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A new protocol for high-yield purification of recombinant human prothymosin α expressed in *Escherichia coli* for NMR studies

Shiluan Yi, Anne Brickenden, Wing-Yiu Choy *

Department of Biochemistry, The University of Western Ontario, London, Ont., Canada N6A 5C1

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Abstract

Human prothymosin α (ProTα) is a small acidic protein (12.1 kDa; pI ~3.5) ubiquitously expressed in a wide variety of tissues. The amino acid composition of this protein is highly unusual. While close to half of its sequence is composed of acidic amino acids, the protein does not contain any aromatic residues. ProTα has been shown to play crucial roles in different biological processes including cell proliferation, transcriptional regulation and apoptosis. Despite the multiple functions this protein has, it does not adopt a stable tertiary fold under physiological conditions. In order to understand how ProTα functions, detailed structural characterization of this protein is essential. Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique for elucidating the protein structure and dynamics at the atomic level. However, milligrams of isotopically labeled protein with high purity are usually required for the studies. In this work, we developed a high-yield protocol for purifying recombinant ProTα expressed in *Escherichia coli* by exploiting the intrinsically disordered and acidic natures of this protein. By combining the heat–cooling extraction, ammonium sulfate precipitation, and anion exchange chromatography, we were able to obtain over 20 mg of ProTα with >97% purity from 1 L of M9 minimal media culture. The new purification protocol provides a cost effective and an efficient way to produce large quantities of high purity recombinant human ProTα in various isotopically labeled forms, which will greatly facilitate the structural studies of this protein by NMR and other biophysical methods.

Keywords: Prothymosin α; Disordered protein; Purification; Heating–cooling extraction; Ammonium sulfate precipitation; Ion exchange chromatography; Isotopic labeling; NMR

Prothymosin α (ProTα) is a small and highly acidic protein ubiquitously expressed in a wide variety of human tissues [1]. The protein has been found to be involved in cell proliferation [2,3], transcriptional regulation [4,5], chromatin remodeling [6,7], and oxidative stress–response [8]. Recent studies demonstrated that ProTα also plays a regulatory role in apoptosis by blocking the formation of apoptosome [9], or via the interaction with an anti-apoptotic protein p8 [10]. Detailed structural characterization of ProTα and its interactions with targets will provide insights into how this protein functions. Previous studies showed that ProTα lacks stable tertiary structure under physiological conditions [11], thus, is classified as an intrinsically disordered protein. Detailed structural information of this protein, however, is still limited. Due to the disordered nature of ProTα, X-ray crystallographic study is not feasible. Therefore, nuclear magnetic resonance (NMR)[1] spectroscopy becomes the only other technique that can be employed to obtain structural and dynamic information at the atomic level. Like many other biophysical experiments, however, large quantities of protein with high purity are needed for the NMR studies. More importantly, costly isotopic labeling of protein sample is usually necessary. For instance, to perform three-dimensional heteronuclear NMR experi-

* Corresponding author. Fax: +1 519 661 3175.
E-mail address: jchoy4@uwo.ca (W.-Y. Choy).

[1] *Abbreviations used:* CD, circular dichroism; *E. coli*, *Escherichia coli*; IPTG, isopropyl-β-D-thiogalactopyranoside; NMR, nuclear magnetic resonance; ProTα, prothymosin α; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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ments for the backbone assignment of ProTα, milligrams of $^{13}C/^{15}N$-labeled protein are required. By using the existing purification protocol in the literature for the recombinant human ProTα [12], however, only a low yield (<3 mg from 1 L of M9 culture) of protein could be obtained in our laboratory. In order to produce isotopically enriched ProTα samples in a more cost effective and efficient way, we have developed a new protocol to purify the recombinant protein over-expressed in *Escherichia coli*. The protein extraction and pre-purification steps of this new approach is based on the heat–cooling strategy, which was proposed by Kalthoff [13] for the purification of two other disordered proteins, epsin 1 and AP180. The protein was then further purified by ammonium sulfate precipitation and anion exchange chromatography [9]. By using this new protocol, we were able to obtain over 20 mg ProTα with high purity from a 1 L of M9 minimal media culture. The protein was subjected to characterization by various biophysical methods. Our results confirm that ProTα is intrinsically disordered as previous reported [11]. In addition, the purified protein adopts a monomeric form under physiological conditions and is competent to interact with its target protein p8.

Materials and methods

Expression of recombinant human ProTα

The pHP12A plasmid encoding human ProTα [12] (a kind gift from Dr. Vartapetian at the Moscow State University, Russia) was transformed into *E. coli* BL21(DE3) cells (Novagen) by heat shock transformation. The cells were plated onto Luria–Bertani (LB)-agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar, pH 7.5) containing 50 μg/ml carbenicillin (US Biological) and incubated overnight at 37 °C. A single colony was then used to inoculate 1 ml of LB media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.5) containing 100 μg/ml carbenicillin. The culture was incubated at 37 °C for 8 h before transferring to a 200 ml of M9 minimal media (6.8 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 μg/ml thiamine, 10 μg/ml biotin, 1 g/L NH_4Cl, 3 g/L glucose, pH 7.4) supplemented with 5 ml of LB media and 100 μg/ml of carbenicillin. After an overnight-incubation at 37 °C, the bacterial cells were pelleted by centrifugation and were added into 1 L of M9 minimal media containing 50 μg/ml carbenicillin to obtain a starting OD_{600} of 0.10–0.15. For the expression of $^{15}N/^{13}C$-labeled protein, 1 g of $^{15}NH_4Cl$ (Cambridge Isotope Laboratories) and 3 g of $^{13}C_6$-D-glucose (Isotec) were added to the M9 media instead as the sole nitrogen and carbon sources, respectively. The cell culture was incubated at 37 °C until the OD_{600} reached 0.7–0.8. The protein overexpression was then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; BioShop). The cells were allowed to grow overnight at 25 °C before harvest by centrifugation. Typically 4–5 g wet-weight pellet can be obtained from 1 L of M9 culture.

Extraction and purification of ProTα

Bacterial cells were treated by the heat–cooling method as described by Kalthoff [13] with modifications. The cell pellet was first resuspended in 5 ml/g of TE buffer (25 mM Tris–HCl, 1 mM EDTA, pH 8.0) at 100 °C. The suspension was incubated in boiling water for 5 min and was immediately transferred to a −10 °C NaCl/ice water bath for another 5 min, followed by a 30 s of sonication on ice (output 4% and 40% duty cycle, Ultrasonic Processor W-375). Cellular debris and protein precipitation were then removed by centrifugation (40,000*g*, 30 min, Beckman JA-30.50 rotor).

Ammonium sulfate (EMD Chemicals Inc.) was added to the supernatant until the salt concentration reached 80% of saturation [9]. The mixture was agitated for 1 h at room temperature. Precipitates were then removed by centrifugation (40,000*g*, 30 min, Beckman JA-30.50 rotor), and the supernatant was desalted by dialyzing (3 × 8 h at 4 °C) against TE buffer using Spectra/Por® 7 dialysis tubing (MWCO 1000 Da). The dialyzed sample was filtered by 0.20 μm low-protein-binding membrane filter (Pall) before subjected to further purification by anion exchange chromatography using an ÄKTA Purifier 10 system (GE HealthCare). Specifically, the protein sample was resolved on a 1-ml HiTrap DEAE Fast Flow column (equilibrated with TE buffer at 4 °C) and was eluted with a linear gradient of NaCl (0–0.4 M) in a total volume of 20 ml at a flow rate of 1 ml/min. Collected fractions were analyzed by SDS–PAGE. Fractions containing ProTα eluted between 0.22 and 0.28 M of NaCl were combined and filtered. The protein sample were then applied to an 8-ml Mono Q 10/100 GL column (equilibrated with TE buffer containing 0.3 M NaCl at 4 °C) and the elution was performed with a linear gradient of NaCl (0.3–0.7 M) in a total volume of 80 ml at a flow rate of 4 ml/min. Collected fractions were analyzed by SDS–PAGE and the ProTα-containing fractions eluted between 0.49 and 0.50 M of NaCl were combined for subsequent analysis. The purified protein sample was then subjected to identification by mass spectrometry, amino acid analysis, and UV–visible spectrophotometry.

Determinations of protein concentration and purity

For SDS–PAGE analysis, protein sample was first dissolved in reducing sample buffer (62.5 mM Tris–HCl, 2% (w/v) SDS, 2.5% (v/v) 2-mercaptoethanol, 0.1% (w/v) bromophenol blue, pH 6.8) and incubated at 100 °C for 4 min. SDS–PAGE was conducted on 17% polyacrylamide gels using an XCell SureLock Mini-Cell electrophoresis system (Invitrogen). Gels were stained with Coomassie Brilliant Blue R-250. Apparent molecular weight of protein was estimated based on the Broad Range Protein Marker standard (New England Biolabs).

Protein concentration was estimated by Peterson's modified Lowry assay [14] with bovine serum albumin used as