An Acidocalcisomal Exopolyphosphatase from *Leishmania major* with High Affinity for Short Chain Polyphosphate

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We report the cloning, overexpression, purification, and characterization of the *Leishmania major* exopolyphosphatase (*LmPPX*). The product of this gene (*LmPPX*), the first related to polyphosphate (polyP) metabolism isolated from an eukaryotic organism different from yeast, has 388 amino acids and a molecular mass of 48 kDa. *LmPPX* differs from other exopolyphosphatases previously investigated. Heterologous expression of *LmPPX* in *Escherichia coli* produced a functional enzyme that was similar to the yeast exopolyphosphatase with respect to its Mg$^{2+}$ requirement, optimum pH, and sensitivity to cations, amino acids, and heparin but that, in contrast to the yeast enzyme and other exopolyphosphatases investigated before, acts on polyP of short chain lengths with higher rates and affinity. *LmPPX* is a processive enzyme and it does not hydrolyze pyrophosphate, ATP, or $p$-nitrophenylphosphate. Confocal immunofluorescence microscopy using affinity-purified antibodies against the recombinant enzyme indicated an acidocalcisomal and cytosolic localization. High levels of short chain (21.4 ± 3.0 mM) and long chain polyP (55.9 ± 5.6 mM) were detected in *L. major* promastigotes. The unique characteristics of *LmPPX* and *L. major* polyP metabolism may facilitate the development of novel antileishmanial agents.

Polyphosphate (polyP)$^1$ is a linear polymer of a few to several hundred orthophosphate residues linked by high-energy phosphoanhydride bonds. This compound is widespread in living organisms and is found in the cells of microorganisms, animals, and plants (1, 2). It has been demonstrated that polyP can function as a phosphate reserve under conditions of phosphate starvation, as an energy source in place of ATP, in cation sequestration and storage, in cell membrane formation and function, in gene activity control, in regulation of enzyme activities, in the stress response and stationary-phase adaptation, and in the formation of channels and pumps (1, 2).

In many organisms the mobilization of polyP is performed primarily by the action of enzymes that catalyze the synthesis and degradation of this polymer, the polyphosphate kinase, and the endo and exopolyphosphatases, respectively (1, 2). These enzymes have been described in many prokaryotic and eukaryotic organisms. In eukaryotes these enzymes are localized in a variety of cellular compartments such as cytosol, mitochondria, vacuoles, and nucleus (1). Genes encoding for an exopolyphosphatase (3) and an endopolyphosphatase (4) from *Saccharomyces cerevisiae* are the only ones that have been cloned from an eukaryotic organism, whereas the identification of genes encoding for polyP kinases in eukaryotes has been elusive (1).

In a number of early branching eukaryotes, such as trypanosomatid and Apicomplexan parasites (5), in the green algae *Chlamydomonas reinhardtii* (6), and in the slime mold *Dictyostelium discoideum* (7), polyP is mainly localized in acidic organelles known as acidocalcisomes. These organelles are characterized by their acidic nature, their high electron density, and their high concentration of calcium, magnesium, and other elements in addition to pyrophosphate (PP$i_i$) and polyP (5). In *Trypanosoma cruzi* polyP is mobilized during cell growth and differentiation and when the parasite is submitted to environmental stresses (8). An exopolyphosphatase activity was detected in their acidocalcisomes (8).

A predicted open reading frame with sequence similarity to the yeast exopolyphosphatase was found to be present in chromosome 1 of *Leishmania major* (9), but the gene product has not been characterized. *Leishmania*, a member of the trypanosomatid protozoa, is responsible for the spectrum of diseases in humans collectively called leishmaniasis. Therapy against leishmaniasis is unsatisfactory, and the need to develop novel chemotherapeutic agents through the identification of biochemical pathways that allow survival of the parasite and are absent in the host is clear. Here we report the cloning, overexpression, purification and characterization of the *L. major* exopolyphosphatase (*LmPPX*). It is demonstrated that the product of this gene, the first related to polyP metabolism isolated from an eukaryotic organism different from yeast, differs from other exopolyphosphatases previously investigated. *LmPPX* has an acidocalcisomal and cytosolic localization and preferentially participates in the degradation of short chain polyPs, which are very abundant in these parasites.

**Experimental Procedures**

*Cultures—* *L. major* promastigotes (Friedlin strain) were grown at 28°C in medium SDM-79 supplemented with 10% heat-inactivated fetal bovine serum for 2–3 days before use.

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$^§$The abbreviations used are: polyP, polyphosphate; $P_i$, orthophosphate; polyP$_x$, pyrophosphate; *LmPPX*, *L. major* exopolyphosphatase; Pipes, piperazine-$N,N'$-bis-$2$-ethanesulfonic acid; MOPS, 3-$N$-morpholino)propanesulfonic acid; MES, 2-$N$-morpholino$ethanesulfonic$ acid; PBS, phosphate-buffered saline; ORF, open reading frame; BSA, bovine serum albumin; polyP$_a$, tetrapolyphosphate; $GP_a$, guanosine 5'-tetraphosphate; polyP$_b$, triplypolyphosphate.
Chemicals—SDM-79 medium was obtained from JRH Biosciences (Lenexa, KS). Fetal bovine serum, Dulbecco’s PBS, protease inhibitor mixture (P8849 and P8340), ampicillin, kanamycin, paraformaldehyde, glutaraldehyde, bovine serum albumin, ATP, PP, p-nitrophenylphosphate, polyphosphates, phosphate glass, protease K, lysozyme, primers, horseradish peroxidase-conjugated anti-rabbit, fluorescein-conjugated goat anti-rabbit, and rhodamine-conjugated goat anti-mouse IgGs were purchased from Sigma. Veratridine was from Vector Laboratories (Burlingame, CA). Restriction enzymes, T4 DNA ligase, Tq polymerase, DNA ladder, and goat serum were from Invitrogen. The pET-28a expression system, nickel nitriotriacetic acid HisBind resin, benzoamide, and the thrombin cleavage kit were from Novagen Inc. (Madison, WI). Penta-His antibody was from Qiagen Inc. (Valencia, CA). pcR2.1-TOPO was generated from Invitrogen’s pUC19 vector in which 8 uM of Nicotinamide N-riboside was included. Briefly, supernatants containing the native or denatured protein solubilized from the inclusion bodies were re-centrifuged under the same conditions and filtered using a 0.45-µm Milipore filter before being mixed with the nickel nitriotriacetic acid resin (4:1) under gentle rotation for 1 h at 4 °C. The mixture was loaded into an empty column, and the flow-through was collected. The column was subsequently washed twice with 5 ml of binding buffer and then once with 10 ml of the same buffer with the concentration of imidazole changed to 10 mM. Recombinant protein was eluted twice with 500 µl (1 ml total) of 500 mM imidazole. Immediately after elution, 2 ml dithiothreitol was added to the purified protein, which was kept frozen at −80 °C for further use. The thrombin cleavage was done as described by the kit manufacturer (Novagen).

Preparation of Antibodies—Purified exopolyphosphatase (100 µg) emulsified in Freund’s complete adjuvant, was injected subcutaneously in a rabbit followed by another injection 2 weeks later with the protein (100 µg) emulsified in Freund’s incomplete adjuvant. At 6 weeks after the initial injection, the rabbit was boosted with another 100 µg of the purified protein, this time in PBS containing a 10 mg/ml suspension of Al(OH)3. Serum was collected before the initial injection (pre-immune serum) and 10 days after each boost. The antiserum was separated into aliquots and stored at −80 °C. Affinity purification of the antibody using a HiTrap protein G Hp column was performed as described elsewhere (10), and according to the instructions of the manufacturer.

SDS-PAGE and Western Blotting—Electrophoresis was performed as described by Laemmli (11) under reducing conditions. Electrophoresed proteins were transferred to nitrocellulose membranes, which were first incubated with polyclonal antibody against recombinant exopolyphosphatase. anti-LmPPX (1:20,000), and then with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:3,000). Pre-immune serum was used as control at the same concentration. For detection of the histidine tag, the anti-His antibody and the horseradish peroxidase-conjugated anti-mouse IgG antibody used were diluted 1:1,000 and 1:10,000, respectively. Immunoblots were visualized on blue-sensitive x-ray film (Midwest Scientific, St. Louis, MO) using the TCL chemiluminescence detection kit according to the instructions of the manufacturer.

Immunofluorescence Microscopy—Parasites were washed with PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl2, pH 7.2) and fixed with 4% freshly prepared paraformaldehyde and 0.1% glutaraldehyde in PHEM buffer for 10 min at room temperature and 50 min at 4 °C. Cells were allowed to adhere to poly-L-lysine-coated coverslips, washed with PBS, and permeabilized with 0.3% Triton X-100 in PBS for 5 min. Coverslips were blocked with PBS containing 3% bovine serum albumin (BSA), 1% cold fish gelatin, 2% normal goat serum, and 0.1% Triton X-100 for 30 min at room temperature. Coverslips were washed with PBS, 3% BSA and incubated with a fluorescein-conjugated goat anti-rabbit antibody and a rhodamine-conjugated goat anti-mouse antibody, both diluted 1:100 in PBS, 3% BSA. Samples were washed with PBS, 3% BSA and incubated with a fluorescein-conjugated goat anti-rabbit antibody and a rhodamine-conjugated goat anti-mouse antibody, both diluted 1:100 in PBS, 3% BSA for 60 min at room temperature. Coverslips were washed with PBS, 5% BSA and then with 1:20 dilutions of antibodies stained being done using laser-scanning confocal microscope using optical sections 0.1 µm.

Synthesis of [32P]Polyphosphate—For extraction of polyP from E. coli, a strain that overexpresses the polyphosphate kinase (NR100 P9630 ppk) was used. Bacterial cells were grown overnight in Luria-Bertani medium containing 50 µg/ml ampicillin and then inoculated (5% inoculum) in fresh medium in the presence of antibiotics and grown for ~60 min to an A600 of 0.5 at 37 °C. This time, 50 µM isopropyl β-D-thiogalactoside was added to the culture to induce the overexpression of the polyphosphate kinase. After 60 min of
growth, the cells were washed $2 \times$ in MOPS minimum medium (13) containing 10 mM K$_2$HPO$_4$. The culture was incubated in the same medium in the presence of the antibiotics and $[^{32}P]$phosphate at a final concentration of 10 Ci/ml for 2 h at 25 °C. After labeling, the cells were washed $2 \times$ in the same medium without the labeled $P$, and the final pellet was resuspended in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, and 4 mM guanidine isothiocyanate. Long chain polyP was isolated according to Ault-Riche et al. (14).

**Exopolyphosphatase Activity and PolyP Analysis**—Exopolyphosphatase activity was assayed by measuring the rate of inorganic phosphate release using the EnzCheck phosphate assay kit as described before (15) with the microtitre plate modification (16) and different polyphosphates as substrates. The sensitivity of this method was calibrated for the different buffers used. In the cases where $\nu$-vanadate and ammonium molybdate were tested, phosphate release was detected by the method of Taussky and Shorr (17). For the assays with $[^{32}P]$polyP, 600 $\mu$M polyP$_{200}$ (8 $\mu$Ci/µmol) was added to a reaction mixture containing 250 mM sucrose, 50 mM Tris-HCl, pH 7.5, 2 mM MgCl$_2$, and 1 $\mu$g/ml purified LmPPX. Aliquots of 50 µl were taken at different intervals of time up to 120 min and added to a mixture of phenol:chloroform to stop
the reaction. PolyP was isolated from the aqueous phase and analyzed by electrophoresis in 20% polyacrylamide, 7% urea gel as described before (18).

**Cell Fractionation**—For analysis of polyP levels in different fractions of *L. major*, the cells were harvested and washed 2× in a buffer containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO_4_, 5.5 mM glucose, and 50 mM Hepes, pH 7.4, and resuspended in the same buffer. The cell suspension was lysed by sonication, and the lysate was centrifuged at 15,000 × g for 10 min at 4 °C. The pellet was separated, and the supernatant was centrifuged again at 100,000 × g to separate a cytosolic fraction. PolyP levels were determined from the amount of P_i released upon treatment with an excess of rPPX1 as previously described (8). The intracellular concentration of polyP in *E. coli* probe. All restriction enzymes used gave single, strong bands and 50 mM Hepes, pH 7.4, and resuspended in the same buffer. The cell described (8). The intracellular concentration of polyP in released upon treatment with an excess of rPPX1 as previously de-

**RESULTS**

**Isolation of the LmPPX Gene**—A gene encoding a protein (accession number AAC2464) with sequence similarity to the yeast exopolyphosphatase was found to be present in chromosome 1 of *L. major* (9). The deduced amino acid sequence of this gene is ~40% identical to the sequences of the exopolyphosphatase of *S. cerevisiae* (*S. cerevisiae* exopolyphosphatase 1, Ref. 3) and the putative exopolyphosphatases of *Schizosaccharomyces pombe* (CAB16287) and *Methanococcus jannaschii* (U67509). Greater sequence identity (~60%) was found with a putative exopolyphosphatase from *Trypanosoma brucei* (AC013485). Translation of the ORF of LmPPX yielded a polypeptide of 388 amino acids with a predicted mass of 48 kDa. No apparent signal sequence was detected in the LmPPX polypeptide using PSORT. Comparison of the amino acid sequence of LmPPX to the NCBI Conserved Domain Data base indicated that the polypeptide consisted of an N-terminal DHH domain followed by a C-terminal DHHA2 (DHH-associated domain type 2) domain. This domain structure is shared by a number of other eukaryotic, archaeal, and eubacterial proteins, several of which have been identified as exopolyphosphatase enzymes by sequence similarity or by experimental evidence. The domain structure of LmPPX and the inferred phylogenetic relationships of other apparently homologous proteins with similar domain structure are illustrated in Figs. 1, A and B, respectively.

Southern blotting was performed with the LmPPX gene as a probe. All restriction enzymes used gave single, strong bands that were distinct from one another, suggesting the presence of a single LmPPX gene in the *L. major* genome (data not shown).

**Purification of Recombinant LmPPX**—LmPPX was expressed in *E. coli* DH5α as a fusion protein with an N-terminal polyhistidine tail. Affinity chromatography on a nickel-chelated agarose column allowed protein purification. After 1–3 h of induction with 1 mM isopropyl-1-β-d-galactopyranoside, the cells were disrupted, and the proteins were separated using SDS-polyacrylamide gels. The protein pattern of the crude extracts (*lanes 1 and 2*) and the eluted fractions from the successive purification steps (*lanes 3–6*) are shown in Fig. 2. LmPPX was purified under denaturing conditions from inclusion bodies (*Fig. 2, lane 5, arrow*) for antibody generation and from the soluble fraction of the cell lysate in its native form (*Fig. 2, lane 6, arrow*), for enzymatic characterization. *Fig. 2, lane 1* shows that the expressed protein appears as a strong band with an approximate molecular mass of 48 kDa, which is similar to the predicted molecular mass (48 kDa). Other bands in *lane 2* also seem to be overexpressed when compared with those in the sample taken before induction (*lane 1*). These bands copurified with LmPPX even in the presence of protease inhibitors and seem to be either fragments originated from proteolysis of the recombinant protein (see *lane 5*) or aggregates that did not migrate properly in the gel due to their very high concentration in the sample in the case of the soluble protein (see *lane 6*). In fact, if this sample was concentrated, the only bands that could be seen were those of high molecular mass (data not shown), suggesting that this is probably an effect caused by aggregation.

**Western Blot Analysis and Localization of LmPPX**—Total homogenates of *L. major* and the recombinant protein were subjected to Western blot analysis with antibodies produced against the protein isolated from inclusion bodies and against the histidine tag (*Fig. 3*). Anti-LmPPX antibodies recognized a band of the expected size (48 kDa) as well as other low molecular weight bands (*Fig. 3A, IS*). No detectable bands were observed when the preimmune serum (*Fig. 3A, PBS*) was used at the same dilution. The protein bands were also recognized by antibodies against the polyhistidine tag (*Fig. 3A, α-HIS*), suggesting that the low molecular weight bands probably originated by proteolysis. Similar results were obtained when total homogenates of *L. major* were used (*Fig. 3B*).

The localization of LmPPX in promastigotes of *L. major* was determined by indirect immunofluorescence assays using affinity-purified anti-LmPPX antibodies (*Fig. 4*). The protein was localized in vacuoles and in the cell cytoplasm (*Fig. 4, left*). Co-localization studies were also done using antibodies against the vacuolar proton-translocating pyrophosphatase, an acido-

calciosomal marker (*Fig. 4, center*). Using confocal microscopy we observed co-localization of the LmPPX and the vacuolar proton-translocating pyrophosphatase in acidocalcisomes (*Fig. 4, right*). No fluorescence was observed in control parasites incubated only in the presence of the secondary rhodamine-labeled goat anti-mouse or fluorescein-labeled goat anti-rabbit IgG (data not shown).

**Reaction Requirements of the Recombinant Protein**—The LmPPX was very unstable in solution after purification, loosing its activity after only a few hours of elution. Instability was also a problem found with other exopolyphosphatases (20, 21). Stability increased when 300 mM NaCl and 250 mM sucrose were included in all steps of the purification procedure and 2 mM dithiothreitol was added after elution of the protein. Under these conditions the purified protein could be separated into aliquots and maintained at −80 °C for several months without significant loss of its activity.
As shown in Table I, the recombinant LmPPX preferentially hydrolyzed short chain polyP. The highest specific activity was found for tetrapolyphosphate (polyP₄), guanosine 5'-tetraphosphate (GP₄), and tripolyphosphate (polyP₃), which were shown to be the best substrates for the enzyme. No evidence of an increase in the affinity for the substrate with the increase of the phosphate chain was found. In fact, we were not able to detect any activity with long chain polyP (up to 200 residues) by measuring phosphate release. We therefore investigated the reaction products formed by LmPPX acting on long chain [³²P]polyP by 20% polyacrylamide, 7% urea gel electrophoresis, which is a more sensitive assay. Fig. 5 shows a time course of hydrolysis of [³²P]polyP₃₀₀. Only after 90 min was it possible to detect some Pi accumulation as a result of hydrolysis of the labeled substrate. Intermediary products could not be detected.

Similar results were found when [³²P]polyP₇₀₅ or [³²P]polyP₅₀₀₀ were used (data not shown). These experiments suggest that the enzyme acts as an exoenzyme in a processive mode, releasing Pi residues from the ends of the chain. The enzyme is not a general phosphatase. ATP, PPᵢ, and P-nitro-
phenolphosphate were not significantly hydrolyzed (data not shown). Because PPi does not act as a substrate for LmPPX, it qualifies as an end product in addition to Pi. Despite the presence of a cleavage site for thrombin, the N-terminal fusion peptide was not removed by the enzyme (data not shown). Presumably, as occurs with the S. cerevisiae recombinant exopolyphosphatase (3), the cleavage site is inaccessible in this protein.

The pH optimum of the recombinant LmPPX was determined for polyP3, polyP4, GP4, and polyP15 using imidazole buffer (for pH 6.0–7.0) and Tris-HCl buffer (for pH 7.5–8.0). The use of MES buffer for the low pH values caused total inhibition of enzyme activity (data not shown). Figure 6 shows the pH curve for the four substrates. For each substrate except polyP3 the highest activity was found in the neutral range between pH 7.0 and 8.0. Hydrolysis of polyP2 was more efficient at pH 6.5. At pH 6.0 the enzyme showed a significant reduction of its activity with all the substrates tested, ranging from 40 to 80% reduction as compared with its optimal activity.

The effect of divalent cations on the exopolyphosphatase activity is depicted in Table II. The recombinant LmPPX, like other exopolyphosphatases (18, 20–25), required the presence of a divalent cation for activity. In the absence of divalent cations the activity was almost negligible. MgCl2 at a concentration of 1 mM or above was the best activator using polyP3, polyP4, or polyP15 as substrate. CoCl2 enhanced the activity at various concentrations up to 1 mM. Heparin, a good inhibitor for other well characterized exopolyphosphatases (23, 25, 26), was also effective against LmPPX (Fig. 8B).

Distribution of PolyP in L. major—LmPPX is mainly located in acidocalcisomes (Fig. 4), where the vast accumulation of acidocalcisomes (Fig. 4), in chains of about 700–800-residues-long and in chains of less than 50-residues-long, respectively, were found in L. major promastigotes. Cell fractionation studies showed that most of the cellular polyP (96–98%) was located in the pellet fractions, whereas only 2.05 and 3.76% of the total amount of long chain and short chain polyP, respectively, was in the cytosolic fraction (data not shown).

**DISCUSSION**

We report here that a gene, LmPPX, present in the L. major genome, encodes a functional exopolyphosphatase. The open reading frame corresponding to LmPPX encodes a protein of 388 amino acids and a molecular mass of 48 kDa (Fig. 1). The LmPPX protein is a member of a family of proteins characterized by the presence of a DHH domain in the N terminus of the sequence. The DHH domain was originally identified as common to a novel family of predicted phosphoesterases including the Drosophila prune protein and bacterial RecJ exonuclease (28). The additional presence of the DHH domain in LmPPX to "subfamily 2" of the DHH family, which includes sequences from eubacteria, Archaea, and eukaryotes (Fig. 1). The consensus sequence for the DHHA2 domain is often found in tandem with the DHH domain, but its function is not known. With the exception of the well characterized exopolyphosphatase from S. cerevisiae, there has been no biochemical examination of the members of the subfamily 2 of the DHH domain family.

LmPPX was overexpressed in a bacterial host, and the addition of an N-terminal histidine tag to the recombinant protein allowed efficient purification by affinity chromatography (Fig. 2). As occurred with the S. cerevisiae recombinant exopolyphosphatase (3), the N-terminal appendix probably interacts with the protein since it could not be removed by thrombin, despite possessing the cleavage site for this protease. The recombinant enzyme was similar to S. cerevisiae exopolyphosphatase 1 with respect to its divalent cation requirement, optimum pH, and sensitivity to salts, amino acids, and heparin (Figs. 6–8), but in contrast to that enzyme (3), LmPPX acts on polyPs of short chain lengths with higher rates and affinity (Table I). Hydrolysis of long chain polyP was negligible. As with most known exopolyphosphatases, LmPPX is a processive enzyme releasing Pi residues from the ends of the chain, with no intermediate products detected upon polyP hydrolysis (Fig. 5). The lack of a signal sequence suggests that it does not enter the secretory pathway. Its cytosolic localization suggests that it is synthesized in free polysomes and then transported into the acidocalcisomes. Confocal immunofluorescence microscopy us-
Influence of divalent cations on the purified recombinant exopolyphosphatase from Leishmania major

Activity in the absence of divalent metals was taken as 100%. Results represent the average of at least four replicates ± S.E.

| Substrate | Cation | Concentration (mM) |
|-----------|--------|-------------------|
|           | 0.05   | 0.1               | 0.5 | 1     | 2     | 3     |
| PolyP₃    | Mg²⁺   | 551 ± 266         | 502 ± 87 | 886 ± 203 | 1180 ± 220 | 828 ± 198 | 901 ± 310 |
|           | Co²⁺   | 780 ± 245         | 411 ± 118 | 687 ± 268 | 1030 ± 298 | 1217 ± 351 | 936 ± 223 |
|           | Mn²⁺   | 231 ± 50          | 372 ± 78 | 415 ± 92  | 376 ± 81  | 432 ± 126 | 400 ± 77  |
|           | Mg²⁺   | 179 ± 40          | 171 ± 25 | 552 ± 93  | 235 ± 377 | 369 ± 403 | 309 ± 380 |
| PolyP₄    | Co²⁺   | 759 ± 179         | 539 ± 125 | 525 ± 25  | 434 ± 54  | 467 ± 50  | 411 ± 78  |
|           | Mn²⁺   | 224 ± 26          | 224 ± 17 | 424 ± 104 | 301 ± 60  | 311 ± 71  | 283 ± 71  |
|           | Mg²⁺   | 307 ± 54          | 581 ± 95 | 942 ± 146 | 1092 ± 168 | 1157 ± 185 | 1179 ± 203 |
| PolyP₁₅   | Co²⁺   | 67 ± 9            | 97 ± 18 | 179 ± 21 | 77 ± 12  | 56 ± 6.8 | 80 ± 15 |
|           | Mn²⁺   | 85 ± 4            | 96 ± 4  | 115 ± 4  | 77 ± 2   | 87 ± 4  | 103 ± 9 |
|           | Mg²⁺   | 377 ± 64          | 626 ± 153 | 820 ± 136 | 747 ± 164 | 665 ± 132 | 624 ± 117 |
| GP₄       | Co²⁺   | 247 ± 22          | 295 ± 13 | 356 ± 46 | 310 ± 64 | 263 ± 21 | 215 ± 33 |
|           | Mn²⁺   | 1125 ± 110        | 1208 ± 117 | 1283 ± 197 | 1021 ± 87 | 793 ± 115 | 714 ± 164 |

Fig. 7. Effect of salts (A) and amino acids (B) on the LmPPX activity. Polyphosphatase activity (with 100 μM polyP₄) was determined as described under “Experimental Procedures” except that increasing concentrations of KCl (closed circles) or NaCl (open circles) were added to the reaction mixture in A and of lysine (open circles) and arginine (closed circles) in B. Activity in the absence of any salt or amino acid was taken as 100%.

Fig. 8. Effect of divalent cations (A) and heparin (B) on LmPPX activity in the presence of MgCl₂. A, polyphosphatase activity (with 100 μM polyP₄) was determined as described under “Experimental Procedures” in the presence of 2 mM MgCl₂ and increasing concentrations of CaCl₂ (closed circles), ZnSO₄ (open circles), and CuSO₄ (closed triangles). Activities in the presence of MgCl₂ but without other cations were taken as control. B, polyphosphatase activity (with 100 μM polyP₄) was determined as described under “Experimental Procedures” in the presence of increasing concentrations of heparin. Activity in the absence of inhibitor was taken as control.
ized. Because the enzyme has an extremely low activity in the hydrolysis of long chain polyP, it is possible that either an endopolyphosphatase or a different exopolyphosphatase is also present in acidicalcosomes. In this regard, endopolyphosphatasas that act on long chain polyP, generating tripolyphosphate, have been detected in several eukaryotes including the protist Giardia lamblia (30). Interestingly, the yeast endopolyphosphatase is localized in vacuoles (31). We cannot rule out the possibility that this low activity is due to an impurity in the preparation because the purification gel shows that other bands co-purify with the recombinant protein. However, the low molecular weight bands are also recognized by the antibody against the histidine tag (Fig. 3A), suggesting that they belong to the same protein that could have been degraded by proteolysis.

The cytosolic localization of the exopolyphosphatase suggests that if short chain polyP is located, at least transiently, in the promastigote cytosol, the enzyme would be able to hydrolyze it. As a polyanion, polyP is able to inhibit a number of enzymes (2), and a low cytosolic level should be required to prevent these toxic effects.

Crucial for the accumulation of polyP in trypanosomatid acidicalcosomes is the presence of an active vacuolar H+ -pyrophosphatase, which acidifies the acidocalcisomal lumen and acidocalcisomes is the presence of an active vacuolar H+ /H1001+-pyrophosphatase (20) and the soluble mitochondrial exopolyphosphatase to distinguish the ends of a long chain from those of a short one is intriguing (21), although multiple binding sites on distant portions of E. coli exopolyphosphatase were determined to be responsible for the polymer length recognition of the enzyme (33).

In conclusion, our results indicate that LmPPX is unique in that it preferably hydrolyzes short chain polyPs, which are abundantly present in the parasite. The lack of a similar enzyme in other eukaryotic cells suggests that it could constitute an attractive target for chemotherapy.

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