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*Ld*ARL-1 His-tagged recombinant protein: purification by immobilized metal affinity expanded bed adsorption

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Abstract

Previously we have cloned three ADP-ribosylation factor-like (ARL) genes from the parasitic protozoan *Leishmania donovani*: *Ld*ARL-3A and 3B, *Ld*ARL-1. *Ld*ARL-3A was previously purified as an active native form, which was able to bind GTP in vitro. In this paper, we have performed the production and the purification of Histidine-tagged (His-tagged) *Ld*ARL-1 recombinant protein by immobilized metal affinity chromatography (IMAC) using expanded bed adsorption (EBA) technology. This protein was purified with more than 95% purity and could be successfully used for GTP-binding assay.

Keywords: *Ld*ARL-1; ADP-ribosylation factor-like protein; His-tagged recombinant protein; Immobilized metal affinity chromatography (IMAC); Expanded bed adsorption

1. Introduction

The ADP-ribosylation factor-like proteins belong to the Sar1/Arf family of the small G protein superfamily which are involved in multiple cellular processes including vesicle trafficking, and cytoskeleton reorganization [1]; however, the function of most ARLs is still unclear. These proteins contain four consensus amino acid motifs responsible for the specific interaction with GDP and GTP, and two switch regions, whose conformation changes drastically when the proteins cycle between the GDP- and GTP-bound forms [1].

We have previously cloned three ARL genes from the parasitic protozoan *Leishmania donovani* (*Ld*ARL-1, -3A and -3B). *Ld*ARL-3A is involved in flagellum biogenesis [2], but not *Ld*ARL-1 and -3B despite close sequence similarities between them. *Ld*ARL-3A was previously purified as a native form from *E. coli* and was able to bind GTP in vitro [2].

In this paper, we propose a one-step purification process for *Ld*ARL-1 using the recombinant His-tagged protein and show its GTP binding activity. A one-step chromatographic purification using immobilized metal affinity chromatography (IMAC) [3–7] was performed. Expanded bed technology was preferred as the capture step because it is an alternative to traditional clarification (centrifugation, tangential micro- and ultra-filtration and the first chromatography step) [8–10] and allowed us to process large volumes of crude feedstock directly after ultrasonic homogenization with high throughput. The short processing time at the capture step just after fermentation was essential because the fast adsorption of the target molecule early in the process prevents damage due to its high instability in the absence of GDP. The eluted fraction

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was desalted, conditioned and sampled for further successful GTP binding assay.

2. Experimental procedures

2.1. Instruments

The chromatographic system used throughout this study was the Biopilot workstation from Amersham Biosciences (Saclay, France). The data were collected and evaluated using the Unicorn Data system.

Electrophoresis apparatus used was the Mini Protean II from Biorad (Ivry-sur-seine, France).

For recovery studies, we used Lamda 12 from Perkin-Elmer (Courtaboeuf, France).

Fluorescence studies were performed with SPEX Fluoromax from Jobin-Yvon SAS (Longjumeau, France).

2.2. Chemicals

The pET-29b (plasmid Expression Tag) and *E. coli* BL21 (bacteria lysogenic for bacteriophage DE3) were from Novagen (Madison, WI, USA).

Streamline chelating gel, Streamline 25 column and PD10 columns were from Amersham Biosciences.

Goat anti-rabbit alkaline phosphatase was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immobilon P was from Millipore (Saint-Quentin-en-Yvelines, France).

2.3. Preparation of the cellular extract

2.3.1. Cloning and expression

The *Ld*ARL-1 ORF was obtained by PCR amplification from *L. donovani* genomic DNA. This PCR product was cloned into the vector pUC-18 giving the plasmid pUC-*Ld*ARL-1. The insert was removed from this plasmid by digestion with *Nde*I and *Kpn*I and ligated into the pET29b vector digested with the same enzymes.

2.3.2. Cell culture

The cells of *E. coli* BL21 (DE3) strain transfected by the plasmid pET 29b containing *Ld*ARL-1 His-tag construction were cultured at 37 °C with vigorous agitation in Luria Bertani (LB) broth containing 50 μg/ml kanamycin. When cultures reached an absorbance of about 0.6 at 600 nm, isopropyl β-D-thiogalactopyranoside was added to a concentration of 1 mM to induce expression of the recombinant *Ld*ARL-1 protein. Culture was grown for an additional 3–5 h.

2.3.3. Ultrasonic homogenization

Fifty ml of cell suspension were diluted in 300 ml of equilibration buffer (0.5 M NaCl, 50 mM Tris–HCl pH 8) and sonication was performed at 20 kHz with a 13 mm probe during four 1-min pulses with 2 min in ice between each pulse. Afterwards, the homogenate was directly used for the expanded bed adsorption.

2.4. Chromatographic procedures

2.4.1. Expanded bed adsorption and immobilized metal affinity chromatography (EBA-IMAC)

An IMAC system using EBA technology (Streamline chelating) was performed with the Streamline 25 column containing 90 ml of Streamline chelating gel corresponding to a sedimented bed height of 19 cm. The Streamline 25 column was linked to a Biopilot workstation. The evaluation of bed stability was performed by visual inspection, and by measuring the degree of expansion according to the manufacturer's instructions.

The bed expansion/equilibration, feed application and washing were performed at an upward flow velocity of 180 cm/h (15 ml/min). The buffer A1 used during expansion/equilibration was 0.5 M NaCl, 20 mM Tris–HCl, pH 8. The crude and unclarified feedstock was applied directly onto the expanded bed followed by washing with the equilibration buffer A1 until UV baseline was reached. Then the pump was turned off and the bed sedimented. Next, the adaptor was moved down towards the sedimented bed surface. The downflow experiments were performed at the same flow rate. After a run of two volumes equivalent to the sedimented gel of buffer A2 (10 μM GDP, 5 mM $MgCl_2$, 0.5 M NaCl, 20 mM Tris–HCl, pH 8), a linear gradient to 8% of buffer B (10 μM GDP, 5 mM $MgCl_2$, 0.5 M NaCl, 0.5 M Imidazole, 20 mM Tris–HCl, pH 8) was performed to wash the column and elution was accomplished by step gradient to 100% of elution buffer B.

2.4.2. Preparation of the sample for the GTPγS binding assay

The eluted fraction was dispatched in several fractions and frozen. Before GTPγS binding assay, the sample was desalted in a 50 mM Hepes, 1 mM DTT, 10 μM GDP and 5 mM $MgCl_2$ buffer using PD-10 column according to the manufacturer's instructions.

2.5. Analytical procedures

2.5.1. Protein concentration

The protein concentration was estimated by the Bradford method [11] using bovine albumin as standard.

2.5.2. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a mini protean II apparatus in a Tris glycine buffer [12] system was used to monitor purification during the chromatographic steps. Electrophoresis was performed for 1 h at 150 V using 12% polyacrylamide gels. Detection was done by Coomassie brillant blue R250

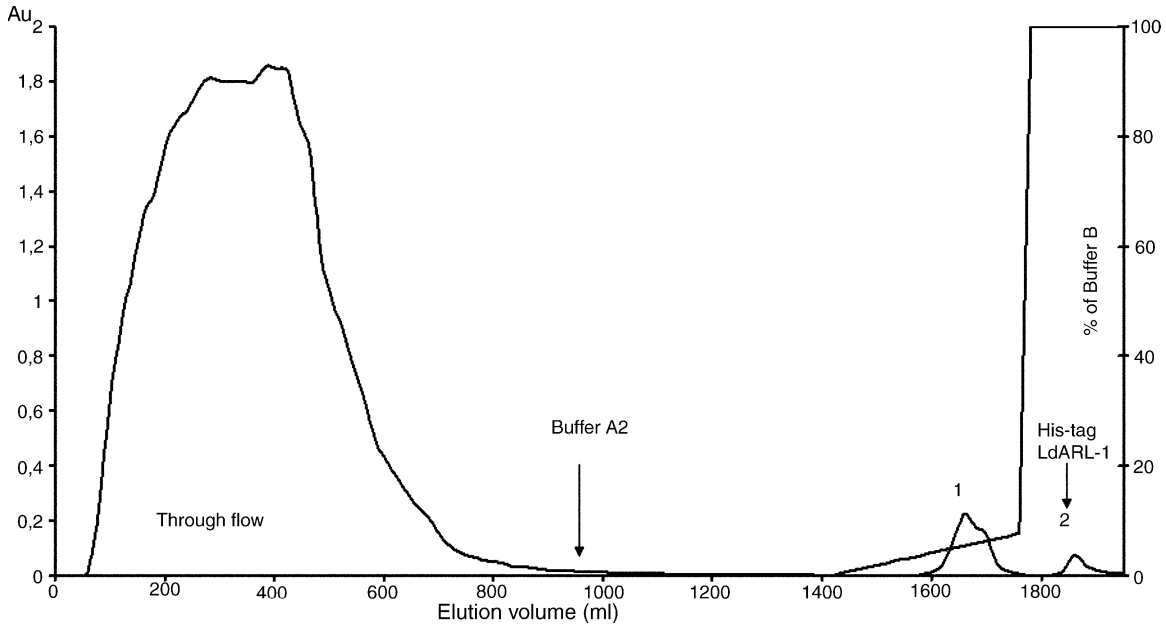

Fig. 1. Purification of recombinant His-tag ADP-ribosylation factor-like protein *Ld*ARL-1 by immobilized metal affinity expanded bed adsorption. Column: Streamline 25 (90 ml of gel). Sample: Unclarified and ultrasonic homogenate of cell culture (300 ml). Equilibration buffer: 0.5 M NaCl, 20 mM Tris–HCl, pH 8. Conditioning buffer (buffer A2): 10 μM GDP, 1 mM $MgCl_2$, 0.5 M NaCl, 20 mM Tris–HCl pH 8 buffer. Elution buffer (buffer B): 10 μM GDP, 1 mM $MgCl_2$, 0.5 M imidazole, 0.5 M NaCl, 20 mM Tris–HCl pH 8 buffer. Flow-rate was 180 cm/h and the detection of the proteins was done at 280 nm.

and analysis performed by gel scanning with NIH Image Software.

2.5.3. Western blotting

Purified proteins in sample buffer (0.1% SDS, 10% glycerol, 50 mM DTT, 90 mM Tris–HCl, pH 6.8) were boiled for 5 min and subjected to 12% SDS-PAGE. Proteins were transferred to polyvinyllidone difluoride membrane (Immobilon P) by semi-dry blotting [13]. Filters were blocked for 30 min with Tris buffered saline (TBS)–Tween–milk (NaCl 150 mM, Tris–HCl 10 mM pH 7.4, EDTA 5 mM, 0.05% Tween 20, 4% milk), and subsequently incubated overnight at 4 °C with solution of polyclonal rabbit immune serum directed against *Ld*ARL-1 C-terminus at dilution 1/1000 in TBS–Tween–1% milk. After several washes, the filters were incubated for 2 h with a goat anti-rabbit IgG conjugated to alkaline phosphatase (Biorad) at a dilution of 1/3000 in TBS–Tween–1% milk. Immunoreactive bands were revealed by incubation in 0.1 M ethanolamine–HCl pH 9.6, 4 mM $MgCl_2$, 6% 5-bromo-4-chloro-3-indolyl phosphate disodium and 0.1% Nitro Blue Tetrazolium.

2.5.4. GTPγS binding assay

Binding assays were performed immediately after purification of the proteins to avoid precipitation. Different concentrations of proteins (1–10 μM) were incubated at 37 °C in 50 mM Hepes pH 7. After addition of 5 mM $MgCl_2$ and 50 μM GTPγS, GTPγS binding was monitored by the enhancement of tryptophan fluorescence intensity concomitant with GDP to GTPγS exchange with a spectrofluorometer, excitation was at 297.5 nm, emission at 340 nm.

3. Results and discussion

3.1. Immobilized metal affinity chromatography-expanded bed adsorption

Expansion, equilibration and washing were performed using a flow velocity of 180 cm/h, which resulted in a degree of expansion of around 2. The application of the unclarified feedstock was performed at the same flow rate. After injection of all the crude feedstock and washing with the equilibration buffer, the gel was sedimented and the adaptor was moved down. A run with new equilibration buffer A2 was performed to stabilize the adsorbed protein (A2 in Fig. 1). Addition of GDP was necessary for a good recovery and activity of the protein. A wash with up to 8% of elution buffer B was performed to eliminate contaminants and elution was performed using a step gradient up to 100% of elution buffer B (Fig. 1).

The different fractions were analyzed by SDS-PAGE (Fig. 2A) and Western blotting (Fig. 2B).

The percentage of recombinant protein were calculated by gel scanning with NIH software.

Fig. 2 shows that in the crude extract, the His-tag *Ld*ARL-1 corresponded to 17% of the total protein. The lane containing the flow-through shows a very low level of *Ld*ARL-1 his-tag recombinant protein (1%). No His-tag protein was in the 8% elution buffer and the majority of the His-tag *Ld*ARL-1 was eluted at 100% of elution buffer without contaminants.

Western blot analysis (Fig. 2B.) indicated that the purified protein really corresponded to the His-tag *Ld*ARL-1. Pure protein was obtained with a purification factor of 6.

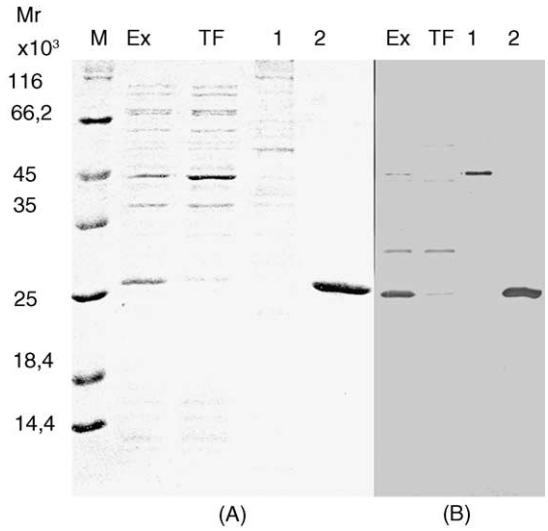

Fig. 2. Analysis of the purification fractions by Coomassie blue staining (A) and Western blotting (B). Electrophoresis was performed using 12% polyacrylamide gels. (A) SDS-PAGE: M: marker, Ex: crude extract (5 μg), TF: through-flow; 1: wash at 8% of buffer B, 2: 2 μg of eluted fraction at 100% of buffer B. (B) Western lot: The proteins were transferred onto PVDF membrane and incubated with 1/1000 diluted polyclonal rabbit antibody against *Ld*ARL-1 C-terminus. Revelation was performed using a goat anti-rabbit antiserum conjugated to alkaline phosphatase (1/3000).

3.2. GTPγS binding assay

The imidazole used to elute the his-tagged protein from IMAC could interfere with the GTPγS binding assay, therefore a desalting column was realized. After equilibration of the PD-10 column, the eluate of EBA-IMAC was applied onto the column and the first fractions were collected. It has been described for small G proteins (ARF-1) [14] that the fluorescence intensity due to tryptophan 78 increased concomitantly to the exchange of GDP by GTPγS depending on the presence of Mg^{2+}. A similar phenomenon was observed for *Ld*ARL-3A using purified native protein [2], the corresponding tryptophan being amino-acid 77. This tryptophan was conserved in *Ld*ARL-1 suggesting that an increase of fluorescence would also occur during the exchange of GDP by GTP. Using purified EBA-IMAC and desalted His-tag *Ld*ARL-1, we found an increase in fluorescence in the presence of 50 μM GTPγS (Fig. 3); this increase occurred within seconds of GTPγS addition, was directly proportional to the protein concentration (1–10 μM) and abolished by the addition of an excess of EDTA (2 mM) (data not shown). Thus, like purified native protein (*Ld*ARL-3A), the His-tagged protein was able to exchange GDP to GTP suggesting that the purification procedure and the presence of 6 histidine at the terminus did not disturb the functionality of this protein.

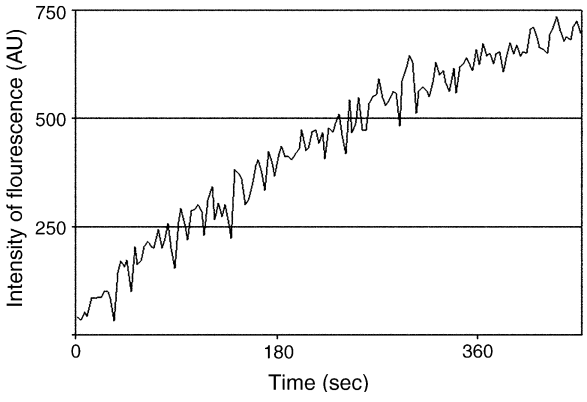

Fig. 3. GTPγS binding assay. The protein *Ld*ARL-1 (5 μM) was incubated at 37 °C in 50 mM Hepes, pH 7. After addition of 5 mM $MgCl_2$ and 50 μM GTPγS (time 0), GTPγS binding was monitored by the enhancement of *Ld*ARL-1 tryptophane 77 fluorescence intensity concomitant with GDP to GTP exchange with a spectrofluorometer. Excitation was at 297.5 nm, emission at 340 nm.

4. Conclusion

In conclusion, from 1 l of cell culture, we are able to produce 10 mg of purified *Ld*ARL-1 His-tag recombinant protein. Expanded bed technology allowed us to process large volume of crude feedstock directly after sonication with high yield. This procedure allows very fast purification which is essential for keeping *Ld*ARL-1 His-tag recombinant protein in a functional state for further detailed kinetic, mechanistic and structural studies.

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