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Short Communication

Measurement of the Pyrophosphate Content of Plant Tissues[1]

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ABSTRACT

Pyrophosphate (PPi) was measured in pea (*Pisum sativum* L.) and corn (*Zea mays* L.) tissues by using an enzymic method based on PPi-dependent phosphofructokinase (PPi-PFK). Different organs of pea and corn seedlings were extracted to determine if PPi is present in sufficient amounts to serve as a substrate for the PPi-PFK activity in these tissues. The amount of PPi is at least 14% to 70% that of the ATP content in shoots and roots of peas and corn; and, for various plant tissues, ranges from 5 to 39 nanomoles of PPi per gram fresh tissue weight. We conclude that PPi is available as a substrate for the glycolytic function of PPi-PFK in plants. Furthermore, the presence of substrate amounts of PPi in plant tissues implies that plant energetics also must be evaluated in terms of PPi as an energy source and phosphate donor.

The discovery of a PPi-PFK[2] in pineapple leaves (2) and the widespread distribution of a high activity of this enzyme in many other plants (3, 6, 9, 13–15) raised the issue of whether plants contain PPi in sufficient amounts to support significant PPi-PFK activity. Theoretically, PPi is produced primarily during polymer synthesis reactions (*e.g.*, DNA, RNA, cellulose, starch, sucrose, and the activation of amino acids and fatty acids). The subsequent hydrolysis of PPi by pyrophosphatases is thought to thermodynamically favor the formation of macromolecules. Other sources of PPi production are less well established. Although PPi-PFK is a reversible enzyme *in vitro* and could participate in gluconeogenesis in tissues such as castor bean endosperm which undergo a rapid conversion of fat to sugar (6), data on fru 2,6-P_2-induced aggregation of PPi-PFK (14, 15) and the kinetic characteristics of PPi-PFK (2, 13–15) support a glycolytic function for the enzyme. Fru 2,6-P_2 promotes the association of a large molecular form of PPi-PFK which favors the glycolytic over the gluconeogenic direction of enzymic activity (14, 15), and fru 2,6-P_2 stimulates the glycolytic direction of PPi-PFK at lower concentrations than the gluconeogenic direction (13–15). Several workers indicate that fru 2,6-P_2 is present in plant tissues in significant amounts (5, 10, 12, 13) and its concentration fluctuates depending on parameters such as physiological condition of the plant (5, 12) or physical factors such as light (10). Clearly, the glycolytic form of PPi-PFK, as indicated by fru 2,6-P_2 (14, 15), requires PPi as its substrate to function.

We are not aware of any measurements of PPi in higher plants, consequently, here we describe a method to detect PPi. Since PPi is the substrate for PPi-PFK, it was clear that the enzyme could be used to assay PPi. Recently a commercial reagent became available using PPi-PFK which is free of contaminating pyrophosphatases and pyrophosphorylases. Using PPi-PFK, we have measured the PPi level in different organs of pea and corn seedlings. We also will consider the implication of a PPi pool in plants relative to other metabolic functions.

MATERIALS AND METHODS

Plant Material. Pea seed (*Pisum sativum* L. cv Alaska) was planted in moist vermiculite and germinated at room temperature (25°C) in the lab. Emerging pea shoots received low light (5 μmol m^{-2} m^{-1} PAR) during the day. Corn seed (*Zea mays* L. cv Merit) was planted in moist vermiculite and germinated at 28°C. Four d after planting, the corn seedlings were transferred to a growth chamber operating with a 15-h photoperiod (200 μmol m^{-2} s^{-1}) and 28°C, and 9 h of darkness at 18°C for an additional 2 d of growth. Plants were watered daily. Plant parts were rinsed with distilled H_2O and blotted dry prior to determining their fresh weights.

Extraction of Plant Organs. Plant samples (3 g) were quickly sliced into small segments and frozen in liquid N_2 in a mortar. The frozen samples were pulverized with a pestle and extracted with 6 ml of 0.45 N $HClO_4$ acid. Insoluble material in the extraction solution was removed by centrifugation at 27,000*g* for 15 min. The supernatant resulting from centrifugation at 27,000*g* was neutralized to pH 7.0 to 7.5 with KOH and any perchlorate formed after cooling the extract to 4°C was removed by centrifugation in a Beckman model B microfuge for 30 s. PPi, glu 6-P, and fru 6-P content was determined immediately, whereas samples used for ATP and Pi were stored at −20°C prior to analysis.

Metabolite Analyses. The PPi present in the neutralized perchlorate extracts was determined enzymically using a commercial reagent (Sigma Chemical Co., P-7275) containing PPi-PFK, aldolase, triose-P isomerase, α-glycerophosphate dehydrogenase, and appropriate substrates and salts to determine PPi as the oxidation of NADH at 340 nm. The PPi assay was done in a 0.7-ml final volume using a Gilford model 240 spectrophotometer. This assay is essentially the same as that used originally to detect PPi-PFK activity in plants (2).

The amounts of ATP, fru 6-P, and glu 6-P in plant extracts were estimated enzymically (1). Assays were done in a 0.5-ml volume using usually 25 to 50 μl of plant extract. The assay medium for ATP contained 0.1 M Hepes-KOH (pH 7.5), 1 mM DTT, 1 mM glycerate-3-P, 2.5 mM $MgSO_4$, 0.16 mM NADH, 4 units of glyceraldehyde 3-P dehydrogenase, and 5 units of phosphoglyceryl kinase to initiate the reaction. The assay medium for fru 6-P and glu 6-P contained 0.1 M Hepes-KOH (pH 7.5), 5 mM $MgCl_2$, 0.5 mM $NADP^+$: 1 unit of glu-6-P dehydrogenase was added to determine glu 6-P, followed by 2 units of phosphoglucose isomerase to determine fru 6-P. The amounts of ATP,

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[2] Abbreviations: PPi-PFK, pyrophosphate-dependent phosphofructokinase; fru 2,6-P_2, fructose 2, 6-bisphosphate; fru 1,6-P_2, fructose 1,6-bisphosphate; glu 6-P, glucose 6-P.

fru 6-P, and glu 6-P were calculated from absorbance changes using an extinction coefficient of 6220 M^{-1} cm^{-1} for NAD(P)H. The Pi content of plant samples was measured using a molybdate reagent (11). All enzymes and substrates were obtained from the Sigma Chemical Co.

RESULTS

Method for Measuring Pyrophosphate. Pyrophosphate could be detected enzymically at concentrations less than 1 nmol/0.7 ml assay volume (Fig. 1) with the Sigma reagent containing PPi-PFK and the coupling enzymes. The absorbance loss at 340 nm with increasing amounts of PPi in the assay system closely correlates with the expected stoichiometry if the NADH concentration is calculated using an extinction coefficient of 6220 M^{-1} cm^{-1}. We, therefore, believe that the PPi assay medium and Sigma reagent are not contaminated by significant amounts of inorganic pyrophosphatase, pyrophosphorylases (see nucleotide test below), or other enzymes which might reduce the efficiency of the assay.

The amount of PPi in some plant extracts necessitated using samples of up to one-third of the total reaction volume. Because of the amount of plant material added in these cases, it was necessary to check for the presence of inhibitory factors or precipitation reactions by adding a known amount of PPi (5 nmol) at the end of each assay as a control. PPi assays on pea cotyledons, corn scutellum, and leaves from mature pea plants were subject to the above influences. All assays were adjusted so that the amount of PPi detected was proportional to volume of plant extract added.

Although a chemical method for PPi analysis is available (4), this method requires the removal of Pi from the extract prior to the PPi determination, then concentrating the PPi by precipitation. Our attempts to precipitate PPi from plant extracts using KF and $CaCl_2$ treatment and subsequent filtration (4) were not successful.

The assay medium was tested for specificity regarding PPi and nucleotides. Nucleotides were tested at 15 nmol/assay or about 10-fold higher concentration that the level of PPi found in plant extracts. Adenosine (ATP, ADP, AMP), cytidine (CTP), guanosine (GTP, GDP), inosine (ITP, IDP), and uridine (UTP) has no effect on the determination of 5 nmol of PPi.

The efficiency of the $HClO_4$ extraction of PPi from plant tissues was tested on duplicate batches of corn roots. One batch of roots was pulverized in the presence of added PPi and the PPi content of the extract was then compared to the batch of untreated roots. Following our standard extraction procedure, we only recovered ~60% of the added PPi in the assay. Thus, we believe the total extraction of PPi from plant tissue is not attained yet and that the PPi values we present, likely, are underestimations of the tissue content.

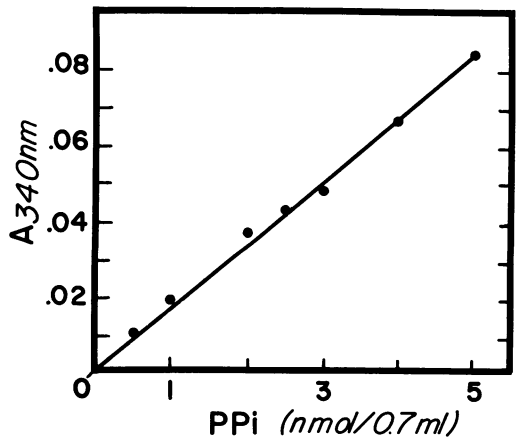

FIG. 1. Relationship between NADH oxidation and PPi content using the PPi-PFK assay.

The question of whether the PPi in plant extracts is due to degradation of nucleotides during tissue extraction was tested by adding a known amount of ATP to a corn root sample before perchlorate extraction. Increasing the amount of ATP in a corn root sample 20-fold did not change the amount of PPi measured.

Pyrophosphate Content of Plant Extracts. PPi was detected in all organs of both pea and corn seedlings (Table I). The amount of PPi measured is substantial relative to the amounts of ATP and fru 6-P, two other potential substrates of the phosphofructokinase reaction. Also, the levels of glu 6-P and Pi are given for comparative purposes; these values are similar to published results. Other experiments show that PPi also is present in mature plant tissues, but care has to be taken to rule out contributions by inhibitory substances on the enzymic assay system. For example, PPi was not detected in mature pea leaves due to inhibitory influences of the extract. Mature pineapple leaves contain 14 nmol PPi/g fresh weight, amounts similar to those in the tissues analyzed for Table I.

DISCUSSION

The hypothesis that PPi-PFK participates in plant glycolysis (2) is now well supported. The large mol wt form of PPi-PFK favors the glycolytic activity of the enzyme (14, 15). The amounts of fru 2,6-P_2 reported, presumably in the cytoplasm (5, 10, 12), would insure that PPi-PFK remained in this glycolytically active form for at least part of a plant's daily cycle. Reconstitution of a soluble PPi-driven glycolysis in pea seed extracts indicates that PPi-PFK can function in the glycolytic sequence in a manner similar to the classical ATP-PFK (unpublished results). The present finding (Table I) that a PPi pool exists in pea and corn seedlings shows that PPi-PFK would have PPi available as a substrate. An estimate of the PPi concentration in these tissues would be 5 to 39 μM if 1 g fresh weight of tissue is equal to 1 ml. The affinity of plant PPi-PFK for PPi correlates well with this minimum estimation of PPi tissue content. Partially purified plant PPi-PFK has K_m values for PPi which are near 10 to 20 μM. Furthermore, the same tissues as in Table I also contain an active PPi-PFK, indeed comparable to their ATP-PFK activity (9, 14). Collectively, these results point to a glycolytic role for PPi-PFK in many plant tissues. However, we realize that the presence of both a PPi and an ATP-dependent phosphofructokinase in the cytoplasm of plant cells raises many unanswered questions about the roles of these two enzymes during sugar

Table I. *Amounts of PPi, ATP, Fru 6-P, Glu 6-P, and Pi in Different Organs of Pea and Corn Seedlings*

Data are the average of two experiments with at least two assays for each determination.

Plant	Organ	Metabolite Amount				
		PPi	ATP	Fru 6-P	Glu 6-P	Pi
		nmol/g fresh wt tissue				
4-d-old pea	Shoots	16	113	89	290	8,452
	Roots	14	98	98	260	7,735
	Cotyledons	5[a]	249	140[a]	268[a]	
6-d-old corn	Shoots	39	158	78	396	11,175
	Roots	23	33	56	301	8,806
	Scutellum	15	391	89	488	28,000

[a] Data from one experiment.

metabolism.

Metabolic Implications of a PPi Pool in Plants. The presence of PPi in plants has broader implications in plant metabolism than solely its role as a substrate for PPi-PFK. The fact that PPi can drive unfavorable reactions, just as ATP does, means that any consideration of the energetics of plant metabolism should include this high energy Pi donor. In some cases, energy charge or the ATP/ADP ratio (8) correlates with the changing metabolic state of a plant tissue. We think that the addition of PPi to these considerations is required and perhaps would increase the correlation between the concentration of high energy Pi donors and the general metabolic state of plant tissues.

The hydrolysis of PPi during polymer synthesis thermodynamically favors the formation of nucleic acids, carbohydrates, lipids, and proteins; usually hydrolysis is through the activity of inorganic pyrophosphatases found in cells which hydrolyze PPi to 2 Pi (7). But it is clear that PPi-PFK also can drive a synthetic phosphorylation reaction thereby partially controlling the PPi content of plant tissues. The existence in plants of a relatively large PPi pool, as measured in these studies (Table I), indicates that the PPi formed during polymer synthesis exceeds the level of pyrophosphatase activity, or that other pathways exist for PPi synthesis at significant rates, or that PPi is in a specific intracellular compartment. How the PPi content is maintained in plant cells remains to be resolved, but its solution implies we will learn more about how the synthesis of macromolecules is integrated with pathways such as glycolysis and gluconeogenesis.

In conclusion, we have measured the PPi content of pea and corn tissues and PPi is present in sufficient amounts to serve as a substrate for PPi-PFK to function in plant glycolysis. Furthermore, the presence of a substantial amount of PPi in plant tissue implies that plant energetics must be reevaluated.

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