BBA 65604

THE REGULATORY PROPERTIES OF A PLANT PHOSPHOFRUCTOKINASE DURING LEAF DEVELOPMENT

D. T. DENNIS AND T. P. COULTATE
Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford (Great Britain)

(Received January 23rd ,1967)

SUMMARY

1. Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) has been extracted from developing Brussels sprout (*Brassica oleracea gemnifera*) leaves. The enzyme from the most immature tissue shows the greatest regulatory control and that from mature and senescent leaves shows the least. This may be an endogenous property of the enzyme or the differences may arise during extraction.

2. The inhibition of the enzyme by citrate and ATP is greater below pH 7.0. In contrast P_i stimulation of the enzyme increases above pH 7.0.

3. The purine nucleoside triphosphates GTP and ITP will act as phosphate donors in place of ATP but are not as inhibitory at higher concentrations. The pyrimidine nucleoside triphosphates UTP and CTP are much less efficient as donors.

4. Unlike other systems AMP and ADP may not be important regulators of plant phosphofructokinase. P_i is probably the positive and citrate the negative effector for the enzyme. The role of ATP is discussed.

INTRODUCTION

The activity of the phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) from carrots has been shown to be regulated in a similar manner to the phosphofructokinase from other sources[1]. Citrate and a high concentration of ATP inhibit the enzyme as they do the enzyme from animals[2–6], yeast[7,8] and ascites tumor cells[9]. The inhibition is reversed by raising the Fru-6-*P* concentration but unlike the enzymes from other sources the inhibition is not reversed by ADP, 5′-AMP or 3′,5′-AMP. In fact ADP and AMP are inhibitory and enhance the inhibitions of ATP and citrate. The phosphofructokinase from parsley leaves is also inhibited by ADP[10]. The inhibition of citrate, ATP, ADP and AMP can be partially reversed by P_i. It was suggested that ATP, citrate and P_i were the important regulators in plant tissue[1]. The importance of P_i and citrate in the regulation of glycolysis in other tissues has also been emphasised[3,8,11].

The work on plant phosphofructokinase has now been extended to the leaf

and bud tissues of the Brussels sprout plant (*Brassica oleracea gemmnifera* var. Cambridge Special or Bedfordshire Prize). The enzyme extracted from different parts of the plants has very different regulatory properties. That from developing sprout buttons shows very pronounced regulatory control whereas the enzyme from leaves loses these properties as the leaf develops and matures. A difference in properties between the enzyme from parsley leaf and avocado fruit has already been described[10]. Whether these differences in regulatory activity are endogenous properties of the enzyme or whether they arise during extraction and storage of the preparation is discussed. Further properties of the enzyme have also been studied.

METHODS

Materials

The sprout plants were grown either in the field or greenhouse. No accurate definition of leaf age was attempted since the properties of leaves can change depending on season and weather conditions[12]. Young leaves were taken from the apex of the plant in a very early stage of expansion. Mature leaves were fully expanded, and senescent leaves were mature leaves which had been kept in the dark at 25° for 5 days until they were fairly yellow. Sprouts were from the top of the stem so that only firm developing sprouts were used. It was considered that the inner yellow leaves would represent active embryonic tissue.

Preparation of extract

Before use leaves were deribbed and the outer (green) leaves of the sprout button removed along with any stem tissue. Typically 100 g of tissue was frozen at −20° in a deep freeze until hard (about 30 min) and while still frozen passed through a kitchen mincer also cooled to −20°. This gave a fairly complete cell disintegration. The mince was passed directly into 265 ml of 50 mM imidazole buffer (pH 7.8) (at 0°) containing 33 g polyclar AT, 2 mM EDTA and 57 mM 2-mercaptoethanol. The semi-frozen mixture was stirred until the temperature had risen above 0°.

All subsequent operations were carried out at around 0°. The mixture was filtered through three layers or muslin and the filtrate centrifuged at 75 000 × *g* for 30 min. Neutralised satd. $(NH_4)_2SO_4$ was slowly added to the supernatant until the solution was 25% satd. After stirring for 20 min the precipitate was removed by centrifugation and the supernatant made up to 40% satn. with $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ precipitation was essential to remove NADH oxidase activity. However, it was usually found difficult to remove all the NADH oxidase activity from mature and senescent leaves. The $(NH_4)_2SO_4$ precipitate was removed by centrifugation and the pellet taken up in 10 ml of 50 mM imidazole buffer (pH 7.0) (at 25°) containing 50 mM CLELAND'S reagent[13]. In some cases the enzyme was used as such but very active preparations had to be diluted 10 or 20 times immediately before use with the resuspending buffer. The undiluted enzyme was stored at 4°. Its stability under these conditions was variable and will be discussed later. In view of this, for most comparative data, the enzyme was used the day after it was prepared.

Assay

The assay is based on that described by UYEDA AND RACKER[3]. The standard

assay contained: 4.0 mM $MgCl_2$, 100 mM 2-mercaptoethanol, 0.60 mM Fru-6-*P*, 1.0 mM ATP, 0.1 mM NADH, 1.8 units of aldolase (EC 4.1.2.7), 1.25 units of α-glycerol-phosphate dehydrogenase (EC 1.1.1.8), 4.0 units of triosephosphate isomerase (EC 5.3.1.1). The final volume was made up to 2.5 ml with 50 mM imidazole buffer (pH 7.0) and all the reagents were made up in the same buffer and adjusted to pH 7.0. The reaction was started by the addition of 0.05 ml of the phosphofructokinase preparation. The decrease in absorbance at 340 mμ was measured in either a Hilger–Gilford or Unicam SP800 recording spectrophotometer.

For pH curves the reaction mixture without the phosphofructokinase was made up in a tube and the pH adjusted with either 4 M NaOH or 4 M HCl. The mixture was then transferred to cuvettes and the phosphofructokinase added to start the reaction. The pH was measured accurately after the incubation period.

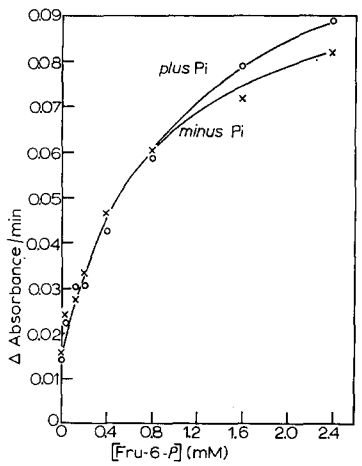
Reagents

The nucleotides and NADH were purchased from Sigma and the auxillary enzymes from Boehringer. The Fru-6-*P* (barium salt) and CLELAND's reagent were purchased from Calbiochem. The Fru-6-*P* was converted to the sodium salt by the addition of sodium sulphate until all the barium precipitated. Polyclar AT was a gift of Fine Dyestuffs and Chemicals, Manchester.

RESULTS

1. The effect of P_i and Fru-6-P concentration

The effect of Fru-6-*P* concentration on the rate is shown in Figs. 1–3. Mature and senescent leaves (Fig. 1) give a curve very similar to that described previously for carrots[1]. P_i has little effect, although there may be an increase in v_{max}. In young leaves there is again a normal curve but in this case there is a repeatable stimulation



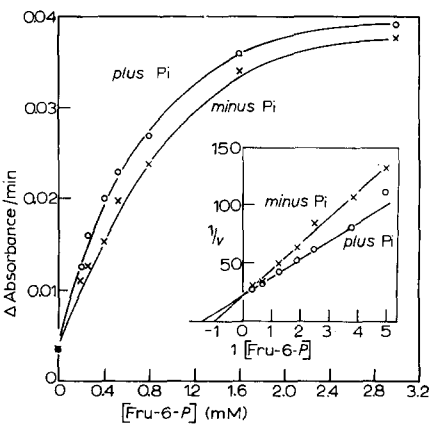
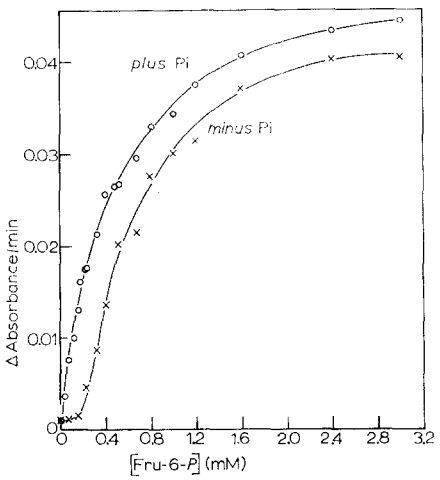


Fig. 1. The effect of Fru-6-*P* concentration and 10 mM P_i on the activity of phosphofructokinase from senescent leaves.

Fig. 2. The effect of Fru-6-*P* concentration and 10 mM P_i on the activity of phosphofructokinase from young leaves.

by 10 mM P_i (sodium salt) especially at the lower concentrations of Fru-6-*P* (Fig. 2). There may also be a slight increase in v_{max}. A plot of $1/v$ against $1/[S]$ (Fig. 2) shows that P_i lowers the K_m for Fru-6-*P* and that the enzyme appears to obey normal kinetics. The apparent K_m in the absence of P_i is approx. 0.9 mM which is much higher than that of phosphofructokinase from heart tissue[4].

The enzyme from sprouts gives a distinctly sigmoid plot (Fig. 3). This is converted to an apparently normal curve by 10 mM P_i. At low concentrations of Fru-6-*P*, therefore, the effect of P_i is very pronounced. All these incubations were performed



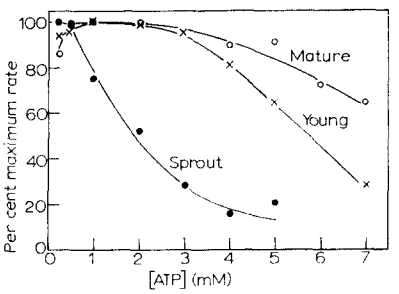


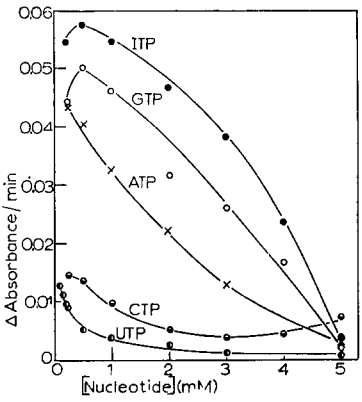
Fig. 3. The effect of Fru-6-*P* concentration and 10 mM P_i on the activity of phosphofructokinase from sprouts.

Fig. 4. The inhibition by ATP of phosphofructokinase from various sources.

with an ATP concentration of 1 mM. It was subsequently found that 1 mM ATP is slightly inhibitory in the case of the enzyme from sprouts but is not inhibitory for the enzymes from young, mature and senescent leaves. This could cause the sigmoid curve observed with the sprout enzyme.

2. The effect of ATP concentration

With the enzyme from young leaves a plot of rate *versus* ATP concentration gives a curve very similar to that given by carrot phosphofructokinase (Fig. 4). Phosphofructokinase from mature leaves shows less inhibition (Fig. 4). Senescent leaves gives an identical curve to mature leaves. In contrast the enzyme from sprouts is very sensitive to ATP concentration and, in some cases, an inhibition occurs at 0.5 mM ATP (Fig. 4) with almost complete inhibition at 5 mM. There is 50% inhibition at 2 mM compared with 6 mM for the enzyme from young leaves, and 5.7 mM for the carrot enzyme[1]. A plot of $1/v$ against $1/[S]$ for ATP at non-inhibitory concentrations gives a K_m for ATP of approx. 0.05 mM for the phosphofructokinase from both young leaves and sprouts. This value is similar to that found for phosphofructokinase from heart muscle[4] but is markedly lower than the K_m for Fru-6-*P*.



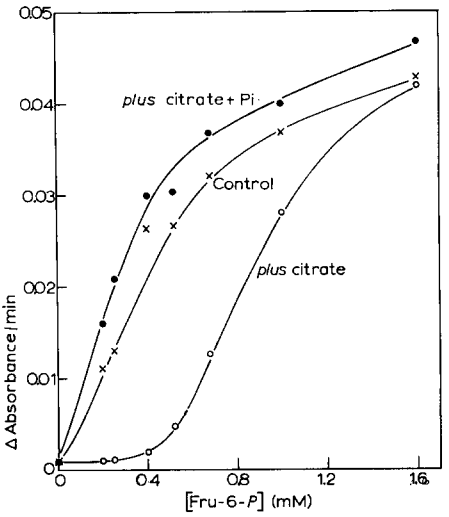


Fig. 5. The ability of various nucleoside triphosphates to act as phosphate donors and inhibitors for phosphofructokinase from sprouts.

Fig. 6. The effect of Fru-6-*P* concentration on the activity of phosphofructokinase from sprouts in the presence of 5 mM citrate and 5 mM citrate *plus* 10 mM P_i.

3. Other nucleoside triphosphates as inhibitors and phosphate donors

Using sprout phosphofructokinase, both GTP and ITP will act as phosphate donors (Fig. 5). They are, however, less inhibitory than ATP at high concentrations. In contrast UTP and CTP are not effective donors even at lower concentrations than those shown in Fig. 5. This is in contrast to muscle where UTP is very similar to ATP[3]. It was thought that UTP may be an extremely effective inhibitor but when 1 mM UTP is used with 0.5 mM ATP, only slight inhibition is observed compared with an incubation without UTP.

It appears, therefore, that in plants, in contrast to muscle, purine nucleoside triphosphates will act as much better phosphate donors than pyrimidine nucleoside triphosphates.

TABLE I

THE INHIBITION OF PHOSPHOFRUCTOKINASE FROM VARIOUS SOURCES BY CITRATE IN THE PRESENCE AND ABSENCE OF P_i

The results are expressed as percentage of the uninhibited rate.

Extract	*Phosphate (mM)*	*Citrate*	
		5 mM	*10 mM*
Sprout	0	14	0
	10	126	—
Young leaves	0	82	54
	10	—	87
Mature leaves	0	96	67
	10	—	78

TABLE II

THE INHIBITION OF PHOSPHOFRUCTOKINASE FROM VARIOUS SOURCES BY ADP
The results are expressed as a percentage of the uninhibited rate.

Extract	*ADP*	
	2 mM	*5 mM*
Sprout	65	18
Young leaves	81	51
Mature leaves	97	72

4. The effect of citrate

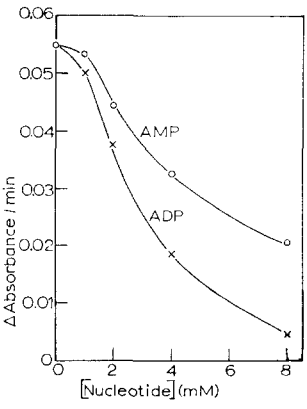
The inhibition of the various enzyme preparations by citrate gives a result very similar to the inhibitions by high concentrations of ATP (Table I). The inhibition of the sprout enzyme is very dependent upon Fru-6-*P* concentration and can be reversed by P_i (Fig. 6). 10 mM P_i reverses the inhibition by 5 mM citrate to a value greater than the uninhibited enzyme (Fig. 6). The citrate inhibition of the phosphofructokinase from young and mature leaves can be partially reversed (Table I).

5. The effect of AMP and ADP

As was the case with carrot phosphofructokinase, AMP and ADP inhibit all the enzyme preparations. The inhibition by ADP is most pronounced in the sprout enzyme (Table II). Both the nucleosides inhibit synergistically with ATP. Over a range of concentrations ADP is a more effective inhibitor than AMP (Fig. 7). Both inhibitions are reversed by P_i.

6. The effect of plant growth regulators

Indole acetic acid, gibberellic acid, kinetin, kinetin riboside and benzyladenine



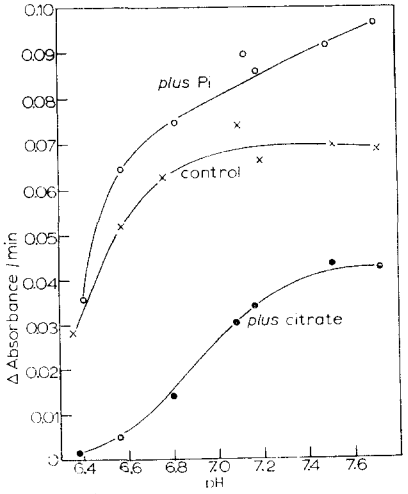


Fig. 7. The inhibition of phosphofructokinase from sprouts by AMP and ADP.

Fig. 8. The effect of pH on the activity of phosphofructokinase from sprouts in the presence of 5 mM citrate and 10 mM phosphate. The ATP concentration in this experiment was 0.5 mM.

will not stimulate or inhibit the enzyme nor relieve the inhibition of 3 mM ATP in the enzyme from sprouts at a concentration of 100 mg/l. Four growth retardants Amo 1618 (2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenyl piperidine-1-carboxylate), phosphon S (tributyl-2,4-dichlorobenzylammonium chloride), β-hydroxyethylhydrazine and B995 (*N*,*N*-dimethylaminosuccinamic acid) are also without effect at the same concentration. Amo 1618 and Phosphon S have been shown to inhibit gibberellin biosynthesis[14] whereas the action of the other two is unknown.

7. The effect of pH

The uninhibited sprout phosphofructokinase has a pH optimum between 7.0 and 8.0 which is similar to the enzyme from other sources (Fig. 8). This enzyme is not very sensitive to pH change compared with enzymes from other sources, for example frog muscle[15]. The inhibition by citrate becomes more pronounced as the pH is lowered below 7.0. The inhibition by 5.0 mM citrate cannot, however, be completely reversed by raising the pH above 7.0. Surprisingly the stimulation by P_i becomes more pronounced as the pH is raised above 7.0. In this work the ATP concentration was 0.5 mM so that an inhibitory concentration was not used. An inhibitory concentration of ATP gives a pH curve similar to the citrate curve.

DISCUSSION

Tissues in different metabolic states can be obtained from the Brussels sprout plant. The inner tissues of the sprout button are a source of embryonic tissue and a sequence of metabolic activity can be followed through developing leaves to mature and senescent leaves. The phosphofructokinase extracted from these tissues shows different regulatory properties. The most regulated phosphofructokinase is from the bud and the least regulated from mature and senescent leaves.

It is possible that the different regulatory properties of the various enzyme preparations may arise during the extraction procedure. On storage for 2–3 days at 4° the phosphofructokinase, particularly from mature and senescent leaves, tends to lose regulatory properties. A loss of regulatory activity in muscle phosphofructokinase by the action of chymotrypsin has been indicated[3]. It is possible that in leaves there is be a relative increase in degradative enzymes, especially in mature and senescent leaves, which could cause the phosphofructokinase to lose regulatory properties during extraction and storage.

Another possibility, however, is that the phosphofructokinase has different regulatory properties within the cell. This could be through the presence of regulated and non-regulated phosphofructokinase with different sensitivities to end-product inhibition as suggested for yeast phosphofructokinase by VINEULA *et al.*[16]. The metabolism of young and mature tissue is different[12] and it is possible therefore that the decline in metabolic activity that accompanies maturity and senscence is paralleled or caused by a decline in enzyme regulatory activity.

Large variations in ATP concentration which would be required for regulation are unlikely to occur since a fairly constant level of ATP is maintained by glycolytic regulation. In fact, a similar concentration of ATP in yeast cells has been demonstrated in both aerobic and anaerobic conditions[8]. It has been suggested that the phosphofructokinase in heart muscle is 90% inhibited in the cell because of the con-

centrations of ATP and Fru-6-*P* (ref. 4). Since the K_m for Fru-6-*P* is high in plant cells it is quite possible that this is also the case here. In the results presented here the magnesium concentration was kept constant at 4.0 mM. It has been shown in brain phosphofructokinase that the ATP:magnesium ratio is very important in determining the amount of inhibition[5] and this is also the case in the enzyme from carrots[1]. Since the magnesium concentration *in vivo* was not measured the actual level of ATP required to cause a substantial inhibition of the phosphofructokinase is not known.

The inhibitory properties of ADP and AMP may be of little importance because of the high stimulatory activity of the P_i which must be produced at the same time as these nucleotides. It is possible that ADP and, to a lesser extent, AMP show a weak binding to the ATP regulator site. A similar anomalous effect of ADP and AMP has been described for citrate synthase (EC 4.1.3.7)[17]. The concept that AMP is the primitive basic regulator of energy metabolism proposed by RAMAIAH, HATHAWAY AND ATKINSON[7] cannot therefore, be applied to plants.

Of much more importance, therefore, may be the levels of P_i and citrate in the cell. The importance of P_i as a coordinated stimulator of glycolysis has already been discussed[3,18]. In plants both the inhibition of starch biosynthesis[19] and the stimulation of an invertase[20] by P_i have been described. The high inhibitory capacity of citrate for phosphofructokinase and the reversibility of this inhibition by P_i suggests that citrate may also be an important regulator of energy metabolism in plants.

Both the synthesis and regulation of enzymes in animal systems are under hormonal control and it is highly probable that the growth regulators fulfil a similar role in plants. The stimulation of citrate synthase by indole acetic acid has already been described[21]. Also the desensitization of some bacterial regulatory enzymes by a product of indole acetic acid metabolism, 3-methyleneoxindole, has been indicated[22]. The lack of effect of the plant growth regulators mentioned above may be due to the inability of the preparation to convert them into active compounds. It has been suggested that 3-methyloxindole is the active auxin[22], and it is possible that a product such as kinetin ribotide is the active kinin. Such products of kinin metabolism have been demonstrated in plants[23].

REFERENCES

1 D. T. DENNIS AND T. P. COULTATE, *Biochem. Biophys. Res. Commun.*, 25 (1966) 187.
2 J. V. PASSONNEAU AND O. H. LOWRY, *Advan. Enzyme Regulation*, 2 (1964) 265.
3 K. UYEDA AND E. RACKER, *J. Biol. Chem.*, 240 (1965) 4682.
4 T. E. MANSOUR, *J. Biol. Chem.*, 238 (1963) 2285.
5 O. H. LOWRY AND J. V. PASSONEAU, *J. Biol. Chem.*, 214 (1966) 2268.
6 P. B. GARLAND, P. J. RANDLE AND E. A. NEWSHOLME, *Nature*, 200 (1963) 169.
7 A. RAMAIAH, J. A. HATHAWAY AND D. E. ATKINSON, *J. Biol. Chem.*, 239 (1964) 3619.
8 M. L. SALAS, E. VINUELA, M. SALAS AND A. SOLS, *Biochem. Biophys. Res. Commun.*, 19 (1965) 371.
9 R. FREYER AND E. HOFMANN, *Biochem. Z.*, 343 (1965) 335.
10 O. H. LOWRY AND J. V. PASSONEAU, *Arch. Exptl. Pathol. Pharmakol.*, 248 (1964) 185.
11 R. M. DENTON AND P. J. RANDLE, *Biochem. J.*, 100 (1966) 420.
12 D. T. DENNIS, M. STUBBS AND T. P. COULTATE, *Can. J. Botany*, in the press.
13 W. W. CLELAND, *Biochemistry*, 3 (1964) 480.
14 D. T. DENNIS, C. D. UPPER AND C. A. WEST, *Plant Physiol.*, 40 (1965) 948.
15 B. TRIVEDI AND W. H. DANFORTH, *J. Biol. Chem.*, 241 (1966) 4110.
16 E. VINUELA, M. L. SALAS, M. SALAS AND A. SOLS, *Biochem. Biophys. Res. Commun.*, 15 (1964) 243.

17 E. BOGIN AND A. WALLACE, *Biochim. Biophys. Acta*, 128 (1966) 190.
18 K. UYEDA AND E. RACKER, *J. Biol. Chem.*, 240 (1965) 4689.
19 H. P. GHOSH AND J. PREISS, *J. Biol. Chem.*, 241 (1966) 4491.
20 D. K. KIDBY, *Plant Physiol.*, 41 (1966) 1139.
21 I. V. SARKISSIAN, *Physiol. Plantarum*, 19 (1966) 328.
22 V. TULI AND H. S. MOYED, *J. Biol. Chem.*, 241 (1966) 4564.
23 D. R. MCCALLA, D. J. MORRE AND D. J. OSBORNE, *Biochim. Biophys. Acta*, 55 (1962) 522.