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Bacterial expression and purification of Interleukin-2 Tyrosine kinase: Single step separation of the chaperonin impurity

Raji E. Joseph, Amy H. Andreotti *

Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, 4208 MBB, Ames, IA 50011, USA

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ABSTRACT

Biochemical and biophysical characterization of kinases requires large quantities of purified protein. Here, we report the bacterial expression and purification of active Itk kinase domain (a Tec family kinase) using ArcticExpress cells that co-express the chaperonin system Cpn60/10 from *Oleispira antarctica*. We describe a simple one step $MgCl_2$/ATP/KCl incubation procedure to remove the co-purifying chaperonin impurity. Chaperonin co-purification is a common problem encountered during protein purification and the simple incubation step described here completely overcomes this problem. The approach targets the chaperonin system rather than the protein of interest and is therefore widely applicable to other protein targets.

Kinases are an important class of enzymes that regulate a wide variety of cellular processes [1–3]. Altered activity of kinases has been implicated in numerous diseases including cancer [1,4]. Structural and functional characterization of these enzymes and development of kinase inhibitors requires large (milligram) quantities of the purified protein [5]. This necessitates the use of an expression system that is simple, rapid and inexpensive. Although bacterial expression systems meet all of the above criteria, the notoriously poor solubility of kinase domains severely hampers the use of this common expression system [5].

Significant effort has been dedicated to the development of strategies to improve the solubility of kinase domains expressed in bacteria. These include the use of solubility tags, co-expression with phosphatases, and co-expression with chaperonins [5–10]. While some of these techniques have been successfully employed in the bacterial expression of certain kinase domains, they are not applicable to all kinases. Moreover, these approaches still pose significant challenges; for example, unwanted co-purification of the chaperonin can stymie the purification of the target kinase [11].

The Tec family kinases (Itk, Btk, Tec, Txk, and Bmx) are non-receptor tyrosine kinases that play a key role in immune cell function [12,13]. Understanding the structural and functional characteristics of this kinase family is of considerable interest due to the importance of immune cell signaling and disease states associated with dysregulation of immune cell function. Currently, efforts to characterize these proteins biochemically and structurally rely on insect cell expression systems that are time consuming and expensive. In fact, to date there are no published methods describing the expression and purification of any Tec family kinase domain from bacteria.

Using Itk as a model for the Tec family, we describe a method to express and purify large quantities of catalytically active kinase domain from bacteria. Specifically, the Itk kinase domain is expressed in the bacterial strain ArcticExpress (Stratagene) that contains the chaperonins Cpn60/10 from a psychrophilic bacterium *Oleispira antarctica*. Expression along with this chaperonin system allows for the production of soluble kinase domain at low temperatures. Importantly, we have developed a single step $MgCl_2$/ATP/KCl incubation procedure for the dissociation of the co-purifying chaperonin. This simple protocol permits purification of the kinase domain away from the co-expressed chaperonin, overcoming a major hurdle in the use of chaperonins as solubilization agents. We also demonstrate that this approach is widely applicable in the separation of co-purifying chaperonins from other systems unrelated to the Tec kinases.

Experimental procedures

Constructs

The mouse wild-type Itk kinase domain (355–619) was PCR amplified and cloned into the pET 28b (Novagen) vector to create the His-tagged Itk kinase domain. (In Fig. 1A and B, the Itk

* Corresponding author. Fax: +1 515 294 0453.
E-mail address: amyand@iastate.edu (A.H. Andreotti).

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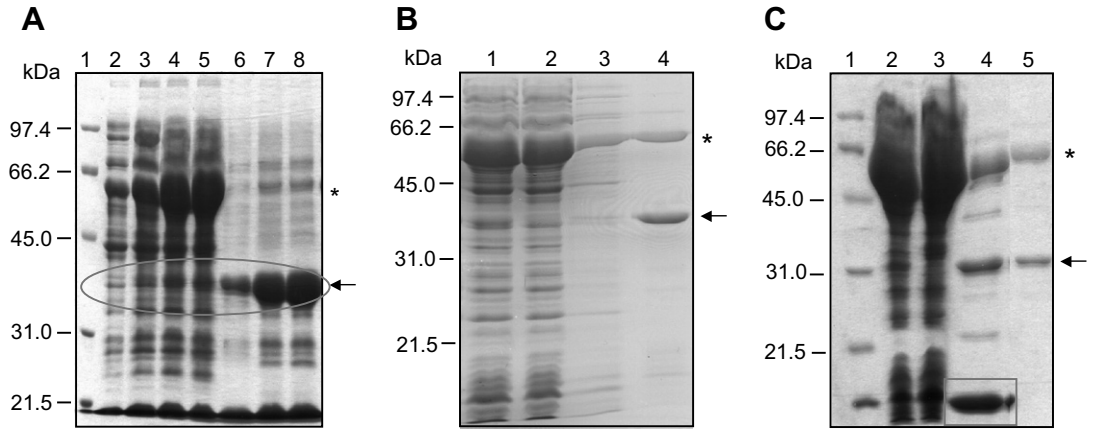

Fig. 1. Bacterial expression of the Itk kinase domain. (A) ArcticExpress bacteria transformed with His-tagged wild-type Itk kinase domain was induced with 0.1 mM IPTG at 12 °C. One milliliter samples of the cell culture were taken at consecutive time points. The cell pellets were re-suspended in 100 μl of lysis buffer. The lysed samples were spun and the supernatant (soluble fraction) was boiled in SDS–PAGE sample buffer. The pellet (insoluble fraction) was re-suspended again in 100 μl of lysis buffer and boiled in SDS–PAGE sample buffer. The samples were separated on a 12% SDS–PAGE gel and stained by Coomassie staining. Lanes 2–5 are the 'soluble' supernatant fraction at 0, 3.5, 18.5, and 23 h, respectively, and lanes 6–8 are the 'insoluble' pellet fractions at 3.5, 18.5, and 23 h, respectively. Molecular weight standards are in lane 1. The band corresponding to the Itk kinase domain is circled across all lanes. Additionally, in (A) through (C), the arrow indicates the position of the Itk kinase domain and the asterisk indicates the position of the chaperonin. (B) The chaperonin Cpn60 co-purifies with the His-tagged wild-type Itk kinase domain. Lane 1, soluble supernatant fraction; lane 2, flow-through from the Nickel column; lane 3, wash from the Nickel column; and lane 4, elution from the Nickel column. (C) Co-purification of the chaperonin Cpn60 with the Strep-tagged wild-type Itk kinase domain. Lane 1, molecular weight markers; lane 2, soluble supernatant fraction; lane 3, flow-through from the Strep Tactin (Novagen) resin; lane 4, washed Strep Tactin resin boiled with SDS loading buffer and loaded directly onto gel. In this process Strep Tactin is released from the resin and appears on the gel (boxed band at low molecular weight); and lane 5, elution from the Strep Tactin resin.

kinase domain carries a His-tag at both the N- and C-termini. In Fig. 2B, the construct was changed to contain a His-tag only at the C-terminus.) The Strep-tagged wild-type Itk kinase domain (356–619) was created by PCR amplification using a reverse primer with a Strep tag II (WSHPQFEK) epitope and cloned into the pET 28a vector (Novagen). The molecular weight of the Itk kinase domain constructs are as follows: Strep-tagged Itk kinase domain is 31.1 kDa, Itk kinase domain with a single C-terminal His-tag is 30.8 kDa and the Itk kinase domain with both an N- and C- terminal His-tag is 34.8 kDa. All constructs were verified by sequencing at the Iowa State DNA synthesis and sequencing facility.

Test expression

Plasmids encoding Itk kinase domain were transformed into the ArcticExpress bacteria (Stratagene) following the manufacturers instructions. For test expression, 50 ml of Terrific broth (TB)[1] media was inoculated with a 2% overnight inoculum. The culture was grown at 30 °C/250 rpm till it reached an OD_{600nm} of 0.8. The temperature was then lowered to 12 °C and the culture was induced with 0.1 mM IPTG. One milliliter samples of the culture were taken at various time points. The cell pellets were re-suspended in 100 μl lysis buffer (0.5 mg/ml lysozyme, 50 mM KH_2PO_4, pH 7.4, 75 mM NaCl, 2 mM DTT, 0.02% NaN_3) and stored overnight at −80 °C. The samples were then thawed, sonicated briefly and spun at 14 K for 10 min at 4 °C. The 'soluble' supernatant fraction was boiled with SDS–PAGE sample buffer. The remaining pellet was re-suspended in 100 μl of the same lysis buffer and boiled with SDS–PAGE sample buffer. The samples were separated on a 12% SDS–PAGE gel and stained by Coomassie staining.

Protein purification

Itk kinase domain was expressed in ArcticExpress bacteria at 12 °C for 23 h as in the smaller scale test expression. For the His-tagged Itk kinase domain, the cell pellets were re-suspended in lysis buffer (0.5 mg/ml lyzozyme, 50 mM KH_2PO_4, pH 8.0, 150 mM NaCl, 20 mM imidazole) and stored overnight at −80 °C. The cell pellets from a 1 l culture were thawed after the addition of 1 mM PMSF and 3000 Units DNase I (Sigma). The lysate was spun at 14 K for 1 h at 4 °C. The supernatant was loaded onto a Nickel NTA resin (Qiagen). The column was washed with 200 ml of wash buffer (50 mM KH_2PO_4, pH 8.0, 150 mM NaCl, 40 mM imidazole) and then eluted with 150 ml of elution buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 250 mM imidazole, 10% glycerol). For the Strep-tagged Itk kinase domain, the cell pellets were re-suspended in lysis buffer (0.5 mg/ml lysozyme, 50 mM KH_2PO_4, pH 7.4, 75 mM NaCl, 2 mM DTT, 0.02% NaN_3) and stored at −80 °C. The cell pellets were thawed, processed as described above for the His-tagged protein and loaded onto a Strep Tactin resin (Novagen). The resin was washed with 200 ml of wash buffer (50 mM KH_2PO_4, pH 7.4, 75 mM NaCl, 2 mM DTT, 0.02% NaN_3) and eluted with 150 ml of elution buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM DTT, 2.5 mM Desthiobiotin (Sigma) and 10% glycerol). Separation of the co-purifying chaperonin was achieved by incubating the resins after the wash step in dissociation buffer (20 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 5 mM ATP, and 150 mM KCl) at 4 °C for 2 h followed by a 200 ml wash with the respective wash buffer.

Activity assay

Kinase assays were carried out using the Itk SH3SH2 domain substrate (containing the Y180 phosphorylation site) as described previously [14]. Phosphorylation on Y180 in the context of the Itk SH3SH2 domain fragment is monitored by Western blotting with a Btk phospho-Y223 (analogous to Itk Y180) specific antibody as reported previously [15]. Briefly, the Strep-tagged Itk kinase domain was incubated with 10 μM purified Itk SH3SH2 domain in an *in vitro* kinase assay buffer (50 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 1 mg/ml BSA, 1 mM Pefabloc, and 200 μM ATP) at room temperature for one hour. The samples were boiled, separated by SDS–PAGE and Western blotted with an anti Btk phospho-Y223 antibody.

[1] *Abbreviations used:* TB, terrific broth; GST, glutathione S transferase; MBP, maltose binding protein.