol involving precipitation with polyethyleneimine (PEI). As our tailored protocol requires only one-step to simultaneously purify tagged proteins and eliminate bound cellular RNA and DNA, it represents a substantial advantage in time, effort, and expense.

Introduction

RNA, in its varied forms, interacts with proteins to carry out fundamental roles in the cell. The understanding of molecular recognition events, including those involved in assembly of macromolecular complexes consisting of both protein and RNA, are a particular challenge for *in vitro* biochemical, biophysical, and structural study. Purification of an overexpressed protein is essential for the subsequent detailed characterization of the protein and its complexes. For this purpose, purity and maintaining the native conformation of the protein are the most important criteria. RNA-binding proteins are known to have a relatively broad specificity in binding to RNA; thus, such proteins may bind to host RNA or DNA during overexpression and co-purify during the purification process. Conventional purification steps to remove nucleic acid contaminants include nuclease digestion, chemical treatments, or ultracentrifugation steps [1]. Additionally, the precipitation of nucleic acids with polyelectrolytes has been proposed and tested for *Escherichia coli* (*E. coli*) [2,3] and wheat germ [4]. Among polyelectrolytes, polyethyleneimine (PEI) has been widely employed [5–7]. PEI is a positively charged polyelectrolyte (pK_a 9.7) with the structural formula $(-CH_2-NH-CH_2-)_n$ [8]. PEI binds negatively charged DNA and RNA, and the resulting precipitates can be removed by centrifugation. Although extensively used, PEI treatment of cellular extract or protein solution is inconvenient. The amount necessary to selectively precipitate non-target proteins and nucleic acids must be determined empirically. The binding of PEI to nucleic acids strongly depends on the ionic strength of the solution. Frequently, the target protein is found in the precipitate and additional purification steps are required to recover the protein of interest. In addition to cost and toxicity, PEI interferes strongly in all standards of protein estimation and prevents the accurate quantification of the protein concentration during purification [9].

We describe an alternative route for the removal of contaminant nucleic acids utilizing chemical denaturation and successive on-column refolding. In particular, we report RNA contamination of the recombinant HIV-1 Rev protein and propose a fast and reliable way to resolve the contamination problem yielding large amounts of native, functional protein. HIV-1 replication critically depends on Rev, which is encoded by a multiply spliced message [10,11]. Rev functions as a sequence-specific RNA-binding protein that activates the nuclear export of intron-containing viral mRNA transcripts to the cytoplasm. A plethora of studies have highlighted the unequivocal importance of oligomeric binding of Rev to the Rev Response Element (RRE) in nuclear export of viral RNA transcripts. Only oligomeric Rev–RRE complexes can interact with the CRM1 (or exportin 1) cellular export factor resulting in Rev-dependent export of full length and partially spliced mRNAs [12–14].

The tight association of RNA-binding proteins such as Rev with contaminating cellular nucleic acid is detrimental to a variety of assays, including RNA-binding analysis, a variety of spectroscopic studies, and structural analysis. Here, we describe a fast and simple one-step affinity-purification method for the isolation of specific RNA-binding proteins. Our protocol is based on chemical denaturation and subsequent on-column renaturation of a hexahistidine (His_6)-tagged HIV-1 Rev protein. Large amounts of soluble protein

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with purity greater than 95% and free of contaminating nucleic acid can routinely be obtained. This purified (His_6)-tagged Rev is homogeneous as judged by its secondary structure content and highly active in gel-mobility RRE stem-loop II binding assays. The adaptable protocol can be a versatile tool for the isolation of unknown RNA-binding proteins.

Material and methods

Bacterial expression of (His_6)-tagged recombinant Rev

Plasmid encoding (His_6)-tagged Rev protein was transformed into *E. coli* strain BL21(DE3). Transformed cells were grown at 37 °C in 1 l of LB media (containing 100 μM/ml ampicillin) to OD_{600} of approximately 0.5. Protein expression was induced by addition of 1 mM isopropylthio-β-D-galactoside (IPTG). After addition of IPTG, the cells were incubated 4 h at 37 °C. The cells were harvested by centrifugation and stored at −80 °C. Wet cell pellets weighted approximately 3 g/1 l of LB medium.

Purification of (His_6)-tagged recombinant Rev using PEI (protocol A)

Frozen pellets were thawed and resuspended in lysis buffer (50 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 1 mM DTT, 0.02% sodium azide, 25 mM imidazole) and lysed by sonication on ice. Insoluble cell debris was removed from the cell lysate by centrifugation at 4 °C for 20 min (15,000*g*); subsequently, the cleared lysate was filtered through a 0.45 μm filter. The filtrate was purified by immobilized metal affinity chromatography (IMAC)[1] on a 1 ml Sepharose HisTrap FF nickel column (GE Healthcare). The protein was eluted with a linear gradient from the His-binding buffer (50 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 1 mM DTT, 0.02% sodium azide, 25 mM imidazole) to the His-elution buffer (50 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 1 mM DTT, 0.02% sodium azide, 500 mM imidazole) at 1 ml/min over 20 min. The fractions containing the protein of interest were collected, pooled, and the ionic strength of the solution was increased to 1 M sodium chloride. All successive steps were performed at 4 °C or on ice. PEI was added to the final concentration of 0.5% (w/v) and the solution was incubated for 1 h. The resulting suspension was centrifuged at 15,000*g* for 20 min to remove PEI–nucleic acid complexes. The protein was recovered from the excess of PEI present in the supernatant by precipitation with 75% ammonium sulfate. After overnight incubation, this suspension was centrifuged, and the pellet containing the protein was resuspended in binding buffer (50 mM sodium phosphate, pH 7.4, 200 mM sodium chloride, 1 mM DTT, 0.02% sodium azide), applied onto a 1 ml HisTrap SP XL column, and eluted with a linear gradient into elution buffer (50 mM sodium phosphate, 2 M sodium chloride, 1 mM DTT, 0.02% sodium azide, pH 7.4). Protein samples were concentrated 8-fold using an Amicon Ultra-15 (Millipore) with a 5 kDa MWCO membrane. Residual traces of imidazole were removed by dialyzing the eluate at 4 °C overnight against 1 l of storage buffer (50 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 1 mM DTT, 0.02% sodium azide) containing 10% glycerol. Aliquots were stored at −80 °C for further characterization. The concentration of proteins contaminated with nucleic acids was determined using a Bradford protein assay (Bio-Rad) while concentrations for the pure protein were determined by measuring the absorbance at 280 nm and an extinction coefficient (ε) of 8600 cm^{-1} M^{-1}, as determined by amino acid sequence data [15]. The purity of Rev was monitored after each purification step by SDS–PAGE using 4–20% gradient gels (Bio-Rad).

[1] *Abbreviation used:* IMAC, immobilized metal affinity chromatography.

Purification of (His_6)-tagged recombinant Rev using urea denaturation/on-column refolding (protocol B)

Frozen pellets were thawed and resuspended in His-binding buffer containing 8 M urea (8 M urea, 50 mM sodium phosphate, 500 mM sodium chloride, 1 mM DTT, 0.02% sodium azide, 25 mM imidazole, pH 7.4) and lysed by sonication on ice. Insoluble cell debris was removed from the cell lysate by centrifugation at 4 °C for 20 min (15,000*g*); subsequently, the cleared lysate was filtered through a 0.45 μm filter. The urea-containing filtrate was purified by IMAC on a pre-equilibrated 1 ml Sepharose HisTrap FF nickel column (GE Healthcare) applied at a flow-rate of 1 ml/min rate. On-column (His_6)-tagged Rev renaturation was achieved at a reduced flow-rate of 0.5 ml/min with a linear gradient from 8 to 0 M urea over 30 column volumes. The renatured protein was eluted from the column with the His-elution buffer (50 mM sodium phosphate, 500 mM sodium chloride, 1 mM DTT, 0.02% sodium azide, 500 mM imidazole, pH 7.4) at 1 ml/min over 20 min. Protein samples were concentrated 15-fold using an Amicon Ultra-15 (Millipore) with a 5 kDa MWCO membrane. Residual traces of imidazole were removed by dialyzing the eluate at 4 °C overnight against 1 l of storage buffer (50 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 1 mM DTT, 0.02% sodium azide) containing 10% glycerol. Aliquots were stored at −80 °C prior to further characterization. The successful renaturation of proteins by gradually reducing the amount of urea potentially presents a serious obstacle for other target proteins. Alternatively, lower, nondenaturing concentrations of urea combined with higher ionic strength can be investigated, e.g. 1 M urea in combination with 1 M NaCl (see Results section).

Circular dichroism measurements

Circular dichroism (CD) measurements of purified (His_6)-tagged Rev were performed on a temperature-controlled AVIV spectropolarimeter. CD spectra were recorded at 5 °C between 198 and 260 nm, with a 0.1 cm path length cell, a wavelength increment of 1 nm, an averaging time of 5 s, and an equilibration time of 5 min. The baseline was corrected by subtracting the spectra of the respective buffers collected under identical conditions. Spectra from three scans were averaged. (His_6)-tagged Rev samples at 10 μM concentration in the storage buffer were centrifuged at 5000*g* for 10 min at 5 °C prior to further analysis. The concentration of the supernatant containing the recombinant Rev protein was assessed by measuring the absorbance at 280 nm. Deconvolution of the CD spectrum was performed using the CDPro suite software package consisting of the CONTIN/LL, CDSSTR, and SELCON3 software packages [16,17]. The chosen IBasis 4 parameter [18] contains a large reference set of 43 soluble proteins. The reported overall secondary structure percentages represent averaged values derived from all three programs.

Urea-induced denaturation

Urea-induced denaturation of (His_6)-tagged Rev was monitored by CD in the wavelength range of 210–260 nm at 25 °C. Rev solutions at 10 μM concentration were mixed with varying amounts of stock solution containing 10 M urea. The buffer in all denaturation reaction was 50 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 1 mM DTT, and 0.02% sodium azide. Unfolding was monitored in the range of 0–8 M urea. Spectra represent the average of three scans for each urea concentration. The urea-unfolding profile of (His_6)-tagged Rev is described by the change of the molar ellipticity value at 222 nm, indicative of an α-helical secondary structure, as a function of denaturant concentration.