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Heterologous expression and kinetic characterization of the α, β and αβ blend of the PPi-dependent phosphofructokinase from *Citrus sinensis*

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KEYWORDS:

Citrus sinensis; PPi-dependent phosphofructokinase; fructose-2,6-bisphosphate; orange; hetero-hexamer; recombinant protein.

ABBREVIATIONS

PPi-PFK	pyrophosphate-dependent phosphofructokinase
ATP-PFK	ATP-dependent phosphofructokinase
Fru6P	fructose-6-phosphate
Fru1,6bisP	fructose-1,6-bisphosphate
Fru2,6bisP	fructose-2,6-bisphosphate

HIGHLIGHTS

- Heterologous expression of a plant pyrophosphate-dependent phosphofructokinase
- Kinetic characterization of orange PPi-PFKase and its regulation by Fru2,6bisP.
- Fru2,6bisP activates the hetero-hexamer while α is not affected and β is inhibited.
-The $\alpha_3\beta_3$ hexamer is three orders of magnitude more active than each single subunits.

ABSTRACT

This work reports the molecular cloning and heterologous expression of the genes coding for α and β subunits of pyrophosphate-dependent phosphofructokinase (PPi-PFK) from orange. When expressed individually, both recombinant subunits were produced as highly purified monomeric proteins able to phosphorylate fructose-6-phosphate at the expenses of PPi (specific activity of 0.075 and 0.017 units.mg^{-1} for α and β subunits, respectively). On the other hand, co-expression rendered a $\alpha_3\beta_3$ hexamer with specific activity three orders of magnitude higher than the single subunits. All the conformations of the enzyme were characterized with respect to its kinetic properties and sensitivity to the regulator fructose-2,6-bisphosphate. A thorough review of current knowledge on the matter indicates that this is the first report of the recombinant production of active plant PPi-PFK and the characterization of its different conformations. This is a main contribution for future studies focused to better understand the enzyme properties and how it accomplishes its relevant role in plant metabolism.

1. INTRODUCTION

Pyrophosphate dependent phosphofructokinase (PPi-PFK, EC 2.7.1.90) is a glycolytic/gluconeogenic enzyme that phosphorylates fructose-6-phosphate (Fru6P) to fructose-1,6-bisphosphate (Fru1,6bisP) using inorganic pyrophosphate (PPi) as a phosphoryl donor in a reversible enzymatic reaction [1-3].

$$\text{Fru6P} + \text{PPi} \leftrightarrow \text{Fru1,6bisP} + \text{Pi}$$

The enzyme was first discovered in 1974 in *Entamoeba histolytica* [3] (a human pathogenic protozoan). It was later found in Gram-positive bacteria [1, 4] and also in higher plants and algae [5, 6]. The occurrence of PPi-PFK in these organisms provides an alternative to the cytosolic ATP-dependent phosphofructokinase (ATP-PFK, EC 2.7.1.11) that also phosphorylates Fru6P to Fru1,6bisP during glycolysis, but using ATP instead of PPi. After many years of research, it is clear that the major regulation point in non-plant glycolysis is exerted at the level of ATP-PFK, which controls the conversion of Fru6P into Fru1,6bisP [7-9]. But in plants, glycolysis is mainly regulated at the level of phosphoenolpyruvate metabolism, being the phosphorylation of Fru6P a secondary control point [8].

The kinetic, regulatory and structural characterization of PPi-PFK from plants was mainly achieved by purifying and characterizing the enzyme from different plant species and tissues [10-23]. These studies established that PPi-PFK in plants is generally composed of α and β subunits arranged in different hetero-oligomeric structures. *Brassica nigra* and *Musa cavendishii* have a PPi-PFK with a heterooctameric structure ($\alpha_4\beta_4$) [21, 23], while the enzyme from *Daucus carota* showed a heterotetrameric ($\alpha_2\beta_2$) conformation [24]. Potato tuber PPi-PFK has been structurally described either as a symmetrical $\alpha_2\beta_2$ tetramer [16] or as a $\alpha_4\beta_4$ heterooctamer [19]. Additionally, in *Ananas comosus* the active enzyme is a dimer [22]. Regulation of the enzyme is exerted primarily by activation by Fru2,6bisP [8, 9, 20, 22]. Based on 3D modelling of grapefruit PPi-PFK and sequence data, several authors [25-27] have suggested that only the β subunit carries the catalytic site, and that the α subunit is essential for activation as the Fru2,6bisP docking site is actually formed in the interface of both subunits [25, 26]. In *Ricinus communis* cotyledons, the profile of PPi-PFK activity during germination closely follows the level of β subunit present [28]. However, the existence of enzyme forms activated by Fru2,6bisP but lacking the α subunit [22] makes things not clear in this subject. Phospoenolpyruvate is an allosteric

inhibitor of the enzyme, and also has the effect of decreasing Fru2,6bisP levels [8]. The relative amount of α and β subunits in different plant tissues depends on factors like nutrient availability, environmental stress or developmental stage of the plant tissue [19, 21, 29]. It has been suggested that a coarse regulation of the enzyme activity would be exerted by such a differential subunit expression [28, 29]. For this and other reasons the role of PPi-PFK in relation to stress response and its function in plant growth has been investigated. Lim *et al.* (2009) [30] showed that Arabidopsis plants with altered PPi-PFK expression (overexpressing or repressing the enzyme) have altered growth patterns, in a manner that high PPi-PFK levels promote earlier development, while decreased expression retards it, in contest to earlier findings showing the opposite (i.e. no effect on growth) in genetically altered potato [31], tobacco [32] and sugarcane plants [15]. Moreover, they showed that these altered expression patterns affect the expression levels of several metabolically important enzymes, which suggests that PPi-PFK has a widespread effect on general metabolism that goes beyond its activity. In a later paper Lim *et al*. (2014)[33], the same group obtained double and quadruple mutants of Arabidopsis deficient in both α1 or α2, β1 or β2 subunits or in all them. They found that Pi or N deprivation did not affect significantly the growth rate compared to controls. However, these authors observed that PPi-PFK is necessary to withstand salt and osmotic stress at germination and during seedling growth and concluded that the enzyme is needed during acclimation to these type of stress conditions, perhaps because of PPi-PFK´s central role in carbohydrate metabolism, that leads to an accumulation of compatible solutes during salt/water stress. Nielsen and Stitt (2001) [32] observed that the base tip of young leaves, an actively growing tissue, contains higher PPi-PFK activity, supporting the hypothesis that the enzyme´s roles in young and mature tissue are different. These authors, as others, also noted that transformants with reduced PPi-PFK (i.e. less than 10% of the wild type) did not show a corresponding metabolic shift. Instead, higher levels of Fru2,6bisP could compensate for the activity loss. Notwithstanding the extensive research dedicated to PPi-PFK, an in-depth examination of the enzyme structure-to-function relationship is still lacking and many questions remain open. Perhaps the main causes are the technical aspects regarding the isolation of the isolated subunits from the plant tissues.

In this work, two *pfp* genes from *Citrus sinensis* (orange) coding for α and β subunits of PPi-PFK were cloned and expressed by a recombinant strategy. Using *Escherichia coli* as a host, the individual expression of the recombinant α and β subunits

and also the coexpression of both was achieved. All the purified proteins were studied to determine its kinetic parameters and comparatively analyzed in order to better understand the role of each subunit of the plant enzyme.

2. MATERIALS AND METHODS

2.1. Chemicals

Protein standards, antibiotics, isopropyl-thiogalactoside (IPTG), Fru6P, Fru2,6bisP, PPi, Pi, and oligonucleotides were obtained from Sigma-Aldrich. All other reagents were of the highest quality available.

2.2. Gene amplification

The *pfpα* and *pfpβ* genes (respectively coding for subunits α and β of PPi-PFK) from *C. sinensis* (sweet orange, Valencia Late variety) were amplified by PCR by using orange endocarp mRNA, prepared by standard protocols [34] and accurate primer oligonucleotides. Since at the moment of these experiments the orange genome was not yet elucidated, oligonucleotides for PCR amplification were designed using the published mRNA sequences from *Citrus x paradise* (pummelo), AF095521 and AF095520. For *pfpα*, the forward primer (5′-CATATGGACTCGGATTTCGGTATTCC-3′) introduced an *Nde*I site (underlined) overlapping the translational initiation codon; whereas the reverse primer (5′-GGTACCTTAAAGTGTTGTCTGGCCTC-3′) introduced a *Kpn*I site (underlined) downstream the stop codon. For *pfpβ* amplification, the forward primer (5′-GGTACCAATGTCTCCGTCCTTGGTGGCAAAC-3′) introduced a *BamH*I site (underlined), while the reverse primer (5′-GTCGACTCATACTTGAGCACCGAGTT-3′) introduced a *Sal*I site (underlined) downstream the stop codon. Amplified genes were cloned in pGEM-T Easy, sequenced and annotated in the NCBI GenBank as EU302909.1 (*Csipfpα*) and EU302910.1 (*Csipfpβ*).

2.3. Bacteria and plasmids

Escherichia coli Top 10 F′ cells (Invitrogen) and pGEMT-Easy vector (Promega) were used for cloning procedures. The *pfpα* and *pfpβ* genes from *C. sinensis* (*Csipfpα* and *Csipfpβ*) were expressed in *E. coli* BL21 (DE3; Novagen) using the pET28c, pRSFDuet and pETDuet (Novagen) expression plasmids. Alternatively, *Csipfpα* was

expressed in *E. coli* BL21 Rosetta (DE3; Novagen). DNA manipulations, *E. coli* cultures as well as transformations were performed according to standard protocols [35].

2.4. Cloning procedures

The pGEM-T Easy plasmid harboring the *Csipfpα* coding sequence was digested with *Nde*I and *Kpn*I restriction enzymes and the gene subcloned into the multiple cloning site 2 (MCS-2) of pETDuet expression vector cut with the same restriction enzymes, allowing the protein expression without tags. Then the *Csipfpα* gene was subcloned from pETDuet into pET28c plasmid, using *Nde*I and *Xho*I (downstream the *Kpn*I in the pETDuet MCS) sites to obtain the [pET28c/*Csipfpα*] construction adding an N-terminal His-tag. The pGEM-T Easy plasmid harboring the *Csipfpβ* coding sequence was digested with *BamH*I and *Sal*I restriction enzymes. The released gene was cloned into pRSFDuet, to obtain the expression vector [pRSFDuet/*Csipfpβ*] that allows expression with an N-terminal His-tag. The constructions [pETDuet/*Csipfpα*] and [pRSFDuet/*Csipfpβ*] were also used for co-expression of the subunits to obtain the hetero-oligomeric form of the PPi-PFK. Figure 1 summarizes the general strategies conducted to produce the PPi-PFK from *C. sinensis* in different arrangements.

2.5. Enzyme expression

E. coli cells were transformed with either [pET28c/*Csipfpα*] or [pRSFDuet/*Csipfpβ*] to respectively express subunit α or β protein individually. For expression of the heteromeric αβ protein, cells were co-transformed with the [pETDuet/*Csipfpα*] and [pRSFDuet/*Csipfpβ*] compatible plasmids. All transformed cells were grown in 1 l of LB medium (10 $g.l^{-1}$ tryptone; 5 $g.l^{-1}$ yeast extract; 10 $g.l^{-1}$ NaCl) at 37 °C and 200 rpm, until an OD_{600} of ~0.8. Then, recombinant expression was induced (during 16 h at 20 °C) with 0.4 mM IPTG. After induction, cells were harvested by centrifugation at 5000 ×*g* for 10 min and store at -20°C until use, obtaining ~5 g FW biomass per liter.

2.6. Purification of the recombinant protein

Purification procedures were done at 4°C. All versions of the recombinant proteins were purified by Immobilized Metal Ion Affinity Chromatography (IMAC). Biomass was suspended in 5 ml of buffer A [HEPES 50 mM pH 7.5; 5% (v/v) glycerol] per g of biomass. Cells were disrupted by sonication on ice (2 s pulse on with intervals

of 4 s pulse off for a total time of 10 min) and centrifuged at 20000 ×*g* for 15 min. Supernatants were loaded in a 1 ml HisTrap column (GE Healthcare) previously equilibrated with buffer A. After sample loading, the column was washed with 10 ml of buffer A and the recombinant proteins were eluted with a linear gradient (0-300 mM) of imidazole in buffer A. Fractions containing the highest activity were pooled and stored at -80°C until use, conditions under which they remained fully active for at least 2 months.

2.7. Protein methods

Protein concentration was determined by the modified Bradford assay [36] using BSA as a standard. Recombinant proteins and purification fractions were defined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [37].

Immunoblotting was performed according to the method of Bollag and Edelstein (1991) [38]. Samples were run on a denaturing PAGE and transferred to nitrocellulose membrane. Anti-potato tuber PPi-PFK serum was obtained as in Moorhead (1991) [39]. The antiserum was immunopurified against the α or β subunits of potato tuber PPi-PFK as described by Plaxton (1989) [40]. For this, the purified potato tuber enzyme was separated by SDS-PAGE, electroblotted onto a nitrocellulose membrane and the bands corresponding to each subunit were excised and used for the purification procedure.

2.8. Molecular mass determination

Proteins molecular masses were determined by gel filtration using a Superdex 200 Tricorn 10/300 (GE Healthcare). A gel filtration molecular mass (high and low) calibration kit (GE Healthcare) with protein standards including thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), ribonuclease (13.7 kDa) and aprotinin (6.5 kDa) were used. The column void volume was determined using dextran blue. A calibration curve was constructed by plotting K_{av} vs $\log_{10}$ molecular mass from standard proteins. $K_{av} = (V_e - V_0) / (V_t - V_0)$, where V_0 = void volume; V_e = elution volume and V_t = total volume. The sample volume used was 100 µl and a flow rate of 0.75 ml/min. Elution was monitored at 280 nm. Collected fractions were subjected to enzyme activity and SDS-PAGE.

2.9. Enzyme activity assays

PPi-PFK activity was determined in the Fru1,6bisP synthesis direction at 37 °C by following the formation of Pi with the highly sensitive colorimetric method of Malachite Green adapted from Fusari *et al*. [41]. The reaction mixture for hetero-oligomeric PPi-PFK and homo-β contained 50 mM HEPES-NaOH pH 7.5, 2 mM $MgSO_4$, 5 mM Fru6P, 2.5 mM PPi, 0.2 $mg.ml^{-1}$ bovine serum albumin and a proper enzyme dilution. For activity assay of the homo-α protein conditions were similar except that the concentration of PPi was 1 mM (since inhibition was observed at higher substrate levels). Assays were initiated by addition of Fru6P in a total reaction volume of 50 µl. Reaction plates were incubated at 37°C for 10 min and stopped by the addition of the Malachite Green reactive. The complex formed by Malachite Green and Pi released from the enzyme reaction was measured at 630 nm in a Multiskan Ascent ELISA microplate photometer (Thermo Scientific). Sodium phosphate was used as standard for the whole procedure.

Alternatively, the production of Fru1,6bisP was tested by assaying PPi-PFK by the coupled enzyme spectrophotometric assay [22] under the same conditions as the discontinuous method.

One unit (U) of activity is defined as the amount of enzyme catalyzing the formation of 1 µmol of product per min, under the conditions above described in each case. In all activity assays, blanks were carried out to determine possible unspecific Pi presence.

Saturation curves were performed by assaying enzyme activity at different concentrations of the variable substrate, with or without effectors, and maintaining the other substrates at a saturating level. Experimental data were plotted as the enzyme activity ($U.mg^{-1}$) versus substrate (or effector) concentration (mM or µM) and kinetic constants were determined by fitting the data to the Hill equation. Hill plots were used to calculate the Hill coefficient (n_H), the maximal velocity (V_{max}), and the kinetic constants that correspond to the activator, substrate or inhibitor concentrations giving 50% of the maximal activation ($A_{0.5}$), velocity ($S_{0.5}$) or inhibition ($I_{0.5}$). All kinetic constants are the mean of at least three independent sets of data, which were reproducible within ± 10%.

3. RESULTS AND DISCUSSION

3.1. Expression and purification of the α, β and αβ PPi-PFK from orange

The sequence of the genes from *C. sinensis* coding for α (*Csipfpα*, NCBI EU302909.1) and β (*Csipfpβ*, NCBI EU302910.1) PPi-PFK subunits were amplified by PCR with specific oligonucleotides and the plant cDNA. The strategies for heterologous expression of the different enzyme conformations are summarized in Figure 1. The scheme shows that the individual expression of His-Tagged α subunit was achieved in *E. coli* BL21 Rosetta™ (DE3) cells transformed with [pET28c/*Csipfpα*] construction, while the β subunit alone (also His-Tagged) was expressed in *E. coli* BL21 (DE3) cells transformed with [pRSFDuet/*Csipfpβ*]. For the expression of the PPi-PFK αβ arrangement, *E. coli* BL21 (DE3) cells were co-transformed with [pRSFDuet/*Csipfpβ*] and [pETDuet/*Csipfpα*]. In the last case, only the β subunit is His-Tagged and allows the purification of the interacting αβ oligomer. Expression assays were performed in LB medium and different time and temperature conditions were tested for production of the soluble recombinant proteins. The different PPi-PFK versions were purified by IMAC-Ni^{2+} chromatography to electrophoretic homogeneity (Figure 2A). Single protein bands of 70 and 63 kDa were detected for the α and β subunits, respectively (Figure 2A, lines 2 and 3), these values in agreement with calculated masses of 69.6 kDa for the His-tagged α subunit and 63.2 kDa for the His-tagged β subunit. Both subunits were present in the chromatographic eluate from the soluble extract of *E. coli* BL21 (DE3) cells harboring [pETDuet/*Csipfpα*] and [pRSFDuet/*Csipfpβ*] plasmids (Figure 2A, line 4; note that in this case the α subunit has not His-tag).

Supplemental Fig. 1 shows a comparison of the sequences of cDNA-deduced polypeptides corresponding to the *Citrus sinensis* PPi-PFK α and β subunits cloned in this work (PPi-PFKα, A9YVC8; PPi-PFKβ, A9YVC9) with that of *Citrus paradisi*, *Ricinus communis*, and *Solanum tuberosum* [42]. A phylogenetic tree comparing the evolutionary relationship of both subunits to those of other dicotiledoneous plants was constructed and showed a clear assignment of both *C. sinensis* PPi-PFK subunits to the corresponding ones of other species (Supplemental Fig. 1 A). Furthermore, similarity at aminoacid level, and protein parameters such as aminoacid number, estimated molecular mass, and isoelectric point reinforced this observation (Supplemental Fig. 1 B,C).

Separate protein alignments were performed for the PPi-PFK α and β subunits (Supplemental Fig. 1 D, E). These evidenced a high degree of conservation among the sequences, being PPi-PFKα slightly divergent at C-terminus, while on the contrary PPi-PFKβ showed more divergence at N-terminus region. The PPi-PFK β subunit alignment

contained a highly conserved sequence corresponding to the PPi binding domain (PKTID), which includes a critical Lys possibly involved in substrate binding and catalysis and that is conserved in plants β subunits (Supplemental Fig. 1 E; light green box). PKTID is replaced by PVTLN in the α subunit (Supplemental Fig. 1 D; light green box). These differences were suggested to be responsible for the catalytic role of the β subunit [21].

3.2. Native structure and activity of the α, β and αβ PPi-PFK

The quaternary structure of the recombinant PPi-PFK from *C. sinensis* in its different versions was determined by size exclusion chromatography assays. The homo-α and homo-β native proteins eluted as single peaks at volumes corresponding with molecular masses of ~75 and ~90 kDa, respectively. In the case of the αβ hetero-oligomer, the determined molecular mass was ~450 kDa (Figure 2B). According to these results, the α and β subunits have a monomeric native structure, while the quaternary arrangement of the active hetero-oligomer corresponds to an $\alpha_3\beta_3$ hexamer. Further information confirming the identity of the purified proteins came from Western blots (Supplemental Fig. 2) probed with anti-potato tuber PPi-PFK serum immunopurified against the α or β subunits of the same enzyme. The anti-α antiserum recognized the recombinant α subunit and the same subunit in the orange recombinant and potato holoenzymes, with only a faint band at the height of the β subunit, possibly owing to an incomplete purification of the antibodies. The same results were observed when the anti-β antiserum was used, only that the β subunits were immunodecorated in this case. The crude antiserum against both subunits recognized both subunits of the heterodimer and both isolated subunits (Supplemental Fig. 2 panel C).

The three versions of the PPi-PFK were active. This is a remarkable finding that shows for the first time that the isolated subunits display activity, albeit with very low levels (0.075 $U.mg^{1}$ for α, 0.017 $U.mg^{-1}$ for β) when compared with the hetero-hexamer (47 $U.mg^{-1}$). Thus, even when both subunits are able to perform the reaction, the association of both is required for a fully active, Fru2,6bisP-activated *C. sinensis* enzyme. How the association of the subunits allows a dramatic increase in activity is still an unanswered question and the data presented here are not enough to propose a hypothesis. Although Van Praag [27] presented a hypothesis based on structural similarities with a *Bacillus stearothermophyllus* ATP-PFK, it must be noted that it is not an exact structural homolog of the plant enzyme. It is also evident that in this case there

is no spontaneous association of the β subunit as it naturally occurs in pineapple [22]. These results were confirmed by the coupled spectrophotometric assay that measures the production of Fru1,6bisP. Both subunits proved to be active and stable after long (more than 30 min) assays, although due to the lower sensitivity of the method and low activities of the isolated subunits this method could not be used to obtain reliable kinetic parameters. The assay by the same procedure of the $\alpha_3\beta_3$ holoenzyme also showed a higher activity (>3500-fold) than the isolated subunits (not shown).

3.3. Kinetic characterization of the different PPi-PFK assemblies

In order to study the structure to function relationship of the different subunits of PPi-PFK, the α, β and $\alpha_3\beta_3$ forms were kinetically characterized under different conditions. Thus, the kinetic parameters and the effect of pH and Fru2,6bisP were determined. Previous reports agree with the notion that Fru2,6bisP is a critical regulator of plant glycolysis that activates PPi-PFK (but not ATP-PFK) in a pH-dependent manner, while it inhibits fructose bisphosphatase [6, 20, 22]. Based on the above, the effect of pH, in the absence and presence of Fru2,6bisP (2 μM), was studied on the purified recombinant α, β, and $\alpha_3\beta_3$ forms of PPi-PFK from *C. sinensis*. The enzymes were assayed in the glycolytic direction of Fru1,6bisP synthesis at variable pH values (Figure 3). When the highly purified versions of the PPi-PFK were assayed at different pH values, they exhibited marked differences in their respective specific activities. Also, the presence of Fru2,6bisP affected dissimilarly and in a pH-dependent manner the activity of the three forms of the enzyme (Figure 3). For the α subunit, the activity assayed in the absence of the effector is maximal at pH 8.0 (Figure 3A, white squares); whereas in the presence of Fru2,6bisP (Figure 3A, black squares) the activity was not significantly affected in the pH range from 7.5-8.5. The latter is a consequence of Fru2,6bisP exerting a slight activating effect on the homo-α form at pH values lower than 7.5. In the case of the β subunit in the absence of Fru2,6bisP, the activity was 3-fold lower at pH 6.5 compared to that observed in the range from 7.0-8.5 (Figure 3B, white triangles); but in its presence the activity displayed at pH 6.5 remained unaltered in the whole pH range (Figure 3B, black triangles). This means that the presence of Fru2,6bisP is detrimental for the 3-fold activation gained by the homo-β form at the pH range between 7.0-8.5. For the $\alpha_3\beta_3$ version of the PPi-PFK, the highest activity was determined in the pH range 7.5-8.0 (Figure 3C, white circles), even in the presence of Fru2,6bisP (Figure 3C, black circles). Fru2,6bisP exerted an activating effect on the

$\alpha_3\beta_3$ heteromeric protein throughout the whole pH range analyzed. It must be noted that the lower the pH, the higher the activation of the $\alpha_3\beta_3$ PPi-PFK by Fru2,6bisP. In the pH range from 7.0 to 8.5 the activity was approximately 2- or 3-fold higher in the presence of Fru2,6bisP, whereas at pH 6.5 such an activation is around 18-fold. A similar effect had been reported before for the PPi-PFKs from black mustard, pineapple and potato tuber [20-22]. This is consistent with the hypothesis that *in vivo* and in slightly acidic environments the activity of the PPi-PFK would prevail over the activity of the ATP-PFK [20]. Fru2,6bisP is a central regulator of carbon metabolism and it has been proposed that its synthesis would balance the starch and sucrose metabolism according to the photosynthetic activity displayed by leaves [9, 43]. Levels of Fru2,6bisP vary greatly in the leaf cytosol, but are generally steadily high in non-photosynthetic tissues, perhaps to maintain an active sink metabolism in non-photosynthetic organs [43]. Fru2,6bisP only activates the PPi-PFK, since plant ATP-PFK is not sensitive to this effector and it inhibits fructose 1,6 bisphosphatase. Interestingly, it has been shown that in orange fruit the PPi-PFK varied its sensitivity towards this signal metabolite upon a frost event in a way that in frost exposed fruit (which displays a sharp decrease in respiration), the affinity for Fru2,6bisP is greatly increased [29]. Moreover, the ratio of α/β subunits changes from 1.7 to 1 in stressed fruit and this is accompanied by an increase in total extractable activity. These observations agreed with the results presented here and underscore the importance of PPi-PFK regulation by Fru2,6bisP and its key role in the control of the central carbon metabolism in orange.

The effect of Fru2,6bisP on the different forms of the recombinant PPi-PFK from *C. sinensis* was monitored at variable concentrations of this effector at pH 7.5 (Figure 4). The presence of Fru2,6bisP did not affect the activity of the α subunit (Figure 4A), but inhibited the activity of the β subunit (Figure 4B) proportionally to its concentration. The $\alpha_3\beta_3$ heteromeric protein activity (Figure 4C) increased sharply with the concentration of Fru2,6bisP. From these data, an $I_{0,5}$ of 340 nM was estimated for the Fru2,6bisP inhibitory effect on the β subunit (Figure 4B). With respect to the activating effect of Fru2,6bisP on the $\alpha_3\beta_3$ hetero-oligomeric PPi-PFK (Figure 4C), Fru2,6bisP produced a 2-fold activation of the enzyme with an $A_{0,5}$ of 70 nM.

The kinetic parameters (V_{max}, $S_{0,5}$ and n_H) for the different oligomeric forms of the PPi-PFK from orange were determined from experimental saturation curves for each substrates (Fru6P and PPi). Data, presented in Table 1, were obtained at pH 7.5 in the absence and in the presence of 2 μM Fru2,6bisP. The heterohexameric complex

displays full activity, actually 3 orders of magnitude higher than any of the isolated subunits. Like with other PPi-PFKs studied before, Fru2,6bisP increases the affinity for Fru6P and also the V_{max} of the $\alpha_3\beta_3$ complex. In this particular case, a rather medium to strong cooperativity effect for Fru6P binding was attenuated in the presence of Fru2,6bisP, while affinity increased 7-fold. Supplemental Figure 3 illustrates the effect of Fru2,6bisP on the catalytic efficiency (measured as $k_{cat}/S_{0.5}$) of the different forms of PPi-PFK.

The affinity values determined for both substrates *in vitro* for the recombinant enzyme suggest that the enzyme would work *in vivo* under non-saturating conditions. PPi levels in plants vary in the range between 10 and 800 µM [44], while those for Fru6P are around 0.03 nmol.g^{-1} fresh weight [45]. Considering that the volume of the cytosol in a plant cell may be (depending on the tissue) around 20% of the cell volume [46], then PPi concentrations would be from 0.25 to 0.5 mM and that of Fru6P around 0.15 mM. It was also estimated by Farré *et al.* [47] that Fru6P could be around 0.17 mM in the cytosol of potato tuber cells. These values are close to the $S_{0.5}$ for PPi but below the $S_{0.5}$ for Fru6P and enable PPi-PFK to vary its activity levels according to substrate (Fru6P) availability. A comparison of the kinetic properties of the orange PPi-PFK studied herein with its homolog from other plants studied so far [21-23], shows a similar response to Fru2,6bisP with an increase in the affinity for Fru6P and a $K_m/S_{0.5}$ values within the same range for both substrates. The other previous report for cooperativity in a PPi-PFK is that of banana fruit, which was also reverted to Michaelian kinetics by Fru2,6bisP [23]. Thus, the recombinant enzyme characterized here shows properties that resemble those obtained by the more canonical methods of protein purification and suggests that its behavior must be similar to the protein synthesized by the plant cells. However, it is worth to note reports showing that Fru2,6bisP increase V_{max} of the enzymes from *Brassica nigra* [21] and *Musa cavendishii* [23] by about 10- and 20-fold, compared with the about 2-fold effect measured with the recombinant $\alpha_3\beta_3$ PPi-PFK.

As pointed above, the regulation of PPi-PFK is pivotal for the control of carbohydrate metabolism in different plant tissues. The results obtained with the recombinant enzyme can be then analyzed in this context and provide a new way of exploring the yet unaccounted for structure-to-function properties. In the first place, it was shown that α subunit is catalytically active, an observation that has not been reported before. This is an unexpected result taking into account former assumptions that this subunit does not have a proper active site, based on sequence comparisons with

the ATP-PFK [25]. Moreover, these authors suggested that, although the Fru6P/Fru1,6bisP site seems conserved, substitution of a critical Asp present in position 127 of the ATP-dependent enzyme thwarted the catalytic function. Instead, they proposed this to be the Fru2,6bisP binding site. The present work shows that the α subunit in fact retains some catalytic activity, albeit at a highly reduced level with respect to the heteromeric enzyme. It also presents evidence that there is activation of the heteromer by Fru2,6bisP, as shown by an increase in the affinity for PPi, but with little effect on $S_{0.5}$. It should also be noted that binding of Fru6P is non-hyperbolic, and that Fru26bisP has no effect on it. Given that there is no indication that the α subunit forms a dimer in solution, and that there are no other plausible binding sites on the structure, it could be hypothesized that Fru6P might induce a transient activated state with higher affinity, constituting a homotropic cooperative effect (Table 1). It is also the first time that an active β subunit has been heterologously expressed. This subunit is much more affected by Fru2,6bisP but in an unexpected way: it is inhibited at pH values between 7 and 8.5, but not affected at pH 6.5. Unlike the α counterpart, it displays cooperativity for PPi but not for Fru6P binding. It is also intriguing how a monomeric enzyme can display cooperative binding, but the same hypothesis proposed for the α subunit (transient modification of affinity upon the catalytic cycle) can be proposed. The activity of this subunit is very low compared with the β dimer of pineapple leaves.

A comparison of the general properties of the isolated subunits and the heteromeric complex can be summarized as follows. The α subunit is little responsive to Fru2,6bisP and is weakly active. This goes against earlier assumptions that this protein is not active but does help the hypothesis that it may convey the activating effect to the complex. The β subunit is also active and more responsive to Fru2,6bisP, although in the opposite, inhibitory way. The heteromer is fully active, much like PPi-PFKs purified from plant tissues, and also displays the same pattern of response towards Fru2,6bisP, albeit to a reduced extent. Thus, the particular kinetic and regulatory properties of the fully assembled complex are not exactly the sum of its parts and is intrinsically linked to its heteromeric conformation.

To the best of current knowledge on the subject, this is the first report on the recombinant production of a plant PPi-PFK and the characterization of its different conformations. Even when many questions are still open, this is a main contribution for future studies focused to better understand the enzyme properties and how these are related with its relevant role in plant metabolism.

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FIGURE LEGENDS

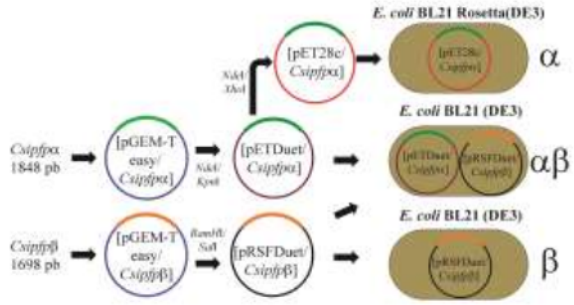

Figure 1: Scheme of the cloning and expressions procedures for *Csipfpα* and *Csipfpβ* genes. **A.** *C. sinensis pfpα* and *pfpβ* genes were amplified by PCR and cloned into pGEM-T easy vector. Then for expression strategies, α subunit was subcloned into pETDuet expression (without tag); while the β subunit was subcloned into pRSFDuet expression (with N-terminal His-tag). To obtain a tagged α subunit, the *Csipfpα* gene was also subcloned in the pET28c expression vector. *E. coli* BL21 Rosetta ™ (DE3) cells were transformed to obtain α subunit with His-tag; while for the expression of PPi-PFK β and αβ arrangements, *E. coli* BL21 (DE3) cells were used.

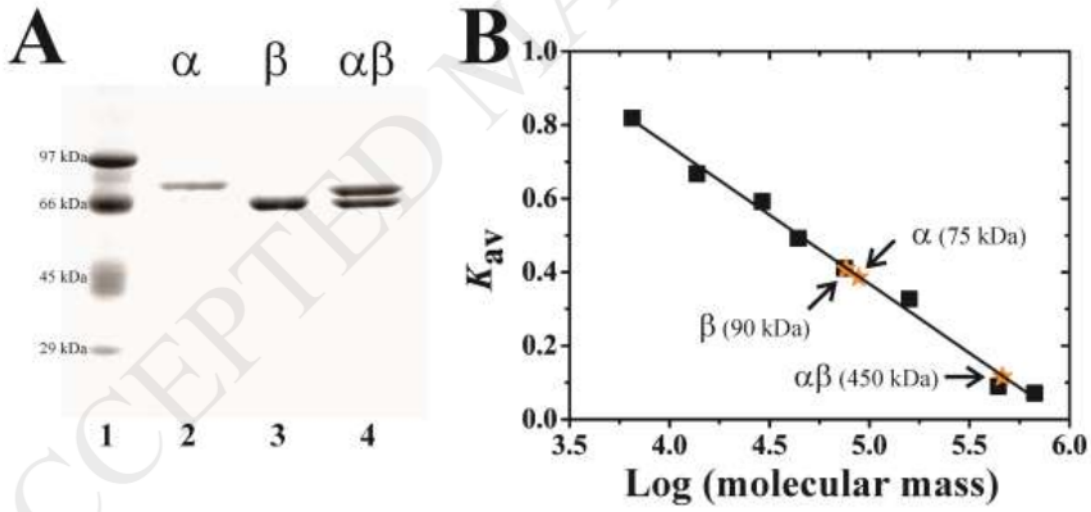

Figure 2: Structure of α, β and αβ PPi-PFKs. A. SDS-PAGE (10%) of purified versions of PPi-PFK of *C. sinensis* (α, β, αβ) by IMAC-Ni^{2+} stained with coomassie blue. **(1)** Molecular mass markers; **(2)** α subunit [1.5 µg, 0.08 $U.mg^{-1}$ when assayed with 2 µM Fru2,6bisP], **(3)** β subunit [2 µg, 0.006 $U.mg^{-1}$ when assayed with 2 µM Fru2,6bisP]; **(4)** $\alpha_3\beta_3$ hetero-hexamer [2 µg, 92 $U.mg^{-1}$ when assayed with 2 µM

Fru2,6bisP]. **B.** Molecular mass (MM) determination performed by size exclusion chromatography on Superdex 200, as detailed at *Material and methods*.

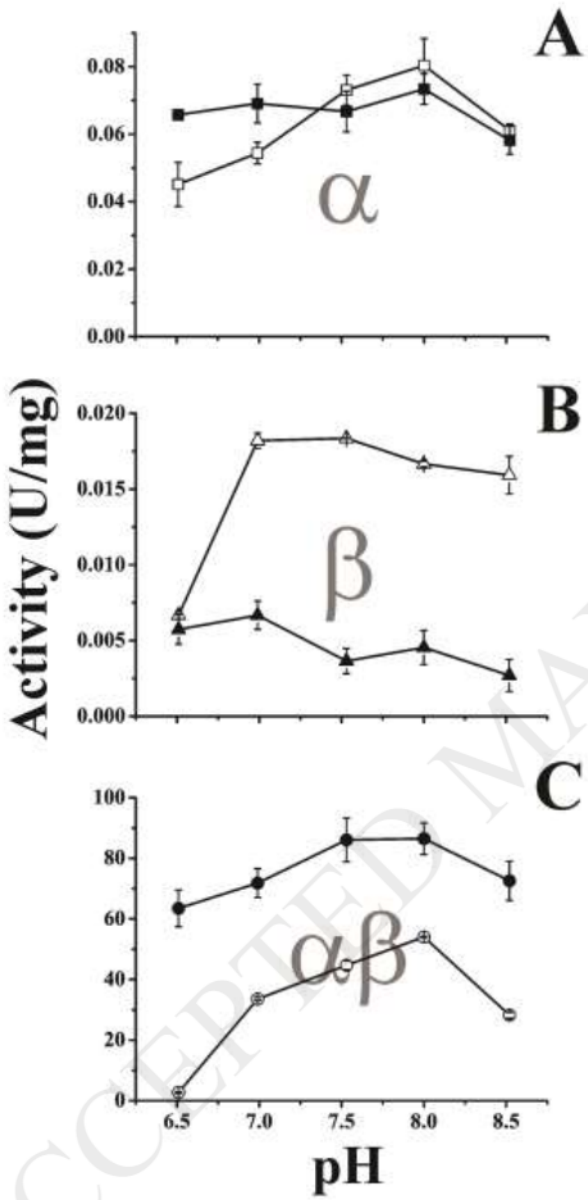

Figure 3: Activity of purified PPi-PFKs (α, β, $\alpha_3\beta_3$) at different pH values. The pH-dependent activity profiles for purified PPi-PFK were assayed in absence (empty symbols) or presence (filled symbols) of 2 μM of Fru2,6bisP. **(A)** α subunit,**(B)** β subunit and **(C)** $\alpha_3\beta_3$ hetero-hexamer.

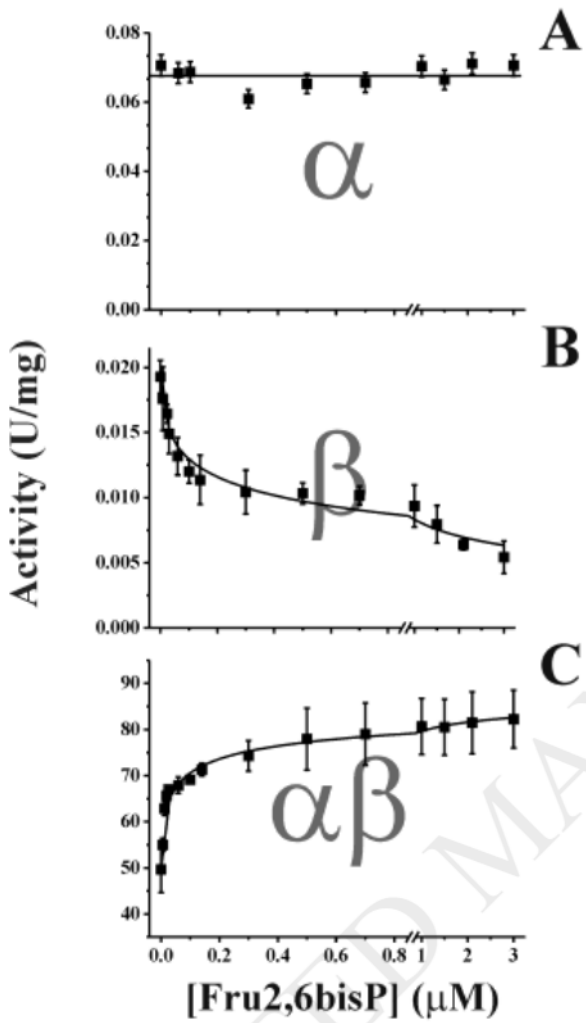

Figure 4: Activity for purified PPi-PFKs (α, β, $\alpha_3\beta_3$) at different concentrations of Fru2,6bisP (μM). Saturation curves for Fru2,6bisP were assayed at pH 7.5 as described at *Material and methods*. **(A)** α subunit, **(B)** β subunit and **(C)** $\alpha_3\beta_3$ hetero-hexamer.

Table 1. Kinetic characterization of different recombinant *Csi*PPi-PFK assembles

		None				Fru2,6bisP			
		$S_{0.5}$ (mM)	n_H	[a]k_{cat} (s^{-1})	$k_{cat}/S_{0.5}$ ($M^{-1}s^{-1}$)	$S_{0.5}$ (mM)	n_H	[a]k_{cat} (s^{-1})	$k_{cat}/S_{0.5}$ ($M^{-1}s^{-1}$)
α	**Fru6P**	1.5 ± 0.1	2.0 ± 0.2	0.09 ± 0.005	60 ± 7	1.52 ± 0.09	1.8 ± 0.1	0.09 ± 0.005	59 ± 6
	PPi	0.034 ± 0.002	1.1 ± 0.1		2650 ± 290	0.019 ± 0.002	0.8 ± 0.1		4737 ± 760
β	**Fru6P**	0.97± 0.07	1.1± 0.1	0.018 ± 0.001	18.6 ± 2	4.5± 0.3	0.8± 0.1	0.006 ± 0.001	1.3 ± 0.3
	PPi	1.33 ± 0.04	2.4 ± 0.2		13.5 ± 1	0.32 ± 0.02	1.7± 0.2		18.8 ± 4
αβ	**Fru6P**	1.32 ± 0.04	2.8 ± 0.2	312.14 ± 0.04	(23.6 ± 0.7) x 10^4	0.21 ± 0.01	1.3 ± 0.1	610.99 ± 0.05	(291 ± 14) x 10^4
	PPi	0.19 ± 0.01	1.0 ± 0.1		(164 ± 8) x 10^4	0.77 ± 0.03	1.5 ± 0.1		(79 ± 3) x 10^4

[a]k_{cat} values were calculated from the respective V_{max} and considering molecular masses for the different active forms of: α, 69.6 kDa; β, 63.2 kDa; and $\alpha_2\beta_2$, 398.5 kDa