Vol. 117, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1983 Pages 37-42

FRUCTOSE 2,6-BISPHOSPHATE AS A CONTAMINANT OF COMMERCIALLY OBTAINED FRUCTOSE 6-PHOSPHATE : EFFECT ON PP_i:FRUCTOSE 6-PHOSPHATE PHOSPHOTRANSFERASE

Nicholas J. Kruger, Erich Kombrink* and Harry Beevers

Biology Department, University of California
Santa Cruz, California 95064

Received October 13, 1983

SUMMARY. Fructose 6-phosphate from several commercial sources was shown to be contaminated with fructose 2,6-bisphosphate. This contaminant was identified by its activation of PP_i:fructose 6-phosphate phosphotransferase, extreme acid lability and behaviour on ion-exchange chromatography. The apparent kinetic properties of PP_i:fructose 6-phosphate phosphotransferase from castor bean endosperm were considerably altered when contaminated fructose 6-phosphate was used as a substrate. Varying levels of fructose 2,6-bisphosphate in the substrate may account for differences that have been observed in the properties of the above enzyme from several plant sources.

Fructose 2,6-bisphosphate is a potent activator of PFP from several plant tissues (1-3). However, the response to Fru-2,6-P_2 seems to be variable. In the absence of Fru-2,6-P_2, PFP from potato tuber (1) and spinach leaf (2) exhibited sigmoid kinetics with respect to Fru-6-P. Fru-2,6-P_2 increased the affinity of the enzyme for Fru-6-P by altering the kinetics from sigmoid to hyperbolic, and increased maximum activity about 2-fold. In contrast, the effects of Fru-2,6-P_2 on PFP from mung bean hypocotyl were much stronger and there was no evidence of sigmoid kinetics (3). The activator decreased the K_m for Fru-6-P by a factor of 67 and increased maximum activity 15-fold. The above variation may merely reflect differences in the source of PFP. However, in a study of PFP from endosperm of germinating castor bean we have observed a similar variation in the kinetics of the enzyme from a single tissue. Here we investigate the cause for such variation and demonstrate that commercially obtained Fru-6-P, used as a substrate for PFP, may contain significant amounts of Fru-2,6-P_2.

* Present address: Max-Planck-Institut für Züchtungsforschung, Abteilung Biochemie, 5000 Köln 30, Fed Rep Germany.

Abbreviations: Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; PFP, PP_i:fructose 6-phosphate phosphotransferase (EC 2.7.1.90)

0006-291X/83 $1.50

MATERIALS AND METHODS

Fru-6-P was obtained from Sigma (batch no. 101F-7181), United States Biochemical Corporation (batch no. 30576), Calbiochem (batch no. 103118), Boehringer (type I, batch no. 1372358; type II, batch no. 1306148). All cofactors and auxillary enzymes were purchased from Sigma. The auxillary enzymes were dialysed before use.

PFP was purified over 500-fold from endosperm of 4-day-old castor beans by a combination of ammonium sulfate precipitation, phosphocellulose and DEAE-cellulose chromatography, and gel filtration (4). The resulting PFP preparation was free from other enzymes that could interfere with the measurement of PFP. Details of the purification and properties of PFP from germinating castor bean endosperm are presented elsewhere (manuscript submitted for publication).

PFP activity was measured as described previously (5). The standard assay contained 100 mM Hepes-NaOH (pH 7.5), 1 mM $MgCl_2$, 5 mM Fru-6-P, 0.1 mM NADH, 0.2 mM PP_i, 1 μM Fru-2,6-P_2, 1 U aldolase, 10 U triosephosphate isomerase, 1 U glycerol-3-phosphate dehydrogenase. The reaction was started with PP_i. For acid treatment of Fru-6-P, samples containing up to 100 mM Fru-6-P were incubated in 1.41 M $HClO_4$, at 25°C. After 30 min each sample was neutralised with 5 M K_2CO_3 and then centrifuged (Beckman microfuge, 3 min). The resulting supernatant was used in the assay described above.

Ion exchange chromatography for separation of Fru-6-P from other sugar phosphates was performed on a column (2.5 x 6 cm) of Bio-Rad AG 1-X10 (200-400 mesh, Cl^- form). A 5 ml sample containing 100 mM Fru-6-P (Boehringer I) and 0.25 μCi [U-^{14}C]fructose 1,6-bisphosphate (specific activity 180 Ci/mol) was applied to the ion exchange column, previously equilibrated with 5 mM triethanolamine-HCl (pH 7.7). The column was washed with 100 ml of equilibrating buffer (75 ml/h) and then sugar phosphates were eluted by a linear gradient (300 ml) of 0-0.4 M KCl in the same buffer. Fractions, each 3 ml, were assayed for PFP activation, Fru-6-P and fructose 1,6-bisphosphate. PFP activation was measured by omitting Fru-2,6-P_2 from the standard PFP assay described above. Sigma Fru-6-P was used in these assays. PFP activity in the presence of 100 μl of each fraction was expressed as a percentage of the maximum activity, obtained with 1 μM Fru-2,6-P_2. Fru-6-P was assayed enzymically as described by Lowry and Passonneau (6) and fructose 1,6-bisphosphate was located by measuring ^{14}C in the scintillation cocktail described in (7).

RESULTS

A comparison of the response to Fru-6-P from two different sources as a substrate for castor bean PFP is presented in fig. 1. In the presence of Fru-2,6-P_2, the two samples of Fru-6-P gave almost identical results. Apparent K_m values, calculated from Eadie-Hofstee plots, were 0.222 and 0.224 mM for Fru-6-P from Sigma and Boehringer, respectively. Corresponding values of V_{max} were 6.32 and 6.19 μmol.min^{-1}.mg protein^{-1}. However, without added Fru-2,6-P_2, PFP activity with the two substrates differed strikingly. With Boehringer Fru-6-P, PFP displayed sigmoid kinetics with a Hill coefficient of 1.5 and V_{max} was 4.30 μmol.min^{-1}.mg protein^{-1}, about 70% of the value in the presence of Fru-2,6-P_2. PFP activity with Sigma Fru-6-P exhibited hyperbolic kinetics

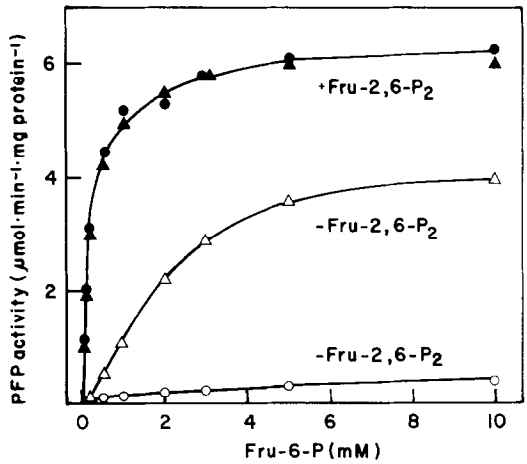

Fig. 1. Effect of Fru-6-P on PFP activity. PFP was measured in the presence of 1 μM Fru-2,6-P_2 (open symbols) and in its absence (closed symbols). PP_i was 0.2 mM, and Fru-6-P from Sigma (● ○) and Boehringer I (▲ △) were varied as shown.

and V_{max}, 0.813 μmol.min^{-1}.mg $protein^{-1}$, was considerably lower. Supplementation experiments suggested that this difference was due to an activator in the Boehringer substrate. Addition of Boehringer Fru-6-P to an assay containing 5 mM Sigma Fru-6-P stimulated PFP activity, whereas Sigma Fru-6-P had no significant effect when added to an assay already containing 5 mM Boehringer Fru-6-P (results not shown).

To estimate the extent of problem we examined Fru-6-P from several sources (table 1). There was no significant difference in PFP activity measured with each of these substrates in the presence of Fru-2,6-P_2, whereas in its absence PFP activity ranged between the two extremes observed above. By incubating Fru-6-P at below pH 3 before use these differences could be considerably reduced. PFP activity with all Fru-6-P samples was slightly below that measured with untreated Sigma Fru-6-P and, with the exception of Boehringer II Fru-6-P, there was no significant difference between enzyme activity measured with any of the substrates after acid treatment. We confirmed that this decline in activity was not due to acid hydrolysis of Fru-6-P. First, the activity with acid-treated Fru-6-P in the presence of Fru-2,6-P_2 was not significantly different from that measured in controls with untreated

Table 1. PFP activity with Fru-6-P from various sources.

Source of Fru-6-P	PFP activity (μmol.min^{-1}.mg protein^{-1})				Amount of Fru-6-P recovered after acid treatment
	Untreated Fru-6-P		Acid-treated Fru-6-P		
	- Fru-2,6-P_2	+ Fru-2,6-P_2	- Fru-2,6-P_2	+ Fru-2,6-P_2	(% of control)
Sigma	0.476 ± 0.018	6.215 ± 0.151	0.158 ± 0.020	5.954 ± 0.111	106.4 ± 0.3
United States Biochemical Corporation	0.896 ± 0.023	6.180 ± 0.190	0.163 ± 0.033	6.170 ± 0.102	109.5 ± 0.6
Calbiochem	3.065 ± 0.065	6.114 ± 0.166	0.137 ± 0.019	5.853 ± 0.086	108.7 ± 1.7
Boehringer I	3.306 ± 0.050	6.154 ± 0.139	0.147 ± 0.013	5.903 ± 0.075	105.6 ± 0.7
Boehringer II	3.170 ± 0.063	6.069 ± 0.142	0.247 ± 0.020	5.903 ± 0.166	107.2 ± 0.8

Acid-treated Fru-6-P was incubated in 1.41 M $HClO_4$ for 30 min at 25°C and neutralised with K_2CO_3 before being used to measure PFP activity. When present Fru-2,6-P_2 was 1 μM. The recovery of Fru-6-P after this acid treatment is expressed as a percentage of Fru-6-P measured in untreated controls. All values represent the mean ± SE of 4 separate measurements.

Fru-6-P. Second, the amount of Fru-6-P recovered after acid-treatment was 106-110% of that measured in untreated samples.

To identify the activator a sample of Boehringer I Fru-6-P was chromatographed on an ion-exchange column (fig. 2). The activating factor separated

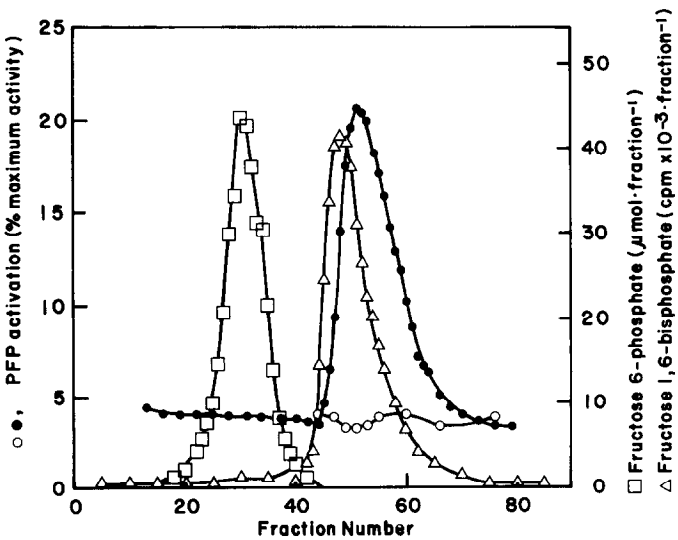

Fig. 2. Ion exchange chromatography of commercial Fru-6-P. A sample of Fru-6-P (Boehringer I) was combined with 0.25 μCi [U-^{14}C]fructose 1,6-bisphosphate and chromatographed on Bio-Rad AG 1-X10. Fractions, 3 ml, were analysed for Fru-6-P (□), fructose 1,6-bisphosphate (△), and for PFP activation before (●) and after (○) acid treatment. PFP activity in the presence of 100 μl of each fraction is expressed as a percentage of the maximum activity, obtained with 1 μM Fru-2,6-P_2.

from Fru-6-P and eluted just after authentic fructose 1,6-bisphosphate, in a position similar to Fru-2,6-P_2 (3). The activation of PFP by the fractions off the column could be totally abolished by the acid treatment described above (fig. 2). This behaviour is identical to that of authentic Fru-2,6-P_2 and differs from that of glucose 1,6-bisphosphate and fructose 1,6-bisphosphate which, at higher concentrations, can also activate PFP (8). Activation of PFP by 1 μM Fru-2,6-P_2 was completely abolished by the acid incubation. In contrast, activation by 0.2 mM glucose 1,6-bisphosphate was not significantly reduced by the above treatment and 1 mM fructose 1,6-bisphosphate, measured enzymically (6), was unaffected. From its strong activation of PFP, extreme acid lability and behaviour on ion-exchange chromatography we conclude that the contaminant is Fru-2,6-P_2. By comparison with PFP activation by 0-10 nM authentic Fru-2,6-P_2, the batches of Fru-6-P examined above probably contain the following amounts of Fru-2,6-P_2, expressed as μmol/mol Fru-6-P: Sigma, 0.029; United States Biochemical Corporation, 0.116; Calbiochem, 1.08; Boehringer I, 1.32; Boehringer II, 1.22.

DISCUSSION

The results demonstrate that commercial preparations of Fru-6-P are commonly contaminated with Fru-2,6-P_2. Although in no instance studied was the level of contamination greater than 0.0002%, even this small amount of Fru-2,6-P_2 is significant, since PFP from higher plants is extremely sensitive to this activator (K_a = 5-50 nM) (1-3). Fig. 1 demonstrates that the contamination is sufficient to distort considerably a kinetic investigation of PFP. The apparent sigmoid kinetics of the enzyme can be abolished and Fru-2,6-P_2 activation can be considerably increased by using a source of Fru-6-P containing less Fru-2,6-P_2. Therefore, for castor bean, the sigmoid kinetics with respect to Fru-6-P observed with some batches of the substrate are apparently an artifact caused by increasing the concentration of Fru-2,6-P_2 in concert with that of Fru-6-P. This contamination could also account for the sigmoid kinetics reported for PFP from potato tuber (1) and spinach leaf (2). In both of these investigations (1,2) Fru-6-P was obtained from Boehringer,

the source which contained the highest Fru-2,6-P_2 contamination in the present study. However, inter-specific differences in the properties of the enzyme may exist. To avoid misleading results similar to those demonstrated in this paper we suggest that in future studies on PFP attention is paid to the contamination of Fru-6-P by Fru-2,6-P_2. Where necessary the activator may be removed from Fru-6-P by acid hydrolysis as described above.

ACKNOWLEDGEMENT

This work was supported by grant PCM-78-19575 from the U.S. National Science Foundation. E.K. was supported by a North Atlantic Treaty Organization Postdoctoral Fellowship administered through the Deutscher Akademischer Austauschdienst.

REFERENCES

1. Van Schaftingen, E., Lederer, B., Bartrons, R. and Hers, H.G. (1982) Eur. J. Biochem. 129, 191-195.
2. Cséke, C., Weeden, N.F., Buchanan, B.B. and Uyeda, K. (1982) Proc. Natl. Acad. Sci. USA 79, 4322-4326.
3. Sabularse, D.C. and Anderson, R.L. (1981) Biochem. Biophys. Res. Commun. 103, 848-855.
4. Kombrink, E., Kruger, N.J. and Beevers, H. (1983) Plant Physiol. 72, S-124.
5. Kruger, N.J., Kombrink, E. and Beevers, H. (1983) FEBS Letts. 153, 409-412.
6. Lowry, O.H. and Passonneau, J.V. (1972) A Flexible System of Enzymatic Analysis, pp. 167-168, Academic Press, New York, London.
7. Robinson, S.P. and Beevers, H. (1981) Plant Physiol. 68, 560-566.
8. Sabularse, D.C. and Anderson, R.L. (1981) Biochem. Biophys. Res. Commun. 100, 1423-1429.