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GIBBERELLIC ACID AND THE CASTOR BEAN FRUCTOSE-1,6-DIPHOSPHATASES

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Abstract—Fructose-1,6-diphosphatase (FDPase) activity increases in the castor bean during germination. The increase is due to *de novo* synthesis of a second FDPase and is induced by gibberellic acid. Isolation and partial purification of these FDPases provide a comparison of their respective properties. FDPase I has a neutral pH optimum, is AMP-sensitive, and is present in the ungerminated bean. FDPase II also has a neutral pH optimum, is AMP-insensitive, and is synthesized in the endosperm during germination as described above. FDPase III has been isolated from the mature leaf, has an alkaline pH optimum and is present only in the photosynthetic tissue.

INTRODUCTION

EXTENSIVE isotope studies by Beevers and collaborators have shown that endosperm of the germinating castor bean utilizes acetate, derived from aliphatic acids for the biosynthesis of sucrose during germination.[1,2] An active glyoxalate cycle exists in the castor bean endosperm, the tissue in which gluconeogenesis occurs.[3] Sucrose is also synthesized in the endosperm and is then translocated to the embryonic axes.[4,5] Since glucose and fructose for sucrose biosynthesis are synthesized via a reversal of the glycolytic pathway,[1,2] the presence of fructose-1,6-diphosphatase (FDPase) in the castor bean endosperm was predicted by Marrè.[6] Bianchetti has demonstrated the presence of FDPase in wheat endosperm.[7] Marrè *et al.*,[8–10] in their studies on developmental changes of several glycolytic and gluconeogenic enzymes of the germinating castor been endosperm, have shown that a rapid increase in enzyme activity occurs during the third day of germination and is sustained for the duration of germination.

The importance of FDPase as a regulatory enzyme in animal and microbial systems is well established.[11–16] In many systems, FDPase is an allosteric enzyme and AMP functions as the allosteric effector. In addition to AMP sensitivity, recent studies by Buchanan *et al.*,

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show that FDPase is regulated by ferredoxin in photosynthetic tissue.[17] A similar phenomenon has been observed in animal tissue in which interactions between FDPase and phosphofructokinase are involved in a complex regulatory system.[18]

In this paper, the changes in FDPase activity during castor bean germination are reported.*

RESULTS

FDPase Activity During Germination

Beans were germinated at 30° ± 0·5° in wet vermiculite, and the endosperm was assayed for FDPase at various intervals. At 40 hr germination time, all beans which showed no signs of germination (about 10 per cent of the total) were discarded. In order to maintain consistency in these experiments, the endosperm was ground in the same weight: volume ratio (1 : 4) after the cotyledons and axes were removed. The mixture was centrifuged at 15,000 ***g*** for 30 min; the supernatant was assayed for total FDPase activity at pH 7·0.

Total FDPase activity increases 3·2-fold between 40 and 80 hr and remains constant for the duration of germination (Fig. 1). AMP-sensitive FDPase does not increase significantly during this period; however, since the total activity increases, it was suspected that a second, AMP-insensitive FDPase was synthesized. Marrè[25] has shown that the increase in activity is observed for the glycolytic and gluconeogenic enzymes in general and is probably *de novo* enzyme synthesis as opposed to activation. Castor beans were therefore deshelled, sterilized in a 5% Clorox solution, washed and divided into five groups. The embryo was removed from each bean in Groups I, II, III, and V, while Group IV served as the deshelled control. Each group was placed in a large covered dish, the bottom of which contained a neoprene grid covered by Whatman No. 1 filter paper. This permitted the test solution to be placed in the dish so as to soak the filter paper and keep the bean in contact with, but not immersed in, the solution. The experiment was arranged as follows:

Group	Treatment to deshelled bean	Addition to test solution
I	Embryo removed	None
II	Embryo removed	GA (10^{-4} M)
III	Embryo removed	GA (10^{-4} M) + CH(10^{-4} M)
IV	Embryo intact	None
V	Embryo removed	Embryo homogenate

* Abbreviations used in this paper are: FDP, fructose-1,6-diphosphate; FDPase I, FDPase from ungerminated beans; FDPase II, FDPase from beans germinated 80 hr; FDPase III, leaf FDPase (cotyledon FDPase); SDPase, sedoheptulose-1,7-diphosphatase; RDP, ribulose-1,5-diphosphate; MB, malonate buffer; G-6-P, glucose-6-phosphate; PMB, *p*-mercuribenzoate; GA, gibberellic acid; CH, cycloheximide.

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At appropriate time intervals, beans from each group were removed, ground in a constant weight of endosperm: volume ratio, centrifuged at 15,000 *g* for 30 min, and the supernatant assayed for FDPase at pH 7·0 by the TPN method (Fig. 2).

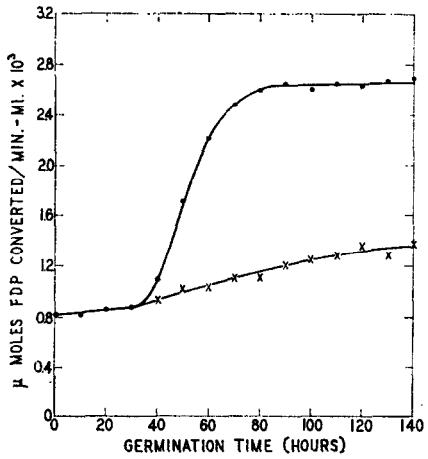

FIG. 1. FDPASE ACTIVITY DURING GERMINATION. TOTAL FDPASE WAS ASSAYED AT pH 7·0 WITH A FINAL FDP CONCENTRATION OF 1·0 mM. AMP SENSITIVITY WAS EVALUATED BY ADDING 5′-AMP TO A FINAL CONCENTRATION OF 1·0 mM.
● = Total FDPase activity in the soluble supernatant during germination. × = Total AMP-sensitive FDPase activity during germination.

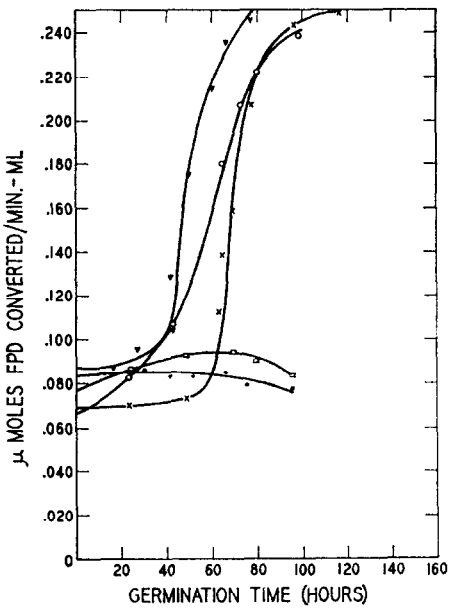

FIG. 2. FDPASE ACTIVITY IN DESHELLED EMBRYOLESS BEANS UNDER THE FOLLOWING CONDITIONS:
□ = Group I—embryo removed
× = Group II—embryo removed + GA(10^{-4} M)
● = Group III—embryo removed + GA(10^{-4} M) + CH(10^{-4} M)
▼ = Group IV—embryo intact, no additions
○ = Group V—embryo removed + embryo homogenate.

Gibberellic acid (Group II) or the embryo homogenate (Group V) restores the FDPase activity to its control levels (Group IV). Comparison of Groups II, III, and V with the others shows that cycloheximide, an inhibitor of protein synthesis,[26] prevents synthesis of the AMP-insensitive FDPase. Other studies have shown that GA induces *de novo* protein biosynthesis, an observation which is supported by these data.[27,28]

From the data in Figs. 1 and 2, it was concluded that more than one FDPase was present in the endosperm during germination, while *a priori* it was assumed that one would be present in the photosynthetic tissue of the plant as part of the carbon cycle enzymes. The FDPases were then partially purified (see Experimental) for the purpose of comparing their properties. Comparison of the three castor bean FDPases so obtained on the Sephadex G-200 column indicates that each enzyme has a molecular weight between 120,000 and 135,000.

Requirements for FDPase Activity

The properties of the enzymes are summarized in Table 2. All three castor bean FDPases have a requirement for 5 mM Mg^{2+}; seventy-five per cent of the activity can be retained when the Mg^{2+} is replaced with 5 mM Mn^{2+}. Addition of 1·8 mM EDTA to the reaction mixture

TABLE 1. PARTIAL PURIFICATION OF CASTOR BEAN FDPASES

Sample	Protein (Mg/ml)	Units/ml	Specific activity	Total units	Recovery (%)
FDPase I					
Crude extract	42·76	0·034	0·0008	41·1	100
29·1% $(NH_4)_2SO_4$-supernatant	15·04	0·400	0·0027	36·8	90
34·8% $(NH_4)_2SO_4$-precipitate	22·20	0·068	0·0031	16·2	41
Sephadex G-150	2·34	0·134	0·0573	13·4	33
Sephadex G-200	0·45	0·060	0·1330	7·0	17
FDPase II					
Crude extract	18·04	0·150	0·0083	60·0	100
29·1% $(NH_4)_2SO_4$-precipitate	18·46	0·201	0·0109	16·1	27
Sephadex G-150	1·82	0·212	0·1165	8·5	14
Sephadex G-200	0·32	0·200	0·6260	4·0	7
FDPase III					
Crude extract	40·80	0·123	0·0030	24·6	100
4% Streptomycin sulfate-supernatant	28·71	0·157	0·0055	18·8	76
29·1% $(NH_4)_2SO_4$-precipitate	15·56	0·359	0·0231	7·2	29
Sephadex G-150	1·96	0·432	0·2204	6·1	24
Sephadex G-200	0·42	0·201	0·4785	2·8	11

of FDPase I is necessary for maximum activity; however, EDTA is not effective on FDPase II and inhibits FDPase III. Therefore, FDPase I requires a chelating agent, FDPase II is unaffected, and FDPase III is inhibited by the chelating agent.

FDPase I is a neutral FDPase and has a broad pH distribution, with a maximum at pH 7·5. FDPase II is also a neutral FDPase, with a sharp pH optimum at pH 6·7. Photosynthetic FDPase III has a very sharp optimum at pH 8·6.

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TABLE 2. ENZYME REQUIREMENTS

FDPase	Mg^{2+}	Requirements		pH Optimum
		EDTA	Substrate	
I	5 mM	1·8 mM	0·1 mM (inhibited by higher concentrations)	7·5
II	5 mM	None	1·0	6·7
III	5 mM	Inhibits	1·0	8·6

Substrate Specificity

Phosphatase activity of the castor FDPases to other sugar mono- and diphosphates is summarized in Table 3. Since the coupling system was ineffective with most substrates, the phosphate method of assay was employed in obtaining these data. Concentrations of substrate were tested from 0·5 to 5·0 mM. Optimum RDP activity (FDPase II) is observed at

TABLE 3. SUBSTRATE SPECIFICITY

Phosphate	Phosphate hydrolysis (μmoles P_i released/min-ml)		
	FDPase I	FDPase II	FDPase III
Fructose-1,6-diphosphate (control)	0·168	0·120	0·243
Fructose-1-phosphate	0·004	None	0·005
Fructose-6-phosphate	0·005	0·003	0·005
Glucose-1-phosphate	None	None	None
Ribulose-1,5-diphosphate	None	0·107	None
Ribulose-1-phosphate	None	None	None
Sedoheptulose-1,7-diphosphate	None	None	None

1·5 mM RDP. According to these data, FDPases I and III are specific FDPases, since neither shows activity greater than 3 per cent of the FDPase activity on other substrates. However, FDPase II, which converts ribulose-1,5-diphosphate to ribulose-5-phosphate (89 per cent of FDPase activity), is very similar to spinach FDPase,[27] which has a pH optimum of 5·5, and which also hydrolyzes RDP to a similar extent.

Substrate Concentration and K_m

Concentrations of FDP greater than 0·1 mM were found to be inhibitory to FDPase I at all pH's. Determination of K_m by the method of Lineweaver and Burk gave a value of $1{\cdot}2 \times 10^{-5}$ M. Although substrate inhibition imposed difficulties in the K_m determinations, the reported value has less than a 6 per cent variation. No substrate inhibition is exhibited by FDPases I and III. The K_m for FDPase II is $3{\cdot}3 \times 10^{-4}$ M and for FDPase III is $1{\cdot}6 \times 10^{-4}$ M.

Inhibitors

5′-AMP inhibited FDPase I competitively with an inhibitor constant (K_i) of $1{\cdot}0 \times 10^{-4}$ M. Deoxy-AMP was 83 per cent as effective as 5′-AMP, but no other purine or pyrimidine mono-, di-, or triphosphate had any influence on the enzyme; however, adenosine 5′-monoacetate

was 10 per cent as active as 5′-AMP. FDPases II and III were not inhibited by AMP, ADP, or ATP at any concentrations, nor by any of the other purine or pyrimidines which were readily available.

Fluoride is inhibitory to all three FDPases, as expected for magnesium-requiring enzymes, and it elicits approximately 50 per cent inhibition at 10^{-2} M on each enzyme; in this respect it is not nearly so strong an inhibitor as AMP is to FDPase I. FDPase I is also inhibited 25 per cent by 1·0 mM Zn^{2+} or Cu^{2+}; however, these same two ions have no effect upon FDPases II and III.

DISCUSSION

During the early stages of castor bean germination FDPase I, a regulatory FDPase, is present in the endosperm. However, after germination is established, FDPase II is induced by the addition of GA from the embryo. This observation is consistent with the observations of Varner and others[28–32] that GA regulates enzyme biosynthesis in germinating seeds. However, it is not clear why FDPase II has none of the regulatory properties of FDPase I. In this respect FDPases I and II provide an interesting comparison with regard to the respective AMP sensitivity of each. The activity of FDPase II on RDP suggests that it participates in more than one metabolic pathway or there is no RDP present in the endosperm and substrate specificity would be an excess. The data of Beevers show an absence of RDP in castor bean endosperm, and therefore support the latter possibility.[1] Since the RDPase activity is 89 per cent of the FDPase activity and all attempts to separate the activities were unsuccessful, it appears that it is not a case of two separate enzymes. FDPase II is very similar to spinach leaf FDPase with regard to RDP activity, but the spinach enzyme is found in the chloroplast and has a pH optimum of 5·5.[27]

FDPase I is similar to animal tissue and wheat embryo FDPases with respect to pH optima, Mg^{2+} and EDTA requirements, AMP sensitivity and substrate inhibition. Within a factor of 10 (10^{-5} M to 10^{-6} M), the K_m's for FDP and the K_i's for AMP are similar.[11, 12, 15, 16]

FDPase III is similar to other photosynthetic FDPases with the exception of the latent FDPase isolated by Buchanan *et al.*[17] These FDPases exhibit no AMP, ADP, or ATP sensitivity and do not have SDPase activity. In general, FDPases of the photosynthetic carbon cycle have not been extensively studied; however, the data which are available suggest that they are not, as a group, AMP-sensitive and therefore not regulatory in this respect.

EXPERIMENTAL

Materials

Castor beans (*Ricinus communis* L.) were purchased locally. Sedoheptulose-1,7-diphosphate was synthesized in these laboratories by the method of Smyrniotis and Horecker.[19]

Preparation of FDPases

All procedures were carried out at 0–4° except when otherwise indicated. Detailed enzyme studies were conducted on FDPases extracted from ungerminated endosperm, endosperm at 80 hr, cotyledons, and mature leaves.

Protein Determination

Protein concentration was determined spectrophotometrically by the method of Warburg and Christian[20] or colorimetrically by the method of Lowry.[21] The procedure of Nobel was followed in order to isolate and break the chloroplasts in the castor leaves.[22]

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Definition of Enzyme Unit

One unit of FDPase catalyzes the conversion of 1 micromole of FDP to F-6-P per minute. Specific activity is the number of units per milligram of protein.

Spectrophotometric Assay of FDPase

FDPase activity was determined by the reduction of NADP at 340 nm after Racker and Schroeder.[23] For the assay system, the following solutions, having a combined volume of 1·0 ml, were pipetted into a micro-quartz cell with 10-mm lightpath: 0·5 ml water; 0·1 ml 2·0 M cacodylate buffer, pH 6·8, for FDPase II, 0·1 ml 2·0 M Tris buffer, pH 7·4, for FDPase I, and 0·1 ml Tris buffer, pH 8·6, for FDPase III; 0·05 ml 1·2 per cent EDTA; 0·05 ml 0·1 M $MgCl_2$; 0·05 ml 0·005 M NADP; 0·05 ml G-6-P isomerase, 3 units/ml; 0·05 ml G-6-P dehydrogenase, 6 units/ml; 0·1 ml enzyme preparation; and either 0·05 ml 20 mM FDP for FDPases I and II or 0·05 ml 100 mM FDP for FDPase III, in the order indicated. This reaction was carried out at 28°; the optical density at 340 nm was recorded for a 4 min interval on a Beckman DK-2A spectrophotometer, equipped with a temperature-controlled cell. The interval began 1–2 min after the addition of the substrate to the cuvette. The coupling system was tested with known amounts of F-6-P and was in no case rate-limiting in the studies reported here.

Inorganic Phosphate Assay of FDPase

The method of Fiske and Subba Row was used to measure the amount of inorganic phosphate release from phosphate esters.[24] For this procedure, tubes containing 1·3 ml water; 0·2 ml 2·0 M buffer (cacodylate, pH 6·8, for FDPase II, Tris, pH 7·4, for FDPase I, and Tris, pH 8·6, for FDPase III); 0·1 ml 1·2 per cent EDTA; 0·1 ml 0·1 M $MgCl_2$; 0·2 ml enzyme preparation; and 0·1 ml substrate, either 20 mM for FDPases I and II or 100 mM for FDPase III, were placed in a 28° water bath for 30 min, and the reaction was then stopped by the addition of 1 ml 10% trichloroacetic acid. After centrifuging these tubes for 20 min, inorganic phosphate was determined on 1-ml aliquots of the supernatant from each reaction mixture.

FDPase I

Ungerminated castor beans were finely ground in an omnimixer containing 4 ml H_2O/g endosperm; the homogenate was cleared by centrifugation at 16,000 *g* for 30 min. The crude supernatant was made 29·1 per cent in $(NH_4)_2SO_4$ and centrifuged at 16,000 *g* for 30 min. To each 100 ml of supernatant, 34·8 g of $(NH_4)_2SO_4$ were added. The mixture was centrifuged at 10,000 *g* for 30 min and the precipitate was redissolved in a minimum amount of water and placed on a Sephadex G-150 column 1·5 × 45 cm. 10 mM malonate buffer (MB), pH 6·0, was used to elute the FDPase. The most active fractions were pooled and loaded on a Sephadex G-200 column 1·5 × 15 cm and 15 mM MB was used to eluate the partially purified enzyme.

FDPase II

The cotyledons and total axis were removed from 3-day-old germinating castor beans, and the endosperm was ground as for FDPase I. The mixture was centrifuged at 16,000 *g* for 30 min. The resulting supernatant was made 29·1 per cent in $(NH_4)_2SO_4$ and centrifuged for 30 min at 16,000 *g*. The 29·1 per cent precipitate, which contained FDPase II, was extracted (× 3) with 28 per cent ammonium sulfate, a procedure which removed any FDPase I from the precipitate. The final precipitate was then redissolved and placed on a Sephadex G-150 column 2·5 × 45 cm. As before, the enzyme was eluted by 10 mM MB, whereupon the most active tubes were pooled and placed on a Sephadex G-200 column 1·5 × 15 cm and eluted by 15 mM MB.

FDPase III

From mature leaves. Fresh, deribbed castor bean leaves were ground in an omnimixer in 2 vol. water and centrifuged for 45 min at 23,000 *g*. Alternatively, the chloroplasts were isolated by the method of Nobel[22] from the deribbed leaves. Since the supernatant fluid from chloroplast isolation had very little FDPase activity, it was discarded. Chloroplasts were then broken by sonication, in a 0·2 M sucrose buffered with 0·02 M N-Tris-(hydroxymethyl) methyl-2-aminoethane-sulfonate (TES)-NaOH (pH 8·2) for 3 min at 75 per cent power with a Bronwill sonicator. After 30 min, the resultant fragments were removed by centrifugation at 30,000 *g*. Since this method gave poorer yields, but identical enzyme properties, it was not used, in favor of the previous procedure of grinding the leaves in an omnimixer. However, it was taken as evidence that FDPase III is a chloroplast system.

The supernatant from Step 1 was made 4 per cent in streptomycin sulfate, and after a digestion period of 60 min was centrifuged at 10,000 *g* for 30 min. Ammonium sulfate was added to the streptomycin sulfate supernatant, making it 29·1 per cent in $(NH_4)_2SO_4$; and after standing 1 hr, the preparation was centrifuged at 10,000 *g* for 30 min. The 29·1 per cent precipitate, was redissolved and placed on a Sephadex G-150 column 2·5 × 45 cm. After elution by 10 mM MB, the most active fractions were combined and loaded on a Sephadex G-200 column 1·5 × 15 cm and the enzyme was eluted with 15 mM MB.

From the cotyledons. The cotyledons at 3 days' germination were ground in 4 vol. water (Sorvall omni-mixer) and the extract was centrifuged at 20,000 *g* for 30 min. The supernatant contained FDPase activity which exhibited the same properties as FDPase III isolated by the above methods.

These methods of partial purification were used because they routinely gave the highest degres of purity and maximum yield of enzyme (Table 1). The final purification, which for FDPase is a 150-fold increase over the crude extract, is very low with respect to crude extracts from other sources.[15] The best preparations of the castor bean FDPases I, II, and III were all labile at 4° (50 per cent loss in activity in 24 hr) and FDPases I and II were moderately stable to freezing (20 per cent loss during 2 weeks at $-10°$); while FDPase III usually denatured within 2 weeks when frozen. Vertical acrylamide gel electrophoresis of the partially purified FDPases showed three bands of protein in each preparation.