

Molecular diversity of the pyrophosphate-dependent phosphofructokinase isoforms differently activated by fructose 2,6-bisphosphate*

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Since the discovery in 1979 of pyrophosphate-dependent phosphofructokinase (PP_i-PFK¹) in pineapple leaves [1], the enzyme has been found in a variety of plant tissues [2, 3]. Most of the work on the enzyme from plant sources shows that it is activated by and sometimes completely dependent on fructose 2,6-bisphosphate (Fru-2,6-P₂) which increases both the rate of enzymic activity and the affinity of PP_i-PFK for its substrates [2, 4 - 7]. However, in some plant tissues, in particular in the Crassulacean (CAM) plants, the enzyme activity is very high and is but slightly activated by Fru-2,6-P₂ at V_{max} [2, 5]. It is evident that different isoforms of PP_i-PFK are present that differ in molecular weight and kinetic properties [8 - 13]. In our earlier paper we have reported on the occurrence of three forms of PP_i-PFK: one from *Sansevieria trifasciata* (a CAM plant) and two from *Phaseolus coccineus* stems [5]. The *S. trifasciata* enzyme displays a significant activity in the absence of Fru-2,6-P₂ and only an about two-fold activation when this activator is included in the assay mixture. The *P. coccineus* enzymes have very low activity in the absence of Fru-2,6-P₂, and consequently a much greater stimulation by Fru-2,6-P₂ expressed as a change of V_{max} and K_m values [5].

In the current studies we have purified *S. trifasciata* PP_i-PFK and the purified enzyme was used as the antigen to produce polyclonal antibodies. We have also initiated studies on the molecular diversity of PP_i-PFK from differ-

ent sources based on the immuno-inactivation and the protein blot techniques.

PP_i-PFK was purified from 300 g *S. trifasciata* leaves homogenized in 1200 ml of 20 mM Tris/HCl buffer (pH 8.2) containing 20 mM CH₃COOK, 5 mM Mg(CH₃COO)₂, 15 mM 2-mercaptoethanol, 1 mM EGTA and 0.5 mM phenylmethylsulfonyl fluoride. The clarified crude extract was subjected to ammonium sulphate fractionation. The protein fraction precipitated between 0.3 and 0.5 saturation was collected, dissolved in 20 mM Tris/acetate buffer (pH 7.2) containing 5 mM 2-mercaptoethanol, 1 mM EGTA, 5 mM Mg(CH₃COO)₂ and 20% glycerol (buffer A), and was further purified by sequential chromatography on the columns of: (a) phosphocellulose (2.5 × 16 cm) eluted with a linear 0 - 0.5 M KCl gradient in buffer A; (b) Sephadex G 200 (1.8 × 65 cm) washed with 50 mM Tris/HCl (pH 7.6) containing 50 mM KCl, 5 mM 2-mercaptoethanol, 1 mM Mg(CH₃COO)₂, 1 mM NaF and 10% glycerol; (c) Toyopearl DEAE-650M (1.2 × 12 cm) eluted with a linear 0 - 0.15 M KCl gradient in 20 mM Tris/HCl buffer (pH 7.6) containing 5 mM 2-mercaptoethanol and 20% glycerol. In each case, the active fractions were collected and concentrated using the Amicon ultrafiltration system fitted with a Diaflo UM 10 membrane. PP_i-PFK activity was assayed spectrophotometrically [5].

As shown in Fig. 1, the purified PP_i-PFK was homogeneous in 8% polyacrylamide gel elec-

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¹Abbreviations: PP_i-PFK, pyrophosphate-dependent phosphofructokinase; Fru-2,6-P₂, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate

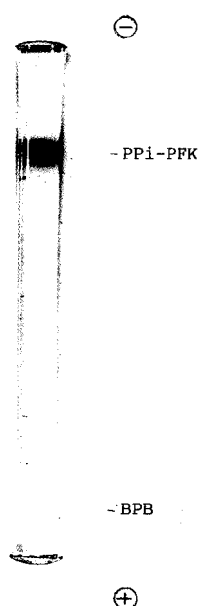


Fig. 1. Analytical disc gel electrophoresis of purified pyrophosphate-dependent phosphofructokinase (PP_i -PFK) in 8% polyacrylamide.

The protein was stained with Coomassie Blue

electrophoresis performed according to Ogita & Markert [14].

Like in our previous experiments [5], an only approximately 1.6-fold maximal stimulation of the purified enzyme by 0.1 mM Fru-2,6-P₂ was observed at 2.5 mM Fru-6-P concentration (Fig. 2) Thus the activity of *S. trifasciata* PP_i -PFK was stimulated to only a small degree in the forward (Fru-1,6-P₂-forming) reaction, as compared with the *P. coccineus* enzyme, as well as with the enzyme from other sources [2, 4 - 7]. The effect of Fru-2,6-P₂ depended on concentration of Fru-6-P (Fig. 2) and orthophosphate in the reaction mixture (not shown).

Several workers have reported that activation of PP_i -PFK is accompanied by an increase in the molecular mass of the enzyme and conversion of the strongly activated enzyme form into the form relatively insensitive to the activator [11, 15]. In our experiments, the purified enzyme from *S. trifasciata* (115 kDa) and the partially purified form A of PP_i -PFK from *P. coccineus* (220 kDa) [5] were incubated with or without Fru-2,6-P₂ for 24 h at 4°C. Then the enzymes were applied to a Sephadex G 200 column (1.6 × 40 cm) and eluted with 50 mM Tris/HCl buffer (pH 7.6) containing 1 mM Mg(CH₃COO)₂, 50 mM KCl, 5 mM 2-mercaptoethanol and 10% glycerol. The enzyme incubated with Fru-2,6-P₂ was eluted with the same

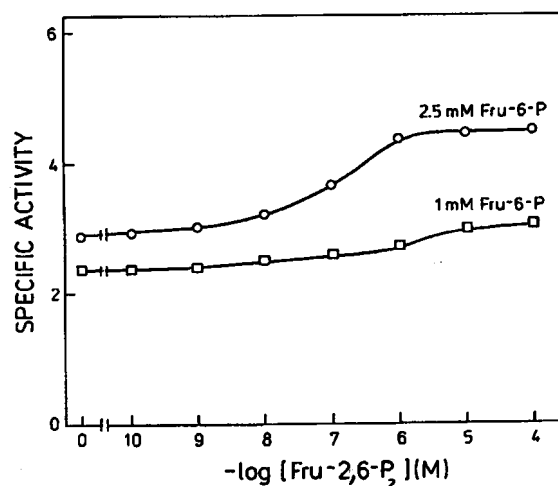


Fig. 2. Activation of the purified PP_i -PFK from *S. trifasciata* by Fru-2,6-P₂ in the forward (Fru-1,6-P₂-forming) reaction.

The reaction mixture contained 2.5 mM (○) or 1 mM (□) Fru-6-P and 0.5 mM PP_i . Specific activity is expressed in $\mu\text{mol Fru-1,6-P}_2/\text{min per mg protein}$

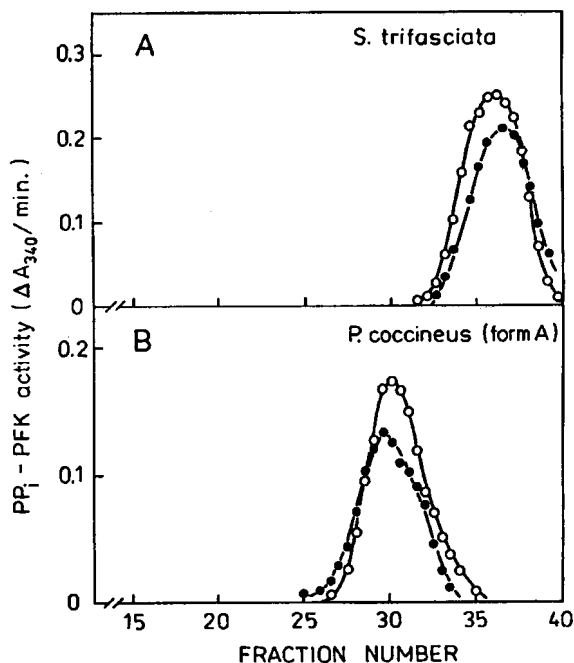


Fig. 3. Sephadex G 200 chromatography of PP_i -PFK.

The purified PP_i -PFK from *S. trifasciata* (A) or partially purified enzyme (form A) from *P. coccineus* (B) were incubated with (○) and without (●) 1 μM Fru-2,6-P₂ for 24 h at 4°C. PP_i -PFK was assayed in the presence of 1 μM Fru-2,6-P₂ with 2.5 mM Fru-6-P and 0.5 mM PP_i

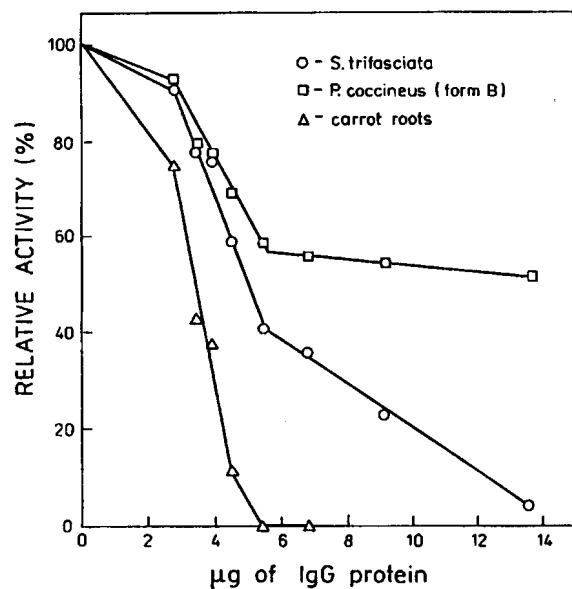


Fig. 4. Immunoprecipitation of PP_i-PFK from *S. trifasciata* (○), *P. coccineus* (□) and carrot roots (Δ). The enzymes were incubated with the antibody raised against *S. trifasciata* PP_i-PFK. After incubation at room temperature for 45 min, insoluble protein A (10 mg) was added to the mixture which was then centrifuged. The activity of PP_i-PFK remaining in the supernatant was measured and expressed as percentage of the activity prior to the addition of antibody. Equivalent amounts of preimmune IgG had no detectable effect on the enzymatic activity

solution containing in addition 1 mM Fru-2,6-P₂. As shown in Fig. 3, the *S. trifasciata* (Fig. 3A) and *P. coccineus* (Fig. 3B) enzymes were eluted in the same fraction irrespective of the presence of Fru-2,6-P₂. Consistently, activation of PP_i-PFK by this activator was not accompanied by any measurable change in molecular mass. These results suggest that the *S. trifasciata* and *P. coccineus* enzyme forms do not result from Fru-2,6-P₂ - induced conversion.

For immunological characterization of the different enzyme forms an antiserum against the purified PP_i-PFK from *S. trifasciata* was prepared by injecting rabbit with about 350 µg of the purified protein (in two portions), emulsified with Freund's complete and incomplete adjuvant. Immunoglobulin G was purified by affinity chromatography on protein A-Sepharose, as described by Harlow & Lane [16]. Immuno-inactivation of PP_i-PFK was performed by the previously described procedure [17].

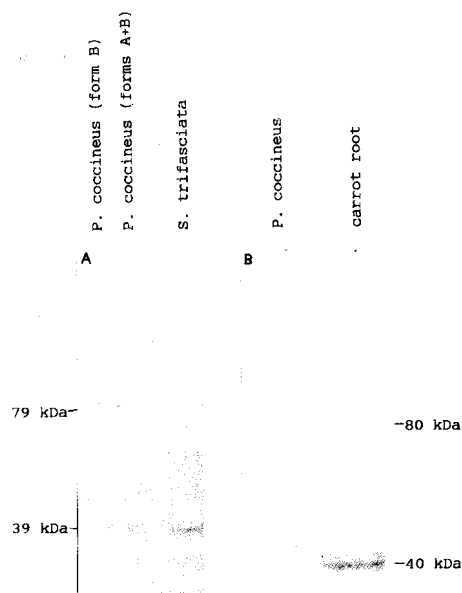


Fig. 5. Protein blot analysis of PP_i-PFK from *S. trifasciata*, *P. coccineus* and carrot roots.

The polypeptides were resolved by SDS-PAGE in 10% polyacrylamide, electroblotted into nitrocellulose and probed with antibody. Bound antibody was detected using the peroxidase-conjugated goat antibody against rabbit IgG. Molecular mass standards were BSA (66 kDa); ovalbumin (45 kDa) and glyceraldehyde 3-phosphate dehydrogenase (36 kDa)

The results of the immunotitration indicate that the obtained antibody is effective against the *S. trifasciata* PP_i-PFK but less effective in inactivating the enzyme from *P. coccineus* (form B; 450 kDa) (Fig. 4). We also investigated the ability of antibody to precipitate the partially purified enzyme from carrots. As shown in Fig. 4, in this case the antibody was very effective in inactivating enzyme activity. The differences observed indicate that plant species differ in the surface antigenic determinants of the native PP_i-PFK.

To investigate whether the antibody cross-reacted specifically with the protein against which it was raised, we used Western blot analysis according to Harlow & Lane [16]. Cross-reacting polypeptides were detected with the peroxidase-coupled goat antibodies using 4-chloro-1-naphthol as the substrate. The initial screening indicates that the obtained antibody was monospecific towards the polypeptide of approximately 39 kDa (Fig. 5A) when the ammonium sulphate fraction of the *S. trifasciata*

extract, precipitated at 0.3 - 0.6 saturation, was blotted. Moreover, the finally purified enzyme exhibited one polypeptide band of approximately 39 - 40 kDa on SDS-PAGE (not shown). These results support the view that the *S. trifasciata* PP_i-PFK is a homotrimer because the estimated relative molecular mass of the native enzyme was about 120 000 [3, 5]. In contrast to the *S. trifasciata* PP_i-PFK, the two different polypeptides of *P. coccineus* enzyme (form B) cross-reacted with the antibody after SDS-PAGE and blotting (Fig. 5B). The same two polypeptides with apparent molecular mass values of 39 and 79 kDa were also recognized in the *P. coccineus* extract containing both forms of PP_i-PFK (Fig. 5A). In the case of partially purified enzyme from carrot roots we have observed the occurrence of two or three closely migrating polypeptides in the 37 - 43 kDa region (Fig. 5B).

To sum up, the results of our study corroborate the structural diversity of PP_i-PFK from different sources. However, we are also taking into consideration the possibility that the differences in polypeptide patterns could be due to rapid proteolytic cleavage. This supposition is currently under investigation.

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