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Lack of fructose-1,6-bisphosphatase in a range of higher plants that store starch

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The aim of this work was to discover whether fructose-1,6-bisphosphatase (FBPase) is present in higher-plant cells that synthesize storage starch. The following were examined: suspension cultures of soybean (*Glycine max*), tubers of potato (*Solanum tuberosum*), florets of cauliflower (*Brassica oleracea*), developing endosperm of maize and of sweet corn (*Zea mays*), roots of pea (*Pisum sativum*), and the developing embryos of round and wrinkled varieties of pea. Unfractionated extracts of each tissue readily converted fructose 1,6-bisphosphate to fructose 6-phosphate in assays for both plastidic and cytosolic FBPase. These conversions were not inhibited by 20 μM-fructose 2,6-bisphosphate. Except in extracts of pea embryos and sweet-corn endosperm, treatment with affinity-purified antibodies to pyrophosphate:fructose-6-phosphate 1-phosphotransferase reduced the above fructose 6-phosphate production to the rate found with boiled extracts. The antibody-resistant activity from sweet corn was slight. In immunoblot analyses, antibody to plastidic FBPase did not react positively with any protein in extracts of soybean cells, potato tuber, cauliflower florets, maize endosperm and pea roots. Positive reactions were found for extracts of embryos of both round and wrinkled varieties of peas and endosperm of sweet corn. For pea embryos, but not for sweet-corn endosperm, the M_r of the recognized protein corresponded to that of plastidic FBPase. It is argued that soybean cells, potato tuber, cauliflower florets, maize (var. White Horse Tooth) endosperm and pea roots lack significant activity of plastidic FBPase, but that this enzyme is present in developing embryos of pea. The data for sweet corn (var. Golden Bantam) are not decisive. It is also argued that, where FBPase is absent, carbon for starch synthesis does not enter the amyloplast as triose phosphate.

INTRODUCTION

Two routes from translocated sucrose to storage starch in non-photosynthetic tissues of higher plants have been proposed. In one the carbon for starch synthesis enters the plastid as triose phosphate (Boyer, 1986). In the other, entry is as hexose monophosphate (Entwistle & ap Rees, 1988; Keeling *et al.*, 1988; Tyson & ap Rees, 1988). A key feature of the latter view is the evidence that there is no significant fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) in the amyloplasts in the developing endosperm of wheat (*Triticum aestivum*) (Entwistle & ap Rees, 1988, 1990). Claims that other non-photosynthetic starch-forming tissues do contain plastidic FBPase have been made for soybean (*Glycine max*) suspension cultures (Macdonald & ap Rees, 1983), potato (*Solanum tuberosum*) tuber (Mohabir & John, 1988), cauliflower (*Brassica oleracea*) florets (Journet & Douce, 1985), developing endosperm of maize (*Zea mays*) (Echeverria *et al.*, 1988), and developing endosperm of rice (*Oryza sativa*) (Nakamura *et al.*, 1989). Each of these claims rests on measurements of the ability of extracts, or fractions thereof, to convert fructose 1,6-bisphosphate (Fru-1,6-P_2) to fructose 6-phosphate (Fru-6-*P*). We (Entwistle & ap Rees, 1988) have shown that such activity is very difficult to distinguish from that of pyrophosphate:fructose-6-phosphate 1-phosphotransferase [PFK(PP_i); EC 2.7.1.90]. This is because such extracts and/or the reagents used in the assay for FBPase, may contain sufficient orthophosphate and fructose 2,6-bisphosphate (Fru-2,6-P_2) to allow extensive conversion of Fru-1,6-P_2 into Fru-6-*P* via PFK(PP_i). The evidence that the starchy tissues contain FBPase was obtained before it was appreciated that PFK(PP_i) could be mistaken for FBPase. The aim of the work described here was to determine whether the reported activities were due to FBPase or to PFK(PP_i). In addition to the tissues already listed, we examined both the roots and the developing embryos of peas (*Pisum sativum*). We did this to see if any FBPase activity varied with the extent of starch synthesis.

We used three methods to distinguish between FBPase and PFK(PP_i). First, we assayed the ability of crude extracts and desalted extracts to convert Fru-1,6-P_2 into Fru-6-*P* in the presence and absence of 20 μM-Fru-2,6-P_2. We did this with assays previously optimized for both cytosolic and plastidic FBPase. Fru-2,6-P_2 inhibits plastidic FBPase competitively (Cadet *et al.*, 1987) and inhibits cytosolic FBPase allosterically (Herzog *et al.*, 1984). With extracts of wheat leaves, the above concentration of Fru-2,6-P_2 caused marked inhibition of the cytosolic activity and slight inhibition of the plastidic activity (Entwistle & ap Rees, 1988). PFK(PP_i) is stimulated by Fru-1,6-P_2, but this stimulation is not always apparent when the enzyme is assayed in the direction of Fru-6-*P* formation (Kombrink *et al.*, 1984; Entwistle & ap Rees, 1988). Extracts were desalted in an attempt to reduce the amount of P_i present and thus discriminate against PFK(PP_i). Our second approach was to see if any ability of the extracts to convert Fru-1,6-P_2 into Fru-6-*P* was specifically inhibited by antibodies to PFK(PP_i). Finally, we determined whether antibody to plastidic FBPase reacted with any protein in an immunoblot analysis of the different plant extracts.

EXPERIMENTAL

Plants

Suspension cultures of soybean (*Glycine max* L. var. Acme) were obtained and grown as described by Macdonald & ap Rees (1983) and were harvested after 7 days growth. Soybean leaves were from 20–28-day-old seedlings grown at 25 °C in natural light. Mature tubers of potato (*Solanum tuberosum* L., var. Record) were harvested, stored at 10 °C and used within 10 weeks; leaves were from tubers sprouted in a greenhouse.

Abbreviations used: FBPase, fructose-1,6-bisphosphatase (EC 3.1.3.11); Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-6-*P*, fructose 6-phosphate; PFK(PP_i), pyrophosphate: fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.90).

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Cauliflowers (*Brassica oleracea* L.) were bought fresh and used at once; the outer 3 mm of the heads were shaved off with a razor blade and formed the experimental material; leaves were from 21–30-day-old seedlings grown in a greenhouse. Seeds of *Zea mays* L. (var. White Horse Tooth and var. Golden Bantam) were sown singly in compost each in a 30 cm-diameter pot and grown in a greenhouse (15–25 °C) in a 16 h daylength and photosynthetically active radiation of 350–500 $\mu E \cdot s^{-1} \cdot m^{-2}$. Plants were pollinated by hand, and cobs were harvested 24–30 days after anthesis. Maize leaves were from 12–20-day-old seedlings grown at 25 °C under natural light in a greenhouse. Pea (*Pisum sativum* L., var. Kelvedon Wonder) roots, the apical 5 cm, were from 5-day-old seedlings grown as described by Smith & ap Rees (1979). Leaves were from 7–14-day-old seedlings grown as described for maize leaves. Developing embryos of round-seeded (var. Birte JI 1068) and wrinkled-seeded (var. Greenshaft JI 1430) peas at the 300–400 mg weight stage were grown as described by Edwards & ap Rees (1986).

Materials

Enzymes, substrates and special chemicals were obtained as described earlier (Entwistle *et al.*, 1988; Entwistle & ap Rees, 1988). Antibody to plastidic FBPase from leaves of spinach (*Spinacia oleracea* L.) was prepared as described by Nishizawa & Buchanan (1981) and obtained from Dr. C. Raines, AFRC Institute of Plant Science Research, Cambridge, U.K. Antibody to PFK(PP_i) was produced with enzyme that we have purified from potato tubers as described by Kruger & Dennis (1987). The preparation obtained from the DEAE-Sephacel column showed a 127-fold purification and a specific activity of 7 μmol/min per mg of protein. The protein band that was obtained by further purification by non-denaturing PAGE and corresponding to the peak of PFK(PP_i) activity was analysed by SDS/PAGE as described by Kruger & Dennis (1987) and the same result was obtained. The two purified polypeptides of PFK(PP_i) were excised and electroeluted in 50 mM-Tris/0.38 M-glycine, pH 8.6, containing 0.1 % (v/v) SDS as described by Allington *et al.* (1978).

Antibodies to the two PFK(PP_i) polypeptides were raised in New Zealand White rabbits by injection of 200 μg of protein emulsified with an equal volume of Freund's complete adjuvant in 1 ml at multiple intramuscular sites along the back at 14-day intervals for 10 weeks. Blood was collected every 14 days, kept at 37 °C for 1 h, and the clot was retracted at 4 °C overnight. The serum was collected by centrifugation and stored at −20 °C. To purify the antibodies from the serum, an affinity column was prepared by dissolving 2.5 mg of purified PFK(PP_i) (from Sigma) in 1.0 ml of 0.1 mM-$NaHCO_3$ (pH 8.3)/0.5 M-NaCl (coupling buffer) and dialysing overnight at 4 °C against 500 ml of coupling buffer. The dialysed enzyme was coupled to CNBr-Sepharose (0.5 g) as described by Entwistle & ap Rees (1990), and then packed into a 2 ml syringe blocked with a glass-microfibre filter. This affinity column was washed with 5 vol. of coupling buffer and then with 5 vol. of starting buffer. Serum (1 ml) was added to 1 ml of double-strength starting buffer and loaded on to the column, which was then washed with 10 vol. of starting buffer, followed by 3 vol. of 0.5 M-NaCl. Bound PFK(PP_i) antibodies were eluted with 5 column vol. of 0.1 M-glycine/HCl, pH 2.5; fractions (1 ml each) were collected and were neutralized immediately with 1 M-Tris. The protein content of each fraction was determined by the Bio-Rad protein assay, with γ-globulin as a standard. We demonstrated the effectiveness of the antibodies by subjecting them to Western-blot analysis in which they were challenged with extracts of potato tuber and wheat endosperm (Fig. 1). The predominant reaction was with two proteins of M_r 60000 and 65000, which we suggest represent PFK(PP_i). The reaction with proteins of much lower M_r is attributed to breakdown products of PFK(PP_i).

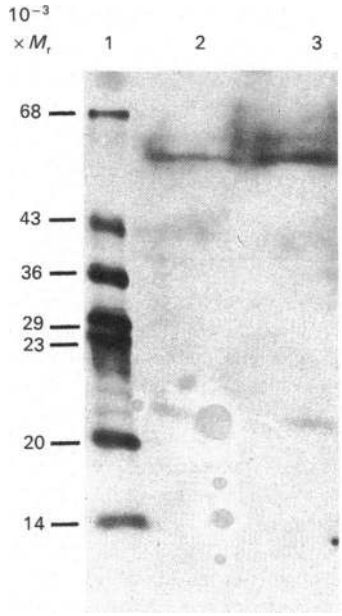

Fig. 1. Use of antibodies to PFK(PP_i) in immunoblot analysis of extracts of wheat endosperm and potato tuber

Samples of the developing endosperm of wheat, 8–12 days from anthesis (Entwistle & ap Rees, 1988), were homogenized in 70 mM-glycylglycine buffer, pH 7.5, that contained 0.3 % (w/v) BSA. Potato tuber was extracted as in the experiments described in Table 1. Extracts were centrifuged at 60000 *g* for 30 min, and samples of the supernatants were subjected to electrophoresis on SDS/polyacrylamide gels. The separated proteins were then transferred to nitrocellulose paper and PFK(PP_i) was detected by immunodecoration with the affinity-purified antibodies to potato PFK(PP_i) and ^{125}I-labelled Protein A, followed by autoradiography. Lane 1, standard proteins (BSA, M_r 68000; ovalbumin, M_r 43000; glyceraldehyde-3-phosphate dehydrogenase, M_r 36000; carbonic anhydrase, M_r 29000; casein, M_r 23000; soybean trypsin inhibitor, M_r 20000; lactalbumin, M_r 14200); lane 2, 100 μg of wheat endosperm protein; lane 3, 100 μg of potato tuber protein.

Enzyme assays

For the direct assay of FBPase, samples (500–900 mg fresh wt.) of tissue were homogenized, first in a pestle and mortar, and then in an all-glass homogenizer, in 4–6 vol. of 70 mM-glycylglycine, pH 7.4, containing 0.4 % (w/v) BSA. For cauliflower this extraction medium also contained 4 mM-cysteine/HCl and 1 mM-EDTA. For potatoes, the extraction medium was 100 mM-Tris/HCl (pH 7.6)/20 mM-EDTA/20 mM-cysteine/HCl/20 mM-diethyldithiocarbamic acid/0.3 % (w/v) BSA/insoluble polyvinylpyrrolidone (250 mg/g fresh wt.). The mixed samples consisted of equal weights of the tissue under consideration and leaf tissue. The homogenates were centrifuged at 60000 *g* for 30 min, and the supernatant was either assayed at once or after desalting with a column of Sephadex G-25. Extracts were prepared and kept at 2–4 °C.

FBPase was measured at 25 °C in the direction of Fru-6-*P* formation by continuous and stopped assays. Separate continuous assays were used for the plastidic and cytosolic enzymes; these were as described by Entwistle & ap Rees (1988), except that the buffer for the cytosolic assay was 50 mM-Tris/HCl, pH 7.1. For the stopped assay for plastidic FBPase, 20–100 μl of extract was incubated in a final volume of 1.0 ml of 50 mM-Hepes/NaOH (pH 8.1)/20 mM-$MgCl_2$/10 mM-Fru-1,6-P_2 for

15 min at 25 °C. Then 100 μl of 1.41 M-$HClO_4$ was added; 80 s later the reaction mixture was neutralized with 20 μl of 5 M-K_2CO_3 and centrifuged at 11600 *g* for 4 min. The supernatant (900 μl) was then added to 50 μl of 0.45 mM-$NADP^+$, 0.7 unit of glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 0.1 unit of glucose-6-phosphate isomerase (EC 5.3.1.9), 0.4 unit of phosphoglucomutase (EC 2.7.5.5) and 0.24 unit of 6-phosphogluconate dehydrogenase (EC 1.1.1.44) to measure total hexose monophosphate. The latter is taken as the measure of Fru-6-*P* produced. PFK(PP_i) was assayed as described by Entwistle & ap Rees (1988).

Immunoprecipitation of PFK(PP_i)

Extracts of the different tissues were prepared and centrifuged as for the direct assay of FBPase. Portions of the supernatants were boiled for 10 min and then samples (20–100 μl) of untreated and boiled supernatants were incubated with antibody for 1 h at 20 °C. The amount of antibody protein added to each sample was 15–20 μg, except that 20–40 μg was used with pea embryos and 50–70 μg for maize endosperm. Next the antibody–antigen complex was precipitated with insoluble Protein A (4 μg/ml) in Hepes-buffered saline [10 mM-Hepes (pH 7.3)/150 mM-NaCl]. After 30 min the mixture was centrifuged at 12000 *g* for 3 min, and portions of the supernatant were assayed for plastidic FBPase by the stopped assay.

SDS/PAGE and immunoblotting

Extracts were prepared as for the enzymic assay of FBPase, and samples of the supernatant, containing 25–100 μg of protein, were taken without prior desalting. The procedures used to separate proteins by SDS/PAGE, and in the subsequent immunoblotting, were exactly as described by Entwistle & ap Rees (1988).

RESULTS

Spectrophotometric assay of unfractionated extracts

We measured the ability of extracts of each tissue to convert Fru-1,6-P_2 into Fru-6-*P*, in assays for plastidic and cytosolic FBPase, and determined the effect of Fru-2,6-P_2 on such activity. We made concurrent measurements on extracts of pea leaves to check that the method does indicate the presence of FBPase where it is known to be present in appreciable activities, and on extracts of mixtures of the tissue under examination plus leaf tissue. We did this to see whether any of the extracts interfered with the abilities of the assays to detect FBPase in the leaf extracts. Each measurement was repeated on samples of the extracts that had been desalted with Sephadex G-25. Desalting did not alter the response to Fru-2,6-P_2 in any assay, and the data for the desalted extracts are not shown, except for potato. In general, desalting reduced activity by 25–60%, but in potato extracts the values for the plastidic and cytosolic assays were 6- and 2-fold higher respectively in the desalted extracts.

Extracts of each tissue produced Fru-6-*P* from Fru-1,6-P_2 at significant rates in each assay (Table 1). The activities found in extracts of leaves responded to Fru-2,6-P_2 in the manner expected of FBPase, in that plastidic activity was slightly decreased, whereas cytosolic activity was severely reduced. Similar evidence of inhibition by Fru-2,6-P_2 was found with extracts of the mixed samples. This suggests that none of the tissue extracts contained substances that prevented the demonstration of FBPase when this was present at appreciable activities. Except for pea embryos, the effect of Fru-2,6-P_2 on Fru-6-*P* production by extracts of the tissues examined for FBPase differed from that found for leaf extracts. The mean values for activity in the presence of Fru-2,6-P_2 were higher than those found in its absence. The differences were often very slight and not statistically significant; the key point is that, pea embryos apart, there was no sign of inhibition

Table 1. Assay of unfractionated extracts of plant tissues for plastidic and cytosolic FBPase

For each experiment we prepared three samples; one of the tissue examined (500–750 mg fresh wt.), one of pea or wheat leaf tissue (500–880 mg fresh wt.) and one that was a mixture of equal weights of the tissue examined and leaf tissue (500–900 mg total fresh wt.). Tissue homogenates were centrifuged at 60000 *g* for 30 min, and portions of the supernatant were assayed for plastidic (pH 8.1) and cytosolic (pH 7.1) FBPase in the presence and absence of 20 μM-Fru-2,6-P_2. Each value is the mean ± S.E.M. for determinations from three different extracts, except that for pea leaves, which is from 21 extracts.

	Activity (nmol of Fru-6-*P*/min per g fresh wt.)			
	Plastidic assay		Cytosolic assay	
Tissue	−Fru-2,6-P_2	+Fru-2,6-P_2	−Fru-2,6-P_2	+Fru-2,6-P_2
Pea leaf	2081 ± 191	1810 ± 156	287 ± 34	23 ± 6
Soybean suspension cells	60 ± 14	67 ± 24	74 ± 4	85 ± 2
Soybean cells + pea leaf	528 ± 124	508 ± 171	57 ± 9	20 ± 11
Potato tubers*	118 ± 18	120 ± 14	38 ± 3	40 ± 8
Potato tubers + pea leaf*	434 ± 95	45 ± 7	186 ± 34	15 ± 4
Cauliflower florets	43 ± 7	54 ± 6	43 ± 15	87 ± 23
Cauliflower + pea leaf	443 ± 54	329 ± 54	150 ± 28	32 ± 17
Endosperm of maize (var. White Horse Tooth)	182 ± 71	216 ± 77	79 ± 21	169 ± 26
Wheat leaf	274 ± 56	231 ± 32	112 ± 24	2 ± 2
Endosperm of maize (var. White Horse Tooth) + wheat leaf	249 ± 48	49 ± 19	201 ± 12	25 ± 17
Endosperm of maize (var. Golden Bantam)	273 ± 38	361 ± 60	155 ± 18	220 ± 38
Endosperm of maize (var. Golden Bantam) + pea leaf	587 ± 133	41 ± 7	134 ± 22	52 ± 15
Pea roots	55 ± 15	57 ± 17	35 ± 1	94 ± 3
Pea roots + pea leaf	557 ± 94	544 ± 81	152 ± 73	45 ± 1
Embryos of round peas	43 ± 11	41 ± 11	24 ± 13	53 ± 14
Embryos of round peas + pea leaf	511 ± 71	398 ± 50	88 ± 25	42 ± 30
Embryos of wrinkled peas	53 ± 19	45 ± 15	21 ± 9	52 ± 7
Embryos of wrinkled peas + pea leaf	897 ± 22	698 ± 41	48 ± 21	12 ± 8

* These extracts were desalted with Sephadex G-25 before assay.

by Fru-2,6-P_2. The activities for the mixed samples were not the sum of those predicted from the measurements made on the separate components of the mixtures. We think that this is due to the measured activities being an unspecified combination of FBPase and PFK(PP_i). The assay is designed and optimized for FBPase; the contribution from PFK(PP_i) will depend, at least in part, on the amounts of P_i and Fru-2,6-P_2 present in the extracts. These will differ according to whether the sample consists of 500 mg of pea leaf or of 250 mg of pea leaf plus 250 mg of some other tissue. Our data are consistent with the view that, except for leaves and pea embryos, the Fru-6-*P* production shown in Table 1 was due to PFK(PP_i) and not FBPase.

To test the above hypothesis, we preincubated extracts of each tissue with our antibodies to PFK(PP_i) and then measured their ability to convert Fru-1,6-P_2 into Fru-6-*P* in the assay for plastidic FBPase (Table 2). In order to check that any loss of activity was due to the antibodies and not to the incubation *per se*, samples of the extracts were incubated under comparable conditions but without the antibodies. To check that any activity that survived treatment with the antibodies was enzymic, we incubated boiled samples of the extracts with the antibodies. Table 2 shows that, as expected, each extract produced appreciable amounts of Fru-6-*P* from Fru-1,6-P_2. This activity was not diminished by the incubation alone: the values for the control samples were equal to those found before incubation. Treatment of the extracts of each tissue with antibodies to PFK(PP_i) reduced Fru-6-*P* production. For extracts of soybean cells, potato tubers, cauliflowers, maize endosperm (var. White Horse Tooth) and pea roots, treatment with the antibodies reduced the rate of Fru-6-*P* production to the very low rate found with boiled extracts. We argue that the antibodies removed all of the enzymic ability of these extracts to convert Fru-1,6-P_2 into Fru-6-*P*. Treatment of the extracts of pea embryos with antibodies had relatively little effect on Fru-6-*P* production and left substantial activities that were completely removed by boiling. The data for the endosperm of the sweet corn (var. Golden Bantam) were intermediate; the antibodies reduced activity markedly, but there was some slight residual activity that was destroyed by boiling (9 nmol of Fru-6-*P*/min per g fresh wt.). This activity was not reduced below this value if the amount of antibody added to the extract was increased beyond the usual 50 μg.

Table 2. Effect of antibody to PFK(PP_i) on conversion of Fru-1,6-P_2 into Fru-6-*P* by unfractionated extracts of plant tissues

In each experiment, samples (650–1000 mg fresh wt.) were homogenized in 3–5 vol. of extraction medium and the homogenates were centrifuged at 60000 *g* for 30 min. Portions of the supernatants were boiled for 10 min. Samples of untreated and of boiled supernatants were incubated at 20 °C for 1 h with 12–70 μg of antibody to PFK(PP_i); a control sample of untreated supernatant was incubated under the same conditions, except that no antibody was present. At the end of the incubation the antigen–antibody complex was precipitated with insoluble Protein A in Hepes-buffered saline and removed by centrifugation. Then the supernatant was assayed for plastidic FBPase with the stopped assay. Each value is the mean ± S.E.M. for determinations from three different extracts, except where the three samples of tissue were of slightly different age: here each value is given. N.D., not detected.

Tissue	Treatment of extract	Activity (nmol of Fru-6-*P*/min per g fresh wt.)
Soybean suspension cells	None	17.0 ± 0.6
	Buffer	18.7 ± 1.9
	Antibody	3.0 ± 1.2
	Boiled	3.1 ± 1.3
Potato tuber	None	8.7, 15.3, 23.9
	Buffer	8.9, 16.7, 22.9
	Antibody	1.8, 6.0, N.D.
	Boiled	1.8, 4.8, N.D.
Cauliflower florets	None	30.4 ± 1.3
	Buffer	29.6 ± 1.7
	Antibody	4.8 ± 0.6
	Boiled	5.5 ± 0.3
Maize endosperm	None	44.5, 70.9, 51.0
var. White Horse Tooth	Buffer	38.9, 74.5, 51.2
	Antibody	4.0, 18.6, 7.5
	Boiled	4.0, 18.6, 7.5
var. Golden Bantam	None	60.6, 64.8, 62.3
	Buffer	66.6, 66.8, 57.5
	Antibody	27.6, 16.4, 12.9
	Boiled	7.5, 10.8, 4.3
Pea roots	None	24.4 ± 1.6
	Buffer	30.3 ± 2.0
	Antibody	7.1 ± 2.4
	Boiled	6.7 ± 2.1
Pea embryo	None	40.6 ± 4.0
Round	Buffer	41.0 ± 2.0
	Antibody	29.3 ± 0.4
	Boiled	N.D.
Wrinkled	None	28.5 ± 1.3
	Buffer	23.1 ± 0.2
	Antibody	22.6 ± 1.4
	Boiled	N.D.

Immunoblotting

We prepared extracts of each non-photosynthetic tissue, of leaves of the same species, and of a mixture of the non-photosynthetic tissue and a leaf tissue. We did this to demonstrate that the antibody would recognize FBPase from the species examined, and to show that the extract of the non-photosynthetic tissue did not prevent the antibody from recognizing FBPase. The extracts were subjected to SDS/PAGE. The separated proteins were then transferred to nitrocellulose paper and FBPase was detected by immunodecoration with antibody to spinach plastidic FBPase and ^{125}I-labelled Protein A, followed by autoradiography. The results are shown in Figs. 2–5; each

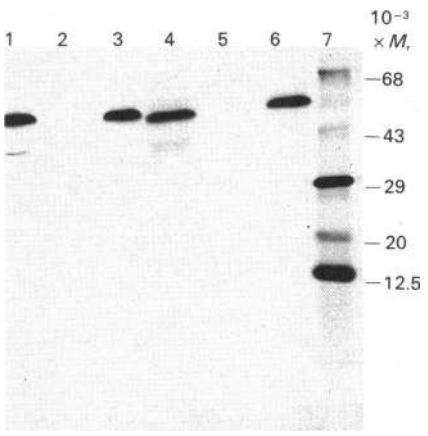

Fig. 2. Western blotting of extracts of soybean suspension cells and pea root apices using antibody to plastidic FBPase

Unfractionated extracts, prepared as for the experiments described in Tables 1 and 2, were treated as described in Fig. 1, except that antibody to plastidic FBPase from spinach leaf replaced the antibodies to potato PFK(PP_i). Lane 1, 25 μg of pea leaf protein; lane 2, 100 μg of pea root protein; lane 3, 100 μg of protein from a mixture of pea leaf and pea root; lane 4, 25 μg of soybean leaf protein; lane 5, 100 μg of protein from soybean suspension culture; lane 6, 100 μg of protein from a mixture of soybean leaf and suspension cells; lane 7, standard proteins (as in Fig. 1, except that cytochrome *c*, M_r 12500, replaced lactalbumin, and glyceraldehyde-3-phosphate dehydrogenase and casein were omitted).

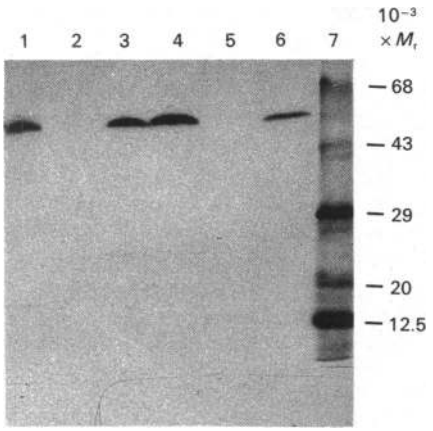

Fig. 3. Western blotting of extracts of potato tuber and the florets of cauliflower using antibody to plastidic FBPase

The procedure was as described in Fig. 2. Lane 1, 25 μg of potato leaf protein; lane 2, 100 μg of potato tuber protein; lane 3, 100 μg of protein from a mixture of potato tuber and pea leaf; lane 4, 25 μg of cauliflower leaf protein; lane 5, 100 μg of cauliflower floret protein; lane 6, 100 μg of protein from a mixture of cauliflower florets and pea leaves; lane 7, standard proteins (as in Fig. 2).

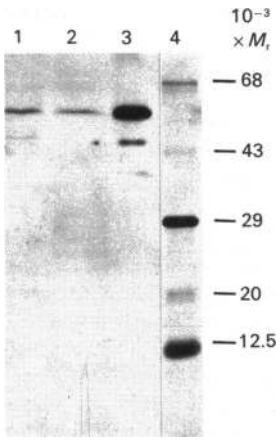

Fig. 5. Western blotting of extracts of the developing embryos of peas using antibody to plastidic FBPase

The procedure was as described in Fig. 2. Lane 1, 100 μg of pea (round) embryo protein; lane 2, 100 μg of pea (wrinkled) embryo protein; lane 3, 25 μg of pea leaf protein; lane 4, standard proteins (as in Fig. 2).

experiment was repeated three times. A different extract was used on each occasion, and similar results were obtained each time.

The antibody to the plastidic FBPase recognized proteins of M_r about 54000 in extracts of leaves of each species examined. These same proteins were recognized in the mixtures of these leaves and the corresponding non-photosynthetic tissues. No protein was recognized in the extracts of the soybean suspension cells, potato tubers, cauliflower florets, endosperm of maize (var. White Horse Tooth), and pea root apices. Extracts of endosperm of sweet corn (var. Golden Bantam) contained a protein of M_r about 45000 that was recognized by the antibody. Extracts of the developing embryos of both varieties of peas contained a protein of M_r about 54000 that reacted with the antibody.

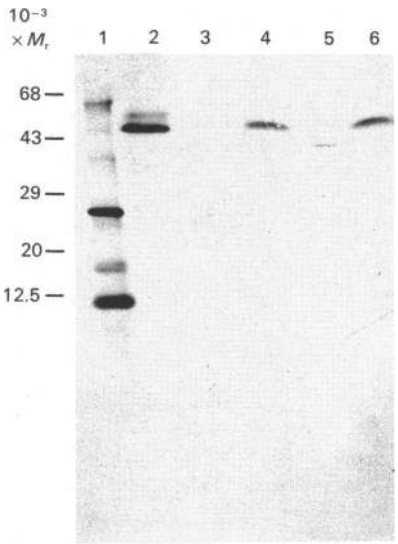

Fig. 4. Western blotting of extracts of the developing endosperm of maize using antibody to plastidic FBPase

The procedure was as described in Fig. 2. Lane 1, standard proteins (as in Fig. 2); lane 2, 25 μg of maize leaf protein; lane 3, 100 μg of maize (var. White Horse Tooth) endosperm protein; lane 4, 100 μg of protein from mixture of endosperm (var. White Horse Tooth) and pea leaf; lane 5, 100 μg of sweet-corn (var Golden Bantam) endosperm protein; lane 6, 100 μg of protein from mixture of sweet-corn endosperm and pea leaf.

DISCUSSION

The spectrophotometric assays showed that extracts of soybean cells, potato tubers, cauliflower florets, endosperm of maize (var. White Horse Tooth) and pea roots all converted Fru-1,6-P_2 into Fru-6-P at appreciable rates. However, this activity did not respond to Fru-2,6-P_2, as would be expected of FBPase, as no inhibition was found. When the assays were applied to extracts of pea leaves or mixtures of leaves and the above-named non-photosynthetic tissues, then evidence of FBPase was obtained. These results suggest that if there were appreciable FBPase in any of the non-photosynthetic tissues listed, then we would have detected it. The results also suggest that the observed production of Fru-6-P was due to PFK(PP_i), not FBPase. This suggestion is supported by our observations that pretreatment of the extracts with antibody to PFK(PP_i) reduced Fru-6-P formation to a value that is indistinguishable from that found in samples of extracts that had been boiled for 10 min. We argue that the activity that resisted the antibody was not due to an enzyme and that the antibody abolished the enzymic production of Fru-6-P. Antibody to plastidic FBPase failed to recognize any protein in extracts of the above-named non-photosynthetic tissues. In contrast, the antibody did recognize a protein of M_r about 54000 in extracts of the corresponding leaves and did so in the presence of the extracts of the non-photosynthetic tissues. We argue that this protein is plastidic FBPase, and that the extracts of the non-photosynthetic tissues do not interfere with detection of plastidic FBPase by the antibody. Collectively, the three experimental approaches that we have used agree, and the results lead us to conclude that soybean suspension cells, potato tubers, cauliflower florets, the developing endosperm of maize (var. White Horse Tooth) and pea root apices lack significant activities of plastidic FBPase. These tissues are thus similar to the developing endosperm of wheat in that they do not import carbon for starch synthesis into the amyloplast as triose phosphate; we think it likely that they use hexose phosphate. The manner in which [1-^{14}C]- and [6-^{14}C]-glucose labelled starch when supplied to

potato tubers and maize endosperm strongly supports this view (Hatzfeld & Stitt, 1990).

Our conclusions are at odds with the claims that FBPase is present in soybean suspension cultures (Macdonald & ap Rees, 1983), potato tubers (Mohabir & John, 1988), maize endosperm (Echeverria *et al.*, 1988) and cauliflower florets (Journet & Douce, 1985). None of these claims take into acount the ease with which FBPase may be confused with PFK(PP_i). The evidence for the first three of the above claims comes from analyses of cell fractionations in which apparent FBPase activity was reported for fractions that contained amyloplasts. We cannot now be sure that the activities attributed to FBPase were not, at least in part, due to PFK(PP_i). Thus arguments that the amyloplast fractions were enriched in FBPase are no longer convincing. In the study of cauliflowers the plastids were very pure, and it is unlikely that the observed activity, which was very small, was due to contaminating PFK(PP_i). However, Journet & Douce (1985) started with a very large amount of tissue, taken from the surface of the head, and finished with a very small yield of plastids (1 %). This procedure may have concentrated plastids, from the very outermost layers of cells, that had received sufficient light to have begun differentiating into chloroplasts, in which case some FBPase activity might be expected. Such plastids would be expected to be a very small fraction of the total population of plastids in the florets, thus FBPase would not be detected when the complete florets were analysed. A similar explanation is offered for the evidence that led to the claim that rice amyloplasts contain FBPase (Nakamura *et al.*, 1989), particularly as their amyloplast preparations contained appreciable activity of ribulosebisphosphate carboxylase.

Our data for the endosperm of sweet corn (the Golden Bantam cultivar of maize) is equivocal. Direct assay for FBPase (Table 1) provided no evidence for the presence of FBPase. However, not all of the observed activity was abolished by antibody to PFK(PP_i), and the antibody to FBPase did recognize a protein in the extracts, although this was smaller than expected for plastidic FBPase. These data are not definitive enough to decide the issue. Our estimate of the maximum activity of FBPase that might be present in Golden Bantam is 9 nmol/min per g fresh wt. This is appreciably below the rate of starch synthesis observed *in vivo*, namely 20 nmol/min per g fresh wt. Thus, even if the activity is FBPase, it does not appear to be on the main route from sucrose to starch.

For the developing embryos of peas the results of all three types of experiment indicate the presence of FBPase activity. Assay of unfractionated extracts for plastidic FBPase produced results comparable with those obtained with leaves, and most of this activity was resistant to antibodies to PFK(PP_i). In addition the antibodies to plastidic FBPase recognized a protein of M_r comparable with that of known plastidic FBPase. We sugget that the developing embryos of peas contain plastidic FBPase. This is not very surprising, as the plastids of the developing embryos of peas retain some of the characteristics of chloroplasts (Smith *et al.*, 1990). The presence of FBPase in plastids of developing embryos of peas may reflect the origin of these plastids from chloroplasts and be largely incidental to starch synthesis. The enzyme may be confined to those plastids that contain little starch (Smith *et al.*, 1990) and, even if it is not, its presence does not preclude entry of the carbon for starch synthesis as hexose monophosphate.

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