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PHOSPHOFRUCTOKINASE OF *SOLANUM TUBEROSUM* TUBER

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; phosphofructokinase; kinetics; regulation.

Abstract—Potato tuber phosphofructokinase was purified 19·6-fold by a combination of ethanol fractionation and DEAE-cellulose column chromatography. The enzyme was very unstable; its pH optimum was 8·0. K_m for fructose-6-phosphate, ATP and Mg^{2+} was $2{\cdot}1 \times 10^{-4}$ M, $4{\cdot}5 \times 10^{-5}$ M and $4{\cdot}0 \times 10^{-4}$ M respectively. ITP, GTP, UTP and CTP can act as phosphate donors, but are less active than ATP. Inhibition of enzyme activity by high levels of ATP was reversed by increasing the concentration of fructose-6-phosphate; the affinity of enzyme for fructose-6-phosphate decreased with increasing concentration of ATP. 5′-AMP, 3′,5′-AMP, 3′-AMP, deoxy AMP, UMP, IMP, CMP, GMP, ADP, CDP, GDP and UDP did not reverse the inhibition of enzyme by ATP. ADP, phosphoenolpyruvate and citrate inhibited phosphofructokinase activity but Pi did not affect it. Phosphofructokinase was not reactivated reversibly by mild change of pH and addition of effectors.

INTRODUCTION

THE KINETIC properties of phosphofructokinase (ATP-D-fructose 6-phosphate 1-phosphotransferase, E.C. 2.7.1.11), which appears to play a regulatory role in carbohydrate metabolism, has been studied mainly with preparation from mammalian tissues and microorganisms.

Lardy *et al.*[1] showed that phosphofructokinase of mammalian tissue was inhibited by high ATP concentration. Inhibition by ATP of enzyme from animal sources, including liver fluke, rabbit muscle, guinea-pig heart and sheep brain, is overcome by 3′,5′-AMP and ADP.[2–4] Phosphofructokinase of yeast and *E. coli* was found to be inhibited at high ATP level and was affected by positive effectors, 5′-AMP and ADP, but 3′,5′-AMP has no effect.[5–7] The difference in response to 3′,5′-AMP of phosphofructokinase from animal sources and microorganisms raises the question of the response of higher plant phosphofructokinases to their effectors.

Less work has been carried out with the enzyme from higher plants. It has been reported that phosphofructokinase of some plants (parsley, avocado and carrots) are susceptible to ADP and phosphoenolpyruvate inhibition and other plant phosphofructokinase are un-

[1] LARDY, H. A. and PARKS, R. E., JR. (1956) in *Enzymes, Units of Biological Structure and Function* (GAEBLER, O. H., ed.), p. 584, Academic Press, New York.
[2] MANSOUR, T. E. and MANSOUR, J. M. (1962) *J. Biol. Chem.* **237**, 629.
[3] PASSONNEAU, J. V. and LOWRY, O. H. (1962) *Biochem. Biophys. Res. Commun.* **7**, 10.
[4] MANSOUR, T. E. (1963) *J. Biol. Chem.* **238**, 2285.
[5] HATHAWAY, J. A. and ATKINSON, D. E. (1963) *J. Biol. Chem.* **238**, 2875.
[6] RAMAIAH, A., HATHAWAY, J. A. and ATKINSON, D. E. (1964) *J. Biol. Chem.* **239**, 3619.
[7] ATKINSON, D. E. and WALTON, G. M. (1965) *J. Biol. Chem.* **240**, 757.

affected by 5′-AMP or 3′,5′-AMP.[8,9] In this paper, we attempt to clarify the general properties and kinetic behaviour towards some effectors of potato phosphofructokinase.

RESULTS AND DISCUSSION

Partial Purification of Phosphofructokinase of Potato Tuber

As indicated in Table 1, a 19·6-fold purification of phosphofructokinase from extract of potato tuber was achieved by the procedure outlined.

TABLE 1. PURIFICATION OF PHOSPHOFRUCTOKINASE FROM POTATO TUBER

Fraction	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification
Crude extract	375	4800	133	0·027	(1)
Supernatant	300	3420	144	0·033	1·2
EtOH precipitation (13–26%)	102	620	52	0·084	3·1
Soluble fraction	97	378	51	0·139	5·1
DEAE-cellulose eluate	50	38	20	0·530	19·6

Stability of Phosphofructokinase

In the course of enzyme purification, the major amount of phosphofructokinase was lost. This loss was due mainly to the instability of the enzyme. Fractionation of crude extracts with ammonium sulphate resulted in a loss of activity of over 90%. Much of the phosphofructokinase activity of crude extracts was lost within 10 hr, as shown in Fig. 1a. The loss was prevented by β-mercaptoethanol.

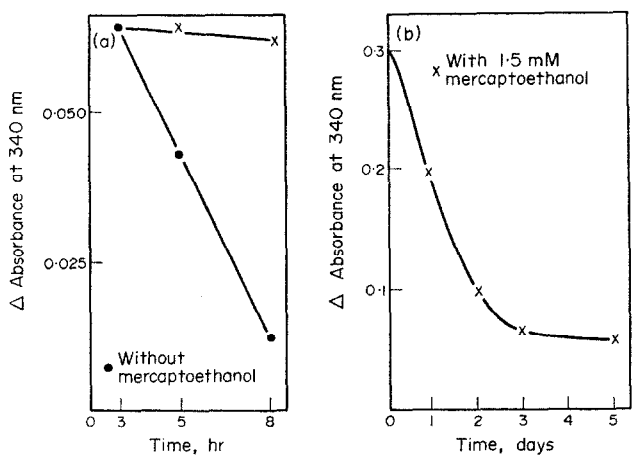

FIG. 1. STABILITY OF PHOSPHOFRUCTOKINASE.
(a) Crude extract; without mercaptoethanol and with 1·5 mM mercaptoethanol. (b) DEAE cellulose fraction, which contained 1·5 mM mercaptoethanol.

[8] LOWRY, O. H. and PASSONNEAU, J. V. (1964) *Arch. Exp. Path. Pharmak.* **248,** 185.
[9] DENNIS, D. T. and CAULTATE, T. P. (1966) *Biochem. Biophys. Res. Commun.* **25,** 187.

β-Mercaptoethanol (1·5 mM) was added to prevent loss of activity in all steps of phosphofructokinase purification. Nevertheless, loss of phosphofructokinase activity after DEAE-cellulose fractionation was observed even when β-mercaptoethanol was used and continued for 3 days after fractionation (Fig. 1b).

The effect of various concentrations of β-mercaptoethanol was tested with enzyme after DEAE-cellulose fractionation, but it was ineffective. EDTA, FDP, F-6-P, $(CH_3COO)_2Mg$, $MgCl_2$ and ATP, tested at different concentrations and in combination, were ineffective in preventing the loss of phosphofructokinase activity.

We have not yet found conditions to prevent loss of enzyme activity. Therefore, all the following experiments were performed within 8 hr (approx. 7% loss of activity) after DEAE-cellulose fractionation.

Properties of the Partially Purified Phosphofructokinase

The enzyme has a rather broad pH optimum at 8·0. The formation of FDP by enzyme was found to be linear with the incubation time for at least 5 min.

In order to determine the Michaelis constants for F-6-P, ATP and Mg^{2+} (as $MgCl_2$), the initial reaction velocities were determined as a function of the substrates and $MgCl_2$ concentrations. The K_m for F-6-P, ATP and Mg^{2+}, derived from Lineweaver–Burk plots of experimental data are $2{\cdot}1 \times 10^{-4}$ M, $4{\cdot}5 \times 10^{-5}$ M and $4{\cdot}0 \times 10^{-4}$ M, respectively.

The relative activity of the enzyme in the presence of different nucleotides was tested. Table 2 shows that ATP was a better phosphate donor than the other nucleotides.

TABLE 2. RELATIVE ACTIVITY OF PHOSPHOFRUCTOKINASE IN PRESENCE OF DIFFERENT NUCLEOSIDE TRIPHOSPHATES

Nucleoside triphosphate	K_m M($\times 10^4$)	V_{max} (mmol/mg/min)	Nucleoside triphosphate	K_m M($\times 10^4$)	V_{max} (mmol/mg/min)
ATP	0·45	330	GTP	1·6	220
ITP	0·79	328	CTP	3·6	275
UTP	1·0	325			

No inhibition by ATP was observed with concentrations below 1 mM at F-6-P and $MgCl_2$ concentrations of 1 and 2 mM respectively (Fig. 2), but ATP at concentrations above 1 mM inhibited phosphofructokinase activity.

The interaction between AMP and ATP on potato phosphofructokinase is entirely different from results for mammalian and yeast phosphofructokinases. It has been reported that inhibition of enzyme by ATP is overcome by 3′,5′-AMP or 5′-AMP in phosphofructokinase of heart, muscle and yeast. However, inhibition of potato phosphofructokinase by ATP was not reversed by 3′,5′-AMP or 5′-AMP. In fact, a slight apparent enhancement of ATP inhibition by AMP was detected under conditions of inhibiting ATP level, although no inhibitory effect of AMP was observed at low concentration of ATP (Fig. 2). Thus, the effector specificity for potato phosphofructokinase is different from that of yeast and mammalian enzymes; 5′-AMP and 3′,5′-AMP do not serve as positive effectors, although ATP serves as a negative effector.

The effect of increasing concentrations of F-6-P on the velocity of the enzyme reaction with two different ATP concentrations is shown in Fig. 3. At an inhibitory concentration of

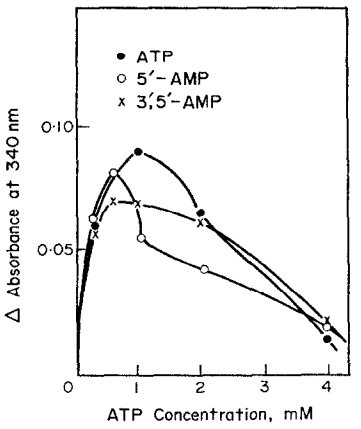

FIG. 2. INHIBITION OF PHOSPHOFRUCTOKINASE BY ATP, AND EFFECT OF 5′-AMP, 3 mM (○——○) AND 3′,5′-AMP, 3 mM (×——×) ON ATP INHIBITION
The concentration of F-6-P was held constant at 1 mM and that of MgC_2 at 2 mM.

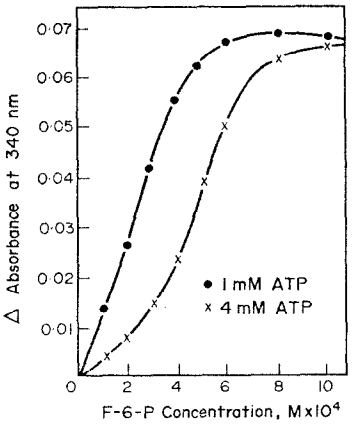

FIG. 3. PHOSPHOFRUCTOKINASE ACTIVITY AS A FUNCTION OF F-6-P CONCENTRATION AT TWO CONCENTRATIONS OF ATP.

ATP (4 mM), the velocity of the enzymic reaction shows a sigmoidol dependence on the concentration of F-6-P and an apparent increase in K_m for F-6-P, typical of regulatory enzymes. Fig. 3 also shows that ATP inhibition of phosphofructokinase is reversed by high concentrations of F-6-P.

TABLE 3. EFFECT OF RIBONUCLEOSIDE MONOPHOSPHATES (XMP) ON POTATO PHOSPHOFRUCTOKINASE

ATP concentration (mM)	XMP	Concentration (mM)	Relative activity	ATP concentration (mM)	XMP	Concentration (mM)	Relative activity
1·0			100	10·0	3′-AMP	0·4	19
5·0			64	10·0	,,	4·0	10
10·0			19				
				10·0	deoxy AMP	1·0	12
10·0	5′-AMP	0·4	19	10·0	UMP	1·0	19
10·0	,,	4·0	19	10·0	IMP	1·0	12
				10·0	CMP	1·0	12
10·0	3′,5′-AMP	0·4	15	10·0	GMP	1·0	17
10·0	,,	4·0	15				

The substrate concentrations were F-6-P, 1 mM and $MgCl_2$, 2 mM.

The effects of various ribonucleoside monophosphates on phosphofructokinase activity at an inhibitory concentration of ATP are shown in Table 3. None of the ribonucleoside monophosphates tested (3′,5′-AMP, 5′-AMP, 3′-AMP deoxy AMP, UMP, IMP, CMP and GMP) released phosphofructokinase from inhibition by ATP. The effect of increasing F-6-P concentration on the velocity of the enzymic reaction in the presence of AMP is shown in Fig. 4. The velocity of the enzyme reaction in the presence of AMP exhibits a hyperbolic

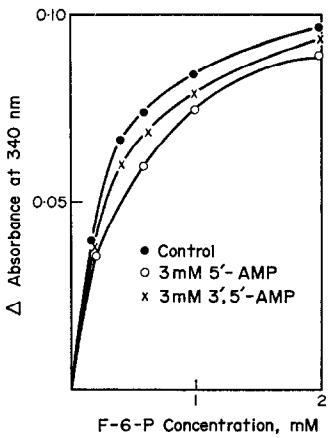

FIG. 4. EFFECT OF 5′-AMP OR 3′,5′-AMP ON PHOSPHOFRUCTOKINASE.
The concentration of ATP was held constant at 1 mM, and that of $MgCl_2$ at 2 mM.

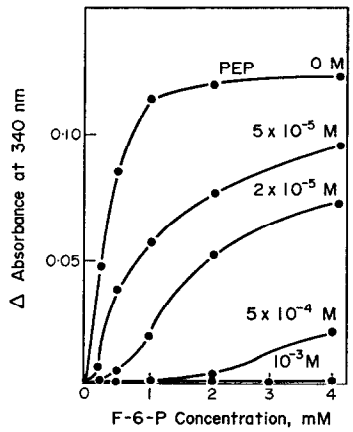

FIG. 5. INHIBITION OF PHOSPHOFRUCTOKINASE BY PHOSPHOENOLPYRUVATE (PEP).
The concentration of ATP was held constant at 1 mM, and that of $MgCl_2$ at 2 mM.

dependence on the concentration of F-6-P, as it does in the absence of AMP, but 3′,5′-AMP or 5′-AMP at 3 mM inhibited the velocity of reaction appreciably.

Of a range of nucleoside diphosphates tested, none reversed inhibition of phosphofructokinase by ATP (Table 4). Thus, positive effectors of mammalian and yeast phosphofructokinase are ineffective on activity of the potato enzyme inhibited by ATP.

TABLE 4. EFFECT OF NUCLEOSIDE DIPHOSPHATES ON POTATO PHOSPHOFRUCTOKINASE

ATP concentration (mM)	Nucleoside diphosphate	Concentration (mM)	Relative activity
1·0			100
10·0			9
10·0	ADP	1·0	7
10·0	CDP	3·0	9
10·0	GDP	3·0	9
10·0	UDP	3·0	9

All assay solutions contained 1 mM F-6-P and 2 mM $MgCl_2$.

After adjustment to pH 6·0, incubation of the enzyme at 20° resulted in a 50% reduction of activity (Table 5), and by readjustment to pH 8·0, further activity was lost. When AMP, cyclic-AMP, FDP, ATP or $MgCl_2$ was present during the incubation after readjustment to pH 8·0, enzyme activity was not restored. This indicates that the enzyme was inactivated under conditions of mild acid pH treatment and differs from the mammalian enzyme which exhibits reversible reactivation.[10]

The effects of ADP, Pi, citrate and phosphoenolpyruvate on ATP-inhibited potato

[10] MANSOUR, T. E. (1965) *J. Biol. Chem.* **240**, 2165.

TABLE 5. IRREVERSIBLE INACTIVATION OF PHOSPHOFRUCTOKINASE

Treatment	Addition	Relative activity
Control (pH 8·0)	None	100
pH 6·0-adjustment	None	54
pH 8·0-readjustment	None	36
	5′-AMP 10^{-4} M	23
	3′,5′-AMP 10^{-4} M	28
	FDP 10^{-5} M	30
	ATP 10^{-4} M	32
	FDP (10^{-5} M) + ATP (10^{-4} M)	28
	ADP 10^{-4} M	20
	$MgCl_2$ 10^{-3} M	36

The pH 6·0-adjusted enzyme, and the pH 8·0-readjusted enzyme solutions were pre-incubated at 37° for 10 min. Then both preparations were assayed by the standard procedure with the indicated amount of nucleotides or other agents.

phosphofructokinase was tested (Table 6). The enzyme was strongly inhibited by phosphoenolpyruvate and ADP, and weakly inhibited by citrate. Pi had no effect. It is considered that ADP is a competitive inhibitor of ATP as reported previously.[11]

TABLE 6. EFFECT OF CITRATE AND PHOSPHOENOLPYRUVATE ON PHOSPHOFRUCTOKINASE

Additions (all at 3 mM)	Relative activity	Additions (all at 3 mM)	Relative activity
Control	100	Citrate	84
ADP	23	PEP	24
Pi	97		

The reaction mixtures were as described for the standard assay (ATP, 1 mM).

Phosphoenolpyruvate gave almost complete inhibition at 1 mM (Fig. 5). Lower concentrations of phosphoenolpyruvate (5×10^{-5} M and 2×10^{-4} M) are less inhibitory; the inhibition was reversed by increasing the concentration of F-6-P. This indicates a decreased affinity of the enzyme for F-6-P at lower phosphoenolpyruvate levels.

These results confirm that the activity of the phosphofructokinase from potato tuber is regulated by ATP in a similar manner to the phosphofructokinase from other sources, in that it is inhibited by excess ATP. It differs, however, in that its inhibition by ATP is not reversed by AMP and ADP.

Phosphoenolpyruvate, ADP and to a lesser extent, citrate may be important regulators of potato phosphofructokinase.

[11] BLANGY, D., BUC, H. and MONOD, J. (1968) *J. Mol. Biol.* **31**, 13.

EXPERIMENTAL

Preparation of crude extract. Potato tubers (var. Danshaku) were purchased from the market and stored at 4°. Tubers selected randomly were washed, peeled and squeezed with a juicer (Fuji Denki Electric Co., Japan). Mercaptoethanol (7·3 mmol) was added to 100 ml homogenate. After filtration through gauze, the pH of the extract was adjusted to pH 7·5 with 0·1 M KOH. A clear supernatant was obtained by centrifugation at 15 000 rpm for 30 min. All the operations were carried out at 2–4°. As much activity of phosphofructokinase of potato tuber was lost by $(NH_4)_2SO_4$ fractionation, other purification methods were tried.

The supernatant was cooled in an ice bath (0°) and stirred continuously while EtOH at −20° was added dropwise. The fraction precipitated between 13 and 26% EtOH was dissolved in 0·1 M Tris-HCl buffer (pH 8·0) containing $1·5 \times 10^{-3}$ M β-mercaptoethanol as the protein concentration was adjusted to about 6 mg/ml, and dialyzed overnight against several changes of the same buffer containing β-mercaptoethanol. The insoluble material produced during dialysis was removed by centrifugation. The insoluble fraction did not have the activity of phosphofructokinase. Activity was increased 5-fold by EtOH fractionation. The supernatant fraction was applied to a column of DEAE-cellulose (1 × 20 cm), equilibrated with 0·1 M Tris-HCl buffer (pH 8·0) containing $1·5 \times 10^{-3}$ M β-mercaptoethanol. The protein was eluted from the column with a linear Tris-HCl gradient at pH 8·0. The concentration of Tris-HCl buffer was 0·1 M in the mixing chamber and 1·0 M in the other chamber. The flow rate was 20 ml/hr and 5 ml fractions were collected. Phosphofructokinase was eluted between 0·45 and 0·6 M Tris-HCl buffer (pH 8·0).

Enzyme assay. Phosphofructokinase activity was measured spectrophotometrically in the coupled system by following the oxidation of NADH.[11] The standard assay solution consisted of 0·5 ml of Tris-HCL buffer (0·1 M, pH 8·0), 2 μmol of $MgCl_2$, 1 μmol of ATP, 1 μmol of F-6-P, 10 μg of aldolase, 10 μg of glycerol-1-phosphate dehydrogenase, 10 μg of triosephosphate isomerase, 0·1 μmol of $NADH_2$ and 100 μg of crude enzyme in a final vol. of 1 ml. The rate of disappearance of NADH at 20° was followed at a wavelength of 340 mμ over 5 min (path length of 1 cm).