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

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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

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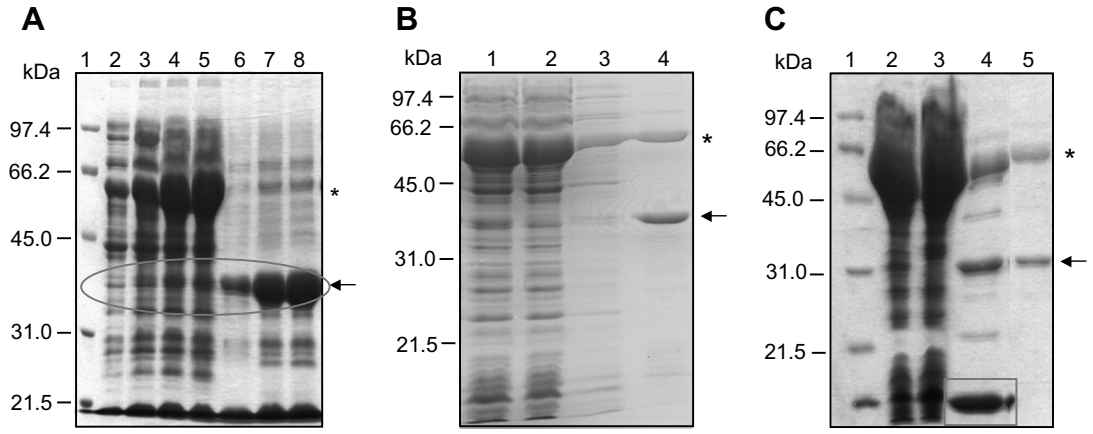

Fig. 1. Bacterial expression of the Itk kinase domain. (A) ArcticExpress bacteria transformed with His-tagged wild-type Itk kinase domain was induced with 0.1 mM IPTG at 12 °C. One milliliter samples of the cell culture were taken at consecutive time points. The cell pellets were re-suspended in 100 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.

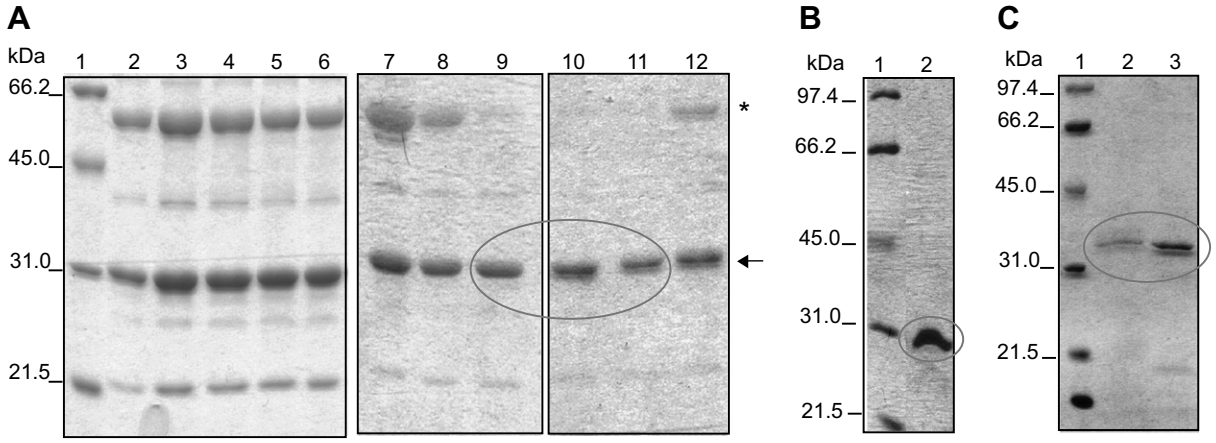

Fig. 2. Purification of bacterially expressed Itk kinase domain. (A) Optimization of conditions for the removal of the co-purifying chaperonin Cpn60 from the Itk kinase domain. The Strep-tagged Itk kinase domain was purified on the Strep Tactin resin and subjected to different wash conditions as indicated below. The samples were then boiled, separated on an SDS–PAGE gel and stained. Lane 1, molecular weight markers; lane 2, Hepes buffer pH 7.2 wash; lane 3, phosphate buffer pH 7.2 wash; lane 4, Tris buffer pH 8.0 wash; lane 5, RIPA buffer wash; lane 6, low salt (10 mM KH_2PO_4, pH 7.2) wash; lane 7, incubation with denatured protein; lane 8, incubation with 5 mM ATP/10 mM $MgCl_2$; lane 9, incubation with 5 mM ATP/10 mM $MgCl_2$/150 mM KCl; lane 10, incubation with 10 mM ATP/10 mM $MgCl_2$/150 mM KCl; lane 11, incubation with 5 mM ATP/10 mM $MgCl_2$/300 mM KCl; and lane 12, incubation with 150 mM KCl. The arrow and asterisk indicates the position of the Itk kinase domain and the chaperonin, respectively, in all the panels. The purified Itk kinase domain resulting from the one step $MgCl_2$/ATP/KCl wash is circled in lanes 9–11. (B) Lane 1, molecular weight markers; lane 2, final purified protein preparation of His-tagged Itk kinase domain. (C) Lane 1, molecular weight markers; lane 2, final purified protein preparation of kinase active His-tagged Lck kinase domain; and lane 3, kinase inactive His-tagged Lck kinase domain. In panels (B) and (C), bands corresponding to purified kinase domains are circled.

Results and discussion

In spite of the reasonable expression levels that can often be obtained for kinase domains in bacteria, the utility of this approach is problematic due to the formation of inclusion bodies and correspondingly low levels of soluble recombinant protein. In an effort to increase the solubility of the Itk kinase domain in bacteria, we tested a variety of strategies that have proven successful in the solubilization of other proteins [5–10]. First, a panel of solubilization tags (glutathione S transferase (GST), maltose binding protein (MBP), thioredoxin, Z-domain from protein A (ZZ domain), and Gb1-domain from protein G (GB1)) covalently attached to the Itk kinase domain failed to produce soluble protein (data not shown). Next, we took advantage of recent reports that co-expression of a phosphatase with the target kinase reduces the cellular toxicity that can be associated with over-expression of kinases in bacteria [5,10]. The YopH phosphatase was obtained from the Kuriyan laboratory, who had demonstrated its use in the production of soluble Src and Abl kinases in bacteria [5]. Unfortunately, co-expression with the phosphatase YopH failed to produce soluble Itk kinase domain (data not shown). In hindsight, this is consistent with data that indicate that the isolated Tec kinase domains exhibit poor catalytic activity when compared to their Src family counterparts [16,17]. Moreover, we observe no differences in the levels of soluble Itk kinase domain when we compare the expression of active or kinase inactive (K390R) Itk (data not shown). These observations suggest that the poor solubility of the Itk kinase domain in bacteria is unlikely due to cellular toxicity effects.

Co-expression with the *Escherichia coli* chaperonin GroEL/ES is another strategy used for the solubilization of a wide variety of proteins in bacteria [6,9]. These chaperonins aid in the folding of the over-expressed proteins and prevent aggregation leading to inclusion bodies. We find however, that co-expression of the Itk kinase domain with *E. coli* GroEL/ES at room temperature also failed to produce soluble protein (data not shown).

Bacterial expression of proteins is often carried out at low temperatures (12–20 °C) to increase the yield of soluble proteins by slowing down the rate of protein expression and allowing time for their folding [18]. The ArcticExpress system is a new line of competent cells that contain the chaperonin system Cpn60/10 from the psychrophilic bacterium *O. antarctica*. The advantage of the ArcticExpress system is that, unlike the *E. coli* chaperonin GroEL/ES, the Cpn60/10 chaperonin remains active at the low temperatures often used for protein expression [19].

We expressed the Itk kinase domain in the ArcticExpress bacteria to assess the feasibility of this approach. Using a low temperature induction (12 °C) yielded a significant improvement in solubility; about 10% of the total Itk kinase domain is in the soluble fraction (Fig. 1A). Although 90% of the Itk kinase domain remains insoluble, the level of soluble protein produced in this system (Fig. 1A, lane 5) is similar to the levels of soluble Src and Abl kinase domains produced in bacteria by co-expression with the YopH phosphatase [5] and should, therefore, yield milligram quantities of purified kinase from a large-scale culture.

Based on the promising results of the small-scale expression of the Itk kinase domain in the ArcticExpress cells, we proceeded with a large-scale (1 l) expression and purification of the His-tagged Itk kinase domain. The soluble Itk kinase domain could be readily extracted from the lysed bacteria using the Nickel-NTA resin but the Cpn60/10 chaperonin co-purifies to an extent that is not acceptable for applications that require greater than 90% purity (Fig. 1B). To eliminate the possibility that the chaperonin is binding non-specifically to the Nickel-NTA resin, we changed the nature of the tag attached to the Itk kinase domain. The Strep tag II affinity purification system (Novagen) is a high affinity tag derived from the biotin:Streptavidin interaction. The high specificity and affinity of the Strep tag II for the Strep Tactin resin allows the use of stringent wash conditions that should eliminate the co-purification of non-specific impurities, in this case the Cpn60/10 chaperonin. We find, however, that the Cpn60/10 chaperonin continued to co-purify with the Itk kinase domain using this alternative affinity tag system (Fig. 1C). This indicates that the chaperonin is most likely interacting directly with the Itk kinase domain rather than non-specifically with the resin.

Co-purification of chaperonins is a common problem encountered during protein purification and to date limits the effectiveness of this solubilization approach [20,21]. Indeed, simple purification procedures using common wash buffers did not yield pure Itk kinase domain (Fig. 2A, lanes 2–4). To address this limitation, a number of different techniques have been reported to remove the co-purifying chaperonin impurity [11,20,21]. In an effort to remove the Cpn60/10 protein impurity from the bacteri-

ally expressed Itk kinase domain, we surveyed these previously reported techniques: detergent washes (Fig. 2A, lane 5), low salt washes (Fig. 2A, lane 6), the addition of denatured bacterial proteins as a competitive strategy (Fig. 2A, lane 7), and $MgCl_2$/ATP washes (Fig. 2A, lane 8). Unfortunately, none lead to complete removal of the co-purifying chaperonin from the Itk kinase domain (Fig. 2A, lanes 2–8).

The failure of the published protocols to remove the co-purifying chaperonin from the over-expressed Itk kinase domain prompted us to consider the mechanistic aspects of chaperonin function. Since the chaperonin is likely to be bound to the Itk kinase domain, we reasoned that reagents that could stimulate substrate release from the chaperonin might permit successful purification of the target kinase domain. Examination of the chaperonin literature reveals that potassium when combined with $MgCl_2$/ATP promotes disassembly of certain classes of chaperonins into their component subunits [22,23]. Potassium has also been shown to stimulate the ATPase activity of some chaperonins [24–27]. Since disassembly into subunits or an increase in ATPase activity can bring about substrate release, we tested whether we could dissociate the chaperonin from the Itk kinase domain by incubating with KCl and $MgCl_2$/ATP.

Incubation of the Itk kinase domain immobilized on the Strep Tactin resin with $MgCl_2$/ATP and KCl for 2 h at 4 °C completely released the co-purifying chaperonin yielding pure Itk kinase domain (Fig. 2A, lanes 9–11). Incubation of the Itk kinase domain with either $MgCl_2$/ATP or KCl alone was only partially able to remove the chaperonin (Fig. 2A, lanes 8 and 12). While the mechanism of action of the potassium ion on Cpn60/10 chaperonin is not clear, it seems to be an ion specific effect as similar concentrations of sodium are not as effective (data not shown). Moreover, this single step incubation is broadly applicable to different purification systems. It is capable of removing the Cpn60/10 chaperonin from His-tagged Itk kinase domain immobilized on the Nickel-NTA resin (Fig. 2B, lane 2) and from kinase active and kinase inactive His-tagged Src family kinase, Lck (Fig. 2C, lanes 2 and 3, respectively).

To ensure that the bacterially produced Itk kinase domain is active, we used a well-established *in vitro* kinase assay to test catalytic function [14,17]. Using the ArcticExpress system and the new $MgCl_2$/ATP/KCl single step separation protocol described here, we are able to purify 7–8 mg of Itk kinase domain per liter of culture. This purified enzyme effectively phosphorylates its substrate (Fig. 3) and these yields are sufficient for detailed biochemical and structural work. The simple procedure developed here to separate the co-purifying chaperonin impurity should be widely applicable to a range of protein systems.

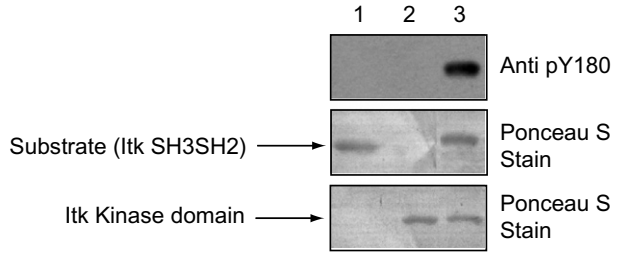

Fig. 3. Bacterially expressed Itk kinase domain is catalytically active. Strep-tagged wild-type Itk kinase domain was incubated with purified substrate (10 μM Itk SH3SH2 domain) in an *in vitro* kinase assay buffer for 1 h at RT. Lane 1, substrate alone control; lane 2, enzyme alone control; and lane 3, enzyme plus substrate. The SH3SH2 substrate is phosphorylated on Y180 and detected with an antibody specific to this site. The middle panel shows substrate levels and the bottom panel shows enzyme levels. Both are detected by Ponceau S stain.

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