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On-column refolding of an insoluble histidine tag recombinant exopolyphosphatase from *Trypanosoma brucei* overexpressed in *Escherichia coli*

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Abstract

An exopolyphosphatase gene has been cloned by polymerase chain reaction (PCR) from *Trypanosoma brucei* and the corresponding protein overexpressed as a recombinant His-tag (histidine tag) exopolyphosphatase in *E. coli* in order to characterize its biochemical activity and to produce antibody to determine its localization. Because overexpression of this protein in bacteria resulted in the formation of inactive inclusion bodies, these structures were first solubilized in denaturant condition (6 *M* urea). Secondly, after a capture step using immobilized metal affinity chromatography (IMAC), a gradual refolding of the protein was performed on-column from 6 *M* to 0 *M* urea in the presence of 1% Triton X-100. Triton X-100 was used to abolish protein aggregation during the refolding step. The purified enzyme was active, demonstrating that at least part of the proteins was properly refolded.

Keywords: *Trypanosoma brucei*; *Escherichia coli*; Refolding; Histidine tag recombinant exopolyphosphatase

1. Introduction

Eukaryotic microorganisms possess polyphosphate pools localized in different cell compartments [1]. Polyphosphate plays significant roles in stress response [2], stationary phase adaptation and in the regulation of different biochemical processes [3]. The implication of this polymer in cellular processes such as energetic metabolism, phosphate storage and divalent cation homeostasis was demonstrated [3]. However enzymes involved in polyphosphate metabolism are still little studied. In the parasitic protozoa *Trypanosoma brucei (T. brucei)*, polyphosphates are mainly located in an acidic compartment named the acidocalcisome. This compartment was shown to contain polyphosphatase activities such as pyrophosphatase and exopolyphosphatase (polyphosphate phosphohydrolase, EC 3.6.1.11). We recently cloned an exopolyphosphatase from *T. brucei*. To characterize its biochemical activity and to produce antibodies to determine its localization, we overexpressed it in *E. coli* as an His-tag recombinant protein [4–8]. However the expression of this protein in bacteria

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resulted in the formation of an inactive protein that intracellularly accumulated. In this paper we propose a method to recover native and active exopolyphosphatase from highly insoluble inclusion bodies.

2. Experimental

2.1. Instruments

The chromatographic systems used throughout this study were the Åkta Explorer 100 from Amersham Biosciences (Saclay, France). The data were collected and evaluated using the Unicorn 4.0 Data system.

The electrophoretic apparatus used was the Miniprotean II from Biorad (Ivry sur Seine, France). A Cary 100 spectrophotometer from Varian (Les Ulis, France) was used for recovery and enzymatic studies.

2.2. Materials

The pET 23a and *E. coli* BL21 were from Novagen (Madison, WI, USA). Urea, Triton X-100, Triton X-100 UV reduced, all salts and mouse anti-poly(His)$_6$ were from Sigma (L'isle d'Abeau Chesnes, France). XK 16/20 column, PD-10 Column, chelating Sepharose Fast Flow and ECL kit were from Amersham Biosciences (Saclay, France). Imidazole was from Merck (Fontenay sous Bois, France). Goat anti-mouse IgG1 conjugated to horseradish peroxidase was from Sanofi-Pasteur (Marnes la Coquette, France). Restriction enzymes were from Takara (Gennevilliers, France). Pfu turbo® DNA polymerase was from Stratagene (Amsterdam, The Netherlands). Molecular mass marker was from Invitrogen (Cergy Pontoise, France).

EnzChek pyrophosphate assay kit was from Molecular Probes (Eugene, USA).

2.3. Preparation of the cellular extract

2.3.1. Cloning and expression

A 1146 bp fragment comprising the entire sequence of the exopolyphosphatase gene was generated by PCR using pfu DNA polymerase. The DNA sequence verified by sequencing was similar to the sequence found in the *T. brucei* genome database. The 5′ primer (5′-GCGGCGCATATGACGGCAGTGGTGAATGAG) contained a 12 nucleotides linker with an *XhoI* restriction site to facilitate cloning and 5′ adjacent N-terminal residues. The 3′ primer, (5′-CAGGACCTCGAGCAAATTGTTCCACACTGAC) included a 12-nucleotide linker with an *NdeI* site for cloning. The PCR product was inserted into the *NdeI*/*XhoI* linearized pET 23a plasmid to create the C-terminal His-tag construction.

2.3.2. Cellular culture

The *E. coli* BL21 strain transformed with the plasmid pET 23a containing the exopolyphosphatase His-tag construction was cultured in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) with ampicillin (100 μg/ml). The volume of the culture medium was 80-fold higher than the inoculated volume.

The culture was grown at 37 °C to 2×10^8 cells/ml ($A_{600nm}=0.7$). Then IPTG (isopropyl β-D-thiogalactopyranoside) was added to a final concentration of 0.5 m*M* to a culture stabilized and incubated overnight at room temperature. These culture conditions did not help for soluble protein production but they allowed a high production of a non-proteolysed protein.

2.4. Ultrasonic homogenization and solubilization

After induction, cells were harvested by centrifugation and resupended in binding buffer (5 m*M* Imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl pH 7.9). Then cells were lysed by three steps of freezing and thawing and a brief sonication (30 s pulse with 2 min in ice between each pulse). The lysate was centrifuged for 30 min at 10 000 *g*. The resulting pellet containing inclusion bodies was resuspended in binding buffer containing successively 2 *M* and 4 *M* urea plus 1% Triton X-100 and placed under agitation during 1 h, after centrifugation the final pellet was treated with buffer containing 6 *M* urea plus 1% Triton X-100 UV reduced in same conditions. After centrifugation (30 min at 10 000 *g*) this solution was applied to the IMAC column.

2.5. Immobilized metal affinity chromatography (IMAC) and refolding

An IMAC system was performed with the XK 16/20 column containing 1.5 ml chelating Sepharose Fast Flow. The XK16/20 was linked to an Åkta Explorer 100 workstation. After loading with 5 ml 50 m*M* $NiSO_4$ in H_2O, the buffer used during equilibration was 5 m*M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 7.9, 6 *M* urea, Triton X-100 UV reduced 1%. The sample (5 ml) was applied onto the column at a flow-rate of 0.5 ml/min followed by washing with 10 ml of the equilibration buffer until UV baseline was reached. Then, refolding of the bound protein was performed on-column by the use of a linear gradient from 6 *M* to 0 *M* urea, starting with the equilibration buffer and finishing with a buffer containing 5 m*M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 7.9, Triton X-100 UV reduced 1%. The refolded protein was eluted with a buffer containing 1 *M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 7.9, Triton X-100 UV reduced 1%.

2.6. Analytical procedures

2.6.1. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [9] using a Tris–SDS–glycine buffer system was used to monitor the fractions obtained after the IMAC.

Electrophoresis was performed for 1 h at 160 V using 12% polyacrylamide gels. Detection was done by Coomassie Brilliant Blue R250.

2.6.2. Western blotting

Purified proteins in sample buffer (0.1% SDS, 10% glycerol, 50 m*M* dithiothreitol, 90 m*M* 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 [10].

Filters were blocked for 15 min with PBS (phosphate-buffered saline)–Tween (0.05%)–milk (5%), and incubated overnight at 4 °C with a 1/200 mouse monoclonal anti-$His_{(6)}$ antibody diluted in PBS–Tween–milk, then incubated for 2 h with a 1/5000 goat anti-mouse IgG1 conjugated to horseradish peroxidase in PBS–Tween–milk. Immunoreactive bands were revealed by the light emitting detection method ECL™.

Mid-range molecular mass marker has been used for size estimations.

2.6.3. Protein concentration

The protein concentration was estimated by Bradford quantitative protein determination assay [11] using bovine serum albumin as standard.

2.6.4. Exopolyphosphatase activity

A rapid desalting and buffer exchange of the eluted fraction was performed on a PD-10 column to avoid no-desired interference with the assay. Exopolyphosphatase activity was measured by using phosphate release assay kit (EnzChek pyrophosphate assay kit) [12] in the presence of 0.1 m*M* tetrapolyphosphate. The Pi released is consumed by the 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG)/purine nucleoside phosphorylase (PNP) reaction and detected by an increase in absorbance at 360 nm.

3. Results and discussion

3.1. Extraction and solubilization of exopolyphosphatase overexpressed in E. coli

In our construction, His-tag exopolyphosphatase is expressed under the dependence of the T7 promoter and is not excreted in the culture medium. Then we have to harvest the cells and to break them. After centrifugation, the protein was recovered in the pellet (data not shown). Solubilization of inclusion bodies was performed with urea (Fig. 2A). The final solubilization was performed with a buffer containing 5 m*M* Imidazole, 0.5 *M* NaCl, 6 *M* urea, 1% Triton X-100 UV reduced, 20 m*M* Tris–HCl pH 7.9. This treatment with 6 *M* urea solubilized the major amount of the recombinant protein but, it should be noted that, even in 8 *M* urea, a large quantity of the recombinant protein remains in the pellet (data not shown).

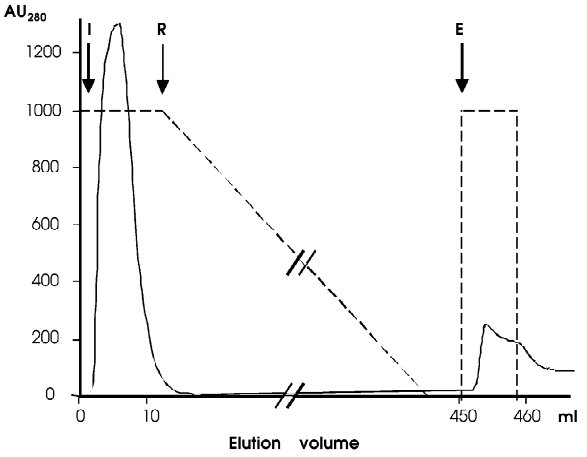

Fig. 1. Purification and refolding of recombinant exopolyphosphatase. Sample fraction in binding buffer: 5 m*M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 7.9, 6 *M* urea, Triton X100 UV reduced 1% (I); linear 6-0 *M* urea gradient in binding buffer (R); elute buffer: 1 *M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 7.9 (E). Detection at 280 nm. Flow-rate: 1 ml/min during equilibration and elution and 0.5 ml/min during sample loading and refolding.

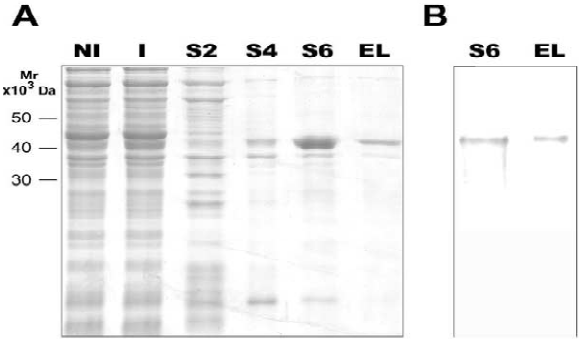

Fig. 2. Exopolyphosphatase expression, solubilization and purification analysis. 12% SDS–PAGE system (A) Coomassie staining. I: IPTG soluble fraction of induced culture (loaded 5 μl, total volume: 5 ml), NI: soluble fraction of non-induced culture (loaded 5 μl, total volume: 5 ml), S2: supernatant after treatment of the previous pellet with 2 *M* urea (loaded 10 μl, total volume: 5 ml), S4: supernatant after treatment of the previous pellet with 4 *M* urea (loaded 10 μl, total volume: 5 ml), S6: supernatant after treatment of the previous pellet with 6 *M* urea (loaded 10 μl, total volume: 5 ml), EL: eluted fraction (2 μg of protein bradford estimated). (B) Western blot analysis of the recombinant His-tag protein. A monoclonal antibody, anti-poly-histidine, was used as first antibody. Revelation was performed using a goat anti-mouse peroxidase-conjugated antibody.

3.2. Immobilized metal affinity chromatography and on-column refolding

The soluble fraction was injected onto the column (I in Fig. 1). The column was equilibrated and washed with the equilibration buffer that eliminated most of the contaminants in the through flow. Preliminary experiments (data not shown) realized in 6 *M* urea, resulted in protein aggregation during the refolding step. This aggregation prevented the elution of the exopolyphosphatase. Since the use of detergent was shown to help for protein refolding [13], we tested their effect in the purification process. We found that addition of Triton X-100 UV reduced allowed proper protein refolding without affecting the exopolyphosphatase activity. In a second step, we evaluated different speed of denaturant removal to improve exopolyphosphatase refolding. The optimum speed of urea elimination was 7 m*M*/min.

After this optimization, the on-column refolding of the protein was performed by the use of a linear gradient from 6 *M* urea to 0 *M* (R in Fig. 1). Elution of the enzyme was performed with 1 *M* imidazole (E in Fig. 1) and soluble protein was recovered. The recovery of the purification is summarized in Table 1, the remaining 18% are either in the flow-through (not collected) or still on-column after the elution step.

Table 1
Purification of 400 ml of culture of recombinant His-tag exopolyphosphatase

	Volume (ml)	Protein (μg/ml)	Total protein (μg)	Recovery (%)
Starting material	5	450	2250	–
Non retained and wash	10	75	750	33
Eluate	5	230	1150	51

Table 2
Exopolyphosphatase activity

Purified recombinant exopolyphosphatase (μg)	0	5	10
Tetrapolyphosphatase activity (μmole Pi released/min)	0.11	1.12	3

Tetrapolyphosphatase activity was measured by the amount of released Pi from 100 m*M* tetrapolyphosphate. Exopolyphosphatase (0 to 10 μg) was incubated at 27 °C in the Enz Check kit buffer.

3.3. Western blot and exopolyphosphatase activity analysis

The Western blot analysis (Fig. 2B) indicated that the purified protein really corresponded to the His-tag exopolyphosphatase protein, since the anti-poly-His recognized the purified protein at a relative molecular mass of 43 000.

The exopolyphosphatase activity was followed by incubating tetrapolyphosphate with 5 and 10 μg of the purified His-tag enzyme. Table 2 shows the presence of an exopolyphosphatase activity compared to the baseline (0 μg of purified enzyme). These enzymatic results indicate that, during the chromatography, part of the proteins were at least properly refolded.

4. Conclusion

The process described in this paper allows the purification of a His-tag recombinant exopolyphosphatase overexpressed in *E. coli*. This overexpression produces inactive inclusion bodies. To overcome the high insolubility of the protein, we have developed a reliable method for recovering recombinant protein by the use of urea for protein solubilization and Triton X-100 during the on-column refolding step. Moreover we obtained an active and purified exopolyphosphatase.

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