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Research Article

Expression, Purification, and Biochemical Characterization of a recombinant protein kinase (CRK1) from *Leishmania mexicana*

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Abstract: The CRKs cyclin-dependent kinases of *Leishmania* are essential for multiplication and survival of the parasite. Previous genetic studies have suggested CRKs as potential drug target. Further analyses were hindered by unavailability of sufficient amount of the kinase. Aim of the study is the production, purification and biochemically analysis of recombinant protein kinase (CRK1) from *Leishmania mexicana*. A system was developed to express and affinity-purify recombinant *L. mexicana* CRK1 protein from *Escherichia coli*, in which 6-histidine tag was added to the C-terminus of the over-expressed CRK1 kinase. Insoluble recombinant *L. mexicana* CRK1his was purified to nearly homogeneity using urea and was renatured by dilution and dialysis methods. Western blot analysis has confirmed the expression of the pure kinase. Work presented by this paper has confirmed the usefulness of the prokaryotic system for production of pure homogenous recombinant protein kinase of *Leishmania* parasite. This protein could possibly be used for inhibitory assays. Generally, same approach could be exploited for production of anti-bodies to be used in diagnosis or vaccine development.

Keywords: *Leishmania mexicana*; bacterial-expression; cdc2-related kinase; CRK1

INTRODUCTION

The protozoan parasite of the genus *Leishmania* causes leishmaniasis in most tropical and sub-tropical areas of the world, affecting 12 million people and rendering 350 million at risk in 88 countries, 72 of which are developing countries [7]. *Leishmania* has a biphasic life cycle: a motile extracellular form, found in the sand fly vector, and a non-motile intracellular form in the mammalian host. After ingestion of infected blood by the sand fly, the parasite initially develops into rapidly dividing promastigotes in the fly midgut from where they migrate to the proboscis where they are found as metacyclic promastigotes, a non-proliferative infective form pre-adapted for survival in the mammalian host. After inoculation into the mammal, the metacyclics invade host macrophage cells where they differentiate into proliferative amastigotes within phagolysosomal vacuoles. The life cycle is completed when infected macrophages or amastigotes free in the blood are ingested by the sand fly. The metacyclic stage is cell cycle arrested, probably in the G1 phase, and release from this block occurs as the parasite differentiates to an amastigote, within the host macrophage.

Cyclin-dependent kinases (cdks) are serine-threonine kinases that play pivotal roles in the control of the eukaryotic cell cycle [18]. In yeast, cell-cycle progression is predominantly regulated by one cdk, $p34^{cdc2}$ in *Schizosaccharomyces pombe* or $p34^{CDC28}$ in *Saccharomyces cerevisiae*, whereas in higher eukaryotes many cdks are involved (cdk1-8) [11]. cdk activity is post-translationally regulated by several mechanisms; positively and negatively by phosphorylation / dephosphorylation at various key residues, by association with cyclins (which are positive regulatory subunits) [9, 2] and cdk inhibitors [17, 10, 8]. This cell-cycle regulatory mechanism is conserved throughout evolution with homologues of $p34^{cdc2}$ and cyclins, determined both by sequence similarity and by complementation of yeast mutants, found in a wide range of eukaryotes [18].

A number of CRK genes have been isolated from trypanosomatids, including *Crithidia fasciculata* (6), *Trypanosoma brucei* [15], *Trypanosoma cruzi* [12], *L. mexicana* [14] and [10], *L. major* [20], and *L. donovani* [1, 3]. *L. mexicana* CRK1 was found in comparable amounts in all life cycle stages, however, its activity was thought to be stage-regulated at the post-translational level, because its histone H1 kinase activity was detected only in the promastigote metacyclic forms [15].

The over-expression of CRKs in the bacterial as well as insect cells is well known approach to study the biochemical and physical characteristics of these molecules, and to look for their associated kinase activity. The recombinant *T. Cruzi* CRK1 (TzCRK1) is a good example [12] that has been shown to interact with the mammalian cyclins. To investigate further the

potentiality of CRK1 as drug target, the availability of a recombinant product would be of great help. Therefore the objectives of this study were to produce recombinant protein kinase (CRK1) from *L. mexicana* and to characterize it biochemically.

MATERIALS AND METHODS

Parasites:

Leishmania mexicana mexicana (MNYC/BZ/62/M379) wild-type promastigotes were grown at 25^0C in HOMEM medium [+10% fetal calf serum (FCS)] to mid-logarithmic phase of growth ($5x10^6$ cells/ml)

Cloning of *L. mexicana CRK1his*:

Originally, the *CRK1* gene was PCR-amplified, cloned and then inserted into the expression vector pQE60 to create a fusion protein with six histidines at the C-terminus as previously described [16]. *L. mexicana* CRK1 was sub-cloned into pET5a (Novagen) that gave a C-terminal histidine-tagged CRK1 to create plasmid pGL399 (Figure 1).

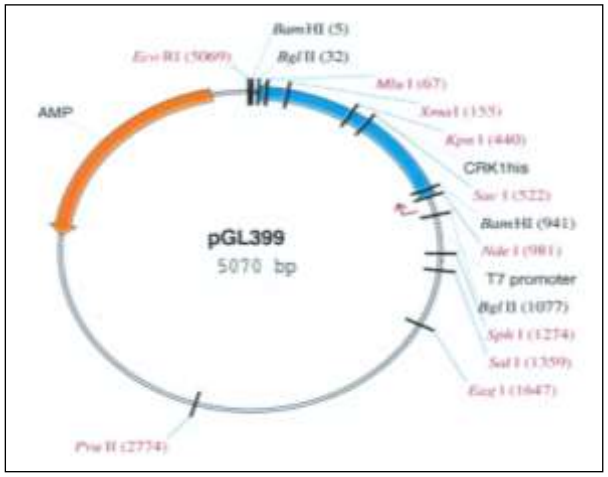

Figure 1: Map of the pGL399 that contains *L. mexicana CRK1his*

The circular molecule was designed by using the appropriate program of the Vector NTI suite. The blue-filled arrow represents the *CRK1his* gene.

Induction of BL-21 (DE3) cells transformed by pGL399 and SDS-PAGE analysis for the sub-cellular localization:

The transformation reaction was heat shocked for 45 seconds at 42^0C, then incubated on ice for 2 min before adding the SOC medium, finally it was incubated for 1 hr at 37^0C. To LB plates that containing ampicillin, 100 µl of X-gal/IPTG mixture were added 15 min before plating. After plating, the Petri-dishes were incubated overnight at 37^0C. Overnight grown cultures of cells transformed with pGL399 were induced with isopropy-β-D-thiogalactopyranoside (IPTG) as previously described [8, 2, 4]. Briefly, expression of the recombinant CRK1his were induced with IPTG to a final concentration of 0.2 mM after cultures reached an A_{600} of ~0.6. Cultures were then maintained for 4 hr at 30^0C 200 rpm rotary shaker, cells were harvested by centrifugation at 4,000 x g for 20 min at 4^0C. The supernatants were discarded and pellets were kept on ice and the cells pellets were resuspended by adding 4 times wet weight volumes sonication buffer (ice-cold), then the suspended cells were disrupted by sonication on ice in short 9 seconds bursts. The sonicates were saved for SDS-PAGE analysis. The cell debris was sedimented by centrifugation at 10,000 x g for 20 min at 4^0C. The supernatants were separated from the pellets and all were then, loaded into a 12.5% SDS-polyacrylamide gels and the separated proteins were visualized by Commassie-Blue staining. Non-induced BL-21 (DE3) was used as a control, together with empty pET5a-infected cells. The loaded protein was given in (µl), however, the quantification was performed according to the used low range SDS protein marker (Bio Rad). The expected CRK1 band size lies between Carbonic Anhydrase (pink colour) with 42 kDa and Myoglobin (purple blue colour) with 30 kDa in size. Different volumes of the CRK1 preparations were loaded for sake of quantification of the expression level (Data not shown).

Ni-NTA purification of *L. mexicana* CRK1his:

This was done using denaturing conditions. The bacterial cells were lysed with binding buffer that contains 8 M Urea or 6 M Guanidine hydrochloride and the lysates were bound to Ni-NTA agarose beads (affinity media) in 10 ml disposable columns, the cell proteins (contaminants) were washed away using the binding buffer (as above) that contained 10-50 mM imidazole. Finally, the pure denatured fusion CRK1his was eluted with binding buffer that contained increasing amount of imidazole. The denatured CRK1his was then subjected to refolding by dilution and dialysis techniques.

L. mexicana CRK1his Western blot analysis:

The induced samples lysates were run on 12.5% SDS-PAGE as before described, and then were transferred to PVDF Nylon membrane. The polyclonal CRK1 antibodies used were generated in rabbits, against a 16-residue peptide corresponding to the C-terminus of the LmmCRK1 (CITAA-DALNHPYFSLQF) [14]. These primary antibodies were used at the final dilution of 1:200. The immune complexes were detected with the horseradish peroxidise (HRP) conjugate secondary antibodies (anti-rabbit IgG) that were used to the final dilution of 1:2000, and HRP was revealed with an ECL kit (Amersham). Anti-His monoclonal anti-bodies (Tetra-His Antibody BSA free Mouse IgG, QiaGen) were used also to check for the expression. Where, bovine serum albumin (BSA) was used instead of milk, because the latter reduce the efficiency of the epitope (6His) binding. Therefore, the blocking buffer was prepared in PBS (1 x PBS, 1% Tween-20, 3% BSA). The primary anti-bodies were used to a final dilution of 1:1000 (v/v) for 1 hour at room temperature. The washing was

carried out as previously described (1, 2, 3), using the Wash buffer (WB: 1 x PBS, 0.1% Tween-20). The secondary anti-mouse anti-bodies (anti-Mouse IgG HRP conjugate, Promega) were incubated at room temperature for 45 min. The membranes were then washed three times as before. The ECL reagents were mixed 1:1 and diluted to 1/5 with PBS, then was used to detect the signals as previously described.

Solubilisation of inclusion bodies and refolding of recombinant *L. mexicana* CRK1his:

The inclusion bodies were isolated from the previous pellets by a further step of sonication, and use of the denaturant 8 M urea. The pellets were suspended in solubilisation buffer SB (8 M Urea; 100 mM TrisHCl; 10 mM DTT pH 8.0) and incubated at 37^0C 1200rpm shaker for 2 hrs, vortexed and spun. This step was repeated twice using same conditions. The solubilised CRK1his was then obtained by removing of the urea and allowing refolding. Thus, the refolding of the solubilised recombinant CRK1his was done firstly, by dilution 1: 100 and pH 8.0, and secondly, by overnight dialysis at 4^0C in DB1 buffer (100 mM TrisHCl; 5 mM EDTA; 2 mM DTT pH 8.0). Another dialysis was performed for 2 hrs at 4^0C in DB2 buffer (20 mM TrisHCl pH 7.0). Finally, the solubilised denatured proteins were dialyzed against ice-cold Na-phosphate buffer at 4^0C pH 8.0 (2 exchanges, 2 litres each).

RESULTS

Over-expression of recombinant *L. mexicana* CRK1his in *E. coli*:

A high level of expression was observed, with almost all the recombinant CRK1his in the insoluble fraction (Figure 2, lane 2), and when different conditions of temperature, incubation periods and IPTG concentrations were used (Data not shown). A protein of approximate of 34 kDa was obtained (Figure 2), when stained with the Coomassie Blue. The size is consistent with the predicted size of the *L. mexicana* CRK1.

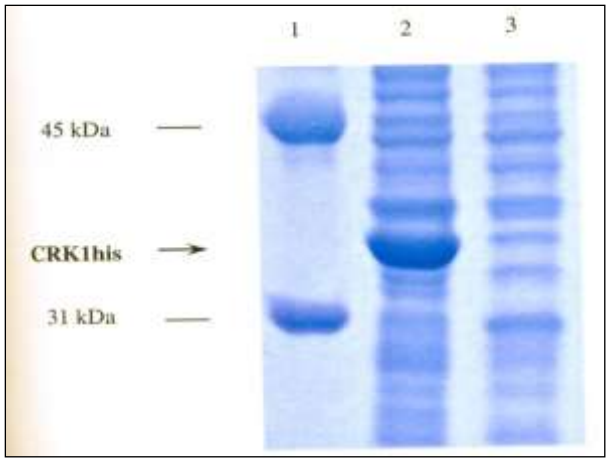

Figure 2: SDS-PAGE analysis of the crude extract of the recombinant *L. mexicana* CRK1his.

The pGL399-transformed BL-21 cells were induced with 0.2 mM IPTG at 37^0C, and the cells were lysed by sonication. Proteins were separated on 12.5% SDS-polyacrylamide gel and visualized by Coomassie-Blue staining. Lane 1; molecular weight marker (Bio-Rad), lane 2; pellet preparations, and lane 3; supernatant preparations.

Characterization of recombinant *L. mexicana* CRK1his by immunoblotting:

In order to confirm that the obtained protein is the over-expressed CRK1his, western blotting analysis was carried out. The anti-CRK1 anti-peptide anti-bodies strongly recognized the over-produced recombinant CRK1his, as a single protein was observed in all the insoluble fractions (Figure 3, lanes 2, 4, 6), the size of which was consistent with the predicted size of *L. mexicana* CRK1, that was approximate 34 kDa. In addition to the fact that the size of the obtained protein was similar to that of the protein detected on SDS-PAGE analysis however, this protein was absent from both, the control non-induced cells (lanes 1, 3, 5, 7, 9, 11) and the soluble fractions (lanes 8, 10, 12). Moreover, this protein was present in all preparations when using temperatures 16^0C, 30^0C, or 37^0C (lanes 2, 4, 6). All together, these results provided a further confirmation that the recombinant *L. mexicana* CRK1his was produced in the insoluble fraction probably inside inclusion bodies. Anti-His monoclonal anti-bodies (Tetra-His Antibody BSA free Mouse IgG, QiaGen) were used also to check for the expression as well as the presence of the histidine tag in the recombinant CRK1his kinase. The ECL was able to detect a strong signal again in the insoluble fractions (Data not shown), the size of which was similar to that recognized by the anti-CITTA anti-bodies as revealed in (Figure 3) by the arrow. These results confirmed that the *L. mexicana* CRK1his was over-expressed in the complete form and that the histidine tag was not degraded, which means affinity purification could possibly be performed on it.

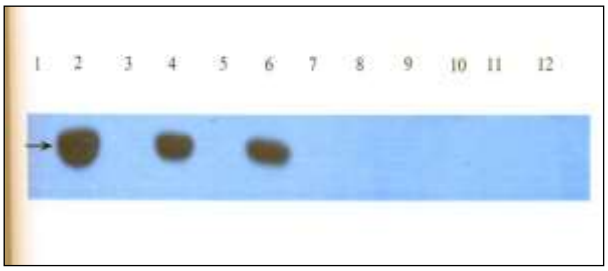

Figure 3: Western blot analysis of the crude extract of the recombinant *L. mexicana* CRK1his.

The pGL399-transformed BL-21 cells were induced at 37^0C (lanes 2 and 8), 30^0C (lanes 4 and 10), and 20^0C (lanes 6 and 12), non-induced BL-21 cells were analysed in parallel (lanes 1, 3, 5, 7, 9, and 11), the lysates (0.6 µl) were then separated by SDS-PAGE. The pellets fractions (lanes 1-6) and the supernatants (lanes 7-12) were transferred to PVDF membrane. The anti-CITAA antibodies were used to detect the signals in an ECL system. The arrow shows the *L. mexicana* CRK1his.

Ni-NTA purification of histidine-tagged *L. mexicana* CRK1his from *E. coli* and confirmation by western blotting:

The recombinant *L. mexicana* CRK1his was affinity purified on nickel-nitrilotriacetic acid (Ni-NTA) resin under denaturing conditions. This method was used in order to extract the recombinant *L. mexicana* CRK1his from the insoluble fractions. Several detergents were used in order to solublize CRK1 fusion protein of histidine. The non-ionic detergents Tween-20, Triton X100 were unable to solubilise the protein (Data not shown). The inability of detergents to solubilise *L. mexicana* CRK1his suggested that it is separated by membrane. Therefore, it was assumed that the protein was deposited in insoluble structures / inclusion bodies. The strong denatuarants, guanidine hydrochloride and urea were used for efficient solublization of the inclusion bodies. The lysates were incubated with the Ni-NTA agarose beads, then by changing the pH, the non-specifically bound contaminants were removed and the bound his-tagged *L. mexicana* CRK1 was finally eluted in the presence of 8 M Urea. Analysis of all the purified samples on SDS-polyacrylamide gels showed the presence of a single protein of 35 kDa (Figure 4). However, the band intensity varied between different fractions, which suggests that different amount of the purified CRK1his was present in each. This result supports the fact that the recombinant *L. mexicana* CRK1his is present in the insoluble fractions and that it could be purified by selection on Ni-NTA agarose beads in soluble, but denatured form. This result was further confirmed by immunoblotting. As shown in the western analysis (Figure 5), a strong signal of 35 kDa was recognized by the anti-CITAA anti-bodies.

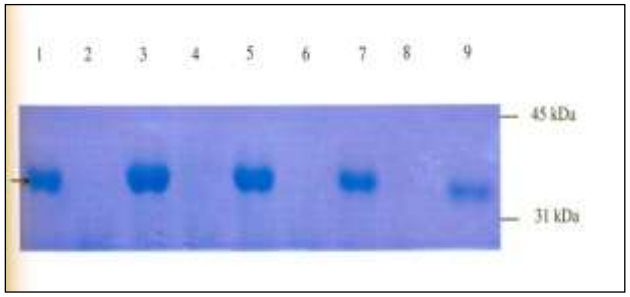

Figure 4: SDS-PAGE analysis of the Ni-NTA purified recombinant *L. mexicana* CRK1his.

The pellets of an induced BL-21 cells that transformed with pGL399, were treated with a buffer containing 6 M guanidium chloride. 5 ml of the lysates were bound to 0.8 ml of 50% Ni-NTA resin, after washing, bound CRK1his was eluted with 8 M urea buffer. The 0.4 ml fractions were collected and 5µl of each were anlysed on 12.5% SDS-polyacrylamide gel and stained with Coomassie-blue (lanes 1, 3, 5, 7, and 9). A single protein of approximte size of 34 kDa appeared in all of these fractions, that represents the denatured purified *L. mexicana* CRK1his. Nothing was purified from the non-induced BL-21 cells (lanes 2, 4, 6, and 8).

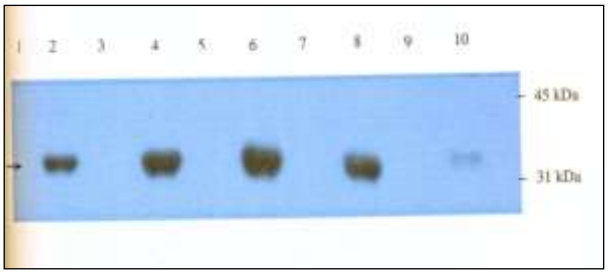

Figure 5: Western blot analysis of the Ni-NTA purified recombinant *L. mexicana* CRK1his.

The Ni-NTA denatured purified *L. mexicana* CRK1his samples were run on 12.5% SDS-PAGE and transferred to a PVDF membrane. Anti-*L. mexicana* CRK1; anti-CITAA antibodies were used; signals were detected by ECL system. These antibodies have recognized a single protein of approximate 34 kDa (lanes 2, 4, 6, and 8). The arrow shows the purified CRK1his.

Renaturation of *L. mexicana* CRK1his / Purification of Inclusion bodies and refolding:

The extraction was carried out as described previously (2, 3, 4), in which Triton X100 was used to lyse the cells. Urea was used in low concentration (2M) to purify the inclusion bodies, and in high concentration (8M) to solubilise the membrane of the inclusion bodies (Figure 6). The refolding was performed by dilution method (1:20, 1:50, 1:100 ratios). Moreover, the refolding was done slowly in order to provide sufficient time for the hydrophobic regions (36%) to associate properly, at low concentration to favour monomer formation, and at 4^0C to avoid protein degradation. Clear solution was obtained by overnight dialysis against a buffer lacking urea and neutral pH. The dialyzed collections were then purified on Ni-NTA resin by increasing pH conditions (lanes 6, 7, 8, 9, 10) and finally were concentrated (lanes 11, 12, 13). As shown in Fig. 4, 5, the electrophoresis analysis of the collected samples on SDS-polyacrylamide gels revealed the presence of only a single protein of 34 kDa. This result provided evidence that insoluble recombinant *L. mexicana* CRK1his can be purified to nearly homogeneity using urea and can be renatured by dilution and dialysis methods

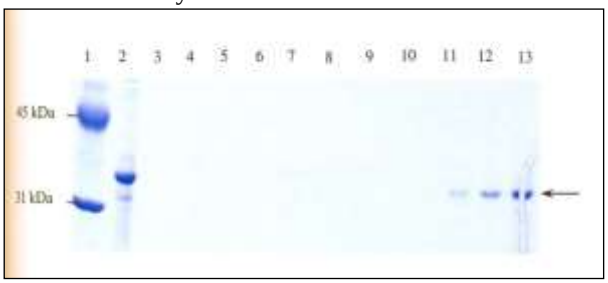

Figure 6: SDS-PAGE analysis of the refolding of the *L. mexicana* CRK1his.

Lane 1 molecular weight marker; lane 2 solubilized sample (inclusion body fraction-washed, solubilized in 8 M urea). Lanes 3, 4, and 5 urea solubilized sample diluted 1:20, 1:50, and 1:100, respectively. Lanes 6 and 7 washes (pH 8.0) of diluted sample, post-dialyzed and loaded onto Ni-NTA column; lane 8 diluted eluate (pH 6.3); lane 9 diluted eluate (pH 5.9; lane 10 diluted eluate (pH 4.5); lane 11 concentrated eluate (pH 6.3); lane 12 concentrated eluate (pH 5.9); and lane 13 concentrated eluate (pH 4.5). The arrow shows the denatured solubilized CRK1his.

DISCUSSION

Leishmanial CRK1 has a potential as drug target. Therefore, we aimed in this study to focus on CRK1 and to biochemically characterize it, and try to produce an active CRK1 that could be able to phosphorylate an exogenous substrate. A recombinant GST fusion TzCRK1 protein was able to phosphorylate histone H1 and retinoblastoma protein [12]. Similarly, His fusion LmmCRK1 protein could possibly be produced as both trypanosomatids CRKs are structurally related. The goal of identifying the function of CRK1 in *Leishmania* was first hampered by the lack of methods of synchronizing the parasites. However, recently, it was shown the possibility to obtain a synchronized *L. mexicana* culture by growing cells lines in a specific concentration of the chemical inhibitor flavopiridol [8]. There are several lines of evidence demonstrate that some of the trypanosomatid CRKs are essential genes for the survival of the trypanosome. Gene-targeting experiments have shown that *CRK1* of *L. mexicana* is an essential gene in the parasite, since it was difficult to produce a *CRK1* null mutant *Leishmania* [16]. Recently the gene function study in *Leishmania* become possible by using transgenic approach which will contribute to the development of novel therapeutics for Leishmaniases [5].

Previous gene disruption experiments have confirmed that CRK1 is an essential gene for the *L. mexicana* growth [16]. Yeast complementation experiments did not work for *L. mexicana* CRK1, however, a yeast can express an active *L. mexicana* CRK1 (14). Moreover, *L. mexicana* CRK1was found inactive in both metacyclic and amastigote forms, reason for that may be as a differentiation prerequisite during transmission from one host to another. It was suggested that CRK1 might have involved in some stage-specific role in the promastigote life cycle. In this study we have chosen the promastigote form according to this fact, such that investigation of activity will be more relevant. However, this activity test is beyond the scope of the work reported in this paper. We only here focus on the provision of pure soluble CRK1 that can be used for further biochemical assay.

In this study we successfully managed to produce recombinant *L. mexicana* CRK1his in bacterial cells in large amounts (few mgs). However, the produced kinase was present in insoluble form as inclusion bodies. Such form is not suitable or even very difficult for activity assay. Therefore, we performed several methods to solubilise the produced kinase. In one method the denaturants were used, the yield of which was a solubilised but denatured CRK1 kinase. Then a modified method of refolding that includes dilution and dialysis steps was used. All steps were carried out at 4^0C to avoid protein degradation. Finally, the produced kinase was affinity purified as it contains the His-tag and was confirmed by western blotting. We used here western blot analysis to test for the presence of the kinase only and not for its activity.

All together, the present study shows that the CRK1his can be purified with high yield by affinity chromatograpgy on Ni-NTA resin as denatured form. The produced kinase can be useful for production of specific antibodies that could be used either in the diagnosis of the disease or in the vaccine development. On the long run, since the recombinant kinase (CRK1) was produced in large quantity (~5 mg), the purification method can be scaled up and that structural crystallographic studies could possibly be carried out in order to elucidate the structure-function relationship of the kinase and its regulatory molecules.

CONCLUSIONS:

Work described in this paper confirm the possibility of studying *Leishmania* cell cycle molecules as new drug targets as it shows the easy way to produce parasite essential kinase *in vitro* as a first step for others investigations. Thus further work can lead to development of a new chemotherapy as the current situation of Leishmaniases worldwide call for an urgent need for new antileishmanial drugs. In general, the approach described in this paper could possibly be used to express any parasitic genes in bacterial cells, and thus perform genetic and biochemical analysis on those genes.

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