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A new method for purification of recombinant human α-synuclein in *Escherichia coli*

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Abstract

α-Synuclein (AS), a major component of Lewy body in Parkinson's disease patients, exists as a natively unfolded protein in physiological buffer. We recently found that the overexpressed AS in *Escherichia coli* bearing the cloned AS cDNA with no signal sequence was actually located inside the periplasm, but not in the cytoplasm as generally recognized. Therefore, a new protocol for preparing recombinant AS has been developed with only two steps: (1) osmotic shock for release of AS-containing periplasm fraction and (2) ion-exchange chromatography for further purification of AS. By using plasmids and *E. coli* strains commonly used the new protocol is much more convenient, faster, and cheaper compared to the current methods established since 1994. About 80 mg AS with 95% purity can be regularly prepared from a 1 L culture in 3 days.

Keywords: α-Synuclein; Purification; *Escherichia coli*; Periplasm; Osmotic shock

Parkinson's disease is the second most common neurodegenerative disease resulting from the loss of dopaminergic neurons in the brain, and affects approximately 2% of the population over the age of 65 [1]. α-Synuclein (AS) in fibrillar form has been characterized to be a major component of proteineous Lewy body in the diseased neurons [2,3]. Recombinant AS has been produced in *Escherichia coli* since 1994 [4] by several methods but with no exception from a whole cell extract obtained by sonication or boiling [5]. The whole cell extract obtained by sonication was boiled, or precipitated with ammonium sulfate, or acid precipitated, to remove most of cytoplasm proteins followed by two successive chromatographic performances of ion-exchange and size-exclusion [6,7]. Later fusion stratagems were developed to improve the preparation, such as the glutathione *S*-transferase (GST) system with a glutathione–Sepharose 4B column and specific enzymes, such as thrombin, for liberating the fused AS [8]. Another stratagem has been commercialized as the "chitin binding domain/intein system" (IMPACT, New England Biolabs, Inc.) with a chitin column and specific reagents for releasing AS protein [9]. Recently, for the first time, we have identified the accurate location of the expressed AS in *E. coli* bearing the cloned AS cDNA without any signal sequence, and found that the expressed AS is located within the periplasm but not in the cytoplasm as has long been recognized (Ren et al., unpublished data). Therefore, we developed a new protocol to purify AS with only two steps, that provides AS preparations with high quality at high yields, while is much simpler, faster, and cheaper compared to the current methods.

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Materials and methods

Materials

The plasmid pET-3a containing human AS cDNA was given generously by Dr. H.Y. Hu, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Thioflavin T (ThT) was purchased from Sigma, economical solid glass beads (5 mm) from Fisher, isopropyl β-D-thiogalactopyranoside (IPTG) from Serva. Other chemicals were local products of analytical grade.

Purification

The overnight culture of transformed BL21 (DE3) with pET-3a was diluted 100-fold and induced at an A_{600} of 0.3–0.4 for 5 h with 100 μM IPTG. All the cultures were performed in LB medium with 100 μg/ml ampicillin at 37 °C. Osmotic shock was carried out basically according to Shevchik et al. [10]. Briefly, the cell pellet from a 1 L culture was resuspended in 100 ml osmotic shock buffer (30 mM Tris–HCl, 40% sucrose, and 2 mM ethylenediaminetetraacetic acid disodium, pH 7.2) and incubated for 10 min at room temperature. The pellet collected by centrifugation at 12,000 rpm for 20 min was resuspended quickly with 90 ml cold water followed by adding 37.5 μl of saturated $MgCl_2$, and kept on ice for 3 min. The supernatant containing periplasm proteins was collected by centrifugation at 12,000 rpm for 20 min, and dialyzed against buffer A (20 mM Tris–HCl, pH 8.0) overnight. After centrifugation at 12,000 rpm for 20 min, the supernatant was loaded onto a Q-Sepharose Fast Flow column (Amersham-Pharmacia Biotech) and eluted with a 0–0.5 M NaCl gradient in buffer A. Chromatography using a DEAE–Sepharose Fast Flow column is also fine. The elution fractions were analyzed by SDS–15% PAGE, and the fractions containing only 18 kDa band were combined for dialysis against 20 mM NH_4HCO_3 and further lyophilization. Centrifugation, chromatography, and dialysis were all carried out at 4 °C.

Determinations

The concentration of AS was determined spectrophotometrically by employing the absorption coefficient $\varepsilon_{280}^{0.1\%}$ of 0.354 [7]. Far-UV circular dichroism (CD) spectra of 11.5 μM AS in buffer B (20 mM Tris–HCl and 0.1 M NaCl, pH 8.0) were measured at 25 °C on a Jasco 720 spectropolarimeter. Intrinsic fluorescence spectra of 5 μM AS in buffer B at 25 °C with 275 nm for excitation, and ThT fluorescence spectra from 460 to 560 nm at 37 °C with 450 nm for excitation, an increment of 0.2 nm, and slits of 5 nm, were measured on a Shimadzu RF5301PC spectrofluorometer. Size-exclusion chromatography was carried out on a Superdex 200 HR 10/30 column (Amersham-Pharmacia Biotech) with buffer B at a flow rate of 0.5 ml/min.

Fibril formation

Following filtration using a 0.13 μm filter membrane, AS solution of 600 μl in buffer B containing 0.05% NaN_3 was shaken at 200 rpm with a shaking diameter of 24 mm in a 5 ml tube with a 5 mm diameter glass bead as a stir bar at 37 °C. At time intervals, an aliquot of 8 μl was taken for measurement of ThT fluorescence spectrum after mixing with 792 μl of ThT solution (790 μl buffer B and 2 μl of ThT stock solution of 8 mM). The kinetics of the AS fibril formation determined by ThT fluorescence was fitted to a sigmoidal curve using an empirical equation [11]

$$F = (F_i + m_i t) + \frac{(F_f + m_f t)}{(1 + e^{(t - t_{50})/\tau})},$$

where F is the fluorescence intensity at the maximum emission wavelength of 482 nm with a blank fluorescence intensity recorded prior to addition of AS to the ThT solution subtracted from the measured ThT intensity; t_{50} is the time to 50% of maximal fluorescence. Thus, the apparent rate constant, k_{app}, for fibril growth is given by $1/\tau$ and the lag time is taken as $t_{50} - 2\tau$. To maximize the reproducibility, the shaking speed and the sample volume were kept constant throughout, and triplet was performed in parallel for each sample.

Electron microscopy

AS (420 μM) fibril suspension of 20 μl at the equilibrium phase was adsorbed onto a glow-discharged carbon support film for 1 min and washed twice with 100 μl of distilled water with excess solution blotted off by a filter paper. Specimen was negatively stained by 2% uranyl acetate (w/v) for 45 s. The image was examined with a Philips Tecnai 20 electron microscope at 120 keV and recorded on a Lekai film at 50,000× magnification.

Results

Purification of AS

AS comprised 80% of the total proteins in the periplasm (Fig. 1A and Table 1), indicating that osmotic shock as the first step of the new protocol is critical for the purification of AS. The peak of AS eluted at 0.3–0.37 M NaCl of Q-Sepharose Fast Flow chromatography was sharp and symmetrical (Fig. 1B), achieving a purity of 95%. On SDS–15% PAGE, AS showed an apparent molecular mass of 18 kDa, which is larger than the theoretical value of 14 kDa. The abnormal slow

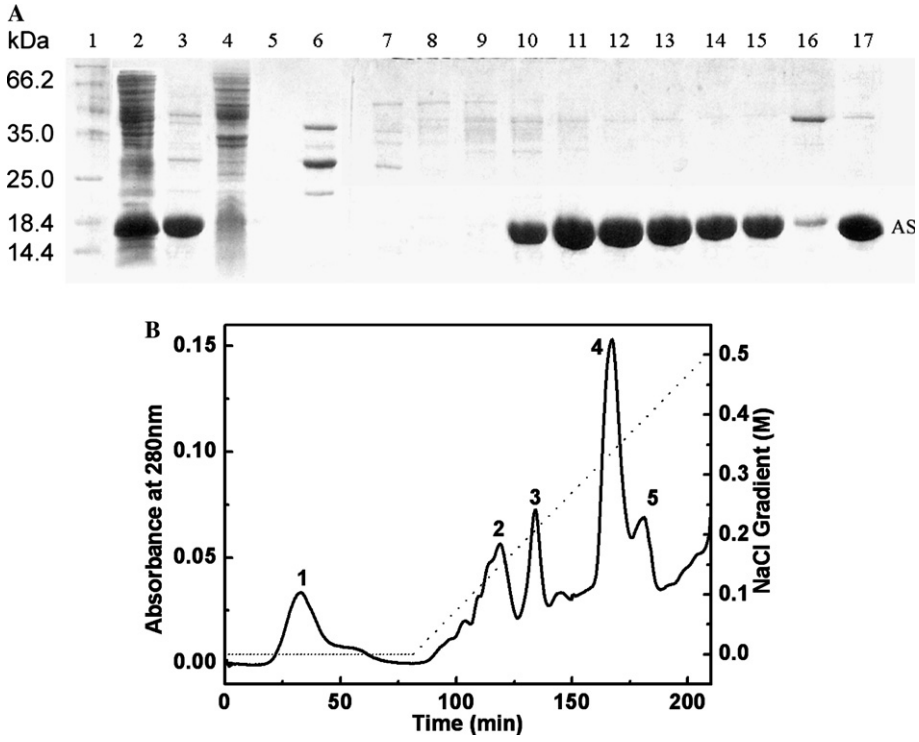

Fig. 1. Purification of AS. (A) SDS–15% PAGE. Lane 1, molecular mass markers (Ferment); lane 2, whole cell extract; lanes 3 and 4, supernatant and pellet after osmotic shock, respectively; lanes 5–7, peaks 1–3 in (B), respectively; lanes 8–15, peak 4; lane 16, peak 5; and lane 17, combination of the lanes 12–15. (B) Chromatography of the supernatant after osmotic shock on a Q-Sepharose Fast Flow column. The dotted line was for NaCl gradient.

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

Purification steps	Total protein (mg)	AS purity[b] (%)	AS (mg)	Yield[c] (%)
Whole cell extract[d]	465[e]	20	93	100
Osmotic shock	100[e]	85	85	91
Q-Sepharose Fast Flow	86[f]	>95	82	88

[a] The data in the table were obtained from a normal performance.
[b] The purity of AS obtained in each step of purification was estimated by SDS–PAGE using "totallab."
[c] Ratio of the amount of AS obtained at each step to 93 mg, the total amount of AS in a 1 L culture.
[d] Supernatant of the cell lysate by sonication.
[e] Determined by Bradford assay [16] with bovine serum albumin as a standard.
[f] Determined spectrophotometrically.

mobility was attributable to the low binding of SDS by the highly acidic C-terminal sequence of AS [6]. From a 1 L culture about 80 mg of AS protein of 95% purity can be obtained.

Characterization of purified AS

The CD spectrum of purified AS presented a strong negative peak at 202 nm and a small shoulder at around 222 nm (Fig. 2A), indicating a random coil structure possibly with short marginally stable α-helices [12]. The intrinsic fluorescence spectrum of AS showed a maximum emission at 306 nm (Fig. 2B) as reported before [13]. AS has been characterized to be a monomer using non-dissociating PAGE [6], however, purified AS preparations were eluted in size-exclusion chromatography as a single symmetric peak at positions corresponding to an apparent molecular mass of 52 ± 2 kDa (expressed as mean $\pm$ SD, $n = 4$. Fig. 2C showed one performance), similar to the reported 54 kDa [14]. The abnormal behavior of AS in size-exclusion chromatography has been ascribed to its natively unfolded conformation in physiological buffer [6].

Fibril formation of purified AS

The fibril formation of the purified AS preparations measured by ThT fluorescence displayed a sigmoidal growth curve (Fig. 3A), which was composed of an initial lag phase, a subsequent exponential growth phase, and a final equilibrium phase. With the increase of AS concentrations, the lag phase was shortened, and both the apparent rate constant of the growth phase and the final fluorescence intensity representing the fibril formation extent were increased. The inverse linear correlation between the lag time and the logarithm of the AS concentration (Fig. 3B), and the linear correlation between the apparent rate constant k_{app}, and the concentration of AS (Fig. 3C) indicated a concentration-dependent kinetics of fibril formation [13]. The apparent

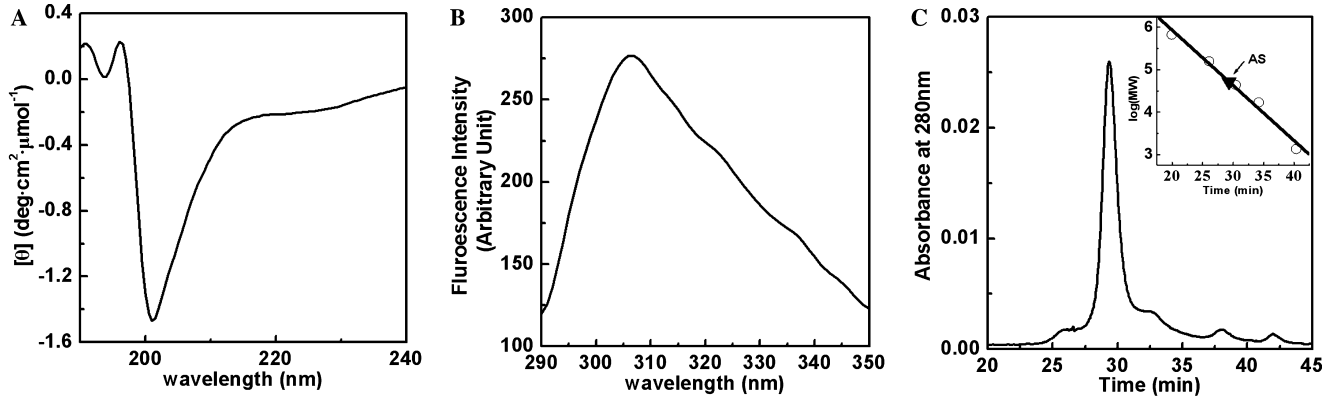

Fig. 2. Characterization of purified AS. (A) CD spectra. (B) Intrinsic fluorescence spectrum. (C) Size-exclusion chromatography on a Superdex 200 HR 10/30 column. The molecular mass markers (○) are bovine thyroglobulin, 670 kDa; bovine γ-globulin, 158 kDa; chicken ovalbumin, 44 kDa; equine myoglobin, 17 kDa; and vitamin B12, 1.35 kDa (Bio-Rad).

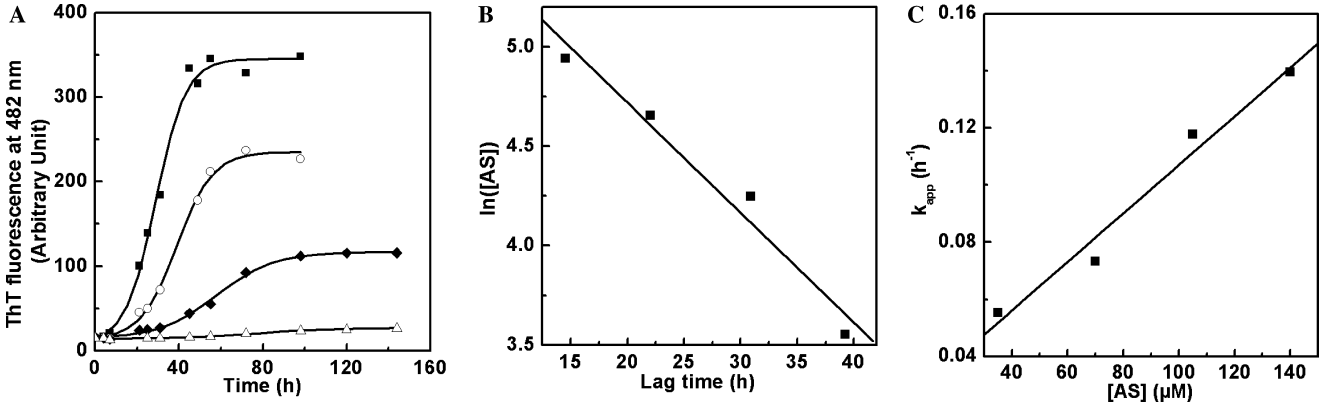

Fig. 3. Kinetics of AS fibril formation. (A) ThT fluorescence intensities of AS at concentrations of 35 (△), 70 (◆), 105 (○), and 140 (■) μM were determined at various times as indicated, and the experimental data were fitted to solid sigmoidal curves as described in the text. (B) Inverse linear dependence of the logarithm of AS concentrations as a function of the lag time. (C) Linear dependence of the rate constant (k_{app}) for fibril growth on the AS concentrations.

rate constant, k_{app}, for fibril growth of 70 μM AS was 0.082, which is similar to the reported k_{app} of 0.1, and the lag time, 28.5 h, close to the reported 30 h, under very similar conditions [9].

Electron microscopy

As shown in Fig. 4, at the equilibrium phase AS formed a large quantity of long fibrils of about 9–12 diameters with no aggregates attached. As the fibrils with diameters of ⩾10 nm are referred to as mature fibrils [15], the AS fibrils formed at the equilibrium phase are mature fibrils.

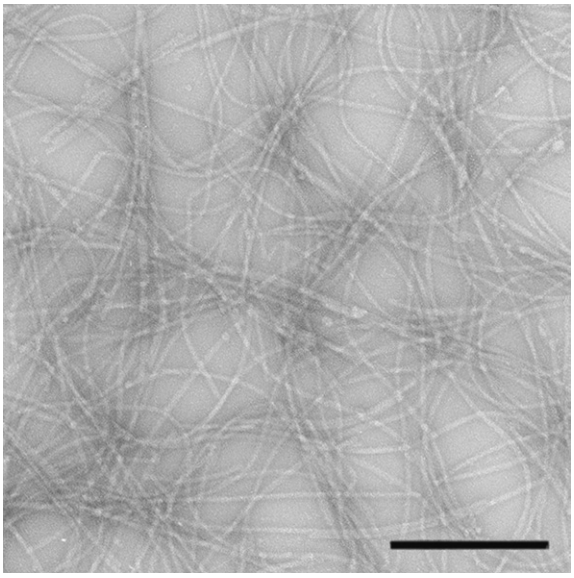

Fig. 4. Electron micrographs of AS fibrils formed at 240 h. Scale bar, 300 nm.

Discussion

All the methods employed to purify recombinant human AS expressed in *E. coli* so far use a whole cell extract as a starting material, therefore one or more kinds of protease inhibitors are usually added to protect

possible degradation of the expressed AS, such as "protease inhibitor cocktail" from Sigma, and "protease inhibitor mixture Complete" from Boehringer–Mannheim [7]. A package of 5 ml protease inhibitor cocktail recommended for the inhibition of proteases extracted from 20 g of *E. coli* costs $40. A tablet of "protease inhibitor mixture Complete" recommended for 50 ml extraction media costs at least $10. With the present protocol no protease inhibitor is required, as AS in a periplasm preparation was not degraded after incubation for 3 days at 4 °C in the absence of protease inhibitor (data not shown).

By using the current methods of boiling, or ammonium sulfate precipitation, or acid precipitation of the whole cell extract followed by two successive ion-exchange and size-exclusion chromatography a cycle of 4–6 days is needed to obtain a purified AS preparation, while our new protocol needs only 3 days. Moreover, a higher yield of about 80 mg AS from a 1 L culture can be regularly obtained using the new protocol compared to the reported yield of 10–80 mg AS by the current methods [6].

GST fusion protein expression system usually provides a AS preparation purified to only 80% purity at a very low yield of only 1 mg AS from a 1 L culture in 2–3 days [8]. IMPACT expression and purification system is a simple protocol, but the efficiency of controlled cleavage of the intein tag could be affected by the target protein sequence, and is therefore variable. Extra non-native residue(s) is thus usually added at the C- or N-terminus of the target protein to increase the cleavage efficiency (IMPACT, New England Biolabs, Inc.).

For the new protocol, only normal *E. coli* strains, normal expression plasmids, and ion-exchange chromatography are needed, which are easily available in all laboratories. If one more step of boiling the periplasm fraction for 10 min was added before ion-exchange chromatography, one more step of size-exclusion chromatography must be applied following the ion-exchange chromatography to remove polymerized forms resulting from boiling. The final AS product thus prepared showed an even higher purity but at a price of decreased yield.

AS preparations purified by the present protocol show the same biochemical properties and the same fibril formation properties as that of AS purified by other methods.

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