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Identification, biochemical characterization, and *in-vivo* expression of the intracellular invertase BfrA from the pathogenic parasite *Leishmania major*

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ABSTRACT

The parasitic life cycle of *Leishmania* includes an extracellular promastigote stage that occurs in the gut of the insect vector. During that period, the sucrose metabolism and more specifically the first glycosidase of this pathway are essential for growth and survival of the parasite. We investigated the expression of the invertase BfrA in the promastigote and amastigote stages of three parasite species representative of the three various clinical forms and of various geographical areas, namely *Leishmania major*, *L. donovani* and *L. braziliensis*. Thereafter, we cloned, overexpressed and biochemically characterized this invertase BfrA from *L. major*, heterologously expressed in both *Escherichia coli* and *L. tarentolae*. For all species, expression levels of BfrA mRNA were correlated to the time of the culture and the parasitic stage (promastigotes > amastigotes). BfrA exhibited no activity when expressed as a glycoprotein in *L. tarentolae* but proved to be an invertase when not glycosylated, yet owing low sequence homology with other invertases from the same family. Our data suggest that BfrA is an original invertase that is located inside the parasite. It is expressed in both parasitic stages, though to a higher extent in promastigotes. This work provides new insight into the parasite sucrose metabolism.

1. Introduction

Leishmaniasis belongs to the group of neglected tropical diseases, as defined by the World Health Organization (WHO), which includes diseases that are endemic in developing countries.[1] The main foci of leishmaniasis are India, Africa, South America, Mediterranean basin and Middle East.[2,3] The microorganism responsible for this widespread zoonosis is a kinetoplastid protozoan parasite of the *Leishmania* genus, transmitted through inoculation by female sandflies. About twenty *Leishmania* species can infect humans, and are not only responsible for various clinical forms (cutaneous leishmaniasis, mucocutaneous leishmaniasis or visceral leishmaniasis) and outcomes, depending mainly on the *Leishmania* species, but also on the immune background of the patient. Whereas cutaneous leishmaniasis is usually a self-healing disease, mucocutaneous leishmaniasis is a more severe non-healing disease, and visceral leishmaniasis is usually fatal in the absence of treatment.[4]

The stages of the parasite life cycle are correlated to the infection stages in the hosts.[5–7] In the vector salivary glands, the parasites are found as extracellular promastigotes. After inoculation by the insect bite, parasites are phagocytosed by mammalian macrophages and differentiate to their amastigote intracellular stage. The adaptation of *Leishmania* to two different environments is thus highly critical for its survival and growth. In sandflies, the parasite load was shown to be correlated with the feeding with plants containing high levels of sucrose,[8–11] which indicates a critical role for the enzymes involved in sucrose transport and metabolism for *Leishmania* survival and growth in insect gut. Several pathways have been described for sucrose metabolism,[12] involving either an extracellular[13,14] or an intracellular[15] invertase. Recently, a report from Lyda et al.[16] identified and characterized a secreted invertase from the leishmania species *donovani* and *mexicana*, highlighting the potentiality of these enzymes as a therapeutic target to disrupt the parasite metabolism.

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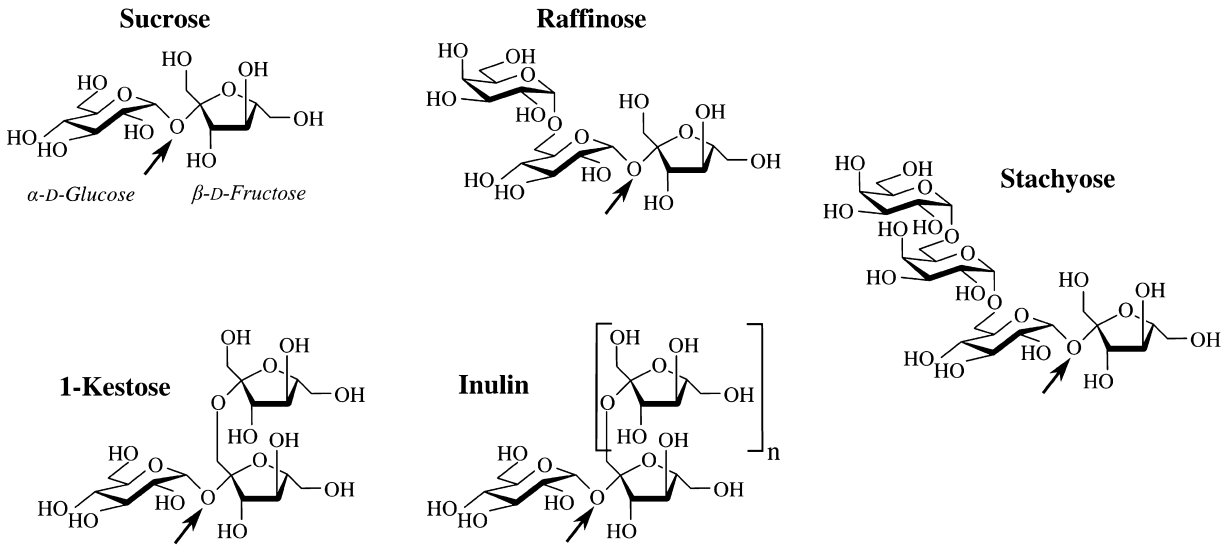

Fig. 1. Sucrose and sucrose-containing substrates tested in this study. The glucoside and fructoside moieties of sucrose are indicated, and the hydrolysis site is shown by an arrow.

Invertases (EC 3.2.1.26), or β-fructofuranosidases, are glycosides hydrolases (GHs) that catalyze the hydrolysis of sucrose into fructose and glucose (Fig. 1) by the recognition of β-D-fructose in the enzyme active site. Invertases belong to the CAZy family GH32 that contains invertases, fructan hydrolases, fructosyltransferases and sucrose-6-phosphate hydrolases.[17] Several X-ray structures of GH32 family from different organisms have been reported in the literature, including bacteria,[18–21] fungi,[22,23] yeast[24–26] and plants.[27–34] Five putative genes encoding invertases have been identified in *Leishmania* genomes,[35–38] but only one protein was cloned and enzymatically characterized as an extracellular invertase.[16] Unlike *Leishmania*, no invertase is present in human genome, but another GH is responsible for sucrose hydrolysis, i.e. sucrase or α-D-glucosidase, which interacts with the α-D-glucosyl moiety of sucrose (Fig. 1).

The importance of *Leishmania* invertases in the survival and growth of the parasite in the insect vector, as well as their absence in humans, make them potential target for the design of anti-*Leishmania* active compounds.[16] Analyzing their enzymatic behavior, as well as their expression level during the parasite life cycle, is thus necessary to better understand their biological role. Herein, we describe the cloning, purification and biochemical characterization of the first intracellular *Leishmania* invertase, named BfrA. Noteworthy, this particular enzyme is only highly expressed in the promastigote stage of the parasite, thus is probably essential for the parasite sucrose metabolism in the insect vector.

2. Materials and methods

2.1. Sequence analysis and primers design

L. major, *L. infantum*, *L. donovani*, *L. mexicana*, and *L. braziliensis* genomes were retrieved from GeneBank database. BlastP[39] was used to identify invertases in these genomes, using CAZy database (www.cazy.org).[40] GH32 protein sequences were retrieved from UniProt database. Clustal Omega EMBL server was used for multiple alignments,[41,42] and Phylogeny server was used for phylogenetic tree generation.[43] Specific primers were designed for RT-qPCR using CLC Workbench® software (Qiagen).

2.2. Promastigote parasite cultures

Leishmania strains were isolated from human patients presenting with visceral leishmaniasis (*L. donovani*) or cutaneous leishmaniasis (*L. major* and *L. braziliensis*). Parasites were grown on NNN medium, then identified and typed by the Centre National de Référence des Leishmanioses (Montpellier, France). *Leishmania* promastigotes were amplified and maintained by serial passages in Schneider's drosophila medium (Sigma®) supplemented with 10% decomplemented fetal calf serum (InVitrogen®) at 27 °C. To study the kinetics of *bfrA* expression in promastigotes, samples of cultures were taken at days 1, 3, 5 and 7.

2.3. Human blood monocytes derived macrophages

Human blood monocytes-derived macrophages were obtained by purifying monocytes from peripheral blood mononuclear cells obtained from buffy coats (supplied by Etablissement Français du Sang, Rennes, France). M-CSF-mediated differentiation of monocytes was conducted for 6 days in 6-well culture plates to obtain 10^6 primary human macrophages per well. Differentiation and culture was performed in RPMI-1640 medium (Gibco®) supplemented with 10% decomplemented fetal calf serum and antibiotics (100 UI/mL penicillin and 100 µg/mL streptomycin), as previously described.[44]

2.4. Leishmania *amastigotes*

Leishmania promastigotes grown at stationary phase (>5 days of culture) were used to infect human macrophages overnight (doi 10:1). After 3 washes, infected macrophages were cultured for another 2, 5 or 7 days, then supernatant was discarded and cells were lyzed for mRNA extraction. All time points were performed in quadruplicates for each parasite species and the whole experiment was repeated at least twice.

2.5. *Quantification of* bfrA *mRNA induction*

Promastigotes were counted and 10^7 promastigotes of each strain was lyzed for RNA extraction at each time point. All time points were

performed in quadruplicates for each parasite species. Kinetics was repeated at least twice for each species. Total RNA were extracted with Qiagen RNeasy mini kit® and eluted in 30 µL. Then, 2 µg of each RNA sample was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit® (Applied Biosystems™). Primers were designed to amplify *bfrA* and actin in 3 species of *Leishmania*. For *bfrA* amplification from *L. major* and *L. donovani*, forward and reverse primers were 5′-GCTATAATGAGGCGACAGGG-3′ and 5′-AAGGATCTGAGCATCGCTGT-3′, respectively. Forward and reverse primers used for *L. braziliensis bfrA* were 5′-TTCTCTTCACCACGCAGGAC-3′ and 5′-CCTGGTGATTTCCGATCGTA-3′, respectively. Actin expression was quantified using the following primers: forward 5′-CGATAAAGCCGAAGGTGGTT-3′ and reverse 5′-CCAGACTCGTCGTACTCGCT-3′ for *L. donovani* and *L. major,* and forward 5′-ATCAAACCAAAGGTGGTTGC-3′, reverse 5′-CCAGACTCGTCGTACTCGCT-3′ for *L. braziliensis*. Amplification was performed in a final volume of 10 µL containing 2 µL cDNA, 8 µL SYBR® green PCR master mix (Applied Biosystems™) and 3 µM of each primer. PCR reaction was performed on a 7900 HT fast real time PCR system® (Applied Biosystems™) and consisted of 2 min at 50 °C, then 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curves were added at the end of each run to check amplification specificity. Each sample was amplified in duplicate. *bfrA* induction was calculated by comparison to actin expression, as previously described.[45] Statistical analysis was performed using Mann–Withney test and GraphPad Prism 5® software.

2.6. Cloning, expression and purification of BfrA protein in E. coli

bfrA gene (locus LmjF.23.0880 in chromosome 23) was amplified by PCR from *L. major* genomic DNA using the following primers: 5′-TTGGATCCAGCTCTCAAGCGCAGCA-3′ (forward) and 5′-TTAAGCTTTCAGTGCCTCACCGTCGC-3′ (reverse). BamHI and HindIII restriction sites (underlined) were respectively added up- and downstream of the gene for subsequent cloning in pET-28a(+) expression vector (Novagen). Rosetta (DE3) *E. coli* cells were used for *N*-terminal His-tagged protein expression. Cells were grown in LB medium at 37 °C until OD600 reached 0.5, and then were induced by 0.5 mM IPTG at 25 °C overnight. Cells were harvested and resuspended in 50 mM Tris buffer (pH 8.5) containing 100 mM of NaCl, lyzed by five freeze–thaw cycles. Lysate was clarified by centrifugation (20,000 *g*, 30 min). Supernatant was filtered (0.45 µm), loaded on a HisPur Ni–NTA column (Thermo), then bound protein was eluted by an imidazole gradient (10–500 mM).

2.7. Cloning, expression and purification of BfrA in Leishmania tarentolae

bfrA gene was initially amplified from genomic DNA by PCR using the following primers: 5′-TTCTAGACTCTTCTCAAGCGCAGCAGCGCG-3′ (forward) and 5′-GTGCCTCACCGTCGCCTTCAGCTTCCACATT-3′ (reverse). This initial PCR enabled the upstream addition of a XbaI restriction site (underlined), and the silent removal of a KpnI restriction site located near the 5′ end of the gene. Then a second PCR was run using the same forward primer and the following reverse primer that added a KpnI restriction site (underlined) downstream of the gene: TGGTACCGTGCCTCACCGTCGCCTT. Amplicon was then inserted in pLEXSY-hyg2 plasmid (Jena Bioscience) using added restriction sites and the insert sequence was checked by sequencing. This construct was designed so that the corresponding recombinant protein was *N*-terminally fused with a secretion signal peptide, and *C*-terminally fused with a polyhistidine tag. The resulting pLEXSY-hyg2-*bfrA* plasmid was then used to transfect *L. tarentolae* P10 cells (Jena Bioscience),[46] following the manufacturer protocol. Briefly, cells were grown in BHI medium and concentrated to 2×10^8 cells/mL. Then, electroporation was run by two pulses at 25 mF, 3.75 V/cm, with a resting time of 10 s between each pulse. Then, after an overnight culture in non-selective BHI medium, selective antibiotic (hygromycin-100 µg/mL) was added and transfected P10 were selected in liquid culture. After 2 transfers in selective medium, genomic integration of the expression cassette into the *ssu* locus was confirmed by diagnostic PCR following the manufacturer protocol. The corresponding $BfrA_{leish}$ recombinant protein was then produced by cultivating transfected P10 in selective medium. After 24 h culture, cells were harvested, and the medium was filtrated and loaded on a HisPur Ni–NTA column. Elution and purification of $BfrA_{leish}$ protein was carried out as described for *E. coli* recombinant BfrA protein.

2.8. Enzymatic assays

Initial screening of hydrolytic activity of BfrA was assayed using sugars linked to paranitrophenol (pNP) as substrates.[47] Enzymatic reaction was carried out at 37 °C for 2 hours in a mixture containing 100 nM BfrA and 1 mM substrate, buffered with phosphate (10 mM, pH 7.5). Reaction was stopped using Na_2CO_3 (0.5 M), and free pNP was detected at 405 nm. For enzymatic reactions using oligosaccharides as substrates, the amount of reduced sugars formed was detected using 3,5-dinitrosalicylic acid assay.[48] Briefly, after reaction of the enzyme (10–100 nM) with the substrate (20 min), DNS reagent was added (DNS 0.5% m/v, NaOH 0.8% m/v, tartrate 15% m/v final concentrations) and the solution was boiled for 10 min. After cooling, reduced DNS was quantified at 540 nm. Calibration curves were obtained using standard solutions of glucose:fructose 1:1 (0.1–1 mM). pH and temperature dependence of BfrA activity were assayed with sucrose as substrate and using respectively citrate–phosphate buffer ranging from pH 4 to 10 and temperature from 20 to 80 °C as experimental conditions. As incubation time for temperature studies is critical, it was set to 20 minutes. For all other enzymatic assays, temperature was set to 37 °C and pH to 6. Kinetic data from three independent experiments were analyzed and plotted using Prism 5 software® (GraphPad).

2.9. Molecular modeling

The amino acid sequence of BfrA was submitted to the ModWed server (https://modbase.compbio.ucsf.edu/scgi/modweb.cgi) using MODELLER® software.[49] Template used for homology modeling was the X-ray structure reported for *Bifidobacterium longum* β-fructofuranosidase[20] (PDB code 3PIJ). NAMD software[50] was used to perform all molecular dynamics (MD) simulations. Oligosaccharide ligands were created using GLYCAM06[51] and Glycam Web server (http://www.glycam.com). BfrA model was initially immersed in a periodic water box (TIP3) and neutralized by adding Na^+ ions. This model was equilibrated with several cycles of minimizations (steepest descent, 10,000 steps) and MD simulations (50–200 K, 500 ps). Final minimization (steepest descent, 10,000 steps) was then run to obtain the final model of free enzyme. Dynamic docking of substrate into BfrA active site was performed as previously reported for other proteins–ligand complexes models.[52–54] Ligands were placed in BfrA model active site using fructose orientation in 3PIJ X-ray structure. The complex was then equilibrated by keeping protein backbone restrained on the model conformation. Finally, a MD simulation (200 K, 1 ns) was run and individual snapshots were randomly extracted and minimized to obtain final complex models. *N*- and *O*-glycosylation site predictions were performed using GlycoEP,[55] NetNGlyc, and NetOGlyc servers.[56]

3. Results and discussion

3.1. Sequence alignment of Leishmania invertases

In order to identify the homologies between *Leishmania* putative invertases and other GH32 enzymes, a sequence alignment was performed. The peptidic sequences of all characterized GH32 enzymes in CaZY database were retrieved and aligned together with the sequences of GH32 enzymes found in *L. major*, *L. infantum*, *L. mexicana*, *L. donovani*, and *L. braziliensis*. Six genes coding for GH32 enzymes were identified in several chromosomes: two on chromosome 4, two on chromosome 23, one on chromosome 27, and one either on chromosome 35 (*L. major*, *L. infantum*, *L. donovani*) or on chromosome 34 (*L. mexicana*, *L. braziliensis*). The corresponding enzymes are relatively close between *Leishmania* species (above 60% identity—except for *L. mexicana* proteins that exhibit lower identity percentage). These six proteins can be classified in two groups. The first group gathers genes located on chromosomes 23 and 27, coding for proteins that are above 45% identity, whereas the second group gathers genes from chromosome 4 and 35 (or 34) exhibiting identity percentage for the corresponding proteins above 35%. Between the two groups, the identity percentage falls to less than 18%.

To identify the sequence relationships for these six proteins, a phylogenetic tree was calculated from the multiple sequence alignment with the GH32 enzymes. For more clarity, a simplified version of the tree is represented in Fig. 2. This tree indicates that the enzymes from the first group (corresponding genes on chromosomes 23 and 27) are close to sucrose-6-phosphate hydrolases whereas the second group of *Leishmania* GH32 enzymes (genes on chromosomes 4 and 35) is closely related to plant invertases.[16]

In the first group, the close proximity (between 20% and 40% sequence identity) of three GH32 *Leishmania* enzymes with sucrose-6-phosphate hydrolases is striking, as no evidence of carbohydrate metabolism involving sucrose-6-phosphate was ever described for *Leishmania* or trypanosomatids. In bacteria, the phosphoenolpyruvate-dependent sugar phosphotransferase system has been widely studied since its discovery in 1964,[57] and several sucrose-6-phosphate hydrolases have been characterized, all of them found in bacteria.[58–63] Thus, the sequential proximity between *Leishmania* invertases from group 1 and sucrose-6-phosphate hydrolases raises the question of their potential involvement in sucrose metabolism.

The localization of catalytic residues of GH32 enzymes has been well documented.[29] Table 1 presents the nature of the amino acids found in conserved catalytic motifs of group 1 *Leishmania* invertases. Three carboxylic acids are necessary in the catalysis by GH32: the canonical nucleophile and acid/base, which are found in all GHs.[64] A third carboxylic residue was found to be involved for the catalysis, and was proposed to act as transition state stabilizer.[65] This particular residue, as well as the acid/base amino acid, is found in all *Leishmania* invertases. However, two invertases lack the critical nucleophilic residue in the corresponding conserved motif. Thus, only the enzyme that exhibited all three catalytic amino acids was selected for further analysis (respectively in *L. major* protein: Asp56, Glu237, and Asp180). The corresponding genes (renamed *bfrA* as the closely related *T. maritima* enzyme[66]) were annotated with the locus tags *LmjF.23.0880*, *LinJ.23.1070*, *LdBPK_231060.1*, *LbrM.23.0990*, and *LmxM.23.0880* in *L. major*, *L. infantum*, *L. donovani*, *L. braziliensis*, and *L. mexicana* genomes, respectively.[35,36] The conservation of *bfrA* gene among the five *Leishmania* species is high, as the sequence homology for BfrA protein was calculated to be above 90%.

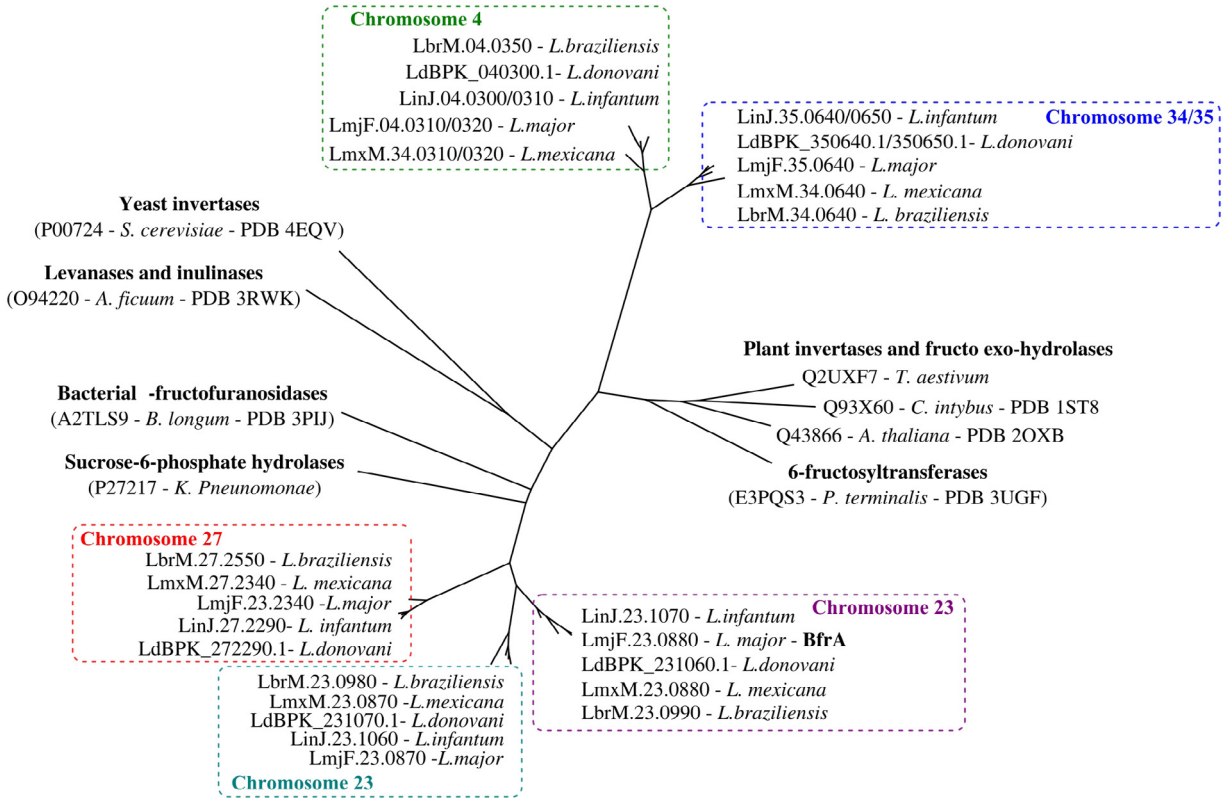

Fig. 2. Phylogenetic tree of GH32 enzymes. GH32 characterized enzymes were retrieved from CaZY and Uniprot databases and aligned using Clustal W2. Leishmania ortholog GH32 enzymes are indicated: BfrA, Lmaj.23.870, Lmaj.27.2340, Lmaj.35.0640, Lmaj.04.0310, and LMaj.04.0320 are the corresponding proteins for *L. major*.

Table 1
Sequence alignment of conserved motifs in the active sites found in resolved GH32 structures and *L. major* invertases related to sucrose-6-phosphate. The 'catalytic carboxylic triad'[29] found in GH32 is underlined: the nucleophile, the transition state (TS) stabilizer, and the acid/base catalyst. The number residue in corresponding sequence is indicated in parentheses. Identical alignments were obtained with other *Leishmania* species

Species		PDB ID/*Locus*	Function	Conserved motifs (*residue number*)		
				Nucleophile	TS stabilizer	Acid/base
Cichorium intybus		1ST8	β-Fructoexohydrolase	WMN**D**PNG (*60*)	R**D**P (*185*)	W**E**CPD (*239*)
Thermotoga maritima		1UYP	Invertase	WMN**D**PNG (*17*)	R**D**P (*138*)	I**E**CPD (*190*)
Arabidopsis thaliana		2AC1	Cell-wall invertase	WMN**D**PNG (*66*)	R**D**P (*192*)	W**E**CPD (*246*)
Bifidobacterium longum		3PIJ	β-Fructofuranosidase	WIN**D**PNG (*54*)	R**D**P (*181*)	L**E**CPD (*235*)
Aspergillus ficuum		3RWK	Endo-inulinase	WMN**E**PNG (*43*)	R**D**P (*176*)	W**E**VPD (*233*)
Saccharomyces cerevisiae		4EQV	Invertase	WMN**D**PNG (*42*)	R**D**P (*171*)	Y**E**CPG (*223*)
L. major	Chromosome 23	*LmjF.23.0870*	—	WMGVPGG (*63*)	R**D**P (*194*)	W**E**CPD (*251*)
	Chromosome 23	*LmjF.23.0880* (BfrA)	—	WMN**D**PTG (*56*)	R**D**P (*180*)	W**E**CPD (*237*)
	Chromosome 27	*LmjF.27.2340*	—	SMSAPCG (*40*)	R**D**P (*162*)	W**E**HPC (*218*)

3.2. *mRNA expression of* bfrA

bfrA mRNA expression was investigated in the promastigote and amastigote stages of three parasite species representative of the three various clinical forms and of various geographical areas, namely *L. major*, *L. donovani* and *L. braziliensis*.

In promastigote cultures, induction of BfrA mRNA increased over time in all species, peaking at day 7 (Fig. 3A). Maximal mRNA induction was observed for *L. major* (×5.7 on day 7, $p < 0.05$), compared to *L. braziliensis* (×3.3, $p < 0.01$) and *L. donovani* (×2, $p < 0.01$). By contrast, *bfrA* was only slightly expressed in amastigotes and did not vary significantly over time in *L. donovani* and *L. braziliensis* (Fig. 3B). In *L. major* amastigotes, *bfrA* was significantly induced on day 5 ($p < 0.05$) and on day 7 ($p < 0.05$), though to a moderate extent (×1.94 and ×1.58 compared to day 2, respectively).

A recent transcriptomic study on *L. major* genome during the promastigote axenic stage also detected the expression of *bfrA* gene.[67] Yet, this study did not compare the evolution of this expression during parasite growth, as well as comparison between species and with amastigotes. Our study demonstrated that BfrA was present in both parasite stages, yet mainly expressed in promastigotes of the three species. This is in agreement with several transcriptomic studies on amastigotes that did not detect this enzyme as up-regulated during this life-stage.[68–71] Its induction increased over time and the strongest mRNA induction was observed at day 7, suggesting that invertase expression is highly expressed by promastigotes at stationary phase and could be important for the development and growth of the parasite in the sandfly gut.

3.3. *Cloning and heterologous expression of* L. major *BfrA protein in* E. coli

bfrA gene was amplified from *L. major* genomic DNA and cloned into expression vector pET28a-(+) for production in *E. coli*. This vector adds a N-terminal His-Tag that was used for protein purification after cell lysis. Purification on Ni–NTA resin yielded good amounts of protein (4 mg/culture liter) (Fig. S1). This purified enzyme was further used to assess its catalytic activity.

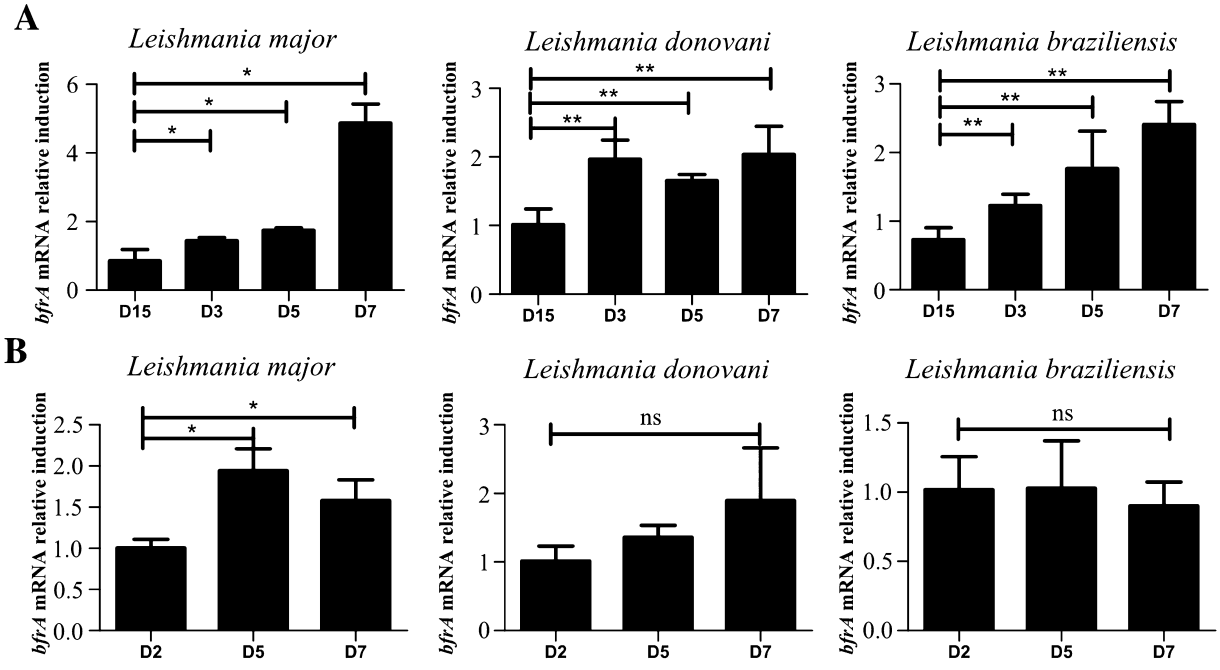

Fig. 3. Quantification of BfrA induction in *L. major*, *L. donovani*, and *L. braziliensis* promastigotes (A) and intracellular amastigotes (B) at the indicated time points. Results were normalized first using actin expression and then using day 1 expression as the 100% reference. Data are representative of two experiments performed in quadruplicates and are expressed as mean ± SEM. Statistical significance is indicated as ns: non significant $p > 0.05$, $^{*}p < 0.05$ and $^{**}p < 0.01$.

3.4. BfrA is a β-fructofuranosidase with a low specificity for sucrose

A first series of assays was performed using several pNP–sugars as substrates (Fig. S2). Potentially cleaved pNP could be easily monitored and quantified at 405 nm. However, BfrA exhibited no hydrolytic activity toward any of the 20 pNP–sugars tested. In particular, the absence of hydrolysis when using pNP-α-D-glucopyranose as substrates confirmed that BfrA is not a sucrase, as it did not recognize the α-D-glucopyranose moiety of sucrose (Fig. 1).

Sucrose hydrolysis by BfrA was then assessed using the classical 3,5-dinitrosalicylic assay[48] to quantify the amount of reduced sugar. After calibration using equimolar concentrations of fructose and glucose, sucrose hydrolysis was detected. It followed the classical Michaelis–Menten model (Fig. 4A) and the catalytic constants were determined and calculated (Table 2). The Michaelis constant K_M observed (152 mM) and the catalytic rate k_{cat} (114 s^{-1}) are similar to those reported for other GH32 enzymes.[25,66,72] Like all glycosidases, the hydrolytic activity is pH-dependent, exhibiting an optimal pH in a narrow range between 5 and 7 (Fig. 4B), significantly decreasing above pH 7. As it will be seen later, this is in agreement with the intracellular localization of BfrA, as it has been estimated that *Leishmania* can maintain an intracellular pH close to 7, whereas the extracellular pH is more alkaline.[73] Optimal temperature for enzymatic activity was 40 °C (Fig. 4C), although remaining high at physiological temperatures of the host organisms (27–28 °C for insects, 37 °C in humans).

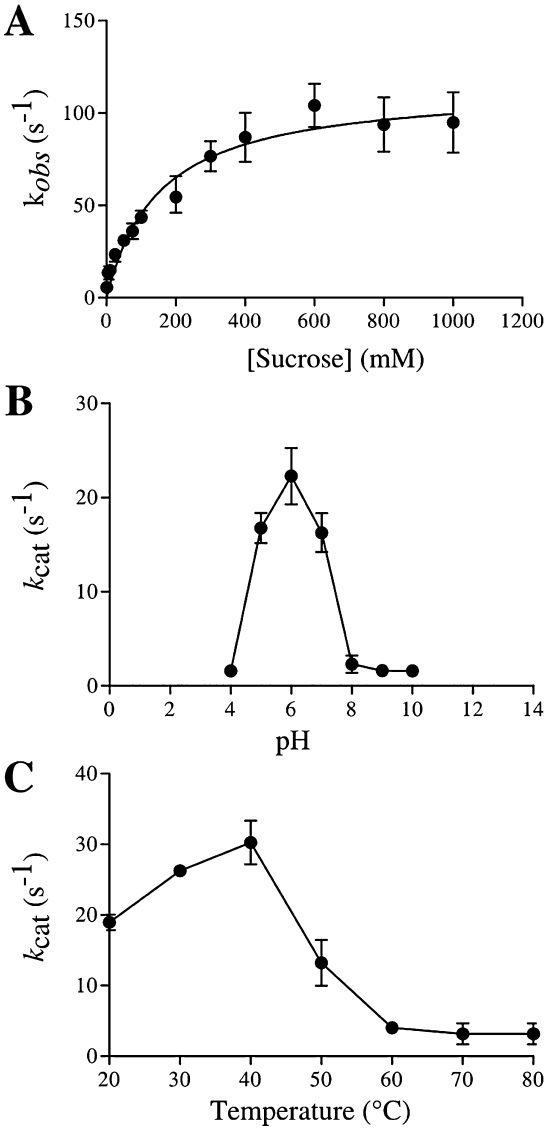

Fig. 4. (A) Michaelis–Menten fit of sucrose hydrolysis catalyzed by BfrA. pH (B) and temperature (C) dependence of sucrose hydrolysis by BfrA. Mean values and SD error bars were calculated from 3 independent experiments.

Table 2
Kinetic parameters of recombinant BfrA for the hydrolysis of several fructose-containing polysaccharides. Substrate structures are depicted in Fig. 1

	Relative activity[a] (%)	K_M[b] (mM)	k_{cat}[b] (s^{-1})	k_{cat}/K_M ($s^{-1}.mM^{-1}$)
Sucrose	100	152 ± 30	114 ± 7	0.75
Raffinose	109	141 ± 30	118 ± 14	0.86
Stachyose	297	168 ± 42	409 ± 27	2.4
1-Kestose	5.6	*n.r.*	*n.r.*	*n.r.*
Inulin	10.9	0.9 ± 0.3[c]	4.2 ± 0.2	4.6[c]

[a] Activity measured for a substrate concentration of 100 mM (18 g/L for inulin).
[b] Mean values and standard deviations were calculated from 3 independent experiments.
[c] For inulin, K_M is expressed in g/L.
n.r.: non relevant.

Raffinose and stachyose are β(1→6)-galactosylated derivatives of sucrose that are commonly found in vegetables. Kestose and inulin are fructan polysaccharides that are found in many plants, and were reported to be specific substrates for several GH32 enzymes.[20,74] For raffinose and stachyose, the measured activity and catalytic constants were of the same order of magnitude, but stachyose exhibited a higher k_{cat} value (409 vs. 114 s^{-1}). Interestingly, the structure of the substrate shows that the number of branched β(1→6)-galactoses to the glucose moiety of sucrose is correlated to an increase of the enzyme specificity (e.g. k_{cat}/K_M). Unlike galactosylated sucrose derivatives, it appeared that fructan polysaccharides are not efficient substrates of BfrA, since the K_M and k_{cat} constants could not be determined for kestose, and inulin was only slowly hydrolyzed (Table 2). This was unexpected, as the closest proteins in the phylogenetic tree have both fructan and sucrose hydrolysis activity.[20,66] GH32 enzymes can be classified according to their specificity for fructan vs. sucrose.[75] Pure invertases will be specific for sucrose (with a K_M value below 10 mM), whereas inulinases and fructoexohydrolases are more versatile and can hydrolyze more compounds, specifically fructan-containing polysaccharides. This puts into question the endogenous activity and substrate of BfrA, as no compounds tested exhibited low K_M constant, neither sucrose nor fructan polysaccharides.

3.5. Modeling of invertase and mechanistical implications

A homology model of the protein was built, and substrate docking simulations were run. So far, eleven X-ray structures of GH32 enzymes have been solved and reported.[18–28,31–34] Among them, the X-ray structure of *B. longum* β-D-fructofuranosidase (PDB code 3PIJ)[20] was chosen, as the sequence homology between the template and target proteins is 55% (35% identity). The optimized model is shown in Fig. S3. It exhibits the classical domains found in GH32 structures: an *N*-terminal five-bladed β-propeller and a *C*-terminal *β*-sandwich module.[20] The identity of catalytic residues of BfrA predicted from sequence alignment (Table 1) was then confirmed by structural alignment with GH32 enzymes: Asp56 acts as the nucleophile, whereas Glu237 is the acid/base residue involved in the reaction. Docking of sucrose into BfrA active site was then performed to identify and localize the active site residues involved in substrate binding (Fig. S3A). As previously reported for other GH32 enzymes, the binding of sucrose is mainly done through a network of hydrogen bonds with the fructose moiety.[19,22,26,29,34,65]

3.6. Production of $BfrA_{leish}$ in L. tarentolae

BfrA sequence was submitted to SignalP server[76,77] and no canonic secretion signal peptides were detected, thus indicating the most likely intracellular localization of the protein. Still, to gain insight about the cellular localization of BfrA, we decided to express it in a eukaryotic host system similar to *L. major*. *L. tarentolae* was chosen as an expression host to produce BfrA because of its close genetic proximity with *L. major*. Unfortunately the cloning and expression of the intracellular form of BfrA could not be successfully achieved in our hands. Therefore, the cloning strategy was selected to produce a recombinant $BfrA_{leish}$ fused with a secretory sequence peptide. The glycosylation pattern of secreted or membrane proteins by *L. tarentolae* was expected to be identical to that of *L. major*.[78] As a consequence, secreted $BfrA_{leish}$ would be used to study the influence of glycosylation on protein activity, as *L. tarentolae* was shown to *O*- and *N*-glycosylate secreted recombinant proteins[79] as well as assessing the potential secretion of the protein in *L. major*. In our case, *N*- and *O*-glycosylation putative sites were predicted using two programs: GlycoEP and NetGlyc.[55,56] These programs identified four potentially *N*-glycosylated asparagines, namely Asn38, 380, 389, and 494, and six *O*-glycosylation putative sites, Ser 36, and Thr 17, 40, 140, and 503 (Fig. S3B).

bfrA gene was cloned in the expression vector, and *L. tarentolae* cells were successfully transfected with the corresponding construct. Recombinant protein was further purified by affinity chromatography from the cultivation medium and SDS–PAGE analysis of the purified extract demonstrated the purity and the glycosylation of $BfrA_{leish}$. However, this recombinant enzyme was not able to hydrolyze any of the substrates recognized by BfrA expressed in *E. coli*. Thus, glycosylation of the secreted protein might prevent the substrates to be efficiently hydrolyzed by $BfrA_{leish}$.

Still, this gives strong clues about the cellular localization of BfrA in *L. major* cells. Sucrose hydrolysis activity was previously detected not only inside *Leishmania* cells[15] but also as an extracellular activity.[14] According to our results, BfrA can be hypothesized to be the intracellular invertase. Moreover, a recent work identified one of the secreted invertase,[16] which gene is located on the chromosome 4 of *L. major* (see Fig. 2). In addition, Gontijo et al. determined the catalytic constants for the secreted sucrose-hydrolyzing enzyme.[14] K_M and k_{cat} constants were respectively 4 mM and 1.6 s^{-1}. These constants are not consistent with the K_M and k_{cat} values determined for BfrA (Table 1). In our case, the low activity of the enzyme outside the pH 5–7 range is also consistent with the intracellular pH reported for *Leishmania*. Moreover, when BfrA sequence was submitted to SignalP server,[76,77] no signal peptides was detected. Experimentally, expression of BfrA as a secreted protein in *Leishmania* cells ($BfrA_{leish}$) yielded an inactive protein. Combining these results leads to the convincing assumptions that BfrA is an intracellular invertase.

4. Conclusion

In this work, we provide the description of an invertase of the GH32 family in the *Leishmania* genus, the first enzyme of the sucrose metabolism and bring new insight into the parasitic metabolic pathways. Despite owing common biochemical characteristics for this family, its poor sequence homology with other GH32 invertases is of high interest in view of medical chemistry development. Our data also suggest that the glycosylated form of BfrA is inactive and that its location is most likely intracellular. It is highly expressed in the promastigote stage, and to a lesser extent in amastigote stage of the three studied parasitic species, making it a putative target for antileishmanian drug development.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.carres.2015.07.001.

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