

## Characterization of Glucokinase-1 from *Phytophthora infestans*: A Versatile Enzyme

Lira Villalobos-Piña<sup>1</sup>, Hermes Balza<sup>1</sup>, Héctor Acosta<sup>1,2\*</sup>, Diego Andrade-Alviárez<sup>2</sup>, Ascanio Rojas<sup>3</sup>, Luisana Avilán<sup>1</sup>, Amaranta Gómez-Arreaza<sup>1</sup>

<sup>1</sup>Laboratorio de Fisiología, Facultad de Ciencias, Universidad de Los Andes, Mérida, Venezuela; <sup>2</sup>Laboratorio de Enzimología de Parásitos, Facultad de Ciencias, Universidad de Los Andes, Mérida, Venezuela; <sup>3</sup>Centro Nacional de Cálculo Científico (CeCalCULA), Universidad de Los Andes, Mérida, Venezuela

### ABSTRACT

*Phytophthora infestans* has seven genes coding for glucokinases, with the [PITG\_06016] (*Pi*GlcK-1) gene being the highest expression at different stages of growth. However, its role in the metabolism of *P. infestans* is still unclear. In this study we cloned, overexpressed, purified and biochemically characterized this enzyme. The purified recombinant protein has a  $K_m$  of 0.66 mM and 2.2 mM for glucose and ATP, respectively. *Pi*GlcK-1 is capable of phosphorylating fructose, although only 48% with respect to glucose, using ADP and pyrophosphate (PPi) as a phosphoryl group donor. These molecules are 50% and 11% less effective than ATP, respectively. In addition, *Pi*GlcK-1 can be activated by PPi increasing its activity 3.6 or 2.5 times when the phosphoryl donor is ATP or ADP, respectively. *Pi*GlcK-1 can form oligomeric complexes and these tend to spontaneously dissociate, with the monomer having the highest activity. This oligomerization can be destabilized by dithiothreitol which increase *Pi*GlcK-1 activity twice. These results reveal that *Pi*GlcK-1 is a versatile enzyme that could play an important role in the use of available sugars during the first stage of infection, as well as in the necrotrophic stage, and suggest a possible regulatory mechanism against possible conditions of oxidative stress.

**Keywords:** *Phytophthora infestans*; Glucokinase; Recombinant protein; Oligomeric complexes; Oxidative stress

### INTRODUCTION

*Phytophthora infestans* is an oomycete that causes the disease called late blight, which affects tomato and potato crops worldwide, causing significant economic losses [1]. Attempts to control this disease include the use of fungicides that are not very effective for the elimination of the phytopathogen and are toxic to humans [2]. Despite having studied *P. infestans* for more than a century to date, it has not been possible to control the disease caused by this phytopathogen, positioning it as a threat to world food security [3]. A detailed characterization of the metabolism of *P. infestans* could contribute to the search of solutions against this threat.

Currently, most studies of *P. infestans* metabolism have been inferred based on mRNA and indicate that glycolysis plays an important role in the infection process levels [4-7]. However, this pathway has been little studied in this organism at enzymatic

level. In common with most eukaryotic organisms, hexokinase (EC 2.7.1.1) is the first enzyme of the glycolytic pathway [8]. However, in *P. infestans* and other stramenopiles, with the exception of *Blastocystis hominis* as well as other unicellular eukaryotes, such as *Trichomonas vaginalis* and *Giardia intestinalis*, this enzyme has been replaced by a glucokinase (EC 2.7.1.2) [6].

Enzymes that catalyze the phosphorylation of D-Glucose and other hexoses through donors of phosphoryl groups such as ATP, ADP, PPi and inorganic Poly-Phosphoryl [Poly (Pi)] have been classified based on their primary structures within two major non-homologous families: the Hexokinase family and the Ribokinase family [9]. The first family is subdivided into three groups: HK, A and B. The HK group harbours hexokinases belonging to eukaryotic organisms that has ATP as a donor of the phosphoryl group, different molecular weights, subcellular distribution and isoenzymes that differ in their kinetic and regulatory characteristics [9,10]. Group A is represented by

**Correspondence to:** Dr. Héctor Acosta, Laboratorio de Enzimología de Parásitos, Facultad de Ciencias, Universidad de Los Andes, Mérida 5101, Venezuela; Tel:+58 2742401302; Fax:+58 2742401390; E-mail: hectoracosta0326@gmail.com

**Received:** November 13, 2020; **Accepted:** November 27, 2020; **Published:** December 04, 2020

**Citation:** Villalobos-Piña L, Balza H, Acosta H, Andrade-Alviarez D, Rojas A, Avilán L, et al. (2020) Characterization of Glucokinase-1 from *Phytophthora infestans*: A Versatile Enzyme. Fungal Genom Biol. S1: 001.

**Copyright:** © 2020 Villalobos-Piña L, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

enzymes belonging to Gram-negative bacteria, cyanobacteria, amytochondriate protists and trypanosomatids with molecular weights between 33 to 42 kDa. Finally, group B is represented by enzymes belonging to Gram-positive bacteria and crenarqueota organisms. These last two groups comprise enzymes known as Glucokinases (GlcKs) capable of exclusively phosphorylating D-glucose [9-11]. On the other hand, the Ribokinase family comprises enzymes that only use ADP as a donor of the phosphoryl group and have molecular weights between 47 to 54 kDa [9,12].

*P. infestans* presents up to seven genes for GlcKs [13] which are differentially transcribed both in nutritional media and in the different life stages of *P. infestans* [6,14,15], which denotes the complexity and adaptability of this organism. One of these glucokinases, [PITG\_06016] available in the National Centre for Biotechnological Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>) (NCBI database) that we will call PiGlcK-1 here, is more expressed in zoospores, germinated cysts and appressorium compared to the other *P. infestans* glucokinases [15], showing eight times greater expression in zoospores than in hyphae [14,15]. The presence of GlcKs in this organism instead of hexokinases could restrict the use of other equally abundant sugars that are present in plants [6] which are usually mobilized using membrane-bound transporters in hyphae or haustoria [16]. In this sense, most of the genes expressed during the development of the haustoria encode for a transporter of sugars (HTX1), specialized for D-glucose and D-fructose, confirming that these sugars are the primary carbohydrates imported into the haustoria [17]. Only a few species, especially bacteria, are known to contain true glucokinases [10]. Therefore, it is important to know if the *P. infestans* GlcKs are true glucokinases or if they are able to use other important sugar in plants.

With the intention of understanding more about the metabolism of *P. infestans*, we have carried out in this study, a biochemical characterization of PiGlcK-1. We describe the kinetic properties of the recombinant enzyme, the effect of dithiothreitol (DTT) on PiGlcK-1 activity, as well as its oligomeric states. We propose a metabolic model where PiGlcK-1 activity can be regulated under conditions of oxidative stress during the progress of *P. infestans* infection.

## MATERIALS AND METHODS

### Cloning of PiGlcK-1 gene

The PiGlcK-1 gen [PITG\_06016] retrieved from NCBI database (<http://www.ncbi.nlm.nih.gov>) was amplified by PCR from genomic DNA obtained from *P. infestans*, isolate SR 1.8-04 (homothallic, originally isolated from potato var. Granola, grown at Santa Rosa, Mérida, Venezuela) [18]. The forward primer was 5'-GCATATGTCTGACTACAGCCTCATCATC-3' (*Nde*I site in bold) and the reverse primer was 5'-CGGATCCCTACGCGCTCGAGTGCAAG-3' (*Bam*HI site in bold). The amplification mixture (50 ml) contained 1 mg of *P. infestans* genomic DNA, 1 mM of each primer, 200 mM of each of four deoxynucleotides, 1.5 mM MgCl<sub>2</sub> and 1.5 U of DNA polymerase (DNA polymerase Platinum, Invitrogen) with the

corresponding PCR buffer. PCR for glucokinase gene was performed as follows: an initial incubation at 95°C for 2 min, 30 cycles of denaturation at 95°C for 2 min, annealing at 55°C for 45's, and extension at 70°C for 90's; the final incubation was at 70°C for 10 min.

The PCR products were ligated into the pGEM-T vector (Promega) cloned and sequenced using the T7 and SP6 primers in an automated sequencer. The gen was transferred to the pET28a vector (Novagen) and used to transform the *Escherichia coli* strain BL21pRARE for the production of the recombinant PiGlcK-1 with a 20 residue-long N-terminal containing a His-tag.

### Multiple sequence alignments and phylogenetic analysis

Sequence data were obtained from the NCBI, which selected sequences homologous to the PiGlcK-1 protein. Multiple alignments were performed using MUSCLE [19] with predetermined parameters. Protein sequences similarity analysis was performed with the ESPript 3.0 software (<http://espript.ibcp.fr/ESPript/ESPript/>).

The sequences for the phylogenetic analysis were obtained as described in the previous section. The alignment was performed with MUSCLE [19] and a subsequent refinement with TCS [20]. To perform the tree, the substitution model that best fit the data was previously evaluated using the Model Finder program [21], with LG+G4 being the best model. The tree was generated by PhyML with the chosen substitution model, with 500 bootstrap non-parametric replicas, the gamma estimate and the optimized distribution parameter of BIONJ. The tree was edited with ITOL v5 [22]. The access numbers of the sequences used were added to the tree legend.

### Overexpression of PiGlcK-1 in *Escherichia coli* and their purification

Cells were grown at 25°C for 48 h in ZYM-5052 auto induction medium (0.5% (w/v) yeast extract, 25 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM KH<sub>2</sub>PO<sub>4</sub>, 50 mM NH<sub>4</sub>Cl, 5 mM Na<sub>2</sub>SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.5% (w/v) glycerol, 0.05% (w/v) glucose and 0.2% (w/v) lactose) [23] supplemented with 50 ml/ml kanamycin and 100 ml/ml chloramphenicol, to O.D 600 nm values of 10. Cells from 50 ml culture were harvested by centrifugation, washed with PBS, and resuspended in 10 ml lysis buffer (100 mM HEPES pH 7.6 and 0.1% Triton X-100) in the presence of a cocktail of protease inhibitors (Sigma). The resulting suspension was broken by sonication and the lysate centrifugated at 12,000 g for 15 min at 4°C. The supernatant contained recombinant His-tagged glucokinase.

PiGlcK-1 was purified using a HisLink column (Amersham Biosciences). The column (2 × 0.8 cm) was equilibrated with the PBS and washing with the same buffer, His-PiGlcK-1 was eluted with 200 mM imidazole. The imidazole was removed from the sample using a PD-10 column (Amersham Biosciences). The purity of the PiGlcK-1 was checked by SDS-PAGE followed by Coomassie blue R-250 staining.

## Antibodies

A polyclonal antiserum against purified recombinant PiGlcK-1 was raised in a rabbit by intradermic injection with 0.2 mg of purified protein in incomplete Freund's adjuvant (1:1) at multiple sites. The immune response of the rabbit was boosted five times, at two-week intervals, using the same conditions as the first injection, except for the last boost which was performed in the absence of the adjuvant. The rabbit was bled by cardiac puncture two weeks after the four immunizations. The sera were stored at 20°C.

## Enzyme assays

Glucokinase activity was assayed by measuring the formation of NADH by coupling the phosphorylation of D-glucose to the reduction of NAD<sup>+</sup> by glucose-6-phosphate dehydrogenase (G6PDH), measured at 340 nm and 25°C using a Hewlett-Packard 8452 diode array spectrophotometer. For the assay of D-fructose phosphorylation, phosphoglucose isomerase (PGI) was added to the reaction mixture instead G6PDH. The assay was performed in a 1-ml cuvette containing 100 mM triethanolamine (TEA), pH 7.6, 5 mM MgCl<sub>2</sub>, 5 mM ATP, 4 mM D-glucose or D-fructose, 0.72 mM NAD<sup>+</sup>, 10 mM DTT in the presence of purified PiGlcK-1 and 1 U.ml<sup>-1</sup> G6PDH (type XXIV from *Leuconostoc mesenteroides*, Sigma) for glucose or 1 U.ml<sup>-1</sup> PGI (*Saccharomyces cerevisiae*, Sigma) for fructose [24]. The Km for glucose was determined by varying its concentration between 0.1 mM and 0.50 mM, whereas the Km for ATP was measured with concentrations varying between 0.5 and 5 mM. The effect of DTT on the glucokinase activity was determined by varying its concentration between 0 and 50 mM. The glucokinase activity with 5 mM ADP or 5 mM PPi (in absence of ATP) and the effect of 1 mM PPi on the glucokinase activity (in presence of ATP or ADP) were determined similarly to glucose assay described above. Values of kinetic parameters were calculated by plotting the data and curve fitting using the appropriate equations in the program SigmaPlot 11.0 SPSS. One unit (U) of enzyme activity is defined as the amount of PiGlcK-1 that catalyzes the formation of 1 mmol of product per minute or consumption of 1 mmol substrate per minute.

The effect of hydrogen ion concentrations on PiGlcK-1 activity was measured using a polybuffer of MES, MOPS, Tris and TEA, each at 25 mM, over a pH range from 7 to 9.5. At each pH condition at which the activity was assayed, the time-dependent absorbance variation at 340 nm was recorded until it reached the steady state.

For the preparation of the β-D-glucose anomer, 10 mM of α-D-glucose (Sigma) was dissolved in cold water (4°C), which was heated for 10 min at 60°C and kept on ice to shift the anomeric equilibrium towards the formation of β-D-glucose [25].

## Protein determination, SDS-PAGE and immunoblotting

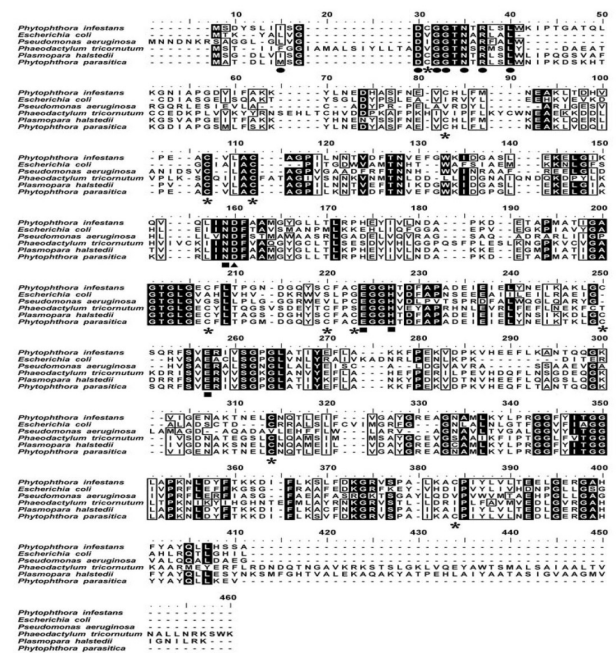
Proteins were quantitatively assayed by the Bradford method [26] with bovine serum albumin as standard. SDS-PAGE was performed in 12.5% polyacrylamide gels according to the method described by Laemmli [27]. Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane

(Amersham Bioscience) and Western blotting was performed as described [28]. The nitrocellulose membrane (Thermo Scientific) was first blocked with PBS containing 10% casein and 0.1% Tween 20 for 2 h. After washing with PBS containing 0.1% Tween 20, the membrane was incubated with the anti-PiGlcK-1 polyclonal obtained in rabbit (1:1000 dilution) for 1 h, then incubated with a rabbit anti-IgG conjugated with alkaline phosphatase (dilution 1:5,000) for 1 h and visualized with BCIP-NBT in PBS.

## RESULTS

### Cloning and sequence analysis of the *P. infestans* glucokinase PiGlcK-1

PiGlcK-1 gene of *P. infestans* was identified in the NCBI database [PITG\_06016] and amplified by PCR using genomic DNA from *P. infestans* and cloned. It encodes a polypeptide of 353 amino acids with a calculated molecular mass and isoelectric point of 38 kDa and 5.2, respectively. A comparison of PiGlcK-1 with homologous sequences identified by MUSCLE [19], showed the following degrees of identity with group-A GlcKs from other stramenopiles organisms: *Phytophthora parasitica* (90.4%), *Plasmopara halstedii* (79.4%) and *Phaeodactylum tricornutum* (38.6%). A low identity was also found with a counterpart in the prokaryote *E. coli* (32.1%) and *Pseudomonas aeruginosa* (28.4%) (Figure 1).

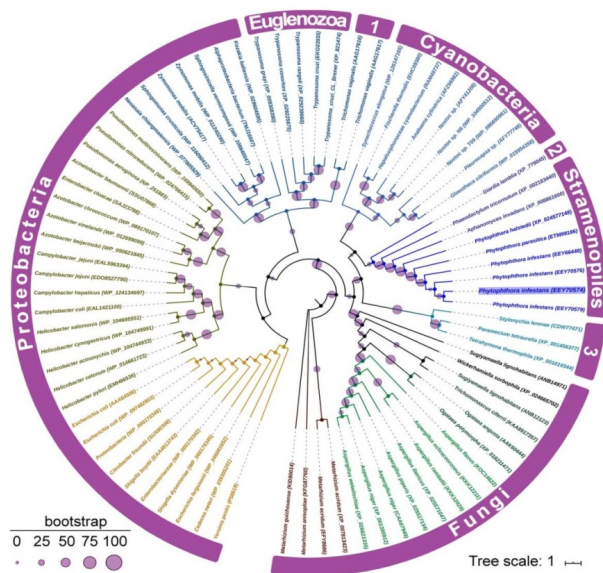


**Figure 1:** Sequence alignment of glucokinase-1 (PiGlcK-1) from *Phytophthora infestans* [PITG\_06016] with representative GlcKs from other species. *Escherichia coli* (AAA64506.1), *Pseudomonas aeruginosa* (NP\_251883.1), *Phaeodactylum tricornutum* (XP\_002183440.1), *Plasmopara halstedii* (XP\_024577148.1), *Phytophthora parasitica* (ETM99186.1). Squares: amino acids involved in glucose binding, black circles: amino acids involved in ATP binding, triangle: catalytic aspartate, asterisks: cysteine residues present in the PiGlcK-1 sequence of *P. infestans*.

Further analysis of the deduced amino acid sequence showed that *PiGlcK-1* contains the characteristic ATP binding site  $^8\text{I-X-X-D-X-G-G-X-N-X-R-X-X-L}^{21}$  (Asp, Gly, Asn, Arg and Leu in bold) and conserved glucose binding residues (Asn101, Glu159, His162 and Glu189) present for GlcK from *E. coli* [29]. The above indicates that *PiGlcK-1* belongs to group A of the Hexokinase family. Interestingly, we also observed that the *PiGlcK-1* sequence shows ten cysteines, with Cys12 within the ATP binding motif and Cys172 and Cys175 near residues involved in glucose binding (Figure 1).

### Phylogenetic analysis

Figure 2 shows the phylogenetic analysis obtained with PhyML for 80 GlcK sequences of *P. infestans* and other organisms. *PiGlcK-1* branches with those from other species of Phytosphthora and other oomycetes in the well supported stramenopiles clade, clearly differentiating it from other eukaryotes. The amino acid identity in this clade is more than 90% in contrast to 50% or less in other groups of bacteria, cyanobacteria and fungi.

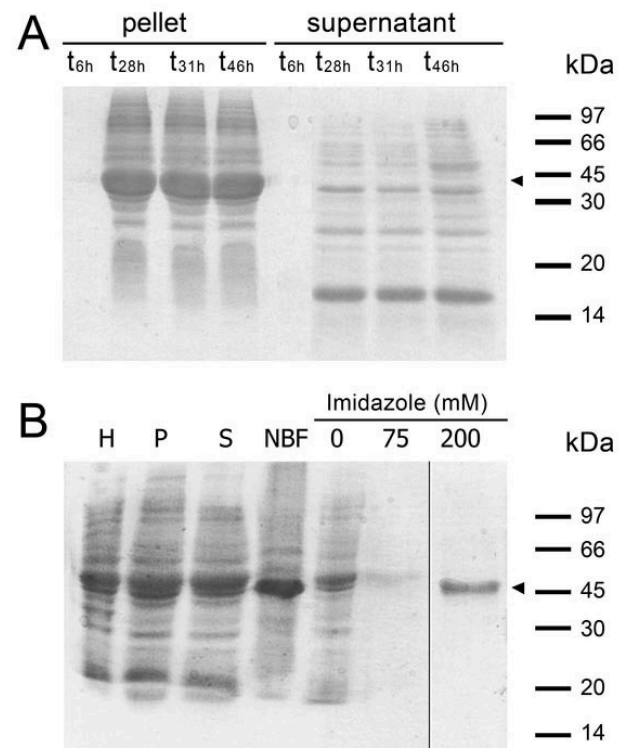


**Figure 2:** Rooted circular phylogenetic tree constructed from 80 GlcKs sequences. The tree was constructed using PhyML as described in Methods. The numbers represent the taxa: Parabasalia (1), Diplomonadida (2) and Alveolata (3). The circles at nodes represent bootstrap the percentages values. Each sequence is marked with its GenBank accession number and *PiGlcK* is highlighted.

### Expression of recombinant *PiGlcK-1* in *E. coli* and purification of the protein

The complete gene for *PiGlcK-1* was expressed in *E. coli* strain BL21pRARE in order to investigate their biochemical and kinetic properties. The coding sequences were ligated to the expression vector pET28a to produce a fusion protein with an amino terminal (His)<sub>6</sub>-tag. Bacteria were grown in autoinduction medium and a high percentage of insoluble enzyme was obtained. However, this expression system allowed us to produce enough amounts of soluble recombinant protein (Figure 3A).

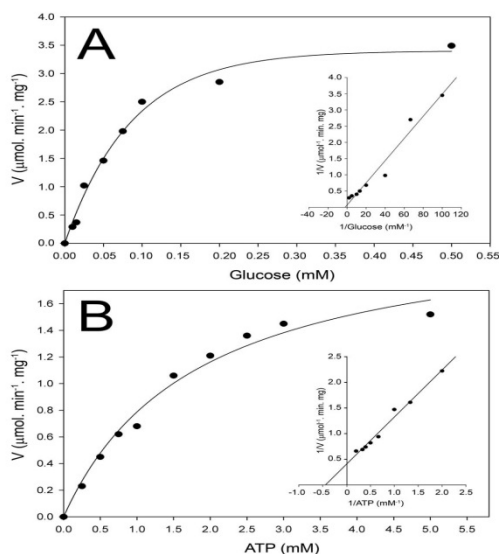
After induction, the soluble fraction was purified to homogeneity by HisLink column, using 200 mM imidazole concentration for the elution (Figure 3B). The purified protein was used to raise antisera, and for its respective kinetic characterization.



**Figure 3:** Expression and purification of *PiGlcK-1*. (A) Expression of *PiGlcK-1*(His)<sub>6</sub> in autoinduction medium with 500 mM NaCl. SDS-PAGE to 12% of pellets: 6 hours (t<sub>6h</sub>), 28 hours (t<sub>28h</sub>), 31 hours (t<sub>31h</sub>), 46 hours (t<sub>46h</sub>) and supernatants: 6 hours (t<sub>6h</sub>), 28 hours (t<sub>28h</sub>), 31 hours (t<sub>31h</sub>), 46 hours (t<sub>46h</sub>). (B) Purification of recombinant *Phytophthora infestans PiGlcK-1* expressed in *Escherichia coli*. H, homogenate; P, pellet; S, supernatant; NBF, not binding fraction, Eluates: 0, not imidazole; 75 mM imidazole; 200 mM imidazole.

### Kinetic characterization of recombinant *PiGlcK-1*

Recombinant *PiGlcK-1* was characterized with respect to its kinetic properties. The obtained progress curve showed a delay (lag) of several minutes until reaching the steady-state velocity, which was used to determine all the kinetic parameters. *PiGlcK-1* showed hyperbolic kinetics for glucose and ATP, indicating classic Michaelis-Menten behavior. The Km values for glucose and ATP were determined according to the values obtained from the Lineweaver-Burk graph, being 0.66 mM and 2.2 mM, respectively (Figure 4).



**Figure 4:** Determination of the  $K_m$  values of recombinant  $PiGlcK-1$ . The reaction mixture contained 100 mM TEA pH 7.6, 5 mM  $MgCl_2$ , 10 mM DTT, 0.72 mM  $NAD^+$  and 1U of G6PDH; with constant concentration of 5 mM ATP and variable concentration of glucose (A) and constant concentration of 5 mM glucose and variable concentration of ATP (B). The data were plotted to calculate the  $K_m$  for each substrate using double inverse graphics (Lineweaver Burk equation).

This  $K_m$  value for glucose is very similar to those reported by other GlcKs from *E. coli* (0.78 mM), *Bacillus stearothermophilus* (0.52 mM) or *T. cruzi* (0.94 mM) [10,11,30]. Interestingly  $PiGlcK-1$  can also use the  $\alpha$ -D-glucose (14  $U \cdot mg^{-1}$ ) and  $\beta$ -D-Glucose (9.8  $U \cdot mg^{-1}$ ) anomers. To determine if  $PiGlcK-1$  was a true glucokinase, we assessed the enzyme's ability to phosphorylate fructose, checking that  $PiGlcK-1$  can phosphorylate this sugar by 48% relative to glucose (Table 1).

Molecules	$U \cdot mg^{-1}$	% Activity
Sugars		
$\alpha$ -D-Glucose (4 mM)	14	100
$\beta$ -D-Glucose (4 mM)	9.8	70
D-Fructose (4 mM)	6.7	48
Donator of phosphoryl group		
ATP (5 mM)	14	100
ADP (5 mM)	7	50
PPi (5 mM)	5.3	11
ATP (5 mM)+PPi (1 mM)	49.7	357
ADP (5 mM)+PPi (1 mM)	35	250
All assays contained 10 mM DTT		

**Table 1:**  $PiGlcK-1$  activity with different substrates.

The enzymes of the large Hexokinase family (groups HK, A or B) catalyse the phosphorylation of hexoses and in some cases only glucose by means of phosphoryl donors (ATP, ADP, PPi and inorganic polyphosphate [poly (P)] [9,10,31-33]. To analyze whether  $PiGlcK-1$  could use other phosphoryl group donors, various enzyme assays were performed in the presence of ADP or PPi. We were able to determine that  $PiGlcK-1$ , like other GlcKs, particularly those from Archaea, can use ATP and ADP. However, ADP was less efficient, that is to say around 50%, concerning ATP. Similarly, a small activity of 11% with respect to ATP was found, when the phosphoryl donor was PPi (Table 1). These results are similar to that of other GlcKs, particularly in GlcKs from Archaea or *B. stearothermophilus*, which can use both ATP and ITP as nucleotide substrate [10]. Even the GlcK of the hyperthermophilic archon *Pyrococcus furiosus* uses ADP as a phosphoryl donor and has no detectable activity with ATP or other potential donors such as GDP or pyrophosphate [34]. Another unusual example is provided by GlcK from the anaerobic bacterium *Propionibacterium shermanii*, in which this enzyme can use ATP as a phosphoryl donor. However, it is much more specific for polyphosphates [35].

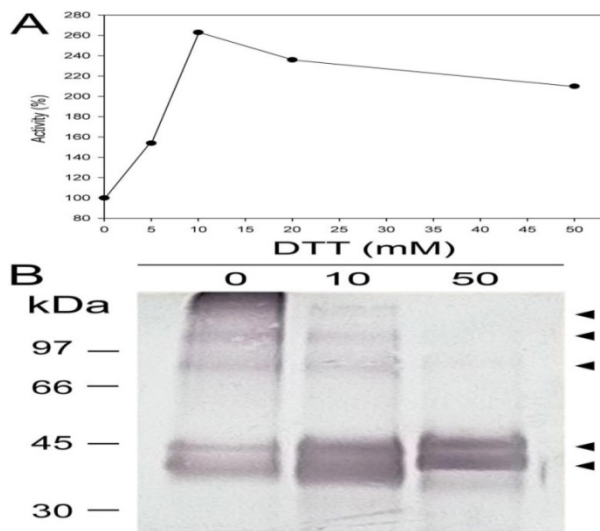
Pyrophosphate has been previously reported to serve as an inhibitor of the activity of some kinases [36-39]. For this reason, we tested PPi as a possible inhibitor of  $PiGlcK-1$  activity in the presence of ATP or ADP. Surprisingly, in the presence of PPi,  $PiGlcK-1$  activity, far from being inhibited, showed an increase of more than three times when the phosphoryl donor was ATP and more than two times when the phosphoryl donor was ADP (Table 1). A study of ATP-dependent  $PiGlcK-1$  activity by varying PPi concentrations showed that it peaked at a PPi concentration between 0.75 and 1 mM. Interestingly, this activation decreased when PPi concentrations were greater than 1 mM (Figure S1). Until now, there are no reports of other eukaryotic GlcKs that are activated by PPi, and when it has exerted some effect it has been as an inhibitor [10].

The effect of the hydrogenation concentration on the  $PiGlcK-1$  activity showed an optimal value for pH between 9.0 and 9.5 (Figure S2). This optimal pH is similar to those obtained for GlcKs from different organisms [11,40,41].

### Effect of Dithiothreitol on the oligomeric forms and the activity of $PiGlcK-1$

Glucokinases are characterized by a high susceptibility to the presence of reducing agents such as DTT or glutathione [42-46]. In our case, the activity of  $PiGlcK-1$  showed an increase (more than double the activity) in the presence of 10 mM DTT. However, higher concentrations of DTT produced an inhibitory effect on its activity (Figure 5A). The effect of ionic strength was ruled out since the activity of  $PiGlcK-1$  in the presence of other salts did not show an increase in the activity of the enzyme. This phenomenon of DTT activation has been reported for several kinases from various organisms [40,47-50]. Interestingly, an analysis of the  $PiGlcK-1$  sequence showed the presence of ten

cysteines that could potentially participate in the formation of multiple inter and intramolecular disulfide bonds, which would be sensitive to reducing agents.



**Figure 5:** Effect of Dithiothreitol on the oligomeric forms and activity of *PiGlcK-1*. (A) Effect of the DTT concentration on the activity. The enzyme activity was measured as described under materials and methods. The reaction mixture contained 100 mM TEA pH 7.6, 5 mM MgCl<sub>2</sub>, 5 mM glucose, 5 mM ATP, 0.72 mM NAD<sup>+</sup> and 1U of G6PDH and variable concentration of DTT (0-50 mM). (B) Western immunoblotting analysis after denaturalizing electrophoresis using anti-*PiGlcK-1*. 30 μg of *PiGlcK-1* purified was incubated with 0 mM Dithiothreitol (DTT), 10 mM and 50 mM.

In order to see if the effect of DTT on *PiGlcK-1* could also be related to the presence of possible oligomeric states of the enzyme, a Western blot was analyzed in the presence and absence of DTT. It was observed that *PiGlcK-1* in the absence of DTT can be found in different oligomeric states (Figure 5B). Although several bands were observed, the most evident corresponds to the monomer (38 kDa) in addition to lower intensity bands corresponding to a dimer (90 kDa) and oligomeric states with masses greater than the latter. Interestingly, the signal from the high molecular weight bands decreased in intensity when the DTT concentration was increased from 10 mM to 50 mM. This result could suggest the presence of oligomeric forms (involving disulfide bridges), in addition to the monomeric form of the enzyme. Interestingly, a double band electrophoretic pattern was observed in the region corresponding to the monomer, both under reducing and non-reducing conditions. This behavior has already been observed for human glucokinase and can be interpreted as an incomplete reduction of disulfide bonds to which DTT would not have had access due to the spatial arrangement of the residues of cysteine in protein [51]. Therefore, this incomplete reduction could give rise to two populations of monomeric *PiGlcK-1* whose conformational states can be evidenced electrophoretically.

## DISCUSSION

In this study we cloned, overexpressed, and kinetically characterized the *P. infestans* *PiGlcK-1* [PITG\_06016]. Based on its sequence, this enzyme belongs to the glucokinase group A from the Hexokinase Family by code IPR003836 [9,52]. However, our kinetic studies showed that *PiGlcK-1* can phosphorylate both glucose and fructose, indicating that it is not a true glucokinase. Another unusual case for group A enzyme that are not specific for glucose is the GlcK from *E. coli* B [40]. Furthermore, the evidence that *PiGlcK-1* can use ATP, ADP or P<sub>i</sub> as donors of phosphoryl groups, suggests that its current classification as a strict glucokinase should be reviewed and this suggests that group A from Hexokinase family can contain a more varied type of enzymes.

Surprisingly, when we included P<sub>i</sub> as possible inhibitors of *PiGlcK-1* activity in the presence of ATP or ADP, the enzyme was activated 3.6 times and 2.5 times when the phosphoryl donor was ATP or ADP, respectively. These results suggest the presence of a P<sub>i</sub> binding site different from that of ATP, as reported for *Zymomonas mobilis* GlcK, which is activated by phosphate [53]. These metabolites could be related with to a low energetic status in cell, contributing in the regulation of this enzyme. In addition, it has already been reported that oomycetes diverge from the classic form of eukaryotic glycolysis by expressing phosphofructokinase (PFK) that use P<sub>i</sub> (EC 2.7.1.90) instead of ATP (EC 2.7.1.11) as a phosphoryl donor [4]. Thus, although several ancestors of oomycete glycolytic enzymes are assumed, *PiGlcK-1* forms a solid clade, along with other species of *Phytophthora* and other oomycetes, reflected by the high identity in their amino acid sequence, perhaps due to the particularity of using ATP, ADP and P<sub>i</sub> as phosphoryl donors.

Another interesting fact was the activation of *PiGlcK-1* by the reducing agent DTT. This and other sulfhydryl compounds play a role in the stability and catalytic activity of GlcK in various organisms [42-48]. Interestingly, *PiGlcK-1* showed ten cysteines in its sequence, of which three are close to the glucose and ATP binding motif (Cys12, Cys172 and Cys175) and could possibly play a role in enzymatic activation. The other cysteines may be involved in oligomeric associations mediated by intermolecular disulfide bridges.

The reduction of disulfide bonds affects the molecular flexibility of proteins [51], which may favours the destabilization of possible oligomeric states. Here we obtained electrophoretic evidence indicating that in the presence or absence of DTT, *PiGlcK-1* can be found in different oligomeric states, whose transition would be associated to a greater or lesser activity of the enzyme and that, could possibly be related to the delay of this activity observed in the progress curve. This aggregation behaviour involving cysteines has been reported for cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Arabidopsis thaliana*, which under conditions of oxidative stress forms inactive aggregation states, which can be reversed in the presence of DTT [54]. It has also been proposed that these disulfide bonds could fulfil the role of redox switches rather than as stabilizers of the native structure [55]. In the case of *PiGlcK-1*, the susceptibility to the reduction of disulfide bonds, under certain extracellular environments of high oxidative

stress, could represent a sensor of such changes, allowing this enzyme to modulate its activity.

In accordance with this, it has previously been reported that in solanaceous plants, infection by *P. infestans* involves the release of Reactive Oxygen Species (ROS) [56]. In response to this, there is a large abundance of enzymes related to redox metabolism in germinated cysts and *P. infestans* appressorium to combat oxidative stress [15]. Therefore, the high expression of PiGlcK-1 in structures related to the establishment of infection could suggest a relationship with high levels of PiGlcK-1 and the need for a high supply of NADPH to fuel this redox metabolism. Thus, PiGlcK-1 would not only be limited to energy generation through glycolysis, phosphorylating available D-glucose and D-fructose, but would also provide glucose-6-phosphate necessary to feed the pentose-phosphate pathway generating NADPH. Actually, PiGlcK-1 can phosphorylate both  $\alpha$ - and  $\beta$ -glucose, giving rise to  $\alpha$ - and  $\beta$ -glucose-6-phosphate (G6P), respectively. In this sense, the anomeric selectivity of G6PDH by  $\beta$ -G6P [57-59] would improve the metabolic flow towards the pentose phosphate pathway. Moreover, the consumption of  $\beta$ -G6P by G6PDH, together with a spontaneous anomerization of the  $\alpha$  to  $\beta$  form, would further contribute to the displacement of this balance, decreasing the concentration of a G6P. This would favour fructose phosphorylation to feed the glycolytic pathway.

The role of various GlcKs that are expressed at all stages of the life cycle of *P. infestans* is still unclear, but differences regarding the specificity of sugars and phosphoryl donors, as well as a diversification of functions, indicate that a mechanism efficient for feeding during the progress of infection should be considered. In this sense, the fact that PiGlcK-1 is actually behaving as a HK, would allow it to use glucose and fructose present in the apoplast of the host cell during the biotrophic phase of infection [7,15]. Additionally, an invertase activity has been reported during infection, which makes use of the sucrose present in the plant to produce D-glucose and D-fructose that can be transported into the phytopathogen [17,60].

This possible participation of PiGlcK-1 during the establishment of the infection to take advantage of other sugars available at this stage coincides with that observed in the transcriptomic models of the dynamics of tomato infection by *P. infestans*, where it is observed that the reactions of *P. infestans* are enriched for glycolysis at the beginning of the infection. However, as the infection progresses, this plant pathogen becomes less dependent on its own metabolism and can continue to obtain its nutrients through the transport of metabolites from the tomato necrotic lesion, including D-fructose [7]. All of the above would allow the maintenance of an optimal glycolytic flow not only for obtaining energy but also for supplying the NADPH necessary for the survival of the pathogen in conditions of oxidative stress as a result of the hypersensitive response of its host.

## CONCLUSION

The characterization of the recombinant enzyme PiGlcK-1 gives us indications of a diversification of functions of this enzyme, as well as of its possible role in the metabolism of *P. infestans*. In

this sense, we propose a versatility of this enzyme to phosphorylate D-glucose and D-fructose present during the first stage of infection. While, during the necrotrophic stage, when ATP concentrations could be lower, PiGlcK-1 would continue to phosphorylate sugars thanks to its ability to use other donors of phosphoryl groups, as well as activation by P<sub>PPi</sub>, ADP or DTT. Furthermore, PiGlcK-1 could respond to variation in a redox environment, modulating its activity and increasing glucose phosphorylation for NADPH synthesis.

## REFERENCES

1. Kamoun S, Furzer O, Jones JDG, Judelson HS, Ali GS, Dalio RJD, et al. The Top 10 oomycete pathogens in molecular plant pathology. *Mol Plant Pathol*. 2015;16(4):413-434.
2. Majeed A, Muhammad Z, Ullah Z, Ullah R, Ahmad H. Late Blight of Potato (*Phytophthora infestans*) I: Fungicides Application and Associated Challenges. *Turkish J Agric Food Sci Technol*. 2017;5(3):261-266.
3. Fry WE. *Phytophthora infestans*: New Tools (and Old Ones) Lead to New Understanding and Precision Management. *Annu Rev Phytopathol*. 2016;54:529-547.
4. Marshall JS, Ashton AR, Govers F, Hardham AR. Isolation and characterization of four genes encoding pyruvate, phosphate dikinase in the oomycete plant pathogen *Phytophthora cinnamomi*. *Curr Genet*. 2001;40:73-81.
5. Giraldo OMD, Jones RW. Characterization of the glucose-6-phosphate isomerase gene in *Phytophthora infestans* reveals the presence of multiple alleles. *Fungal Genet Biol*. 2003;40(3):197-206.
6. Abrahamian M, Kagda M, Fong AAMV, Judelson HS. Rethinking the evolution of eukaryotic metabolism: Novel cellular partitioning of enzymes in stramenopiles links serine biosynthesis to glycolysis in mitochondria. *BMC Evol Biol*. 2017;17:241.
7. Rodenburg SYA, Seidl MF, Judelson HS, Vu AL, Govers F, Ridder DD. Metabolic model of the *Phytophthora infestans*-tomato interaction reveals metabolic switches during host colonization. *Am Soc Microbiol*. 2019;10(4): e00454-e00519.
8. Gilmore FLA, Michels PA. Evolution of glycolysis. *Prog Biophys Mol Biol*. 1993;59(2):105-235.
9. Kawai S, Mukai T, Mori S, Mikami B, Murata K. Hypothesis: Structures, evolution, and ancestor of glucose kinases in the hexokinase family. *J Biosci Bioeng*. 2005;99(4):320-330.
10. Cárdenas ML, Bowden CA, Ureta T. Evolution and regulatory role of the hexokinases. *Biochim Biophys Acta-Mol Cell Res*. 1998;1401(3):242-264.
11. Cáceres AJ, Quiñones W, Gualdrón M, Cordeiro A, Avilán L, Michels PAM, et al. Molecular and biochemical characterization of novel glucokinases from *Trypanosoma cruzi* and *Leishmania* spp. *Mol Biochem Parasitol*. 2007;156(2):235-245.
12. Guixé V, Merino F. The ADP-dependent sugar kinase family: Kinetic and evolutionary aspects. *IUBMB Life*. 2009;61(7):753-761.
13. Haas BJ, Kamoun S, Zody MC, Jiang RHY, Handsaker RE, Cano LM, et al. Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature*. 2009;461(7262):393-398.
14. Ah-Fong AMV, Kim KS, Judelson HS. RNA-seq of life stages of the oomycete *Phytophthora infestans* reveals dynamic changes in metabolic, signal transduction, and pathogenesis genes and a major role for calcium signaling in development. *BMC Genomics*. 2017;18(1):198.

15. Judelson HS. Metabolic Diversity and Novelty in the Oomycetes. *Annu Rev Microbiol.* 2017;71(1):21-39.
16. Mendgen K, Hahn M. Plant infection and the establishment of fungal biotrophy. *Trends Plant Sci.* 2002;7(8):352-356.
17. Voegele RT, Struck C, Hahn M, Mendgen K. The role of haustoria in sugar supply during infection of broad bean by the rust fungus *Uromyces fabae*. *Proc Natl Acad Sci U S A.* 2001;98(14):8133-8138.
18. Calcagno LPE, Moreno J, Cedeño L, Labrador L, Concepción JL, Avilán L. Cloning, expression and biochemical characterization of mitochondrial and cytosolic malate dehydrogenase from *Phytophthora infestans*. *Mycol Res.* 2009;113(6-7):771-781.
19. Edgar RC. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 2004;32(5):1792-1797.
20. Chang JM, Di Tommaso P, Notredame C. TCS: A new multiple sequence alignment reliability measure to estimate alignment accuracy and improve phylogenetic tree reconstruction. *Mol Biol Evol.* 2014;31(6):1625-1637.
21. Kalyanamoorthy S, Minh BQ, Wong TKF, Haeseler AV, Jermini LS. ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nat Methods.* 2017;14(6):587-589.
22. Letunic I, Bork P. Interactive Tree Of Life (iTOL): An online tool for phylogenetic tree display and annotation. *Bioinformatics.* 2007;23(1):127-128.
23. Studier FW. Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif.* 2005;41(1):207-234.
24. Lenzen S, Tiedge M, Panten U. Glucokinase in pancreatic B-cells and its inhibition by alloxan. *Acta Endocrinol (Copenh).* 1987;115(1):21-29.
25. Cordeiro AT, Cáceres AJ, Vertommen D, Concepción JL, Michels PAM, Versées W. The Crystal Structure of *Trypanosoma cruzi* Glucokinase Reveals Features Determining Oligomerization and Anomer Specificity of Hexose-phosphorylating Enzymes. *J Mol Biol.* 2007;372(5):1215-1226.
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72(1-2):248-254.
27. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227:680-685.
28. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual.* Second Edition. Cold Spring Harbor Laboratory Press. 1989. P68.
29. Lunin VV, Li Y, Schrag JD, Iannuzzi P, Cygler M, Matte A. Crystal structures of *Escherichia coli* ATP-dependent glucokinase and its complex with glucose. *J Bacteriol.* 2004;186(20):6915-6927.
30. Meyer D, Schneider-Fresenius C, Horlacher R, Peist R, Boos W. Molecular characterization of glucokinase from *Escherichia coli* K-12. *J Bacteriol.* 1997;179(4):1298-1306.
31. Hansen T, Reichstein B, Schmid R, Schönheit P. The first archaeal ATP-dependent glucokinase, from the hyperthermophilic crenarchaeon *Aeropyrum pernix*, represents a monomeric, extremely thermophilic ROK glucokinase with broad hexose specificity. *J Bacteriol.* 2002;184(21):5955-5965.
32. Dörr C, Zaparty M, Tjaden B, Brinkmann H, Siebers B. The hexokinase of the hyperthermophile *Thermoproteus tenax*: ATP-dependent hexokinases and ADP-dependent glucokinases, two alternatives for glucose phosphorylation in Archaea. *J Biol Chem.* 2003;278(21):18744-18753.
33. Mukai T, Kawai S, Mori S, Mikami B, Murata K. Crystal structure of bacterial inorganic polyphosphate/ATP-glucomannokinase: Insights into kinase evolution. *J Biol Chem.* 2004;279(48):50591-50600.
34. Kengen SWM, Tuininga JE, De Bok FAM, Stams AJM, De Vos WM. Purification and characterization of a novel ADP-dependent glucokinase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Biol Chem.* 1995;270(51):30453-30457.
35. Phillips NFB, Horn PJ, Wood HG. The polyphosphate-and ATP-dependent glucokinase from *Propionibacterium shermanii*: Both activities are catalyzed by the same protein. *Arch Biochem Biophys.* 1993;300(1):309-319.
36. Wilson JE. An introduction to the isoenzymes of mammalian hexokinase types I-III. *Biochem Soc Trans.* 1997;25(1):103-107.
37. Cáceres AJ, Portillo R, Acosta H, Rosales D, Quiñones W, Avilán L, et al. Molecular and biochemical characterization of hexokinase from *Trypanosoma cruzi*. *Mol Biochem Parasitol.* 2003;126(2):251-262.
38. Acosta H, Dubourdiou M, Quiñones W, Cáceres A, Bringaud F, Concepción JL. Pyruvate phosphate dikinase and pyrophosphate metabolism in the glycosome of *Trypanosoma cruzi* epimastigotes. *Comp Biochem Physiol B Biochem Mol Biol.* 2004;138(4):347-356.
39. Pabón MA, Cáceres AJ, Gualdrón M, Quiñones W, Avilán L, Concepción JL. Purification and characterization of hexokinase from *Leishmania mexicana*. *Parasitol Res.* 2007;100(4):803-810.
40. Fukuda Y, Yamaguchi S, Shimosaka M, Murata K, Kimura A. Purification and characterization of glucokinase in *Escherichia coli* B. *Agric Biol Chem.* 1984;48(10):2541-2548.
41. Milanes JE, Suryadi J, Abendroth J, Van Voorhis WC, Barrett KF, Dranow DM, et al. Enzymatic and structural characterization of the *Naegleria fowleri* Glucokinase. *Antimicrob Agents Chemother.* 2019;63(5):e02410-e02418.
42. Salas J, Salas M, Vinuela E, Sols A. Glucokinase of rabbit liver. *J Biol Chem.* 1965;240:1014-1018.
43. Parry MJ, Walker DG. Purification and properties of adenosine 5'-triphosphate-D-glucose 6-phosphotransferase from rat liver. *Biochem J.* 1966;99(2):266-274.
44. Pilks SJ. Rat hepatic glucokinase: Improved purification and some properties. *Arch Biochem Biophys.* 1972;149(2):349-360.
45. Grossman SH, Dorn CG, Potter VR. The preparation and characterization of pure rat liver glucokinase. *J Biol Chem.* 1974;249(10):3055-3060.
46. Holroyde MJ, Allen MB, Storer AC, Warsy AS, Chesher JM, Trayer IP, et al. The purification in high yield and characterization of rat hepatic glucokinase. *Biochem J.* 1976;153(2):363-373.
47. Tippett PS, Neet KE. Interconversions between different sulfhydryl-related kinetic states in glucokinase. *Arch Biochem Biophys.* 1983;222(1):285-298.
48. Vogel WK, Keenan RP, Gelev CW, Neet KE. The regulatory kinetic properties of porcine hepatic glucokinase. *Mol Cell Biochem.* 1989;86(2):171-179.
49. Lenzen S, Freytag S, Panten U, Flatt PR, Bailey CJ. Alloxan and Ninyhydrin Inhibition of Hexokinase from Pancreatic Islets and Tumoural Insulin-Secreting Cells. *Pharmacol Toxicol.* 1990;66:157-162.
50. Avilán L, Gontero B, Lebreton S, Ricard J. Memory and imprinting effects in multienzyme complexes I. Isolation, dissociation, and reassociation of a phosphoribulokinase-glyceraldehyde-3-phosphate dehydrogenase complex from *Chlamydomonas reinhardtii* chloroplasts. *Eur J Biochem.* 1997;246(1):78-84.
51. Tiedge M, Richter T, Lenzen S. Importance of cysteine residues for the stability and catalytic activity of human pancreatic beta cell glucokinase. *Arch Biochem Biophys.* 2000;375(2):251-260.
52. Mitchell AL, Attwood TK, Babbitt PC, Blum M, Bork P, Bridge A, et al. InterPro in 2019: Improving coverage, classification and



- access to protein sequence annotations. *Nucleic Acids Res.* 2019;47(D1):D351-D360.
53. Scopes RK, Bannon DR. Kinetic analysis of the activation of *Zymomonas mobilis* glucokinase by phosphate. *Biochim Biophys Acta (BBA)/Protein Struct Mol.* 1995;1249(2):173-179.
  54. Zaffagnini M, Marchand CH, Malferrari M, Murail S, Bonacchi S, Genovese D, et al. Glutathionylation primes soluble glyceraldehyde-3-phosphate dehydrogenase for late collapse into insoluble aggregates. *Proc Natl Acad Sci U S A.* 2019;116(51):26057-26065.
  55. Wouters M, George R, Haworth N. "Forbidden" Disulfides: Their Role as Redox Switches. *Curr Protein Pept Sci.* 2007;8(5):484-495.
  56. Doke N, Miura Y, Sanchez LM, Park HJ, Noritake T, Yoshioka H, et al. The oxidative burst protects plants against pathogen attack: Mechanism and role as an emergency signal for plant bio-defence- A review. *Gene.* 1996;179(1):45-51.
  57. Salas M, Vinuela E, Sols A. Spontaneous and enzymatically catalyzed anomerization of Glucose. *J Biol Chem.* 1965;240:561-568.
  58. Smith JE, Beutler E. Anomeric specificity of human erythrocyte glucose-6-phosphate dehydrogenase. *Proc Soc Exp Biol Med.* 1966;122(3):671-673.
  59. Malaisse-Lagae F, Malaisse WJ. Anomeric specificity of d-glucose phosphorylation and oxidation in human erythrocytes. *Int J Biochem.* 1987;19(8):733-736.
  60. Voegelé RT, Mendgen K. Rust haustoria: Nutrient uptake and beyond. *New Phytol.* 2003;159(1):93-100.