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AN INDUCED FRUCTOSE-1,6-DIPHOSPHATASE FROM CULTURED CELLS OF *ACER PSEUDOPLATANUS* (ENGLISH SYCAMORE)

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Abstract—Fructose-1,6-diphosphatase (FDPase) activity is induced by growing English sycamore (*Acer pseudoplatanus*) suspension culture cells on a medium with glycerol as the carbon source. The induction rates of this FDPase are evaluated vs. time. The enzyme exhibits a neutral pH optimum, is AMP insensitive and is inhibited by EDTA.

INTRODUCTION

ATTEMPS to grow *Acer pseudoplatanus* with glycerol as the sole carbon source in this laboratory have been successful. Since growth on glycerol requires gluconeogenesis for hexose and pentose synthesis, FDPase would have to be present either as a constitutive enzyme or as an induced enzyme. Our studies show that it is an induced enzyme and we have characterized its properties.

The induction of fructose-1,6-diphosphatase in the castor bean has been considered by Beevers *et al.*[1,2] and by Scala *et al.*,[3] and its induction is similar to that observed in many plant systems. The general induction of enzymes in plant systems has recently been reviewed by Filner *et al.*[4] The importance of FDPase as a regulatory enzyme has been considered in many laboratories, but it has not been considered in great detail as an induced enzyme. In this laboratory at least three FDPases in the castor bean and its leaves have been studied,[3] as well as an FDPase from navy bean leaves.[5] In this study, FDPase has been prepared in plant cells grown in suspension culture and the kinetics of induction of the enzyme have been determined.

RESULTS

Growth Characteristics

After transfer to glycerol-containing medium (final concentration, 20 g/l.), the cells were observed to enter a lag phase for about 2 days, after which they grew with a generation time of 3·5 days. The parent cultures grown on sucrose exhibited a generation time of 70 hr. This parent culture has been maintained in this laboratory for 3 yr on the sucrose medium after having been obtained from Dr. D. T. A. Lamport. In the glycerol-containing medium, the cells grew in small clumps of about 1–4 mm dia.

[1] D. T. CANVIN and H. J. BEEVERS, *J. Biol. Chem.* **236,** 988 (1961).
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[3] J. SCALA, C. PATRICK and G. MACBETH, *Arch. Biochem. Biophys.* **127,** 576 (1968).
[4] P. FILNER, J. WRAY and J. E. VARNER, *Science* **165,** 358 (1969).
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Enzyme Induction

Figure 1 is a plot of the enzyme activity vs. time after introduction to a glycerol-containing medium after having grown on a sucrose-containing medium. After about 20 days, the induction of the enzyme is complete; it has reached 0·35 units of activity per mg of cell protein. The induction is 50 per cent complete at about 5 days and in all experiments

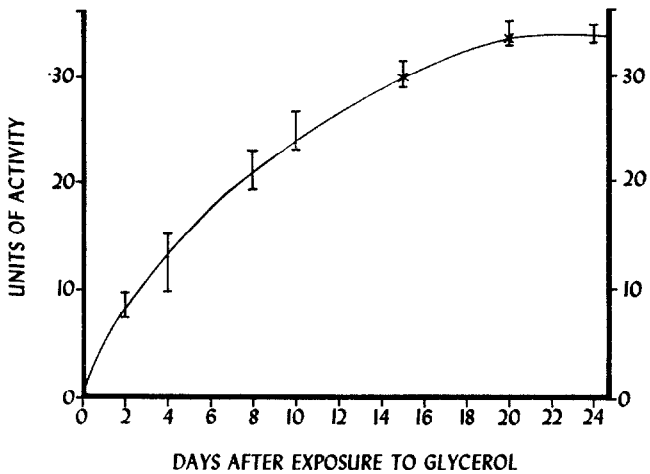

FIG. 1. PLOT OF INDUCTION OF NON-REGULATORY FDPASE VS. TIME OF INITIATION INTO GLYCEROL-CONTAINING MEDIUM.

Each bar on the curve was determined by three or four measurements of activity. All the assays fall within the length of the bar.

the maximum activity leveled off at about 0·35 units of enzyme activity per mg of protein in the cells.

Isolation of the Enzyme

The isolation of the enzyme was not pursued to high purity. A partial purification of the crude extract was used to evaluate the parameters of the enzyme, as seen in Table 2.

(1) Crude extract (As defined in Experimental).

(2) Polyclar AT. One g of purified Polyclar AT[6] was added per ml of crude extract. The slurry was stirred for 30 min and centrifuged at 30,000 *g* for 15 min.

(3) Streptomycin Sulfate. The supernatant from Step 2 was made 4 per cent in streptomycin sulfate, and after a digestion period of 1 hr, was centrifuged at 150,000 *g* for 20 min.

(4) Ammonium Sulfate-Fraction I. The supernatant from Step 3 was taken to 25 per cent $(NH_4)_2SO_4$ by the addition of crystalline ammonium sulfate, stirred for 1 hr and centrifuged at 30,000 *g* for 20 min. The precipitate was resuspended in 2 ml distilled H_2O, stirred, recentrifuged, and the supernatants were combined.

(5) Ammonium Sulfate-Fraction II. The 25 per cent supernatant was raised to 40 per cent in ammonium sulfate, stirred for 1 hr, and centrifuged as above. The precipitate was dissolved in a minimum amount of distilled water and cleared by recentrifugation.

[6] R. RITZERT and B. TURIN, *Phytochem.*, **9**, 1701 (1970).

Properties of the Enzyme

EDTA was found to inhibit the enzyme (Table 1). This was similar to that observed for FDPase II of the castor bean,[3] and the K_m was $5{\cdot}1 \times 10^{-4}$ M (see Table 1). V_{max} was 2·2 μmoles/min, which is very high for an FDPase. The pH optimum of the enzyme exhibits a sharp maximum at pH 6·6, making it similar to other plant FDPases.[3]

TABLE 1. COMPARATIVE PROPERTIES OF THE FDPASE OF *Acer pseudoplatanus*

Carbon source	Optimal $[Mg^{2+}]$	Optimal [EDTA]	Optimum pH	K_m	Inhibition by 0·25 mM 5′-AMP	Substrate inhibition
Glycerol	7 mM	None (Inhibits)	6·6	$5{\cdot}1 \times 10^{-4}$ M	None	None (up to 2 mM)

In contrast to other FDPases,[7,8] this FDPase exhibits no inhibition by AMP or by any other purine or pyrimidine mono-, di-, or triphosphate. It is inhibited by fluorine and by parachloromercurobenzoate. The enzyme has a requirement for magnesium which is optimal at 7 mM. Manganese can replace magnesium, but with 80% of the magnesium activity. At concentrations of magnesium higher than 7 mM, inhibition becomes obvious. This has been considered in previous papers by Mendicino *et al.*[9,10]

Substrate Specificity

The activity of the enzyme with fructose diphosphate is very high. However, little or no activity was found with glucose-diphosphate, paranitrophenyl phosphate, adenosine monophosphate, adenosine diphosphate, and other mono-, di-, and triphosphates (Table 3). Therefore, with the exception of a small amount of RuDP activity, the enzyme is specific for FDP.

TABLE 2. PARTIAL PURIFICATION OF FDPASE

Preparation	Total units	Specific activity*	Purification
I Crude extract	19·1	0·35	1·0
II Polyclar AT	18·2	0·41	1·17
III Streptomycin sulfate	17·7	0·65	1·86
IV $AmSO_4$ Fraction I	9·8	1·78	5·1
V $AmSO_4$ Fraction II	2·2	3·26	9·3
Total yield = 11·5%			

* Units/mg protein.

[7] K. TAKETA and B. POGELL, *J. Biol. Chem.* **240**, 651 (1965).
[8] M. SARNGADHARAN, A. WATANABE and B. POGELL, *Biochemistry* **8**, 1411 (1969).
[9] J. MENDECINO, H. PRIBAR and F. SALAMA, *J. Biol. Chem.* **243**, 2710 (1968).
[10] J. MENDECINO, C. BEAUDREAU, L. HSU and R. MEDICUS, *J. Biol. Chem.* **243**, 3703 (1968).

TABLE 3. SUBSTRATE SPECIFICITY OF FDPASE

Substrate	Units/mg prot.
Fructose-1,6-diphosphate	0·35
Glucose-1,6-diphosphate	0·00
p-Nitrophenyl phosphate	0 03
Ribulose-1,5-diphosphate	0·09
AMP, ADP, or ATP	none

DISCUSSION

Since it has been shown in this laboratory that glycerol serves as a substrate on which plant cells can grow in tissue culture, it was assumed that the cells would produce an FDPase. Indeed, this hypothesis was supported in the observation that FDPase is an induced enzyme in these cells and reaches a relatively high activity. For comparison, one might consider that in other plant systems, 0·40 units of enzyme per mg of protein would be very high. Indeed, this is about 20 times the activity over that observed in the navy bean leaves or in the castor bean FDPase. The induced FDPase reported here is different from most other FDPases since it is not inhibited by AMP and is inhibited by the chelating agent EDTA which is required for maximum activity by other FDPases. Therefore, one would assume that in opposition to its regulatory properties, it does not exhibit any allosteric inhibition since it is regulatory through induction. It is assumed that the induction is sequential; therefore, that glycerol is not the direct inducer of the enzyme. Rather, we have assumed that the presence of glycerol as a metabolite requires the reversal of glycolysis for synthesis of glucose, other hexoses and pentoses. In most gluconeogenic pathways, FDPase is a required enzyme.

EXPERIMENTAL

English sycamore (*Acer pseudoplatanus*) was grown as callus and in suspension culture on a modification of White's medium,[11] supplemented with 10% deproteinized coconut milk, 200 mg/l. casein hydrolysate, and 0·5 ppm 2,4-dichlorophenoxyacetic acid. The suspensions were maintained in 250-ml nippled flasks and rotated at 1 rev/min, as described by Steward [11] These suspension cells were harvested by filtering through one layer of 97 mesh silk bolting cloth and placing in a Sorvall Omnimixer with equal amounts of purified Polyclar AT.[6] Approximately 2·5 vol. of extraction medium, 0 02 M cacodylate buffer, pH 6·6, 0·15 M sodium ascorbate, and 0 02 M 2-mercaptoethanol were added until the mixture was pasty.

This mixture was then homogenized in a Sorvall Omnimixer for 30-sec intervals with a total running time of 2 min at 0° The brei was squeezed through a single layer of bolting silk and centrifuged for 30 min. at 30,000 *g* in a Sorvall RC-2B refrigerated centrifuge. The supernatant was immediately decanted and refrigerated. This procedure is denoted as the crude extract. FDPase activity was assayed spectrophotometrically by recording the rate of reduction of NADP at 340 nm in the presence of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase.[12] Due to the u.v. absorption of our extraction medium, protein determinations by the Lowry method[13] were made after extensive dialysis against water. A unit of activity is defined as that amount of enzyme which hydrolyzed 1 μmole of FDP/min. The specific activity is expressed as units/mg protein.

Enzyme induction was studied by removing the flasks from the rotating wheel, resting them on their sides, allowing the cells to settle to the bottom and become entrapped in the nipples, pouring off the medium and transferring sterile medium to the cells. In each induction study, the sedimentation was done four times after which it could be assumed that the previous medium, that containing sucrose, was definitely dilute and therefore, there was not enough sucrose in the new medium to interfere with the metabolism of the cells. This method was used in order to maintain sterility and each flask was routinely assayed for bacterial contamination. Any cells that were contaminated were discarded; however, contamination was only rarely observed with this method

[11] S. CAPLIN and F. C. STEWARD, *Nature* **163**, 920 (1949).
[12] E. RACKER, in *Methods in Enzymology* (edited by S P. COLOWICK and N O. KAPLAN), Vol. 5, p. 272, Academic Press, New York (1965).
[13] E. LAYNE, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 3, p. 448, Academic Press, New York (1963).