Available online at www.sciencedirect.com
ScienceDirect
Protein Expression and Purification 57 (2008) 1–8
Protein Expression & Purification
www.elsevier.com/locate/yprep

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

Received 27 July 2007, and in revised form 28 August 2007
Available online 14 September 2007

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.

1046-5928/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2007.09.005

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

the standard. To have a more accurate quantitation of the purified protein, six samples with different amounts of ProTα were sent for amino acid analysis (the Advanced Protein Technology Center of SickKids, Hospital for Sick Children, Toronto, Canada) and the concentrations of these samples were also measured using the modified Lowry assay in our laboratory. A correction factor for the Lowry assay measurements was determined based on the results obtained from these two methods.

Since ProTα does not contain any aromatic residues, the UV absorption at 260–280 nm can be used to estimate the level of protein/nucleic acid contaminations of the sample. UV–visible spectrophotometry was performed using an Agilent 8453 UV–visible spectrophotometer (Agilent Technologies). Spectrum of the purified protein sample (with ~0.3 mg/ml in 40 mM Hepes buffer at pH 7.0) was recorded from 190 to 1100 nm using a 50 μl quartz cell with a path length of 10 mm.

NMR spectroscopy

^{1}H–^{15}N HSQC experiments were performed at 22 °C on a Varian Inova 600 MHz spectrometer equipped with a xyz-gradient triple resonance probe. NMR samples with 0.1 mM ^{15}N-labeled ProTα in 40 mM Hepes (pH 7.0) buffer and 10% (v/v) D_2O (Cambridge Isotope Laboratories) were used. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, Sigma) (1 mM) was added to each NMR sample as internal standard for the chemical shift referencing.

Circular dichroism (CD) spectropolarimetry

CD experiments were performed at 22 °C on a Jasco J-810 spectropolarimeter (Easton, MD), equipped with a Peltier temperature control unit, using a cuvette with 0.1 mm in path length. ProTα samples with 50 μM in concentration (in 10 mM Hepes at pH 7.0) were used. CD data were converted to mean residue ellipticity (θ) by standard procedures using the software provided by the manufacturer.

Analytical ultracentrifugation

Analytical ultracentrifugation technique was employed to study the interaction between purified ProTα and its target protein, p8. The pDEST17 expression vector harboring the human p8 sequence was used to generate the N-terminal His-tagged fusion protein. His_6-p8 was over-expressed in *E. coli* BL21(DE3) cells and purified by affinity chromatography using nickel sepharose. Sedimentation equilibrium experiments were performed using a Beckman Optima XL-A Analytical Ultracentrifuge (Beckman Coulter, Palo Alto, CA) equipped with absorption optics. An An-60Ti 4-hole rotor and six-channel cells with Epon–charcoal centerpieces were used. Protein samples were dialyzed into buffer containing 50 mM phosphate and 100 mM NaCl at pH 6.0. Data were collected at 23,000 rpm following an 18-h equilibration period at 22 °C. Absorbances at 280 nm (for p8 and lysozyme) or at 230 nm (for ProTα) were measured in 0.002 cm radial steps and averaged over 10 readings. Two consecutive scans separated by a 2-h interval were performed. The experiments were then repeated at 27,000 and 31,000 rpm. Attainment of equilibrium at each rotor speed was judged based on the overlaid of the two scans. Data obtained at different rotor speeds were then fitted globally to a single species model using Microcal Origin 6.0 software. Partial specific volumes of 0.700 ml/g for the disordered p8 and 0.714 ml/g for the globular lysozyme were calculated based on their amino acid compositions [15]. The solvent density was measured to be 1.010 g/ml.

Results

Over-expression of ProTα in E. coli

The pHP12A plasmid harboring the human ProTα gene was specifically designed for over-expressing the protein in *E. coli* [12]. To further optimize the conditions for producing ProTα in M9 minimal media, the effects of different parameters such as temperature, IPTG concentration and induction time on the protein expression level were investigated (data not shown). Our result indicates that optimal protein over-expression can be obtained with the cell culture growth at 37 °C, followed by induction with 0.1 mM of IPTG at OD_{600} ~0.7–0.8, and harvested after an overnight-incubation at 25 °C (Fig. 1, lane 2).

Extraction and pre-purification of ProTα by heat–cooling method

The heat–cooling treatment lysed the bacterial cells and led to denaturation and precipitation of globular proteins

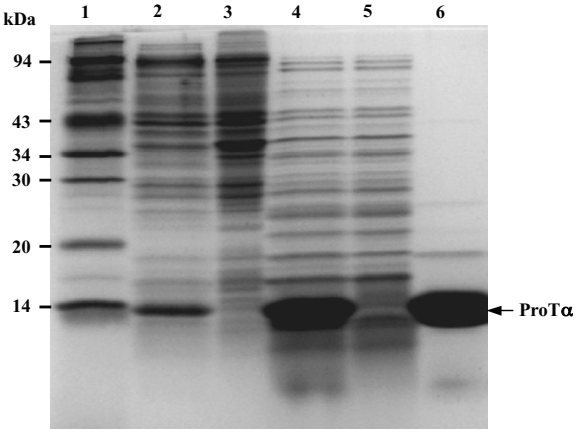

Fig. 1. SDS–PAGE analysis of the ProTα purity at different purification steps. Samples were run on a 17% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250 dye. Lane 1, molecular weight markers; lane 2, cell lysate, extracted by French-press (AMINCO) for 3 times at 20,000 psi; lane 3, precipitation of the heat–cooling treatment; lane 4, supernatant after the heat–cooling treatment; lane 5, precipitation of the 80% $(NH_4)_2SO_4$ saturation; lane 6, supernatant of the 80% $(NH_4)_2SO_4$ saturation.

in the lysate. Our result shows that 76% of the protein contaminants were being precipitated while 90% of the ProTα remained in the supernatant (lanes 3 and 4 in Fig. 1 and Table 1), indicating that the heat–cooling treatment is a critical step in this purification protocol. Compared to other conventional methods used for cell disruption, the heat–cooling treatment is more effective since both cell lysis and protein pre-purification can be achieved in a single step. A brief period of sonication after the heat–cooling step is included in this purification procedure to further disrupt the nucleic acids released from the cells.

Purification of ProTα by ammonium sulfate precipitation and anion exchange chromatography

Ammonium sulfate precipitation is a technique widely used in protein separation and purification. At high concentrations of $(NH_4)_2SO_4$, the ions disrupt the interaction between water and proteins, leading to a decrease in protein solubility and resulting in precipitation. This phenomenon is commonly referred to as the salt-out effect. With an extremely low hydrophobicity and a high net charge, ProTα remained soluble in solution up to 80% saturation of $(NH_4)_2SO_4$, while most other proteins were salted-out (lanes 5 and 6 in Fig. 1). Through the combination of heat–cooling extraction and ammonium sulfate precipitation, 97% of the protein contaminants were removed while 79% of the target protein remained in the supernatant (Table 1). Lane 6 in Fig. 1 shows that besides ProTα, only a few proteins with relatively faint Commassie Blue-stained bands were still present in the supernatant after the ammonium sulfate precipitation step.

The extremely acidic nature of ProTα allows the protein to be purified effectively by anion exchange chromatography. In this work, DEAE and Mono Q columns were used to further separate ProTα protein from the contaminants remained after the ammonium sulfate precipitation. Since ProTα has no aromatic amino acid, the protein is "invisible" at the wavelength of 260 and 280 nm. Therefore, UV-absorption at 225 nm was used to monitor the elution of ProTα instead. However, the absorbances at 260 and 280 nm were still employed to detect for contaminants that are invisible on SDS–PAGE (i.e. nucleic acids). The FPLC elution profile shown in Fig. 2 clearly demonstrates that ProTα (peak IV) can be separated effectively from the nucleic acids and other proteins by DEAE Fast Flow anion exchange chromatography [19]. After the DEAE column,

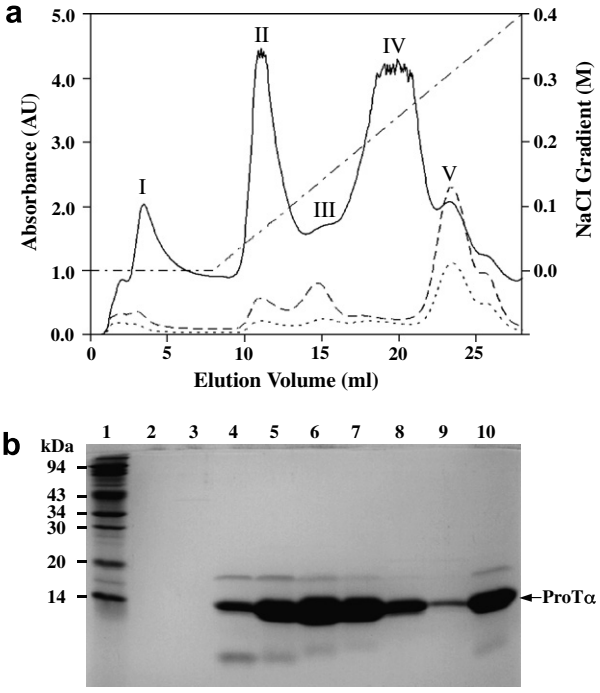

Fig. 2. Purification of ProTα by DEAE anion exchange chromatography. (a) Elution profile obtained on a HiTrap DEAE Fast Flow column (1 ml). Absorbances at 225 nm (—), 280 nm (···), and 260 nm (– –), as well as the NaCl gradient (–·–) are shown. Five major peaks I–V are indicated. (b) SDS–PAGE (17% gel) analysis of the fractions collected under peaks I–V indicated in (a). Lane 1, molecular weight markers; lane 2, peak I; lane 3, peak II; lane 4, peak III; lanes 5–7, peak IV; lanes 8–9, peak V; and lane 10, pool of all fractions collected under peak IV.

Table 1
Purification of ProTα from *E. coli*[a]

Purification step	Total protein (mg)	Purity of ProTα (%)[e]	Yield of ProTα (mg)	Recovery of ProTα (%)	Contaminant (mg)	Contaminant removed (%)
Cell lysate[b]	193[c]	22	42	100	151	0
Heat–cooling extraction	74[c]	51	38	90	36	76
$(NH_4)_2SO_4$ precipitation	38[d]	86	33	79	5	97
DEAE Fast Flow	29[d]	93	27	64	2	99
Mono Q	24[d]	98	23	55	1	99

[a] Obtained from 1 L of M9 culture, containing 4 g of wet-weight cells.
[b] Extracted by French-press at 20,000 psi for 3 times.
[c] Measured by Peterson's modified Lowry assay [14].
[d] Measured by Peterson's modified Lowry assay and corrected by amino acid analysis.
[e] Estimated by densitometric scanning of SDS–PAGE bands.

the ProTα-containing fractions were subjected to further purification using a Mono Q column. Fig. 3 shows that after this final purification step, the ProTα sample was purified to nearly homogeneity.

By using this new purification protocol, approximately 23 mg of ProTα protein with high purity can be obtained from a 1 L of M9 culture. Table 1 summarizes the percentage of recovery and the purity of ProTα at different stages of the purification procedure estimated by SDS–PAGE densitometric analysis (Table 1). Since ProTα does not contain any aromatic amino acid, the low absorbances at 260 and 280 nm are good indicators of the purity level of the target protein. Fig. 4 shows an UV–visible spectrum of the purified ProTα sample. The minimal absorptions at 260 and 280 nm agree with the result of SDS–PAGE densitometric analysis that ProTα protein with >97% purity can be obtained. Amino acid analysis (Supplementary Material Table S1) and electrospray ionization mass spectrometry (ESI-MS; Supplementary Material Fig. S1) were used to confirm the identity of the purified protein sample. A well match in the measured and calculated amino acid compositions was observed from the amino acid analysis. The ESI-MS data also agree with the result of previous studies [16] showing that the first methionine of ProTα was excised and the N-terminal serine is acetylated.

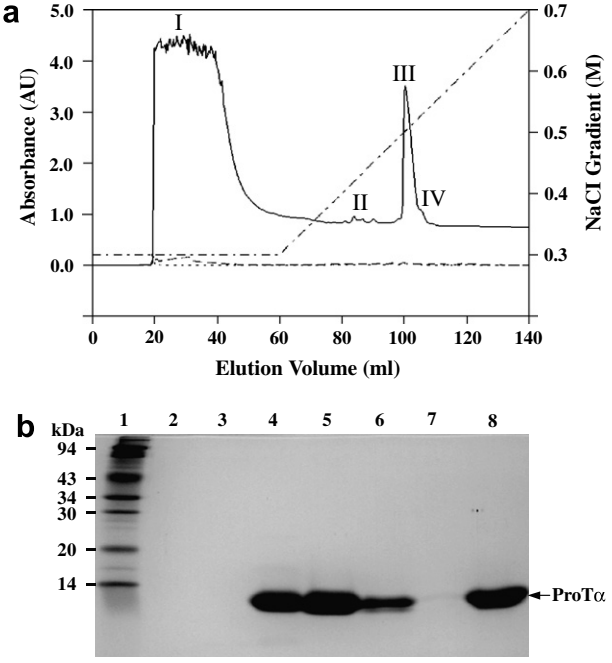

Fig. 3. Purification of ProTα by Mono Q anion exchange chromatography. (a) Elution profile on a Mono Q 10/100 GL column (8 ml). Absorbances at 225 nm (—), 280 nm (···), and 260 nm (– –) as well as NaCl gradient (–·–) are displayed. Four major peaks I–IV are indicated. (b) SDS–PAGE (17% gel) analysis of eluted fractions under peaks indicated in (a). Lane 1, molecular weight markers; lane 2, peak I; lane 3, peak II; lanes 4–5, peak III; lanes 6–7, peak IV; and lane 8, pool of all fractions collected under peak III.

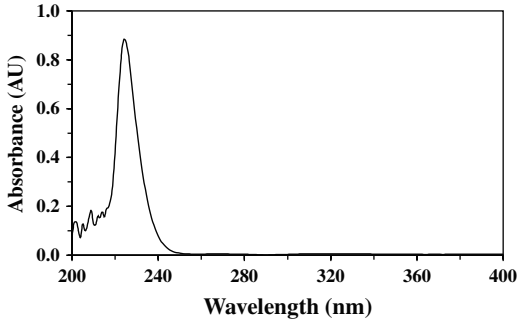

Fig. 4. UV–visible absorption spectrum of purified ProTα. The spectrum was recorded in 40 mM Hepes (pH 7.0) with protein concentration of 0.3 mg/ml, using a 50 μl quartz cell with 10 mm of path length.

Quantitation of purified recombinant ProTα

Due to the unusual amino acid composition of ProTα (Table S1, Supplementary Material), many traditional methods such as Bradford assay [17] and UV-absorption measurement at 280 nm are not applicable for the quantitation of ProTα. Even though it has been suggested that concentration of ProTα can be determined spectrophotometrically by measuring the absorptions at 215 and 225 nm [11], we found that the absorbances of ProTα at these two wavelengths are highly dependent on the buffer conditions. Therefore, accurate protein quantitation cannot be achieved by this approach either.

In this work, the Peterson's modified Lowry assay [14] was employed for the estimation of ProTα concentration. However, we are aware that the quantity of protein can be underestimated due to the existence of systematic errors in protein–dye binding assays for intrinsically unstructured proteins [18]. With this in mind, the concentrations of six purified ProTα samples, with concentrations ranging from 0.1 to 1.0 mg/ml, were measured both by the modified Lowry assay and amino acid analysis. A good correlation ($R^2 = 0.998$) between the results obtained from these two methods was observed. An empirical equation ($C_{AAA} = 1.28C_{Lowry} - 0.008$, where C_{AAA} and C_{Lowry} represent the protein concentrations in mg/ml measured by amino acid analysis and by the modified Lowry assay, respectively) was established which can be used to correct the result obtained from the modified Lowry assay in the future.

Characterization of purified ProTα

Purified ProTα samples were subjected to characterization by different biophysical methods. Sedimentation equilibrium analysis reveals that the protein exists in a monomeric form (12.2 ± 0.4 kDa) under the conditions used. Fig. 5a (blue) shows the ^{1}H–^{15}N HSQC spectrum of purified ProTα. The narrow dispersion of amide proton chemical shifts confirms that the protein is intrinsically disordered [11]. The large negative CD signal at 198 nm and the near-zero ellipticity around 220 nm (Fig. 5b; blue)

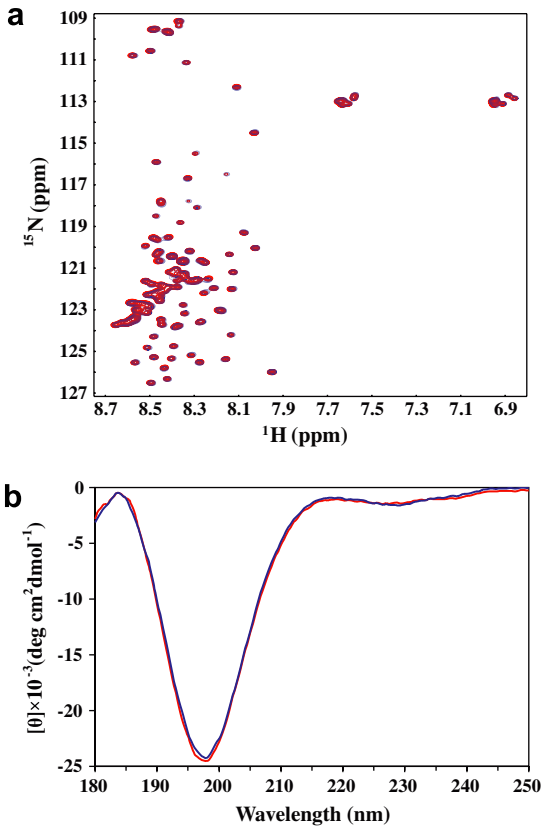

Fig. 5. Overlaid of ^{1}H–^{15}N HSQC spectra (a) and CD spectra (b) of ProTα samples prepared with heat–cooling (blue) and cell lysis by French-press (red) as the initial protein extraction steps.

further support this finding. In addition, protein sample prepared using this new protocol has been used in studying the metal binding properties of ProTα by NMR. The results demonstrate that ProTα is capable to bind specifically to Zn^{2+} ions as previously reported [19,20] (data will be reported elsewhere).

We have also examined the competency of the purified ProTα to bind to its protein target in vitro. Recent studies have demonstrated that ProTα interacts with the protein p8 to form a stable complex in regulating apoptosis [10,21]. By using sedimentation equilibrium technique, we have measured the change in apparent molecular weight (M_{app}) of p8 upon addition of ProTα. Fig. 6a shows that in the absence of ProTα, p8 adopts a monomeric form under the conditions used. Upon addition of an equal molar of ProTα, however, a significant increase in the M_{app} of p8 was observed (Fig. 6a). The experiments were then repeated with lysozyme, which has molecular weight and pI similar to that of p8, as a control (Fig. 6b). Our results clearly demonstrate that purified ProTα can specifically interact with its target p8, but not lysozyme, under the experimental conditions used.

Discussion

The unique amino acid composition of ProTα renders it highly soluble in the aqueous phase during phenol extraction, a method that had been used for separating ProTα from other proteins [12,22]. However, by using this protocol, we were only able to obtain ~3 mg of ProTα from 1 L of M9 culture. In contrast, with the new protocol proposed here, over 20 mg of ProTα with high purity can be obtained. Since the execution of this protocol is very straightforward and all the reagents and instruments required are commonly used for protein purification, it is apparent that this new method can be particularly useful in producing ProTα samples for biophysical studies, where milligrams of protein with high purity are required.

The heat–cooling step is relatively simple to carry out, yet extremely effective for isolating unstructured proteins from the more heat-sensitive globular proteins [23–25]. Recently, this strategy has been applied to a large-scale

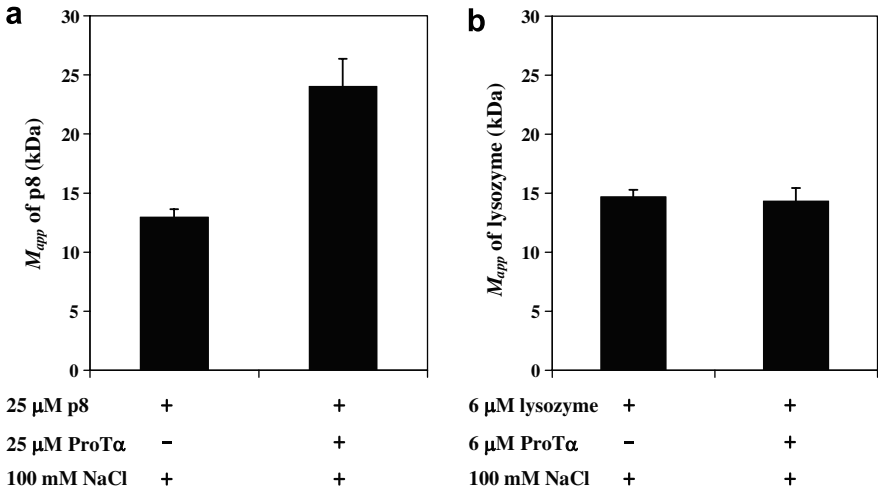

Fig. 6. Sedimentation equilibrium analysis of the interactions between ProTα, p8 and lysozyme. Apparent molecular weight (M_{app}) of p8 (a) and lysozyme (b) in the absence and the presence of an equal molar of ProTα.

proteomic study of intrinsically disordered proteins in the mammalian proteome [26]. Unlike the functions of globular proteins or enzymes which can be disrupted by heat denaturation, the activities of disordered proteins are less sensitive to heat treatment. Evstafieva et al. have demonstrated that ProTα purified under denaturing conditions including phenol extraction and boiling still possesses immunoregulatory activity [12]. Karetsou et al. have also shown that ProTα remains active in modulating the interaction of histone H1 with chromatin even after boiling and acidic extraction steps [6]. In this work, we demonstrated that ProTα extracted by the heat–cooling method is competent to bind to p8. Further, we have also compared the NMR (Fig. 5a) and CD (Fig. 5b) spectra of ProTα samples prepared with heat–cooling and cell lysis by French-press as the initial protein extraction steps. The fact that identical spectra were obtained through these two distinct protein extraction methods strongly suggests that the final structure of ProTα is independent of the purification procedure used.

In summary, by exploiting the intrinsically disordered and acidic natures of ProTα, we have developed a high-yield (>20 mg/L of M9 culture) purification protocol for the recombinant ProTα expressed in *E. coli*. This method greatly enhances our ability to perform NMR and other biophysical experiments for studying the structure–function relationship of this protein.

Acknowledgments

We are grateful to Dr. A. Vartapetian for kindly providing us the pHP12A plasmid, to Dr. L. Konermann's group for recording the mass spectrum, and to Kathryn Barber for her technical help. This work was supported by the Canadian Institutes of Health Research (CIHR), Canada Foundation for Innovation (CFI), and the Province of Ontario. W.Y. Choy is recipient of a CIHR New Investigator Award and an Ontario Early Researcher Award.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pep.2007.09.005.

References

[1] A. Pineiro, O.J. Cordero, M. Nogueira, Fifteen years of prothymosin α: contradictory past and new horizons, Peptides 21 (2000) 1433–1446.
[2] J. Gomez-Marquez, F. Segade, M. Dosil, J.G. Pichel, X.R. Bustelo, M. Freire, The expression of prothymosin α gene in T lymphocytes and leukemic lymphoid cells is tied to lymphocyte proliferation, J. Biol. Chem. 264 (1989) 8451–8454.
[3] O.E. Tsitsiloni, J. Stiakakis, A. Koutselinis, J. Gogas, C. Markopoulos, P. Yialouris, S. Bekris, D. Panoussopoulos, V. Kiortsis, W. Voelter, et al., Expression of α-thymosins in human tissues in normal and abnormal growth, Proc. Natl. Acad. Sci. USA 90 (1993) 9504–9507.
[4] M.A. Cotter 2nd, E.S. Robertson, Modulation of histone acetyltransferase activity through interaction of epstein-barr nuclear antigen 3C with prothymosin α, Mol. Cell Biol. 20 (2000) 5722–5735.
[5] Z. Karetsou, A. Kretsovali, C. Murphy, O. Tsolas, T. Papamarcaki, Prothymosin α interacts with the CREB-binding protein and potentiates transcription, EMBO Rep. 3 (2002) 361–366.
[6] Z. Karetsou, R. Sandaltzopoulos, M. Frangou-Lazaridis, C.Y. Lai, O. Tsolas, P.B. Becker, T. Papamarcaki, Prothymosin α modulates the interaction of histone H1 with chromatin, Nucleic Acids Res. 26 (1998) 3111–3118.
[7] Z. Karetsou, G. Martic, S. Tavoulari, S. Christoforidis, M. Wilm, C. Gruss, T. Papamarcaki, Prothymosin α associates with the oncoprotein SET and is involved in chromatin decondensation, FEBS Lett. 577 (2004) 496–500.
[8] R.N. Karapetian, A.G. Evstafieva, I.S. Abaeva, N.V. Chichkova, G.S. Filonov, Y.P. Rubtsov, E.A. Sukhacheva, S.V. Melnikov, U. Schneider, E.E. Wanker, A.B. Vartapetian, Nuclear oncoprotein prothymosin alpha is a partner of Keap1: implications for expression of oxidative stress-protecting genes, Mol. Cell Biol. 25 (2005) 1089–1099.
[9] X. Jiang, H.E. Kim, H. Shu, Y. Zhao, H. Zhang, J. Kofron, J. Donnelly, D. Burns, S.C. Ng, S. Rosenberg, X. Wang, Distinctive roles of PHAP proteins and prothymosin α in a death regulatory pathway, Science 299 (2003) 223–226.
[10] C. Malicet, J.C. Dagorn, J.L. Neira, J.L. Iovanna, p8 and prothymosin α: unity is strength, Cell Cycle 5 (2006) 829–830.
[11] K. Gast, H. Damaschun, K. Eckert, K. Schulze-Forster, H.R. Maurer, M. Muller-Frohne, D. Zirwer, J. Czarnecki, G. Damaschun, Prothymosin α: a biologically active protein with random coil conformation, Biochemistry 34 (1995) 13211–13218.
[12] A.G. Evstafieva, N.V. Chichkova, T.N. Makarova, A.B. Vartapetian, A.V. Vasilenko, V.M. Abramov, A.A. Bogdanov, Overproduction in *Escherichia coli*, purification and properties of human prothymosin α, Eur. J. Biochem. 231 (1995) 639–643.
[13] C. Kalthoff, A novel strategy for the purification of recombinantly expressed unstructured protein domains, J. Chromatogr. B 786 (2003) 247–254.
[14] G.L. Peterson, A simplification of the protein assay method of Lowry et al. which is more generally applicable, Anal. Biochem. 83 (1977) 346–356.
[15] E.J. Cohn, J.T. Edsall, Proteins, amino acids and peptides as ions and dipolar ions, Reinhold, New York, 1943.
[16] J. Wu, S. Chang, X. Gong, D. Liu, Q. Ma, Identification of N-terminal acetylation of recombinant human prothymosin α in *Escherichia coli*, Biochim. Biophys. Acta 1760 (2006) 1241–1247.
[17] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[18] E. Szollosi, E. Hazy, C. Szasz, P. Tompa, Large systematic errors compromise quantitation of intrinsically unstructured proteins, Anal. Biochem. 360 (2007) 321–323.
[19] N.V. Chichkova, A.G. Evstafieva, I.G. Lyakhov, A.S. Tsvetkov, T.A. Smirnova, R.N. Karapetian, E.M. Karger, A.B. Vartapetian, Divalent metal cation binding properties of human prothymosin α, Eur. J. Biochem. 267 (2000) 4745–4752.
[20] V.N. Uversky, J.R. Gillespie, I.S. Millett, A.V. Khodyakova, R.N. Vasilenko, A.M. Vasiliev, I.L. Rodionov, G.D. Kozlovskaya, D.A. Dolgikh, A.L. Fink, S. Doniach, E.A. Permyakov, V.M. Abramov, Zn^{2+}-mediated structure formation and compaction of the "natively unfolded" human prothymosin α, Biochem. Biophys. Res. Commun. 267 (2000) 663–668.
[21] C. Malicet, V. Giroux, S. Vasseur, J.C. Dagorn, J.L. Neira, J.L. Iovanna, Regulation of apoptosis by the p8/prothymosin α complex, Proc. Natl. Acad. Sci. USA 103 (2006) 2671–2676.
[22] A.R. Sburlati, R.E. Manrow, S.L. Berger, Human prothymosin α: purification of a highly acidic nuclear protein by means of a phenol extraction, Protein Expr. Purif. 1 (1990) 184–190.

[23] M.P. Lisanti, L.S. Shapiro, N. Moskowitz, E.L. Hua, S. Puszkin, W. Schook, Isolation and preliminary characterization of clathrin-associated proteins, Eur. J. Biochem. 125 (1982) 463–470.
[24] P.H. Weinreb, W. Zhen, A.W. Poon, K.A. Conway, P.T. Lansbury Jr., NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded, Biochemistry 35 (1996) 13709–13715.
[25] C. Kalthoff, J. Alves, C. Urbanke, R. Knorr, E.J. Ungewickell, Unusual structural organization of the endocytic proteins AP180 and epsin 1, J. Biol. Chem. 277 (2002) 8209–8216.
[26] C.A. Galea, V.R. Pagala, J.C. Obenauer, C.G. Park, C.A. Slaughter, R.W. Kriwacki, Proteomic studies of the intrinsically unstructured mammalian proteome, J. Proteome Res. 5 (2006) 2839–2848.