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# Enzymic capacities of amyloplasts from wheat (*Triticum aestivum*) endosperm

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Lysates of protoplasts from the endosperm of developing grains of wheat (*Triticum aestivum*) were fractionated on density gradients of Nycodenz to give amyloplasts. Enzyme distribution on the gradients suggested that: (i) starch synthase and ADP-glucose pyrophosphorylase are confined to the amyloplasts; (ii) pyrophosphate:fructose-6-phosphate 1-phosphotransferase and UDP-glucose pyrophosphorylase are confined to the cytosol; (iii) a significant proportion (23–45%) of each glycolytic enzyme, from phosphoglucomutase to pyruvate kinase inclusive, is in the amyloplast. Starch synthase, ADP-glucose pyrophosphorylase and each of the glycolytic enzymes showed appreciable latency when assayed in unfractionated lysates of protoplasts. No activity of fructose-1,6-bisphosphatase was found in amyloplasts or in homogenates of endosperm. Antibody to plastidic fructose-1,6-bisphosphatase did not react positively, in an immunoblot analysis, with any protein in extracts of wheat endosperm. It is argued that wheat endosperm lacks significant plastidic fructose-1,6-bisphosphatase and that carbon for starch synthesis does not enter the amyloplast as a C-3 compound but probably as hexose phosphate.

## INTRODUCTION

The pathway from translocated sucrose to storage starch in the non-photosynthetic cells of higher plants is not known. The initial metabolism of sucrose almost certainly occurs in the cytosol, but subsequently some derivative(s) of sucrose must cross the amyloplast envelope to support starch synthesis therein. Evidence has been presented that amyloplasts from suspension cultures of soybean (*Glycine max*) cells contain all the enzymes required to convert triose phosphate into starch via fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) and ADP-glucose (Macdonald & ap Rees, 1983). A similar argument has been made about the lipid- and starch-rich plastids from cauliflower (*Brassica oleracea*) florets (Journet & Douce, 1985). These observations are consistent with the view that, in non-photosynthetic cells, carbon for starch synthesis enters the amyloplast as a C-3 compound via the phosphate translocator.

In contrast with the above evidence, studies of the endosperm of developing wheat (*Triticum aestivum*) suggest that, in this tissue, carbon for starch synthesis enters the amyloplast as a C-6 compound. Keeling *et al.* (1988) supplied [1-$^{13}$C]- and [6-$^{13}$C]-glucose to developing wheat grains and determined the distribution of $^{13}$C between the carbon atoms of the glucosyl residues of starch and sucrose. The degree of equilibration between C-1 and C-6 in the glucosyl residues of starch was very similar to that in the glucose moiety of sucrose and was much less than would be expected if hexose had to be converted into triose phosphate for entry into the amyloplast. The aim of the work described in the present paper was to use our recently developed method for isolating wheat amyloplasts (Entwistle *et al.*, 1988) to determine which of the enzymes of carbohydrate metabolism are located in these amyloplasts, and to compare the enzymic capacities of the amyloplasts with the distribution of label reported by Keeling *et al.* (1988).

## EXPERIMENTAL

### Materials

Enzymes, substrates, isotopes and special chemicals were obtained as described by Entwistle *et al.* (1988); those not mentioned previously were from either Boehringer Corp., Lewes, East Sussex, U.K., or Sigma Chemical Co., Poole, Dorset, U.K., except that $^{125}$I-labelled Protein A was from Amersham International, Amersham, Bucks., U.K. Antibody to chloroplastic FBPase from leaves of spinach (*Spinacia oleracea* L.) was prepared by Nishizawa & Buchanan (1981) and obtained from Dr. C. Raines, AFRC Institute of Plant Science Research, Cambridge, U.K. Antibody to pyrophosphate:fructose-6-phosphate 1-phosphotransferase [PFK(PP$_i$); EC 2.7.1.90] from tubers of potato (*Solanum tuberosum* L.) was prepared by Kruger & Hammond (1988) and obtained from Dr. N. J. Kruger, Rothamsted Experimental Station, Harpenden, Herts., U.K. Winter wheat (*Triticum aestivum* L. var Mardler) was grown as described by Entwistle *et al.* (1988). Endosperm was obtained from developing grains 8–12 days after anthesis. For studies of leaves we took the basal leaves of 8-week-old plants.

### Amyloplasts

Amyloplasts were obtained from the endosperm of developing grains taken 8–12 days after anthesis. Protoplasts were prepared, purified, and then lysed with dextran and dextran sulphate. The resulting 'unfraction-

Abbreviations used: PPase, alkaline inorganic pyrophosphatase (EC 3.6.1.1); 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); PFK(ATP), 6-phosphofructokinase (EC 2.7.1.11).

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ated lysate' was placed on a continuous density gradient of Nycodenz, which was then left on ice for 4–6 h before being fractionated from the top. The first fraction corresponded to the volume of lysate added to the gradient (4.8–7.0 ml), the next eight fractions were each of 2 ml, and the bottom fraction was of 5 ml. Alkaline inorganic pyrophosphatase (PPase; EC 3.6.1.1) was used as a marker for amyloplasts and assayed in each fraction; that with the highest yield (generally the fifth or sixth 2 ml fraction from the top) was taken as the amyloplast fraction. Details of the above procedures were precisely as in Entwistle *et al.* (1988).

**Enzyme assays**

For determination of the enzymic properties of the amyloplasts, each fraction of the gradient, and the unfractionated lysate, were assayed as described in the following references, except for the variations given (assays from the *same* reference are separated by semicolons; from *different* references by colons): UDP-glucose pyrophosphorylase (EC 2.7.7.9) (Entwistle *et al.*, 1988): PFK($PP_i$), standard assay (ap Rees *et al.*, 1985): starch synthase (EC 2.4.1.21), 0.61 $\mu$mol of ADP-[U-$^{14}$C]glucose (0.25 Ci/mol), no GSH, in 200 $\mu$l at 30 °C (Hawker *et al.*, 1972): alkaline PPase, for control samples the trichloroacetic acid was added to the extract before the $PP_i$ (Gross & ap Rees, 1986): ADP-glucose pyrophosphorylase (EC 2.7.7.27), 100 mM-Hepes, pH 8.0, 5 mM-$MgCl_2$, 1.2 mM-ATP, 7 mM-3-phosphoglyceric acid, 50 $\mu$g of bovine serum albumin, 0.02 unit of inorganic pyrophosphatase, 0.675 mM-[U-$^{14}$C]glucose 1-phosphate (0.7 Ci/mol) in 200 $\mu$l and incubated at 37 °C for 6 min (Shen & Preiss, 1964): phosphoglucomutase (EC 2.7.5.5); glucose-6-phosphate isomerase (EC 5.3.1.9), 0.4 mM-$NAD^+$, 1 mM-$MgCl_2$, 0.7 mM-fructose 6-phosphate, no 6-phosphogluconate dehydrogenase; fructose-1,6-bisphosphate aldolase (EC 4.1.2.13); triosephosphate isomerase (EC 5.3.1.1); glyceraldehyde phosphate dehydrogenase($NAD^+$) (EC 1.2.1.12), 1 mM-EDTA, 7.5 mM-sodium arsenate, 0.8 unit of fructose-1,6-bisphosphate aldolase; fructose-1,6-bisphosphatase (Macdonald & ap Rees, 1983): pyruvate kinase (EC 2.7.1.40), 4 mM-$MgSO_4$, 12 mM-KCl, 30 mM-phosphoenolpyruvate; enolase (EC 4.2.1.11), 4 mM-$MgSO_4$, 12 mM-KCl; phosphoglycerate mutase (EC 2.7.5.3), 4 mM-$MgSO_4$, 12 mM-KCl (ap Rees *et al.* 1975): phosphoglycerate kinase (EC 2.7.2.3) (Scopes, 1975): PFK(ATP) (Scott *et al.*, 1964).

Latency of enzymes in unfractionated lysates was measured by assaying samples in reaction mixtures that contained 0.5 M-sucrose to prevent rupture of the organelles. For each enzyme, the value obtained is compared with that found for a comparable sample of the same lysate assayed under the same conditions, except that the organelles had been ruptured either by the addition of Triton X-100 to 0.1% (v/v) or, in the case of PPase, one cycle of freezing in liquid $N_2$ and thawing at 25 °C, followed by vortex-mixing. The difference between activity in the intact and ruptured samples is expressed as a percentage of that in the ruptured sample to give latency (Stitt & ap Rees, 1979).

For assay of FBPase in unfractionated extracts of endosperm and of leaves, samples of tissue (400–700 mg fresh wt.) were homogenized as described by ap Rees *et al.* (1985) in 4–6 vol. of 70 mM-glycylglycine buffer, pH 7.5, that contained 0.3% (w/v) bovine serum albumin. The homogenates were centrifuged at 60000 *g* for 30 min and the supernatant was assayed either without further treatment or after de-salting by passage through a column (1 cm × 13 cm) of Sephadex G-25 (Coarse grade). Activity was measured as fructose 6-phosphate (Fru-6-*P*) production at 25 °C in assay mixtures of 1.0 ml. For the plastidic FBPase the latter contained 50 mM-Hepes (pH 8.1), 1 mM-Fru-1,6-$P_2$, 20 mM-$MgCl_2$, 0.4 mM-$NAD^+$, 0.7 unit of glucose-6-phosphate dehydrogenase (from *Leuconostoc*, reduces $NAD^+$) and 0.1 unit of glucose-6-phosphate isomerase. For cytosolic FBPase the assay was similar, except that the buffer was 50 mM-imidazole/HCl, pH 7.1, and $MgCl_2$ and Fru-1,6-$P_2$ were 4 mM and 0.1 mM respectively. The Fru-1,6-$P_2$ used in these assays was pretreated to remove any traces of fructose 2,6-bisphosphate (Fru-2,6-$P_2$): solutions of 1 mM-Fru-1,6-$P_2$ were adjusted to pH 2.0 with 2 M-HCl, boiled for 10 min and then neutralized with 2 M-NaOH. This procedure did not cause significant loss of Fru-1,6-$P_2$. For assessment of the effects of antibody to PFK($PP_i$) on the apparent activity of FBPase, 40 $\mu$l of the supernatant obtained by centrifuging the tissue homogenate at 60000 *g* was incubated at 20 °C for 30 min with 80 $\mu$l of 150 mM-NaCl/10 mM-$NaH_2PO_4$, pH 7.2, containing 160 mg of either pre-immune serum, or antibody to PFK($PP_i$). The antibody–antigen complex was then precipitated by adding 40 $\mu$l of 150 mM-NaCl/10 mM-Tris/HCl (pH 7.2), containing 8 mg of Protein A–Sepharose. After incubating at 20 °C for 30 min the mixture was centrifuged and the supernatant was assayed for FBPase. Protein was measured by using the Bio-Rad protein assay.

**SDS/polyacrylamide-gel electrophoresis**

Samples of endosperm (540 mg fresh wt.), leaf (734 mg fresh wt.), and a mixture of endosperm (267 mg) and leaf (258 mg) were homogenized and centrifuged as described for the assay of FBPase in unfractionated extracts. Portions (40–80 $\mu$g of protein) of the supernatant obtained after centrifugation at 60000 *g* were boiled for 2 min with equal volumes of sample buffer [62.5 mM-Tris/HCl (pH 6.8)/2% (w/v) SDS/10% (v/v) glycerol/5% (w/v) 2-mercaptoethanol/0.001% (w/v) Bromophenol Blue] and then subjected to SDS/polyacrylamide-gel electrophoresis in 15% (w/v) gels, as described by Laemmli (1970), for 16 h at 90 V. The marker proteins were bovine serum albumin ($M_r$ 68000), ovalbumin ($M_r$ 43000), carbonic anhydrase ($M_r$ 29000), soybean trypsin inhibitor ($M_r$ 20000) and cytochrome *c* ($M_r$ 12500) and were labelled with [$^{14}$C]acetimidate.

**Immunoblotting procedure**

After electrophoresis the polypeptides were transferred to nitrocellulose paper by a combination of the methods of Vaessen *et al.* (1981) and Renart *et al.* (1979). The gels were immersed in 250 ml of 25 mM-Tris/glycine, pH 8.3, containing 20% (v/v) methanol and 0.1% (w/v) SDS (blotting buffer) and incubated at 25 °C with gentle shaking. The polypeptides were then transferred electrophoretically to nitrocellulose paper (Schleicher und Schüll BA 85) as described by Vaessen *et al.* (1981). Electrophoresis was carried out for 3 h at 250 mV at 4 °C. The nitrocellulose paper was then washed in 100 mM-Tris/HCl (pH 7.8)/300 mM-NaCl and incubated

**Table 1. Activities of enzymes of carbohydrate metabolism in preparations of amyloplasts**

Lysates of protoplasts were fractionated on continuous gradients of Nycodenz; 2 ml fractions were taken successively from the top of the gradient to leave a final fraction of 5 ml. Enzyme activities in each fraction were measured. Results are means ± S.E.M. for the number of different lysates analysed. For each gradient we used the paired-sample *t* test to compare the percentage of the cytosolic marker enzyme recovered in the amyloplast fraction with that of the enzyme whose location we wished to establish. Abbreviation: GPDase(NAD$^+$), glyceraldehyde-phosphate dehydrogenase(NAD$^+$).

| Enzyme | No. of lysates analysed | Activity in the unfractionated lysate (nmol/min per ml of lysate) | Percentage of activity in unfractionated lysate that was recovered in: Amyloplasts | Percentage of activity in unfractionated lysate that was recovered in: Sum of all gradient fractions | Fisher's *P* value for paired *t* test for enzyme activity in amyloplasts versus activity of cytosolic marker |
|---|---|---|---|---|---|
| UDP-glucose pyrophosphorylase | 8 | 541 ± 96 | 1.9 ± 0.4 | 102 ± 12 | – |
| PFK($PP_i$) | 12 | 62 ± 11 | 2.5 ± 0.3 | 100 ± 5 | – |
| PPase | 19 | 191 ± 20 | 17.8 ± 1.2 | 97 ± 6 | < 0.001 |
| Starch synthase | 4 | 2.7 ± 0.3 | 24.4 ± 6.4 | 77 ± 14 | < 0.05 |
| ADP-glucose pyrophosphorylase | 4 | 26 ± 9 | 20.3 ± 5 | 84 ± 13 | < 0.05 |
| Phosphoglucomutase | 5 | 62 ± 15 | 4.5 ± 0.9 | 90 ± 10 | < 0.05 |
| Glucose-6-phosphate isomerase | 5 | 514 ± 146 | 7.4 ± 1.2 | 94 ± 9 | < 0.01 |
| PFK(ATP) | 4 | 2.4 ± 0.3 | 7.0 ± 0.3 | 106 ± 9 | < 0.001 |
| Aldolase | 4 | 15 ± 5 | 7.6 ± 1.9 | 67 ± 8 | < 0.05 |
| Triosephosphate isomerase | 5 | 12449 ± 3988 | 6.2 ± 1.5 | 100 ± 12 | < 0.05 |
| GPDase(NAD$^+$) | 4 | 119 ± 12 | 5.5 ± 0.5 | 80 ± 9 | < 0.02 |
| Phosphoglycerate kinase | 4 | 508 ± 130 | 5.6 ± 0.8 | 98 ± 8 | < 0.05 |
| Phosphoglycerate mutase | 5 | 716 ± 134 | 5.5 ± 1.5 | 96 ± 7 | < 0.05 |
| Enolase | 5 | 397 ± 99 | 6.8 ± 2.1 | 96 ± 6 | < 0.05 |
| Pyruvate kinase | 4 | 86 ± 13 | 9.5 ± 2.8 | 74 ± 12 | < 0.01 |

for 2 h at 25 °C in 250 ml of 100 mM-Tris/HCl (pH 7.8)/300 mM-NaCl/2% (v/v) bovine serum albumin. The nitrocellulose sheet was then sealed in a polythene bag with 20 ml of the last-mentioned solution, which contained 10 $\mu$l of antibodies, and incubated on an orbital shaker at 200 rev./min for 16 h at 25 °C. Next the paper was washed three times, each for 30 min in 100 mM-Tris/HCl (pH 7.8)/300 mM-NaCl; the second of the washing solutions contained 1% (v/v) Nonidet P40. After it had been washed, the paper was sealed in a polythene bag with 1 $\mu$Ci of $^{125}$I-labelled Protein A in 15 ml of 100 mM-Tris/HCl (pH 7.8)/300 mM-NaCl/2% bovine serum albumin and incubated on an orbital shaker at 200 rev./min at 25 °C for 2 h. The nitrocellulose paper was then washed three times in 100 mM-Tris/HCl, pH 7.8, and once in the same solution containing 1% Nonidet P40 before being blotted dry on Whatman 3MM paper, air-dried and subjected to autoradiography against Kodak X-Omat S film at −80 °C.

## RESULTS AND DISCUSSION

### Enzymes of amyloplasts

We have described the effectiveness of our procedure for isolating amyloplasts (Entwistle *et al.*, 1988). No contamination by microbodies was found, and that from mitochondria was less than 5% of the number present in the unfractionated lysate. Contamination by the cytosol was less than 3%, and the total yield of amyloplasts was 19%. The data in Table 1 confirm this picture and show the distribution of enzymes in the present work when protoplast lysates were fractionated on density gradients. We stress that, for each gradient, we assayed each enzyme in each fraction. The sum of the activities recovered from the gradients is comparable with the activities added to the gradients; thus there were no serious losses during analysis. Regardless of whether they are present in amyloplasts, many of the enzymes that we studied are known to be present in the cytosol. Thus it was essential to assess cytosolic contamination of the amyloplasts. We did this for every gradient analysed by determining the distribution of a cytosolic marker, either PFK($PP_i$) or UDP-glucose pyrophosphorylase (Entwistle *et al.*, 1988). In order to decide whether an enzyme co-purified with the amyloplasts, we used the paired-sample *t* test to assess whether the activity in the amyloplast fraction was significantly greater than that expected from cytosolic contamination.

From Table 1 we suggest the following. First, the behaviour of PFK($PP_i$) and UDP-glucose pyrophosphorylase is consistent with their being confined to the cytosol. Secondly, the very similar patterns found for alkaline PPase, starch synthase and ADP-glucose pyrophosphorylase strongly suggest that all three are confined to the amyloplast, and that we recovered 20% of the amyloplasts. Finally, a significant fraction of each of the glycolytic enzymes co-purified with the amyloplasts. The precise fraction varied with the enzyme from 4.5 to 9.5% of the activity present in the unfractionated lysate. As the yield of amyloplasts was 20%, our data suggest that 20–45% of the total glycolytic capacity of wheat endosperm is in the amyloplasts.

If the above suggestion is correct, then a fraction of the total activity of each glycolytic enzyme in the unfractionated lysate should be latent. Assay of the enzymes in the lysate under conditions that kept the amyloplasts intact would reveal activity outside any organelles present but

**Table 2. Latency of enzymes in unfractionated lysates of protoplasts of wheat endosperm**

Lysates were divided into two halves. Samples of the first half were assayed in reaction mixtures that contained 0.5 M-sucrose (intact); samples of the other half were assayed under identical conditions, except that the organelles were first ruptured. Latency is the difference between the activities in the intact and ruptured samples as a percentage of that in the ruptured sample. The latency of each enzyme is compared with the latency of the cytosolic marker, UDP-glucose pyrophosphorylase or PFK($PP_i$) measured in the same lysate, by the paired-sample *t* test. Values are means ± S.E.M. for *n* samples. Abbreviation: GPDase($NAD^+$), glyceraldehyde-phosphate dehydrogenase.

| Enzyme | Number of lysates assayed (*n*) | Activity (nmol/min per ml of lysate) | | Latency (%) | Fisher's *P* value for latency versus that of the cytosolic marker |
|---|---|---|---|---|---|
| | | Intact | Ruptured | | |
| UDP-glucose pyrophosphorylase | 21 | 218 ± 24 | 234 ± 26 | 6.5 ± 1.4 | – |
| PFK($PP_i$) | 5 | 29.2 ± 5 | 30 ± 5 | 4.5 ± 3.1 | – |
| Starch synthase | 4 | 1.0 ± 0.1 | 2.6 ± 0.5 | 55 ± 8.5 | > 0.01 |
| ADP-glucose pyrophosphorylase | 14 | 12.1 ± 3.0 | 24.4 ± 5.3 | 53 ± 3.0 | > 0.001 |
| Phosphoglucomutase | 2 | 5.2, 4.6 | 7.1, 5.5 | 27, 16.8 | > 0.02 |
| Glucose-6-phosphate isomerase | 3 | 135 ± 4 | 196 ± 14 | 30.5 ± 3.8 | > 0.05 |
| PFK(ATP) | 3 | 33.9 ± 7.9 | 51.6 ± 10.7 | 34.5 ± 7.5 | > 0.05 |
| Aldolase | 3 | 9.6 ± 3.6 | 11.7 ± 4.6 | 16.6 ± 2.0 | > 0.02 |
| Triosephosphate isomerase | 3 | 5184 ± 851 | 7140 ± 451 | 28.3 ± 7.0 | > 0.05 |
| GPDase($NAD^+$) | 4 | 8.4 ± 1.7 | 10 ± 1.5 | 18.0 ± 4.3 | > 0.05 |
| Phosphoglycerate kinase | 4 | 84 ± 24 | 106 ± 31 | 19.6 ± 3.5 | > 0.02 |
| Phosphoglycerate mutase | 4 | 258 ± 50 | 323 ± 50 | 21.6 ± 4.1 | > 0.05 |
| Enolase | 4 | 195 ± 45 | 255 ± 51 | 24.8 ± 5.2 | > 0.05 |
| Pyruvate kinase | 3 | 13.5 ± 3.5 | 17.1 ± 3.9 | 29.9 ± 4.7 | > 0.01 |

not that within the organelles. This is because at least one of the components of each assay mixture does not readily cross the amyloplast envelope. Assay of enzymes in lysates in which all the organelles had been ruptured would reveal the total activity and permit calculation of latency. The presence of unbroken protoplasts in the unfractionated lysate would result in latency. Cytosolic enzymes in unbroken protoplasts will be latent, but such enzyme will not be latent in preparations in which the plasmalemma, but not the amyloplast envelope, has been ruptured. Thus for each test of latency for a glycolytic enzyme we made a comparable estimate of the latency of a cytosolic marker enzyme, UDP-glucose pyrophosphorylase or PFK($PP_i$). Table 2 shows that each of the enzymes that co-purified with the amyloplast markers showed appreciable latency, which exceeded that shown by the cytosolic marker. We conclude that amyloplasts of the endosperm of developing wheat grains contain between 25 and 45% of each glycolytic enzyme from phosphoglucomutase to pyruvate kinase inclusive. Thus wheat amyloplasts contain the complete glycolytic sequence and differ from chloroplasts from young leaves of pea (*Pisum sativum*), which lack phosphoglycerate mutase (Stitt & ap Rees, 1979), but resemble the fat-synthesizing plastids from castor bean (*Ricinus communis*; Dennis & Miernyk, 1982) and cauliflower (Journet & Douce, 1985).

## FBPase

Assay of the purified amyloplasts for FBPase did not reveal any activity that could be demonstrated consistently. A plastidic FBPase is a key feature of the current view (Boyer, 1986) that sucrose must be converted into triose phosphate to permit carbon for starch synthesis to enter the amyloplast. Without a plastidic FBPase there would be no known route from triose phosphate to hexose 6-phosphate in the amyloplast. Thus we made a detailed search for FBPase in wheat endosperm. In order to obtain more concentrated extracts in greater quantity, we worked with unfractionated extracts of endosperm taken from grains at the same stage of development as those used to prepare amyloplasts.

The appreciable activity of PFK($PP_i$) in endosperm extracts (Table 1) made it difficult to measure FBPase in such extracts, because production of Fru-6-*P* from Fru-1,6-$P_2$ could have been due to PFK($PP_i$) using $P_i$ present in the extracts or the Fru-1,6-$P_2$. Attempts to separate FBPase from PFK($PP_i$) before assay are open to the criticism that FBPase activity might be lost during the separation. Two forms of FBPase are present in plants. One is inhibited allosterically by Fru-2,6-$P_2$ and is found in the cytosol (Herzog *et al.*, 1984). The other does not respond allosterically to Fru-2,6-$P_2$, but is inhibited competitively and is found in the plastid (Gottschalk *et al.*, 1982; Cadet *et al.*, 1987). Stitt *et al.* (1982) used the higher $K_m$, pH optimum and $Mg^{2+}$ requirement of the plastidic enzyme to develop assays for both FBPases that could be used with unfractionated extracts. We optimized these assays for plastidic and cytosolic FBPase for extracts of wheat leaves. We varied, at pH 7.1, 7.5 and 8.5, $MgCl_2$ (2–20 mM) and Fru-1,6-$P_2$ (0.05–2 mM) in the presence and absence of 20 μM-Fru-2,6-$P_2$. Fru-6-*P* formation by PFK($PP_i$) could be stimulated by Fru-2,6-$P_2$ (Van Schaftingen *et al.*, 1982; Kombrink *et al.*, 1984). Thus the conditions we chose as optimum were those that gave the highest rates of Fru-6-*P* formation in the absence of Fru-2,6-$P_2$ and the greatest inhibition in its presence.

We applied the optimized assays for plastidic (pH 8.1) and cytosolic (pH 7.1) FBPase to extracts of leaves and of endosperm (Table 3). Measurements were made in the presence and absence of 20 μM-Fru-2,6-$P_2$ with both

**Table 3. Assay of unfractionated extracts of wheat leaf and endosperm for FBPase**

Tissue homogenates were centrifuged at 60000 *g* for 30 min, and portions of the supernatants were desalted with Sephadex G-25. Samples of untreated (−) and desalted (+) 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 on three different extracts.

| Tissue | pH of assay | Desalting of extract | Activity (nmol of Fru-6-*P*/min per g fresh wt.) Without Fru-2,6-$P_2$ | With Fru-2,6-$P_2$ |
|---|---|---|---|---|
| Leaf | 8.1 | − | 273 ± 16 | 220 ± 25 |
| | | + | 251 ± 18 | 195 ± 23 |
| | 7.1 | − | 79 ± 24 | None detected |
| | | + | 34 ± 5 | 10 ± 3 |
| Endosperm | 8.1 | + | 204 ± 21 | 295 ± 47 |
| | | − | 95 ± 12 | 125 ± 24 |
| | 7.1 | + | 45 ± 7 | 159 ± 43 |
| | | − | 13 ± 4 | 83 ± 27 |

unfractionated and desalted extracts in order to distinguish between FBPase and PFK($PP_i$). Fru-2,6-$P_2$ should stimulate the latter and inhibit FBPase. Desalting would be expected to decrease PFK($PP_i$) activity by removing $P_i$ from the extract. The assays detected both plastidic and cytosolic FBPase in leaf extracts. Activity of the plastidic enzyme was high, not significantly affected by desalting and was slightly inhibited by 20 μM-Fru-2,6-$P_2$. The latter is expected from the evidence that the chloroplast enzyme has an $I_{0.5}$ (concentration causing 50% inhibition) for Fru-2,6-$P_2$ of 0.1 mM (Gottschalk *et al.*, 1982). Appreciable cytosolic FBPase was found in the leaf extracts, and this was inhibited by Fru-2,6-$P_2$. A very different picture was obtained with the endosperm extracts. There was high apparent activity of plastidic FBPase, but, as it was decreased by desalting and stimulated by Fru-2,6-$P_2$, it cannot be attributed to FBPase. It is likely that this activity was due to PFK($PP_i$). Similar results were obtained with the assay for cytosolic FBPase.

We investigated whether extracts of endosperm inhibited FBPase. We prepared extracts of mixtures of leaf and endosperm and assayed for FBPase in the absence of Fru-2,6-$P_2$. The rates obtained were compared with those predicted from measurements made on the separate components of the mixtures. The values found for the mixtures, as percentages of those predicted, were 99 ± 4% and 92 ± 11% for unfractionated and desalted extracts respectively, assayed at pH 8.1. The corresponding values at pH 7.1 were 105 ± 13% and 130 ± 51%. Each value is the mean ± S.E.M. for results from three experiments. These results provide no evidence that endosperm extracts inhibit FBPase. We suggest that our assays for FBPase activity in endosperm extracts would have detected significant FBPase activity had it been present.

We tested our suggestion that the apparent activity of plastidic FBPase in endosperm extracts (pH 8.1, Table 3) was due to PFK($PP_i$) by adding to the extracts antibody to PFK($PP_i$) purified from potato (*Solanum tuberosum*) tubers (Kruger & Hammond, 1988). Controls were incubated with preimmune serum and showed appreciable Fru-6-*P* production that was stimulated by Fru-2,6-$P_2$ (Table 4). However, we found no activity at all in extracts treated with antibody. Evidence that the antibody did not affect our ability to detect plastidic FBPase is provided by the fact that FBPase was readily measured in leaf extracts treated with PFK($PP_i$) antibody (Table 4). We conclude that the ability of endosperm extracts to convert Fru-1,6-$P_2$ into Fru-6-*P*, under conditions optimal for the assay of plastidic FBPase, was due entirely to PFK($PP_i$). These results highlight how PFK($PP_i$) can complicate the measurement of FBPase, and support our suggestion that FBPase activity in developing endosperm of wheat is minimal or absent.

Antibody to plastidic FBPase from spinach leaves is available (Nishizawa & Buchanan, 1981) and provides another means of determining whether such an enzyme is present in wheat endosperm. We prepared unfractionated extracts of samples of wheat leaf, endosperm and a mixture of equal weights of the two tissues. These extracts were subjected to SDS/polyacrylamide-gel electrophoresis. 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 (Fig. 1). The antibody recognized protein in extracts of wheat leaves and did so in the presence of

**Table 4. Effect of antibody to PFK($PP_i$) on conversion of Fru-1,6-$P_2$ into Fru-6-*P* by extracts of endosperm and leaves of wheat**

Tissue homogenates were centrifuged at 60000 *g* for 30 min, and portions of the supernatant were incubated at 20 °C for 30 min with either preimmune serum or antibody to PFK($PP_i$). The antibody–antigen complex was precipitated with Protein A–Sepharose in buffered saline and the supernatant assayed for plastidic FBPase at pH 8.1 in the presence (+) or absence (−) of 20 μM-Fru-2,6-$P_2$. Values for endosperm are means ± S.E.M. from three extracts; values for leaves are from two separate extracts.

| Tissue | Fru-2,6-$P_2$ ... | Activity (nmol of Fru-6-*P*/min per g fresh wt.) With pre-immune serum − | With pre-immune serum + | With antibody − | With antibody + |
|---|---|---|---|---|---|
| Endosperm | | 373 ± 6 | 521 ± 9 | None detected | − |
| Leaf | | 316, 478 | 282, 452 | 394, 485 | 332, 452 |

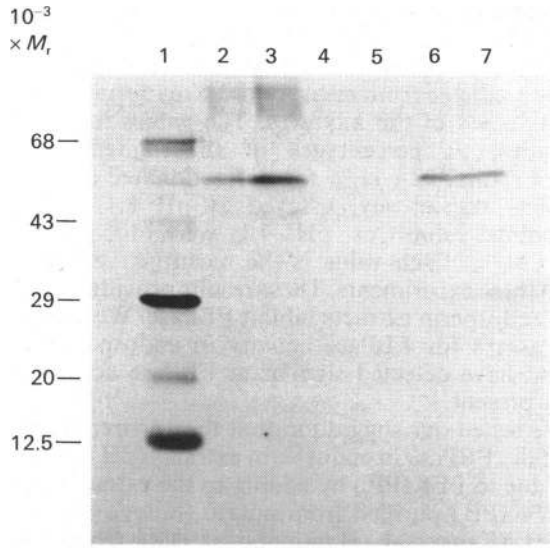


**Fig. 1. Identification of plastidic FBPase in wheat extracts by immunoblotting**

Unfractionated extracts were subjected to electrophoresis on SDS/polyacrylamide gels. The separated proteins were then transferred to nitrocellulose paper and FBPase was detected by immunodecoration with the antibody to the plastidic FBPase from spinach leaf and $^{125}$I-labelled Protein A followed by autoradiography. Lane 1, standard proteins (bovine serum albumin, $M_r$ 68000; ovalbumin, $M_r$ 43000; carbonic anhydrase, $M_r$ 29000; soybean trypsin inhibitor, $M_r$ 20000; cytochrome *c*, $M_r$ 12500); lane 2, 50 $\mu$g of wheat leaf protein; lane 3, 100 $\mu$g of wheat leaf protein; lane 4, 50 $\mu$g of endosperm protein; lane 5, 100 $\mu$g of endosperm protein; lane 6, 50 $\mu$g of mixture of leaf and endosperm protein; lane 7, 100 $\mu$g of mixture of leaf and endosperm protein.

endosperm extract. No reaction at all was found with the endosperm proteins. These results were found with three separate sets of extracts.

The complete agreement between our two methods for detecting FBPase leads us to suggest that amyloplasts in endosperm of developing grains of wheat lack significant activity of this enzyme. This means that these amyloplasts do not possess the conventional means of converting triose phosphate to hexose monophosphate. Our observation that PFK($PP_i$) (Table 1) is absent from amyloplasts precludes this enzyme from fulfilling the above role. Consequently we suggest that these amyloplasts lack the ability to convert triose phosphate to hexose 6-phosphate at the rate required to sustain starch synthesis, and that carbon for starch synthesis does not enter the amyloplast as triose phosphate. The labelling patterns of starch and sucrose made *in vivo* from specifically labelled glucose (Keeling *et al.*, 1988), the fact that amyloplasts isolated from wheat endosperm incorporated glucose 1-phosphate, but not triose phosphate, into starch (Tyson & ap Rees, 1988), and our present results strongly suggest that, in wheat, a C-6 compound enters the amyloplast to support starch synthesis. The extent to which this is the general situation in starch-storing non-photosynthetic tissues will only become clear when more species are examined. Particular attention will need to be paid to the extent to which observed ability of extracts to convert Fru-1,6-$P_2$ into Fru-6-*P* is due to FBPase or PFK($PP_i$).

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