Investigating a galactokinase orthologue from *Leishmania donovani*

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Abstract

Leishmania donovani is a causative organism of visceral leishmaniasis. We have identified an enzyme in *L. donovani* known as a galactokinase-like protein (*Ld*GalK), which could be a novel target for drug development. In the human host, two GalK paralogues are expressed, GalK1 and GalK2, which metabolise galactose in the Leloir pathway and N-acetyl galactosamine pathway (Figure 1), respectively. Both kinases instigate the first committed step in their respective pathways and phosphorylate the carbon-1 position in their carbohydrate ligand[1].

We have expressed recombinant *Ld*GalK in *E. coli* and purified the protein to high purity and yield. The recombinant enzyme is catalytically active with a substate affinity to galactose in the low micromolar range. Interestingly, size exclusion chromatography of *Ld*GalK suggests either an open/closed conformation of the enzyme or dimerization. Future research will test potential inhibitors against recombinant *Ld*GalK in vitro and against *L. donovani* parasites in vivo.

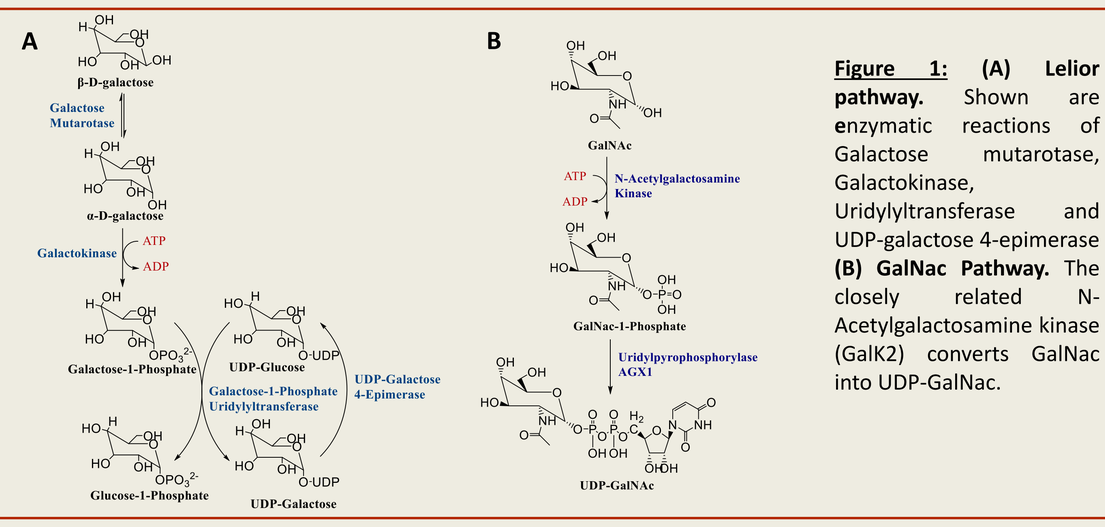

Figure 1: (A) Lelior pathway. Shown are enzymatic reactions of Galactose mutarotase, Galactokinase, Uridylyltransferase and UDP-galactose 4-epimerase **(B) GalNac Pathway.** The closely related N-Acetylgalactosamine kinase (GalK2) converts GalNac into UDP-GalNac.

Results

1 Protein Purification

*Ld*GalK was expressed in *E. coli* to good yield (method adapted from [2]) and purified to high purity by IMAC nickel column (Figure 2A). Western Blot identifies successful purification of His-tagged protein (Figure 2B). Gel filtration of *Ld*GalK depicts two apparent molecular weight peaks (Figure 3). Calculated molecular weights for the peaks are ~115-160kDa and ~52-73kDa which could represent different protein conformations.

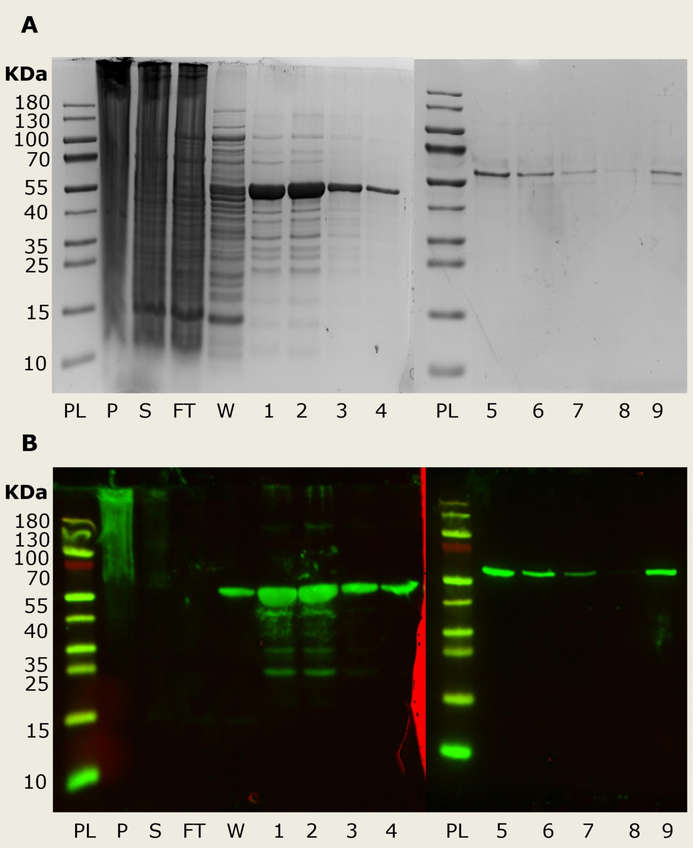

Figure 2: A: IMAC purification of *Ld*GalK protein. SDS-PAGE with molecular weight protein ladder (PL), cell pellet after centrifugation (P), flowthrough (FT), wash (W), imidazole gradient fractions (1-9). **B: Western Blot of His-tagged *Ld*GalK protein.** Identical fractions as SDS-PAGE gel, visualized with mouse anti-His antibody and rabbit anti-mouse secondary (DyLight 680).

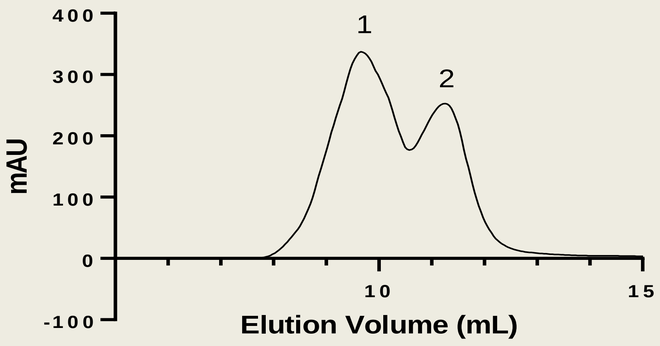

Figure 3: Gel filtration of *Ld*GalK. Recombinant *Ld*GalK elutes in two separate peaks at apparent molecular weights of 115-160kDa (peak 1) and 52-73kDa (peak 2), due to either conformational differences of the enzyme, or dimerization. Corresponding SDS-PAGE analysis only shows *Ld*GalK protein.

2 Circular Dichroism

Circular dichroism (CD) shows slight structural changes in secondary structure elements of *Ld*GalK with different carbohydrate ligands. Secondary structure deconvolution reveals similarities of *Ld*GalK from gel filtration peak 1 compared to non-filtrated protein with 10mM galactose (Figure 4, Table 1). A significant increase in apparent alpha helical content was observed in peak 2 and ATP-bound *Ld*GalK. This could indicate a conformational change in *Ld*Galk when the protein is bound to its ligands galactose or ATP.

Wavelength spectra of *Ld*GalK bound to ATP or galactose-1-phosphate give similar molar ellipticity signals as *Ld*GalK without ligand. However, this is significantly different from galactose-bound *Ld*GalK (Figure 5), although all three ligands bind in the active site during catalysis.

Table 1: Secondary structure deconvolution using BestSel. A significant change was observed between the alpha helical content and beta strand content when *Ld*GalK is present with different ligands.

	No ligand	10mM galactose	10mM G-1-P	Peak 1	Peak 2	400uM ATP
Helix (%)	38.1	25.3	32.9	28.7	53.7	40.5
Strand (%)	13	28.1	16.4	21	5.9	13.1
Others (%)	48.8	46.6	50.6	50.3	40.4	46.4

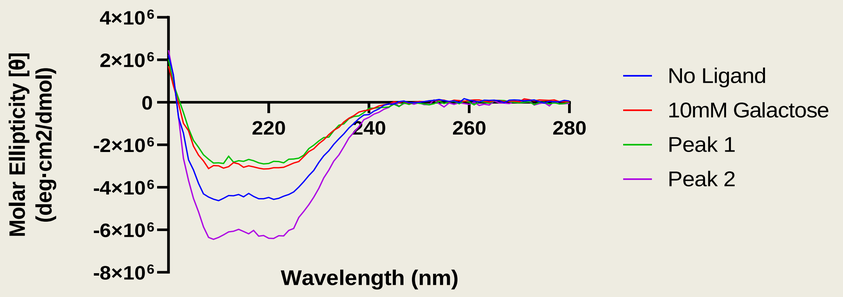

Figure 4: CD Wavelength spectra of *Ld*GalK. Changes in molar ellipticity are observed when the protein is separated into the two apparent molecular weight peaks and in the presence of 10mM galactose.

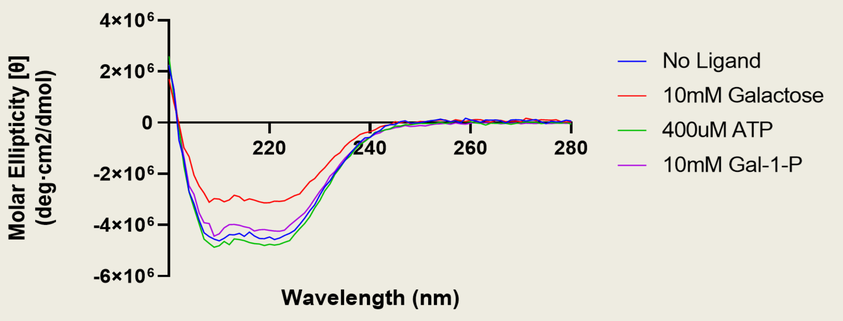

Figure 5: CD Wavelength spectra of *Ld*GalK with different ligands. *Ld*Galk ligand with galactose-1-phosphate or ATP shows similar spectra, as compared to 10mM galactose. Protein concentrations in all samples are the same.

3 Enzyme Kinetics

*Ld*GalK can be studied in Michalis-Menten kinetics by using a three-enzyme reaction with pyruvate kinase and lactate dehydrogenase, measuring the absorbance of NADH turnover at 340nm (Figure 6).

*Ld*GalK is catalytically active at a low micromolar range due to product inhibition (Gal-1-P). Substrate concentrations above 1000-fold excess do not result in an increase of turnover rate (Figure 7). K_m value of galactose ranges from 0.1 to 1.8uM, the apparent maximum velocity (apparent V_{max}) ranges from 0.01 to 0.05uM/s and apparent turnover number (apparent K_{cat}) value ranges between 0.002 to 0.02s^{1}. These values are in the magnitude of other published galactokinase data (Brenda-enzymes.org). Investigation of the two apparent molecular weight peaks in the kinetics assay showed peak 2 *Ld*GalK is slightly more active than peak 1 *Ld*GalK (Figure 8). This may indicate a dynamic conformation of the protein which does not affect catalytic performance.

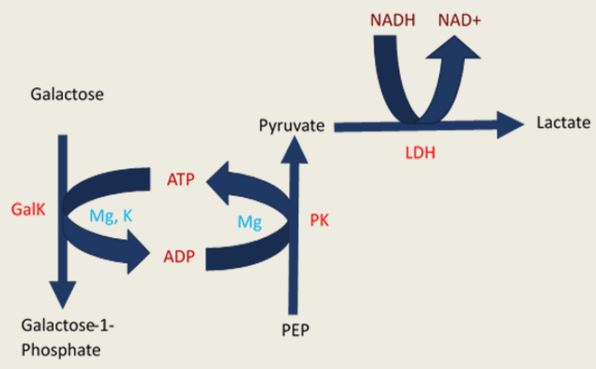

Figure 6: Kinetics assay reaction. This assay uses three enzyme reactions, the turnover of NADH to NAD is measured at 340nm [3,4]. The galactokinase reaction in this assay is the rate limiting step, giving an accurate output of *Ld*GalK activity.

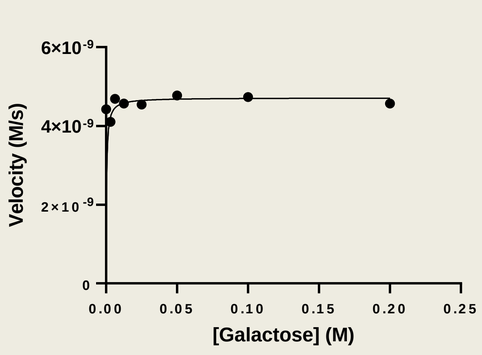

Figure 7: Michalis-Menten fit of *Ld*GalK. 2µM *Ld*GalK was used for all kinetics experiments, substrate concentrations above 1000-fold excess (2mM) saw no increase in velocity.

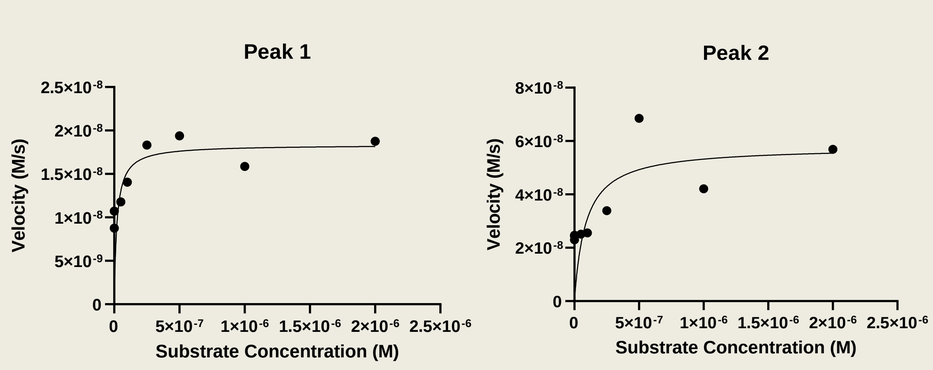

Figure 8: *Ld*GalK protein from both apparent molecular weight peaks is catalytically active. Catalytic activity of the protein from the two peaks differ slightly, K_m, apparent V_{max}, and apparent K_{cat} values of peak 1 are 0.0207µM, 0.0184uM/s and 0.00918s^{-1} respectively. K_m, apparent V_{max}, and apparent K_{cat} values of peak 2 are 0.874µM, 0.0579uM/s and 0.0289s^{-1} respectively.

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Next Steps

- Compound screening for potential inhibitors against recombinant *Ld*GalK
- Viability assays to determine effects of *Ld*GalK inhibitors on *L. donovani* amastigotes
- Generation of *Ld*GalK deletion and complemented parasite lines for further analysis