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The expression of NTPDase1 and -2 of *Leishmania infantum chagasi* in bacterial and mammalian cells: Comparative expression, refolding and nucleotidase characterization

M.S. Bastos [a, b, c], A. Tremblay [c], J.M. Agripino [a], I.L.A. Rabelo [a], L.P. Barreto [a], J. Pelletier [c], J. Lecka [c, d], A. Silva-Júnior [e], G.C. Bressan [a], M.R. Almeida [a], J. Sévigny [c, d], J.L.R. Fietto [a, b, *]

[a] Biochemistry and Molecular Biology Department, Universidade Federal de Viçosa, Viçosa, MG, Brazil
[b] Instituto Nacional de Biotecnologia Estrutural e Química Medicinal em Doenças Infecciosas- INBEQMeDI, Brazil
[c] Département de microbiologie-infectiologie et d'immunologie, Faculté de Médecine, Université Laval, Québec City, QC G1V 0A6, Canada
[d] Centre de recherche du CHU de Québec – Université Laval, Québec City, QC G1V 4G2, Canada
[e] Veterinary Department, Universidade Federal de Viçosa, Viçosa, MG, Brazil

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ABSTRACT

Visceral Leishmaniasis (VL) represents an important global health problem in several warm countries around the world. The main targets in this study are the two nucleoside triphosphate diphosphohydrolases (NTPDases) from *Leishmania infantum chagasi* that are the main etiologic agent of VL in the New World. These enzymes, called LicNTPDase1 and -2, are homologous to members 5 and 6 of the mammalian E-NTPDase/CD39 superfamily of enzymes. These enzymes hydrolyze nucleotides and accordingly can participate in the purine salvage pathways and in the modulation of purinergic signaling through the extracellular nucleotide-dependent host immune responses. They can therefore affect adhesion and infection of host cells and the parasite virulence. To further characterize these enzymes, in this work, we expressed LicNTPDase1 and -2 in the classical bacterial system *Escherichia coli* and mammalian cell system COS-7 cells. Our data demonstrate that changes in refolding after expression in bacteria can increase the activity of recombinant (r) rLicNTPDase2 up to 20 times but has no significant effect on rLicNTPDase1. Meanwhile, the expression in COS-7 led to a significant increase in activity for rLicNTPDase1.

1. Introduction

Leishmaniasis disease is caused by at least 20 pathogenic species of flagellate protozoa of the genus Leishmania [1]. Leishmaniasis diseases are zoonotic diseases and their transmission occurs naturally via the bite of infected females of specific sand fly species (order Diptera: family Psychodidae: sub-family Phlebotominae, genus Phlebotomus and Lutzomyia) [2]. These diseases have been classified in different clinical forms, depending mainly on the species of Leishmania involved in the infection and on the host immune response. Simplified, Leishmaniasis can be classified into two different forms: the Visceral Leishmaniasis (VL) and the Cutaneous Leishmaniasis (CL) [3]. VL is the most severe form of the disease and the main organs affected are the liver, spleen and bone marrow. If not treated, this infection could result in immunosuppression and subsequent death of the host. VL is mainly caused by *Leishmania infantum* in the Old World and by *L. infantum chagasi* in the New World. Despite this, *L. infantum chagasi* could be considered synonymous with *L. infantum.* There are different opinions concerning this classification in the Leishmania research community. In this work, we will use *L. infantum chagasi* to discriminate this species that is implicated as the main agent of VL in the New World [1,4–7]. The majority of VL cases reported worldwide (90%) are concentrated in Bangladesh, Brazil, India, Nepal and Sudan [1,8]. In addition to the high number of infected people, another worrying factor is the growing number of infected dogs in endemic areas, where dogs are the major domestic and peridomestic reservoir of these parasites. Thus, the Canine Leishmaniasis is a major problem for public and animal health because of the severity

* Corresponding author. Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, Viçosa, MG, Brazil.
E-mail address: jufietto@ufv.br (J.L.R. Fietto).

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of this disease in dogs and the zoonotic character of the disease [1,4]. The VL diagnosis is made by a combination of clinical signs and parasitological, molecular (PCR) or serological assays. Nevertheless, there is not a gold-standard, easy, cheap and noninvasive method. PCR is still too expensive to be used routinely and serological assays have the disadvantage of presenting significant false positive results [3,4,9]. There are a limited number of drugs to treat VL. Antimony therapy has been used for a long time, but currently, amphotericin B and pentamidine have been used more frequently. However, the effectiveness of medication varies widely around the world and these drugs are very toxic and must be administered with caution [10]. Consequently, efforts are still needed to improve diagnosis and treatment of this disease and the development of vaccines to control transmission is urgent.

In mammals, the NTPDase members are enzymes from the CD39 family that hydrolyze tri- and diphosphate nucleotides and have multiple functions [11,12]. All NTPDases have five conserved domains called apyrase conserved regions (ACR) [13]. These enzymes use both triphosphate and diphosphate nucleosides as substrates, are dependent on divalent cations and are insensitive or partially sensitive to common ATPase inhibitors [14–16]. In addition, they can be intracellular, secreted or integral membrane proteins [12,17]. Enzymes with NTPDase activities are ubiquitously expressed from lower eukaryotic cells to mammals [18]; however, these enzymes are not common in bacteria. NTPDase homologous enzymes have been reported in pathogenic bacteria *Legionella pneumophila* [19–21]. These enzymes are important for infection and virulence for many pathogens, such as *L. pneumophila* [18,20,21], *Toxoplasma gondii* [22–24], *Trypanosoma cruzi* [25–27] and distinct species of *Leishmania* [18,28–31]. Previous studies by our group demonstrated that *L. infantum chagasi* has two NTPDase homologous enzymes (LicNTPDase1 and-2) that are similar to mammalian NTPDases 5 and 6 at the molecular level and at least one of them, LicNTPDase-2, is important for adhesion to host cells and is naturally expressed in infected dogs [32]. In addition, NTPDase activity inhibits macrophage activation throughout the modulation of purinergic signaling and favors *L. amazonensis* infection [30].

Taking into account the importance of NTPDases for Leishmania infection, it is possible that these enzymes could be used in numerous biotechnological applications, such as diagnosis, prognosis, vaccination and the development of target-based chemotherapy [33,34]. In fact, the recombinant NTPDase2 from L. *infantum chagasi* (rLicNTPDase2) purified after expression in a bacterial system (*E. coli)* was successfully applied to serological immunodiagnosis of Canine Visceral Leishmaniasis [34].

In the context of optimization of biotechnological applications and enzyme characterization of recombinant NTPDase1 and -2 from *L. infantum chagasi*, it is important to produce the best bioactive enzymes and the nature of the heterologous expression system could be an important factor that affects the bioactivity of recombinant proteins. In this context, in this study, we evaluated the activity of the recombinant NTPDase1 and -2 expressed by the classical bacteria *E. coli* pET system and the eukaryotic mammalian COS-7 system.

2. Materials and methods

2.1. Organisms

In this study, we used two strains of *E. coli*: the DH5α strain was used in cloning and plasmid production, and the BL21 codon plus-RIL strain (Stratagene) was used for heterologous expression assays. For expression in mammalian cells, we used the COS-7 cell line, which is derived from the kidney of *Cercopithecus aethiops* (African green monkey).

2.2. Culture medium

For assays with *E. coli*, we used two different media, liquid or solid Luria-Bertani medium (LB) with or without 50 μg mL^{-1} ampicillin. This medium was used in cloning experiments and expansion of the BL21 strain, before induction of expression. The expression medium was SOC medium, which is a modification of SOB medium [35]. The COS-7 cultures were maintained in DMEM medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg mL^{-1} penicillin and 100 μg mL^{-1} streptomycin.

2.3. Cloning of LicNTPDase1 and -2 for expression in a bacterial system

The two LicNTPDases genes from *L. infantum chagasi* (strain M2682) had been previously analyzed and only the regions predicted as the bioactive domain of both enzymes were amplified by PCR and cloned into the pET21b plasmid. This strategy eliminated part of the amino terminal coding region of the predicted proteins and excluded the signal peptide of LicNTPDase1 and the amino-terminal predicted transmembrane domain of LicNTPDase2. These regions coding the ectodomains of LicNTPDases genes were then cloned into the vector pET21b Novagen® [32].

2.4. Cloning of LicNTPDase1 and -2 from L. infantum chagasi into vector pcDNA3 for expression in mammalian cells

The genes of LicNTPDase1 and LicNTPDase2 previously cloned into the pET21b vector Novagen® were used as a template to perform the amplification and cloning in the pcDNA3 vector (Invitrogen). The pcDNA3 vector used in this work was previously used to express the NTPDase1 from mouse on the surface of COS-7 cells [36]. The first step to express LicNTPDases in this mammalian system was the amplification of the entire vector plus the transmembrane domain of mouse NTPDase1. This strategy was necessary to ensure that the enzymes would be expressed on the membrane surface of COS-7 cells. To perform this cloning, we used the In-Fusion HD kit (Clontech). We made three pairs of primers that were used to amplify the target and perform the cloning (Supplementary Table 1). The cloning was performed according to manufacturer's recommendations.

2.5. Expression of recombinant rLicNTPDases in a bacterial system

The constructions pET21 b/rLicNTPDase1 and pET21 b/rLicNTPDase2 were used to transform *E. coli* BL21 codon-plus RIL (Stratagene) using a heat shock protocol. The transformed cells were inoculated in SOC medium supplemented with 50 μg mL^{-1} ampicillin and then incubated overnight at 37 °C and 180 rpm. Subsequently, the cells were transferred to new medium with 50 μg mL^{-1} ampicillin as a 1:20 dilution and allowed to grow for 2 h. Then, the material was transferred to 500 mL of SOC medium without antibiotic. Incubation was performed as described above. The cultures were incubated until they reached DO_{600} of approximately 0.6. Then, we induced the recombinant protein expression by the addition of 0.25 mM IPTG (Fermentas). The cell suspension was incubated for an additional 2 h for rLicNTPDase1 and 1 h for rLicNTPDase2 using the conditions described above.

2.6. Purification of rLicNTPDase1 and -2 expressed in a bacterial system

After induction, the cultures were fractionated into aliquots of 100 mL in conical tubes and centrifuged at 12,500 *g* at 4 °C for 10 min. The pellets were frozen and stored at −80 °C, and these

samples were used independently to purify the recombinant proteins. In the purification step, the frozen pellets were suspended in 4 mL of lysis buffer containing protease inhibitors (50 mM Tris pH 8, 100 mM NaCl, 1 mg mL^{-1} lysozyme, 1 μg mL^{-1} aprotinin, 1 μg mL^{-1} pepstatin and 1 μg mL^{-1} leupeptin). The suspensions were kept on ice for 30 min. Subsequently, the samples were sonicated for 6 cycles for 10 s at 10 W of power, always on ice. Thereafter, the inclusion bodies were washed and the lysates were centrifuged at 12,500 *g* at 4 °C for 30 min. The pellets were washed twice (alternating between the suspension and centrifugation as described above) with 20 mL of wash buffer (2 M urea, 50 mM Tris pH 7.2, 500 mM NaCl and 10 mM β-mercaptoethanol). After the washing, the pellets were solubilized with 12 mL of solubilization buffer (8 M urea, 50 mM Tris pH 7.2, 500 mM NaCl and 10 mM β-mercaptoethanol) [32,37]. To increase the solubilization of the inclusion bodies, the samples were incubated for 10 min at 60 °C [38]. Then, the samples were centrifuged again at 12,500 *g* at 4 °C for 30 min and applied onto a FPLC AKTA PURIFIER UPC10 (GE Healthcare) using the affinity column 1 mL HisTrap Crude FF. The purification was performed in three steps. In the first step, the sample was applied to the machine equilibrated with buffer A (8 M urea, 50 mM Tris pH 7.2, 500 mM NaCl and 12 mM imidazole) and this buffer was used until we achieved the baseline again when we determined that all of the proteins that did not adhere to column were eluted. The second step was a wash with 5 column volumes of buffer B (8 M urea, 50 mM Tris pH 7.2, 500 mM NaCl and 25 mM Imidazole) to wash lowly bound proteins. The third step was the elution of the recombinant enzyme with buffer C (8 M urea, 50 mM Tris pH 7.2, 500 mM NaCl and 250 mM Imidazole). The eluted sample was aliquoted in samples of 100 μL and stored in a freezer at −80 °C.

2.7. Refolding of the purified protein expressed in the bacterial system

To evaluate the influence of different refolding on the activities of the enzymes expressed in the bacterial system, we used three different protocols. It is important to highlight that this step is crucial to obtain bioactive rLicNTPDase1 and -2 because these enzymes are mostly expressed in inclusion bodies.

2.7.1. Refolding protocol 1

This protocol was based on the conditions used for the refolding of rat NTPDase1, -2, and -3 ectodomains in our previous experience producing and studying rLicNTPDase1 and -2 [32,39]. Briefly, the purified enzymes were diluted 10 times in buffer containing 50 mM HEPES pH 7.2, 50 mM Tris pH 7.2, 116 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM L-oxidized glutathione (GSSG) and 2 mM reduced L-glutathione (GSH). After dilution, the samples were incubated without agitation at 4 °C for 24 h. Then, the enzymes were used in enzymatic assays.

2.7.2. Refolding protocol 2

This protocol was based on the conditions used to study an apyrase from *Cryptosporidium parvum* with modifications [40]. The purified proteins were renatured by dialysis in two steps. In the first step, we used a buffer volume 50 times greater than the volume of the sample and in the second step the buffer volume was 200 times greater than the sample. The first step buffer contained 100 mM Tris, pH 8.0, 1 M L-arginine, 2 mM EDTA, 1 mM GSH, 0.1 mM GSSG and 5% glycerol. The samples were dialyzed against this buffer for 16 h at 4 °C with gently stirring. The second step buffer was the activity buffer that contained 50 mM HEPES pH 7.2, 50 mM Tris pH 7.2, 116 mM NaCl, 5 mM KCl and 5 mM $MgCl_2$. Both dialysis steps were carried out with only one buffer exchange.

2.7.3. Refolding protocol 3

This protocol was based on mixed conditions of NTPDases refolding [39–41] with modifications. The purified samples were diluted 10 times in activity buffer supplied with 1 mM GSSG, 2 mM L-GSH, 2 mM calcium chloride and 1 M L-arginine and incubated for 48 h at 4 °C. Then, the samples were dialyzed in activity buffer without 1 mM GSSG, 2 mM L-GSH, 2 mM calcium chloride and 1 M L-arginine. In this step, we used a volume of dialysis buffer 200 times larger than the volume of the sample. The dialysis was performed for 24 h at 4 °C.

2.8. The expression of rLicNTPDase1 and -2 in COS-7 cells

The COS-7 cells (ATCC) were thawed and maintained in culture for two passages before the transfection. The cells were maintained following the instructions of ATCC bank cells. The COS-7 cell transfection was performed with Lipofectamine (Gibco BRL: 2 mg/mL) in a 10 cm diameter dish. The dishes were prepared 48 h before the transfection with 1.5×10^6 cells per dish. After 48 h, the cells were washed 3 times with DMEM medium and then were immediately placed in the B.O .D at 37 °C, 5% CO_2. The transfection was performed by addition of 6 μg of DNA per dish using solution A (containing 6 μL of DNA 1 μg/μL plus 244 μL of DMEM) and solution B (24 μL of Lipofectamine plus 926 μL of DMEM) previously mixed and incubated without agitation for 45–60 min at room temperature. The mixed A/B solution was added in the dish containing 4.8 mL of DMEM and incubated for 5 h in biological oxygen demand (BOD) using the same condition above. Then, the transfection medium was removed and new DMEM supplemented with 10% fetal bovine serum and 2 mM glutamine was added. Then, the cells were incubated in the same condition above for 3 days. After 3 days, the cells were used to prepare protein extracts [36].

2.9. Protein extracts from transfected COS-7 cells

The cells used in transfection experiments were initially washed 3 times with ice-cold Tris-saline in a 4 °C buffer (45 mM Tris pH 7.5 and 95 mM NaCl). Then, the cells were removed by scraping and 1 mL of Tris-saline buffer containing a protease inhibitor (0.1 mM PMSF). After complete removal of the cells, the sample was placed in a 15 mL conical tube and centrifuged at 520 x *g* for 10 min at 4 °C and two additional washes with Tris-saline plus protease inhibitor (0.1 mM PMSF) was performed. After washing, the cells were suspended in 1 mL Tris-saline buffer containing protease inhibitors (0.1 mM PMSF and 10 μg/mL aprotinin) and immediately sonicated for 4 cycles of 1 s, using 10 W of power, always on the ice. The lysate was transferred to a 1.5 mL microtube and centrifuged at 300 x *g* for 10 min at 4 °C. The supernatant was collected and used to measure the protein concentration. Then, we added 7.5% glycerol and made aliquots to store in a −80 °C freezer [36].

2.10. Nucleotidase activity assays

Nucleotidase assays were performed using the malachite green method [36,42]. The reactions were performed in microfuge tubes with a 1.5 mL final volume containing a 200 μL reaction at 37 °C. The tubes were prepared with the activity buffer plus 1 mM substrate (ATP, ADP, GTP, GDP, UTP or UDP). The microtubes were placed in the incubator (adjusted to 37 °C) for 5 min before the reaction was started with the addition of the purified enzyme at a concentration of 0.5 μg to rLicNTPDase2 or 1 μg of rLicNTPDase1 and stopped by the addition of 200 μL of colorimetric reagent after 10 min of incubation with rLicNTPDase2 and 20 min with rLicNTPDase1 (the differences in amount of recombinant protein and time of assay are necessary to achievement of stable activity of

both enzymes as rLicNTPDase-1 is lower active than rLicNTPDase2). The colorimetric reagent was prepared by mixing 10 mL of malachite green solution (0.122% of malachite green dissolved in sulfuric acid 6 M) plus 0.2 mL of Tween 20 solution (11% of Tween 20 solubilized in water) and 2.5 mL of ammonium molybdate solution (7,5% of ammonium molybdate solubilized in water) according to references [36,42]. After the addition of colorimetric reagent 200 μL of each reaction tube was transferred to a 96 well microplate and the reads were done at 630 nm in a microplate reader. To convert absorbance to nmol of phosphate, a phosphate standard curve was made using a stock solution of Na_3PO_4 $12H_2O$ (1.5 mg/mL). COS-7 cell extracts were prepared as described previously [36]. The rLicNTPDase1 assays were performed for 15 min and 3 μg of COS-7 cell extract per vial, and the rLicNTPDase2 assays were performed for 5 min and 1.5 μg of COS-7 cell extracts per vial. The protein extract of non-transfected COS-7 cells was used as a control.

3. Statistical analyses

All of the experiments were performed in triplicate, with independent experiments. The data were analyzed statistically using Student's *t*-test. *P* values of 0.05 or less were considered significant. The data were expressed as the means ± standard error. Statistical analyses were performed by Prism software.

4. Results

4.1. Expression, purification and storage of rLicNTPDase1 and -2 expressed in bacteria

L. infantum has two NTPDase encoding genes, also known as guanosine diphosphatase (gi | 146079010) and nucleoside diphosphatase (gi | 146 081 774). The proteins coded by these genes, is called LicNTPDase1 (*L. infantum chagasi* NTPDase1) and LicNTPDase2 (*L. infantum chagasi* NTPDase2) in strain JPCM5 of *L. infantum chagasi* [32]. Although our analysis showed that both protein belong to the NTPDase family, they share only 20% amino acid sequence homology (Supplementary Fig. 1).

rLicNTPDase2 was previously produced as a recombinant protein in a bacterial system and its biochemical characterization, application to canine Leishmaniasis diagnosis and role as a pro-adhesion molecule in an *in vitro* infection was previously described by our group [32,34]. In contrast, the biochemical characterization of LicNTPDase1 as a genuine nucleotidase was not previously demonstrated. Considering the biological roles and potential of biotechnological applications of these proteins, it is important to search for an adequate heterologous expression system to produce them.

The first approach of this work was to produce rLicNTPDase1 under the same production conditions of rLicNTPDase2 in a bacterial system and compare the nucleotidase activity of these recombinant proteins. After purification, the samples were analyzed by SDS-PAGE 12% and silver staining (Fig. 1). We observed that the expression and purification led to only one protein band in accordance with the predicted molecular weights of monomeric proteins: 72 kDa for rLicNTPDase1 and 45 kDa for rLicNTPDase2 [32].

These proteins were expressed mainly as insoluble recombinant proteins in the inclusion bodies but rLicNTPDase2 was previously recovered as a functional nucleotidase/apyrase after its renaturation [32]. In the present work, after purification from inclusion bodies and renaturation using refolding protocol 1, both proteins were stored at 4 °C and GTPase activity was measured over time. GTP was chosen in this step because rLicNTPDase1 showed higher and consistent activity with this nucleotide. As depicted in Fig. 2, after 96 h of renaturation, rLicNTPDase1 showed a loss of 50% of its activity and after 72 h rLicNTPDase2 showed a 59% loss of its activity.

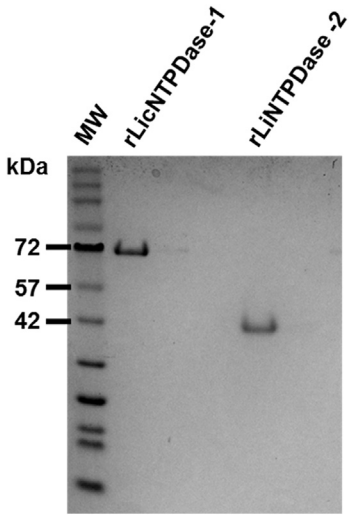


Fig. 1. SDS-PAGE of purified rLicNTPDase1 and rLicNTPDase2. Analysis of recombinant rLicNTPDases expressed in *E. coli*. The gel was subjected to silver staining.

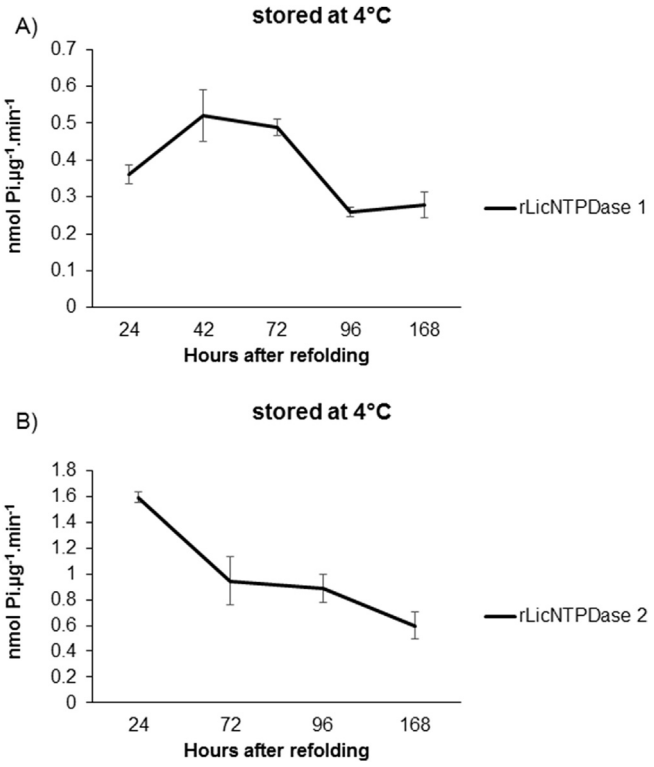


Fig. 2. Storage test at 4°C after expression in bacterial system, purification and renaturation. The results represent the mean ± SD from one independent biological replicate with internal quadruplicates.

A) GTPase activity of rLicNTPDase1 and B) GTPase activity of rLicNTPDase2. The refolding protocol used in this test was the protocol 1 previously described [39].

These results demonstrated that these proteins are unstable. We then decided to study improved storage conditions for the enzymes to improve their nucleotidase stability. We froze the proteins at −30 °C after refolding, but the enzymes lost all GTPase activity

(data not shown).

We then froze the samples at −30 °C prior to the refolding step and evaluated the activity 24 h after renaturation, using refolding protocol 1. We observed that rLicNTPDase1 showed stable activity at least 23 days, and the rLicNTPDase2 was stable at least 129 days (Fig. 3).

Despite the conditions used before the nucleotidase activity assay, it is important to notice that in all assays, rLicNTPDase2 had higher activities. Concerning this comparison in Fig. 2, rLicNTPDase2 showed a 4 time greater GTPase activity than rLicNTPDase1 at 24 h. This superiority in maximum activity of rLicNTPDase1 is up to 7 times greater if the analysis is performed using data from Fig. 3 at the same time (24 h or 1 day). The freeze step after the purification of enzymes and posterior renaturation just 24 h before the activity assay improved the stability of nucleotidase activity. Therefore, this procedure was applied to all of the samples for this work.

4.2. Substrate specificity and divalent cation dependence of rLicNTPDase1 expressed in a bacterial system

rLicNTPDase1 was refolded by dilution in 50 mM HEPES pH 7.2, 50 mM Tris pH 7.2, 116 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM L-oxidized glutathione (GSSG) and 2 mM reduced L-glutathione (GSH) at 4 °C for 24 h and then used in enzymatic assays (the refolding protocol used in this test was the protocol 1). The $MgCl_2$ was removed from the refolding buffer for the divalent cation dependence experiment. The enzyme was used in a substrate nucleotidase assay to assess the activity using different nucleotides that are processed by the enzymes from the CD39/GDA1/Apyrase family [38,43]. The results demonstrated that rLicNTPDase1 was able to hydrolyze different tri- and diphosphate nucleosides. Higher activities were observed with the nucleoside triphosphates and with GDP (Figure 4) with the following preferences for rLicNTPDaseI: GTP > ATP = GDP = UTP > ADP = UDP. Similar to other CD39/GDA1/apyrase family members, AMP was not hydrolyzed by rLicNTPDase1 (Fig. 4).

Next, the biochemical characterization of the divalent cation dependence was performed, which is a general marker of the CD39/Apyrase family. To perform this assay, we chose GTP as a substrate, which is one of the more hydrolysable substrates as previously shown (Figure 4), and calcium and magnesium, which are the main divalent cations used by the CD39/apyrase family [38,43]. The results showed no significant differences between the GTPase activity

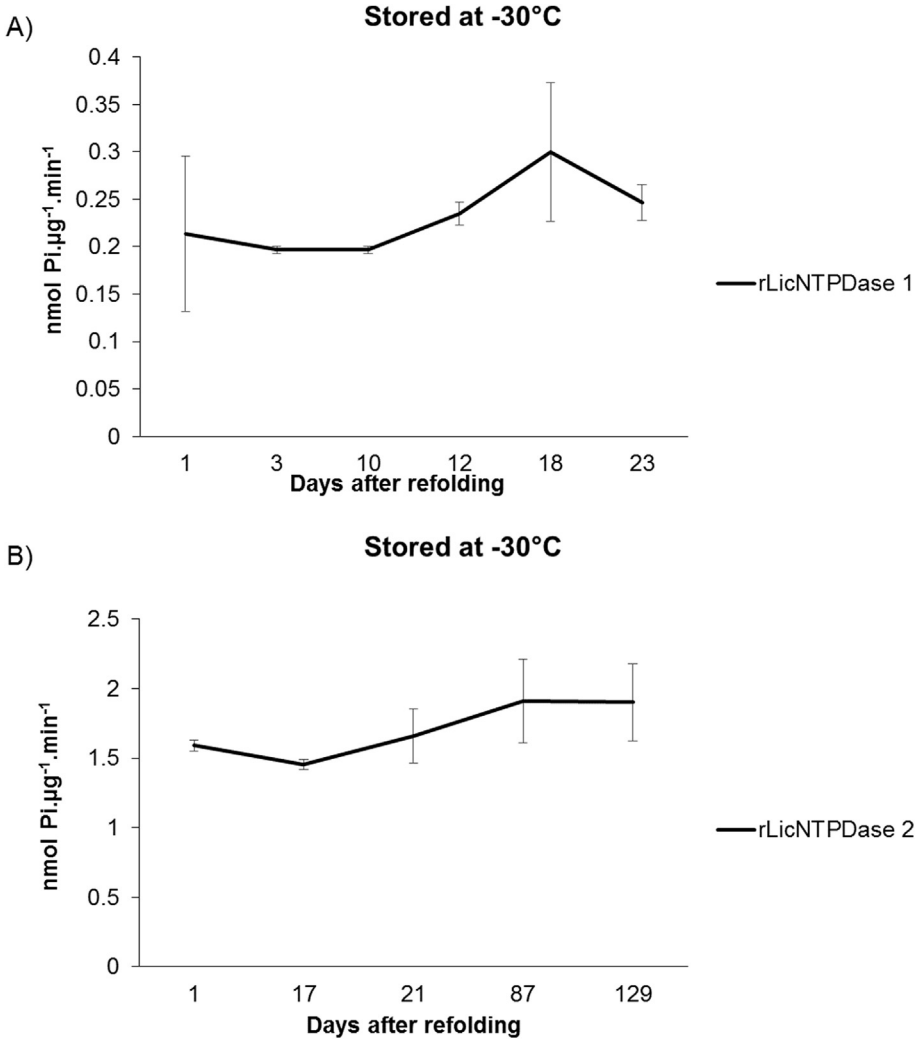
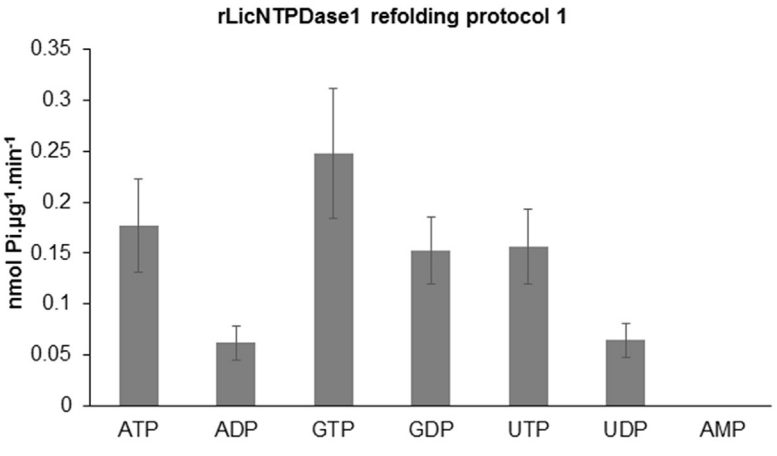


Fig. 3. Storage test at -30°C after expression in a bacterial system, purification and renaturation. The enzyme was purified, aliquoted and immediately frozen at −30 °C. The samples were thawed, refolded and the activities were recorded 24 h after refolding. The results represent the mean ± SD from one independent biological replicates with internal quadruplicates. A) GTPase activity of LicNTPDase1 and B) GTPase activity of LicNTPDase2. The refolding protocol used in this test was the protocol 1 previously described [39].



Substrate	nmol Pi^{*}-1.µg.mim-1
ATP	0.177± 0.046
ADP	0.062 ± 0.017
GTP	0.248 ± 0.064
GDP	0.152 ± 0.033
UTP	0.156 ± 0.0371
UDP	0.064 ± 0.017
AMP	0

Fig. 4. rLicNTPDase1 substrate specificity. The enzyme was purified, aliquoted and immediately frozen at −30 °C. The samples were thawed, refolded and the activities were measured 24 h after refolding. The results represent the mean ± SD from three independent replicates in quadruplicates, except for AMP which was tested once in quadruplicate (The refolding protocol used in this test was the protocol 1 previously described).

of rLicNTPDase1 in the presence of calcium or magnesium (Fig. 5). The addition of calcium and magnesium chelating agents EGTA and EDTA abolished GTPase activity, suggesting that the enzyme activity is dependent of the presence of a divalent cation (the refolding protocol used in this test was the protocol 1). In addition, the data from Fig. 5 shows that the presence of a divalent cation influenced the GTPase activity in a dose dependent manner, with a maximum at 2.5 mM. In fact, NTPDases use nucleotide-divalent cations (usually $-Mg^{2+}$ or $-Ca^{2+}$) as substrates and not only nucleotides. These results demonstrated that this enzyme is a canonical NTPDase from the CD39/apyrase family [15].

4.3. Refolding of rLicNTPDase2 expressed in a bacterial system

It is important to note that the activities of rLicNTPDase1 are lower and result in limitations in the characterization of this enzyme using a colorimetric assay. Due to these difficulties, we decided to test new refolding conditions to improve the activity of both enzymes. We tested a refolding condition used to study an apyrase from *C. parvum* [40] with small modifications. The purified proteins were renatured by dialysis at 16 °C in two steps: one using a buffer containing Tris, pH 8, L-arginine, EDTA, GSH, GSSG and 5% glycerol and the other against the activity buffer (HEPES pH 7.2, Tris pH 7.2, NaCl, KCl and $MgCl_2$).

Unfortunately, this approach was not successful, and the recombinant proteins precipitated in the dialysis step (Refolding protocol 2).

Next, we tested another refolding condition based on mixed conditions of NTPDase refolding [39–41] with modifications. The purified protein was diluted 10 times in activity buffer supplied with L-GSSG, L-GSH, calcium chloride and L-arginine and incubated for 48 h at 4 °C. Then, the samples were dialyzed in activity buffer for 24 h and used in nucleotidase assays. This new protocol (Refolding protocol 3) did not improve the activity of rLicNTPDase1 but improved the activity of rLicNTPDase2 more than 10-fold without significant changes in its substrate specificity (Fig. 6).

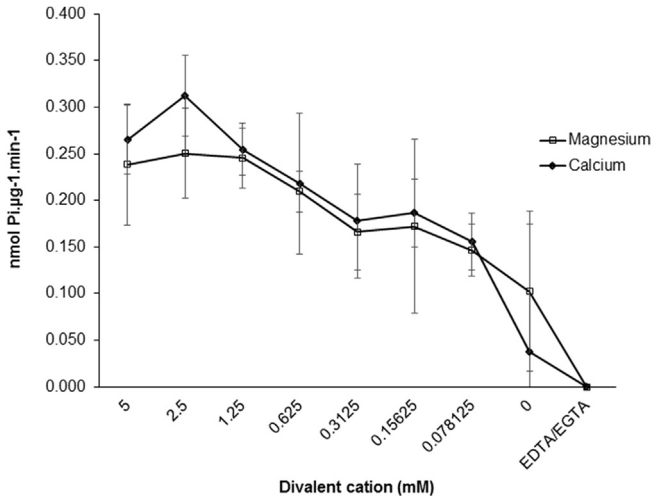


Fig. 5. Divalent cation dependence assay of rLicNTPDase1. The enzyme was purified, aliquoted and immediately frozen at −30 °C. The samples were thawed, refolded and the activities were measured 24 h after refolding in the presence (EDTA/EGTA) of different concentrations of Mg^{2+} or Ca^{2+} and in the absence of residual amounts of these cations (0 mM). The results represent the mean ± SD from two independent replicates in quadruplicate. The magnesium chloride was removed from the refolding buffer for this test. The refolding protocol used in this test was the protocol 1 previously described.

4.4. Expression and nucleotidase activity of rLicNTPDase1 expressed in COS-7 cells

As demonstrated in the above data, the activity of rLicNTPDaseI expressed by *E. coli* system was quite low. This lower level of activity was difficult to characterize for this enzyme, and none of the different refolding methods resulted in significant improvements in rLicNTPDase1 nucleotidase activity. Therefore, we decided to change the heterologous expression system. It is possible that this enzyme needs any co- or post-translational changes or support of the eukaryotic machinery to achieve higher levels of nucleotidase activity. To test this hypothesis, rLicNTPDases were expressed using the mammalian cell line COS-7. This cell has low ectonucleotidase activity at its membrane surface; therefore, COS-7 cells have often been used to study ecto-nucleotidases from the CD39/apyrase family [36]. To guarantee the targeting of rLicNTPDases to the plasma membrane of COS-7 cells, we constructed an expression cassette containing the coding region of the soluble domain of rLicNTPDase1 or 2 in front of the coding sequence of the transmembrane of NTPDase1 from mouse (Fig. 7A). Then, membrane extracts were used to evaluate the nucleotidase activity of rLicNTPDases-1 and -2 and all of the data were compared with the extracts of control COS-7 cells. As shown in

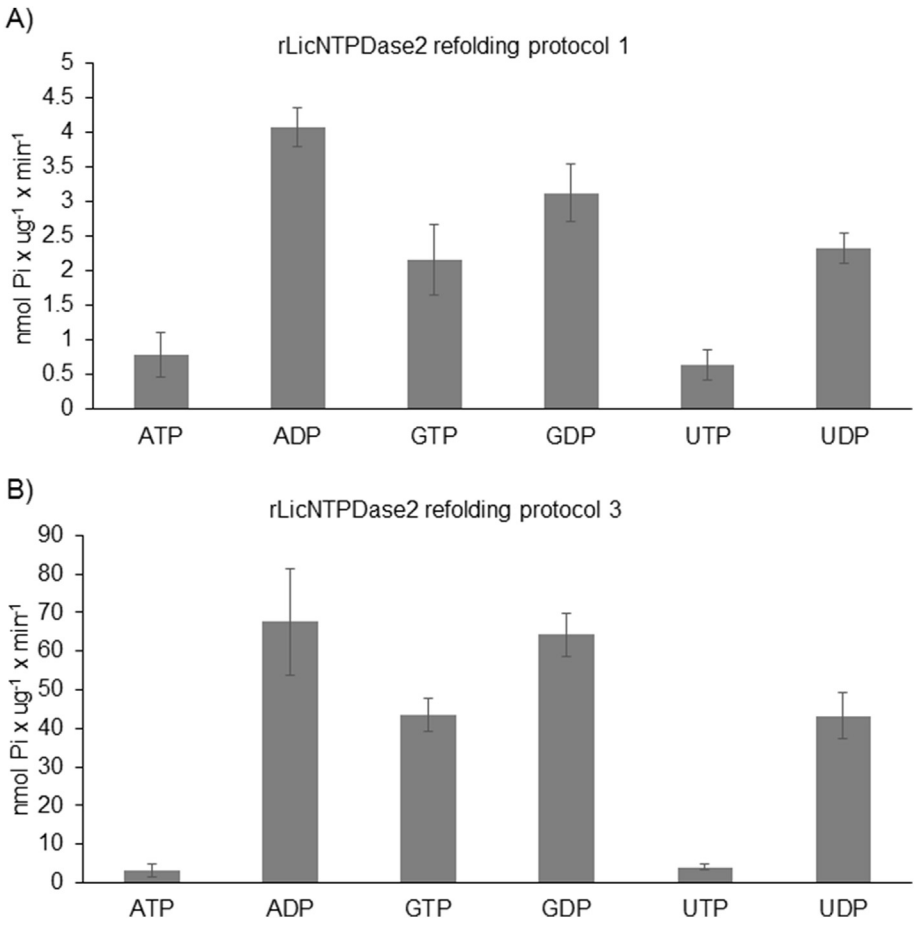


Fig. 6. Comparison of the activity of rLicNTPDase2 from different refolding protocol. The enzyme was purified, aliquoted and immediately frozen at −30 °C. The samples were thawed and refolded, and the activities were measured after refolding. A) The purified enzyme was diluted 10 times in activity buffer containing 1 mM L-glutathione oxidase and 2 mM L-glutathione reduced. The sample was incubated at 4 °C for 24 h. B) The purified enzyme was diluted 10 times in activity buffer containing 1 mM L-glutathione oxidase, 2 mM reduced L-glutathione, 2 mM $CaCl_2$ and 1 M L-arginine. The sample was incubated at 4 °C for 48 h and then immediately dialyzed for an additional 24 h using 200 vol of activity buffer without L-glutathione buffer and L-arginine.

Fig. 7, rLicNTPDase1 expressed in COS-7 hydrolyzed GDP > ATP > UDP > GTP = ADP = UTP and the activity with GDP is double of that found for ATP, which in turn is double the activity obtained for UDP, which is double the activity obtained for GTP. In addition, the activity for ADP and UTP were very low (Fig. 7A). These data are very different from that obtained from rLicNTPDase1 expressed in *E. coli* (GTP > ATP = GDP = UTP > ADP = UDP). Alternatively, LicNTPDase-2 showed a very similar pattern of activity between its expression in *E. coli* and in COS-7 (Fig. 7B). Note that as the proteins were not purified from the membrane fractions, the specific activity of rLicNTPDases 1 and 2 expressed in this system are therefore accordingly lower.

5. Discussion

The main known roles of NTPDases in pathogens/host relationships are their actions as virulence molecules by modulating the immune response-dependent purinergic signaling and the action as pro-adhesion molecules that can facilitate the infection [18,26,28,29,31,40,44–48]. Another potential role of these enzymes is the participation in the purine salvage pathway, mainly in cells such as trypanosomatids that do not have the *de novo* pathway [23,28]. These important functions identify these enzymes as potential targets for biotechnological applications, such as target drug design, vaccines and antigens for diagnosis. To learn more about these enzymes and their role in infections caused by *L. infantum chagasi*, our research group has been studying the two isoforms called LicNTPDase1 and -2.

In this work, we focused on the heterologous expression and refolding of these enzymes in a prokaryotic expression system (*E. coli* BL21/pET21b) and a eukaryotic mammalian cell expression system (pcDNA3). The expression in *E. coli* was previously described and rLicNTPDase2 was determined to be bioactive. Now, we evaluated the effect of storage conditions and refolding of both enzymes expressed in *E. coli*. We tested the use of different buffers and storage temperatures (4 °C or −30 °C) and our results demonstrated that the enzymes need to be stored at −30 °C to stabilize them (Fig. 3). We observed that if the enzymes are storage at −30 °C prior to the refolding they can be stored longer without losing any significant activity. Based on the fact that it was not yet possible to maintain the stability for many days if the proteins were refolded right after the purification step, the storage as purified an not refolded protein can be advantageous because allows researchers to stock many different samples at the same time ready to be refolded and then to be used in enzymatic assays.

We admit that the assays were less precise for rLicNTPDase1 as the activity was very low and close to the background level. Despite that, it is important to note that we are sure that rLicNTPDase1 produced in *E. coli* is bioactive: many replicates were performed, the enzyme was able to hydrolyze different tri- and diphosphate

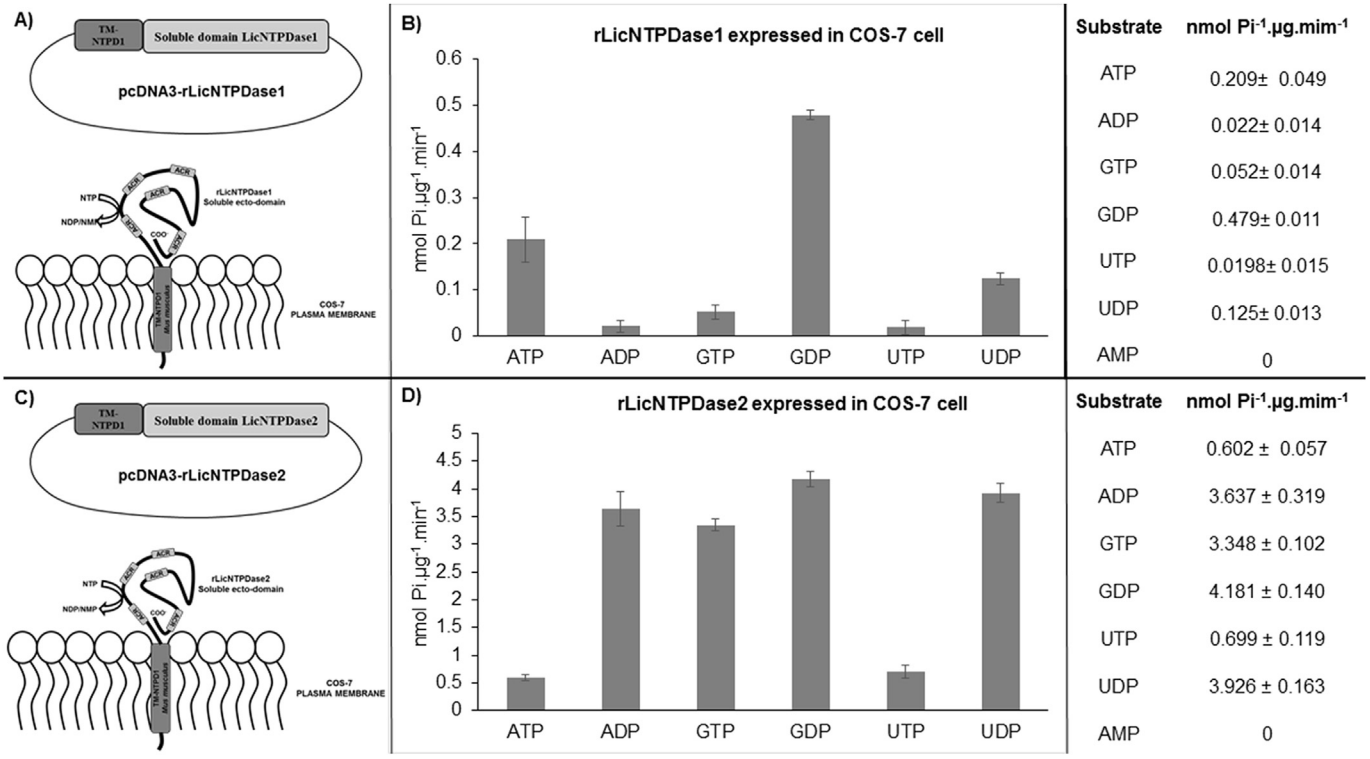


Fig. 7. Nucleotidase activity of rLicNTPDase1 and -2 expressed on the membrane of COS-7 cells. A) Design of constructions used to transform COS-7 cells to express rLicNTPDase1 (pcDNA-rLicNTPDase1) or rLicNTPDase2 (pcDNA3-rLicNTPDase2) at the plasma membrane. TM-NTPDase1 is the coding region of the amino terminal transmembrane domain of *Mus musculus* NTPDase1 [36]. B) rLicNTPDaseI nucleotidase activity. C) rLicNTPDase2 nucleotidase activity. The results represent data from two independent assays with internal quadruplicates. The specific activities represent the difference between the activity obtained from extract of COS-7 cells transfected with pcDNA3 plus rLicNTPDases minus the activity obtained from the control COS-7 cells extract. The activity of COS cell extracts is always below 5% of the activity from the transfected cells.

nucleosides and was not able to use AMP as a substrate, and it is dependent on divalent cations (Figs. 4 and 5). These results confirmed that the rLicNTPDase1 produced using *E. coli* system was bioactive but highlighted the need to improve the conditions to obtain higher enzyme activity. Alternatively, rLicNTPDase2 nucleotidase activity was consistent in both systems, suggesting that this enzyme could be produced by a bacterial system in a superior manner (Fig. 6).

By changing the refolding conditions, we obtained higher activity from rLicNTPDase2 (10–20 times more activity). However, rLic-NTPDase1 activity remained low. These results suggest that the new refolding could increased the folding efficiency of rLicNTP-Dase2. L-arginine used in the new protocol was probably crucial component to this improvement because the basic difference between the protocols was the addition of L-arginine. Although the exact mechanism of action of L-arginine is unknown, one hypothesis is that L-arginine interacts with denatured proteins, decreasing the interaction between proteins and preventing aggregation during refolding. This effect in turn favors could improve folding of the recombinant protein [49].

After several attempts with different renaturation protocols, we realized that it was not possible to improve the activity of rLicNTPDase1 expressed in the *E. coli* system used here. Thus, an alternative would be to produce rLicNTPDase1 in a eukaryotic system, such as in mammalian cells, including the COS-7 lineage. The use of a mammalian cell line that has eukaryotic machinery could lead to appropriate post-translational modifications in eukaryotic proteins. With this approach, it was possible to obtain more stable and consistent rLicNTPDase1 activity (Fig. 7). We also verified that the activity of the enzyme expressed in *E. coli* is quite different from the enzyme activity observed in the COS-7 cell membrane (Fig. 7). These results suggest that rLicNTPDase1 requires some post-translational modification present in eukaryotes to achieve better folding and activity and that this modification is most likely missing or is performed improperly in a bacterial system. This idea is supported by *in silico* analyses of glycosylation sites on both enzymes where we can observe that LicNTPDase1 has three putative N-glycosylation sites and LicNTPDase2 has only one putative site (Supplementary Fig. 1).

Alternatively, rLicNTPDase2 showed a very similar pattern of activity between the enzyme expressed in *E. coli* and the enzyme expressed in COS-7. These results indicate that it was possible to obtain a properly folded enzyme after expression in a bacterial system and most likely this enzyme does not require significant post-translational modifications for its nucleotidase activity. This result gives us confidence regarding the characterization of the enzyme expressed in the bacterial system.

6. Conclusions

In this paper, we performed a comparative analysis of recombinant rLicNTPDases expressed in bacteria and mammalian cells. The data revealed that rLicNTPDase2 is an enzyme more suitable and stable to work than rLicNTPDase1. Nevertheless, we could improve the conditions to evaluate the nucleotidase activity of both enzymes. Our results demonstrated that the expression of rLicNTPDase1 in *E. coli* may not be the best option because the enzyme activity is higher and more stable when it was expressed in COS-7 cells.

Our results offer new ways to work with NTPDases from Leishmania and may be applicable for other parasites because we demonstrated new ways to obtain active enzymes. These advances

in the production of bioactive parasitic NTPDases could help improve their biotechnological applications, such as the search for inhibitors to be used in chemotherapy.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pep.2016.11.004.

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