Appl Microbiol Biotechnol (1985) 22:217-221

Applied and Microbiology Biotechnology

Properties of a pentulose-5-phosphate phosphoketolase from yeasts grown on xylose

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Summary. Phosphoketolase activity from nine yeasts grown on xylose occurred with both xylulose 5-phosphate (Xu5P) and ribulose 5-phosphate (Ru5P) as substrates. With extracts from five yeasts *(Candida curvata, C. famata, Lipomyces starkeyi, Rhodotorula glutinis* and *Pachysolen tannophilus)* activity was approximately the same with either substrate; with *C. boidinii, Pichia media* and *Yarrowia lipolytica* Ru5P was the preferred substrate; and with *Rhodosporidium toruloides* Xu5P was the better substrate. Partial purification of the phosphoketolase from *C. famata* was attempted: although activity of phosphoketolase towards Ru5P was decreased it was not eliminated and it is concluded that either (i) the phosphoketolase does have dual substrate specificity, in which case it should be referred to as a pentulose-5-phosphate phosphoketolase (Pu5PPK) or (ii) Ru5P-3-epimerase activity, which can interconvert Xu5P and Ru5P, may be closely associated with phosphoketolase activity. The Pu5PPK has a K_m of 2.4 mM for Xu5P, a pH optimum of 7.2—7.4 and a M_r of 5×10^5 daltons. It is not sensitive to inhibition by citrate or acetyl-CoA at physiological concentrations.

Introduction

Although there is currently a great deal of interest in the utilization of xylose as a fermentation substrate (see Gong 1983 for a recent review), evidence for its metabolism being solely via the pentose phosphate pathway rests on doubtful assumptions. We have recently pointed out that such a metabolic route cannot account for the efficiency by which xylose is used by most microorganisms and have reported the occurrence of an inducible phosphoketolase in various yeasts when grown on xylose (Evans and Ratledge 1984). As phosphoketolase carries out the following reaction:

xylulose 5-phosphate → glyceraldehyde 3-phosphate + acetyl phosphate, there is no loss of CO_2 as would occur if metabolism proceeded with the pentose phosphate pathway.

Although xylulose 5-phosphate (Xu5P), which is produced from xylose via xylitol, was previously used as the substrate in the assay for this enzyme, there is no *a priori* reason why ribulose 5-phosphate (Ru5P) should not also be considered as a substrate. The difference between Ru5P and Xu5P lies in the stereospecific orientation at C-3 which is lost when this carbon atom becomes the C-1 of glyceraldehyde 3-phosphate (G3P). Ru5P would readily arise from Xu5P via ribulose-5-phosphate-3-epimerase but, as cell extracts will inevitably contain this enzyme, no matter which substrate was used in the assay of phosphoketolase, both would engender activity.

We have attempted to resolve this difficulty but have indeed found that phosphoketolase activity in yeasts can be demonstrated with both Xu5P and Ru5P. To ascertain the true substrate, we have carried out a partial purification of the enzyme from a xylose-utilizing yeast originally regarded as a strain of *Saccharomyces cerevisiae* (strain NCYC 33) but which now is thought to be a strain of *Candida famata* (Barbara Kirsop, NCYC, Norwich personal communication). It is designated in this paper as *C. famata*.

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Materials and methods

Yeasts and growth

Yeasts were obtained from culture collections as designated (CBS = Centraalbureau voor Schimmelcultures, Delft, Netherlands; NCYC = National Collection of Yeast Cultures, Norwich, England). *Candida curvata* D was from Professor E. G. Hammond, Ames, Iowa, USA. They were grown on medium as previously described (Evans and Ratledge, 1984) using xylose as sole carbon source. They were harvested after 46 h.

Preparation of cell-free extracts and assay of Pu5PPK

Cell-free extracts were prepared as previously described (Evans and Ratledge 1984) except that cells were passaged twice through the French press. The assay of Pu5PPK [EC 4.1.2.9; D-xylulose-5-phosphate D-glyceraldehyde-3-phosphate lyase (phosphate-acetylating)] was assayed by following the formation of glyceraldehyde 3-phosphate from either xylulose 5-phosphate or ribulose 5-phosphate using the Method A of Goldberg et al. (1966). Occasionally the enzyme was assayed by Method B of Goldberg et al. (1966) which measures the formation of acetyl phosphate (see Evans and Ratledge 1984; for full details).

Partial purification of Pu5PPK from C. famata NCYC 33

The yeast was grown, harvested and disrupted as described above. Ammonium sulphate fractionation was carried out collecting the precipitate forming between 50 and 70% saturation. The protein was dissolved in the minimum volume of 10 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.0, passed through a column of Sepharose 6B (2.5 cm × 42 cm) using the same buffer as eluant Active fractions were collected and pooled, heated to 50° C for 15 min, centrifuged at 5000 × *g* for 10 min and the supernatant applied to a DEAE cellulose column (2.5 cm × 40 cm) equilibrated with the same buffer as before. The enzyme was eluted by adding a linear gradient of KCl (from 0 to 0.75 M) to the buffer. The enzyme was eluted at approximately 0.45 M KCl; the active fractions were combined, the ammonium sulphate precipitation was repeated with the 70 to 100% fraction being retained. This was dialysed overnight against phosphate buffer and then stored at −20° C until required.

Determination of the Mr of Pu5PPK

The above-mentioned column of Sepharose 6B was used with the following proteins (all *ex* Sigma) being used as standards: β-galactosidase, MW = 520,000; urease, MW = 483,000; phosphorylase a, MW = 370,000; pyruvate kinase, MW = 237,000; and thyroglobin, MW = 695,000.

Results and discussion

Substrate specificity

The apparent dual substrate specificity of phosphoketolase in nine yeasts is indicated in Table 1

Table 1. Specific activity of phosphoketolase in cell free extracts of yeasts grown on xylose as sole carbon source. Enzyme assays were performed according to Goldberg et al. (1966) using assay Method A

Yeast strain	Specific activity of phosphoketolase (nmol min^{-1} mg $protein^{-1}$)	
	Xylulose 5-phosphate	Ribulose 5-phosphate
Candida boidinii CBS 5777	13.0	46.1
Candida curvata D	37.4	40.8
*Candida famata**	39.0	38.0
Lipomyces starkeyi CBS 1809	72.0	64.1
Pachysolen tannophilus NCYC 614	25.0	17.2
Pichia media CBS 5521	29.0	41.0
Rhodotorula glutinis NCYC 59	25.2	24.1
Rhodosporidium toruloides CBS 14	55.0	27.0
Yarrowia lipolytica CBS 2074	2	23.1

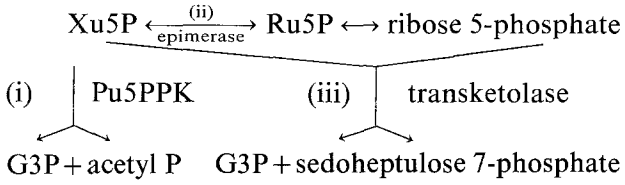
* Originally classified as *Saccharomyces cerevisiae* NCYC 33

where the assay was carried out using either Xu5P or Ru5P as substrate. In five cases *(Candida curvata, C. famata, Lipomyces starkeyi, Rhodotorula glutinis* and possibly *Pachysolen tannophilus)* the enzyme was approximately equally active with either substrate. With *C. boidinii, Pichia media* and *Yarrowia lipolytica,* there was a decided preference for Ru5P as substrate although previously (Evans and Ratledge 1984) each of these yeasts had shown moderate to high activities of Pu5PPK with Xu5P. The reason for this change is not obvious to us though all cell extracts were now prepared by double passage of yeasts through a French press rather than just once as previously. Only with *Rhodosporidium toruloides* did there appear to be a decided substrate preference for Xu5P over Ru5P.

This apparent dual substrate specificity of the Pu5PPK in the various yeasts could be either genuine or due to the presence of Ru5P-3-epimerase interconverting the two substrates. The purification of the enzyme from *C. famata* was therefore undertaken to resolve this question and to ascertain some of the properties of the enzyme.

Partial purification of Pu5PPK

Xylulose 5-phosphate, the putative substrate for Pu5PPK, can react with three enzymes: (i) Pu5PPK; (ii) Ru5P-3-epimerase, and (iii), in the presence of ribose 5-phosphate, with transketolase.



The assay for the epimerase (Williamson and Wood 1966) is dependent upon having a preparation of phosphoketolase which is not reactive with Ru5P; the epimerase cannot therefore be assayed in preparations which may already contain a phosphoketolase with dual specificity. The assay for transketolase is also impossible in extracts containing a phosphoketolase as both enzymes produce glyceraldehyde 3-phosphate which forms the basis of the former assay. Hence, of the three enzymes capable of reacting with Xu5P, it is only possible to assay the Pu5PPK; absence of epimerase activity could only be inferred if purified Pu5PPK proved incapable of reacting with Ru5P. Competing activity of transketolase for Xu5P would disappear if either the epimerase or the pentose phosphate isomerase (converting Ru5P to ribose 5-phosphate) could be eliminated: however for there to be interfering activity from transketolase, both Xu5P and R5P must be simultaneously present which requires activity of two enzymes no matter which substrate is used in the Pu5PPK assay.

The partial purification of Pu5PPK is shown in Table 2. It can be seen that as purification proceeded, the specific activity towards Xu5P increased over four fold in comparison with the specific activity towards Ru5P. The activity of the enzyme towards Xu5P appeared to be more heat-tolerant than the activity displayed with Ru5P as substrate (Fig. 1) and a heat treatment step was therefore introduced into the purification procedure. Enzyme activity emerged as a single peak following passage through Sepharose 6B (Fig. 2). Although the final purification step given in Table 2 increased the specific activity of Pu5PPK by a further 50%, this did not result in any significant change in the ratio of activities with the two different substrates. The final activity of the Pu5PPK was 591 nmol min^{-1} (mg protein)$^{-1}$ by Method A (see Table 2) and 463 nmol min^{-1} (mg protein)$^{-1}$ by Method B i.e. based on formation of acetyl phosphate. Further attempts involving hydroxyapatite and isoelectric focusing were made to increase the purity of the Pu5PPK preparation still further but without success. The final preparation given in Table 2 was the one used in all subse-

Table 2. Results of a typical purification of phosphoketolase from *Candida famata** grown on xylose as carbon source

Fraction	Volume (ml)	Total protein (mg)	Activity with xylulose 5-phosphate				Activity with ribulose 5-phosphate				Specific activity Ratio $\frac{Xu5P}{Ru5P}$
			Total units (nmol min^{-1})	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]	Purification factor	Yield %	Total units (nmol min^{-1})	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]	Purification factor	Yield %	
Crude	23	402.5	10788	27	1	100	12317	31	1	100	0.87
50—70% $(NH_4)_2SO_4$	3.2	193.3	7720	41	1.5	72	10979	56	1.86	89	0.73
Pooled Sepharose 6B eluate	68.0	36.8	7018	191	7.12	65	6245	170	5.5	50	1.1
Heat treatment at 50°C for 15 min	65.0	30.0	5910	197	7.3	55	3540	118	4.4	28.7	1.7
Pooled DEAE cellulose eluate	51.0	14.8	5842	395	14.6	54	1406	95	3.5	11.4	4.2
70—100% $(NH_4)_2SO_4$	53.0	9.5	5601	591	22	52	1444	152	5.6	13	3.9

* Originally classified as *Saccharomyces cerevisiae* NCYC 33

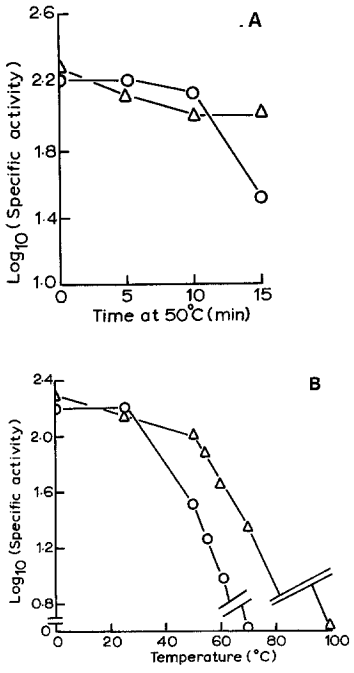


Fig. 1. Denaturation profiles of partially purified phosphoketolase from *Candida famata*. (**A**) Effect of time of incubation at 50°C on specific activity. (**B**) Effect of incubation for 15 min at various temperatures on specific activity. ○ Ribulose-5-phosphate as substrate; △ Xylulose-5-phosphate substrate

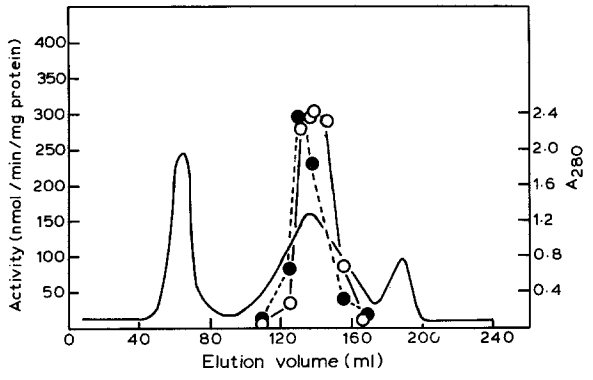


Fig. 2. Gel filtration pattern of Pu5PPK from *Candida famata* on Sepharose-6B. *Continuous line:* protein concentration (A_{280}); ●, Enzyme Activity with ribulose-5-phosphate as substrate; ○, Enzyme Activity with xylulose 5-phosphate as substrate

quent work. [Due to the high cost of assaying Pu5PPK, only Xu5P was used as substrate in this work.]

Properties of Pu5PPK

A Lineweaver-Burk plot of $\frac{1}{V}$ against $\frac{1}{S}$ between 0.1 mM and 10 mM Xu5P gave a straight line relationship from which the K_m and V_{max} were calculated as 2.4 mM and 0.71 μmol min^{-1} (mg protein)$^{-1}$, respectively.

The enzyme showed maximal activity between pH 7.2 and 7.4 with half-maximal activity at approximately pH 6.4 and pH 8.2, using KH_2PO_4/Na_2HPO_4 buffer, 30 mM, below pH 8 and Tris/HCl 30 mM above pH 8.

The enzyme was not affected by citrate at concentrations (< 10 mM) which are likely to prevail intracellularly (Evans and Ratledge, 1983). Inhibition with high concentrations of citrate was however observed (K_i approximately 70 mM) though this is unlikely to be of physiological significance and was attributed to the chelating activity of citrate for magnesium which is a co-factor in the enzyme assay. The enzyme was not sensitive to inhibition by acetyl-CoA; at concentrations above 0.1 mM, which is some ten fold higher than concentrations normally found in yeasts, slight stimulation (~25%) was observed though again no physiological significance is attached to this.

The approximate molecular size (*Mr*) of the Pu5PPK was calculated from its elution volume from a column of Sepharose 6B (see Fig. 2) where it emerged approximately mid-way between β-galactosidase (MW = 520,000) and urease (MW = 490,000). A molecular size of 5×10^5 daltons is accordingly indicated for this enzyme.

The purification of the Pu5PPK as presented in Table 2 does not resolve between the two possibilities:

(a) that the enzyme has dual substrate specificity for both Xu5P and Ru5P, with the present evidence suggesting that the former is the preferred substrate in *C. famata* at least;
(b) that the enzyme has single substrate specificity for Xu5P but activity towards Ru5P is due to the continuing presence of Ru5P-3-epimerase converting Ru5P (when used as substrate) to Xu5P.

As will be appreciated from the argument given above, it is not possible to assay for the epimerase as this depends on a coupled enzyme assay to which phosphoketolase is added. Thus, any enzyme preparation of the epimerase which already contains phosphoketolase activity cannot be assayed. In view of this *impasse* we have not been able to resolve between the two alternatives and for the time being it would be more appropriate to regard the enzyme as a pentulose-5-phosphate phosphoketolase.

The enzyme appears to be of large size, $Mr \sim 5 \times 10^5$, and is thus similar to the phospho-

ketolase from *Lactobacillus plantarum* (Heath et al. 1958) which has a mol. wt. of 550000. The bacterial enzyme, however, had no activity towards any substrate other than Xu5P and thus might prove to be different from the yeast enzyme. There was no evidence of product inhibition (acetyl-phosphate and glyceraldehyde 3-phosphate) or by citrate or acetyl-CoA with the yeast enzyme. Some regulation of the Pu5PPK pathway or of the attendant pentose phosphate pathway enzymes might though be anticipated: when an organism grows on xylose some Xu5P must of necessity be recycled into the hexose monophosphate pathway to produce the required NADPH to catalyse the first step of xylose metabolism (to xylitol via NADPH-dependent xylose dehydrogenase) (Bruinenberg et al. 1983). This will involve the conversion of Xu5P to Ru5P by Ru5P-3-epimerase as well as the reaction of Xu5P with either R5P or E4P in the transketolase reaction. How a xylose-utilizing organism then regulates its metabolism of pentulose phosphates between the various routes, remains, for the time being, an intriguing and open question.

Acknowledgements. This work was supported by an SERC-CASE studentship to Miss J. E. Holdsworth and operated in conjunction with Cadbury-Schweppes plc to whom we are greatly indebted for considerable financial assistance in the purchase of all the biochemicals used in this work.

References

Bruinenberg PM, deBot PHM, van Dijken JP, Scheffers WA (1983) The role of redox balances in the anaerobic fermentation of xylose by yeasts. Eur J Appl Microbiol Biotechnol 18:287—292

Evans CT, Ratledge C (1983) A comparative study of citrate efflux from mitochondria of oleaginous and non-oleaginous yeasts. Eur J Biochem 130:195—204

Evans CT, Ratledge C (1984) Induction of xylulose-5-phosphate phosphoketolase in a variety of yeasts grown on D-xylose: the key to efficient xylose metabolism. Arch Microbiol 139:48—52

Goldberg M, Fessenden JM, Racker E (1966) Phosphoketolase. In: Wood WA (ed) Methods in enzymology, vol 9 Academic Press, New York, pp 515—520

Gong CS (1983) Recent advances in D-xylose conversion in yeasts. Ann Rep Ferment Proc 6:253—297

Heath EC, Hurwitz J, Horecker BL, Ginsburg A (1958) Pentose fermentation by *Lactobacillus plantarum* I The cleavage of xylulose 5-phosphate by phosphoketolase. J Biol Chem 231:1009—1029

Williamson WT, Wood WA (1966) D-Ribulose 5-phosphate 3-epimerase. In: Wood WA (ed) Methods in enzymology, vol 9 Academic Press, New York, pp 605—611

Received February 27, 1985