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Multiple forms of xylose reductase in *Pachysolen tannophilus* CBS4044

(Xylose reductase; xylose fermentation; yeast; wood sugar; ethanol)

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1. SUMMARY

Cell-free extracts of xylose-grown *Pachysolen tannophilus* exhibited xylose reductase activity with both NADPH and NADH. The ratio of the NADPH- and NADH-dependent activities varied with growth conditions. Affinity chromatography of cell-free extracts resulted in a separation of two xylose reductases. One was active with both NADPH and NADH, the other was specific for NADPH. Apart from this coenzyme specificity, the two enzymes also differed in their affinities for xylose and NADPH. The role of the two enzymes in xylose metabolism is discussed in relation to attempts to use *P. tannophilus* for the alcoholic fermentation of wood sugars.

2. INTRODUCTION

The ability of certain yeasts to convert the wood sugar D-xylose to ethanol has been intensively studied over the past 10 years. Most reports in this field have dealt with cultivation conditions in relation to the rate and efficiency of alcoholic fermentation. Recent enzymological studies have indicated that rapid anaerobic fermentation of xylose by yeasts is correlated with the presence of an NADH-linked xylose reductase [1]. This NADH-linked enzyme has been purified from the xylose-fermenting yeast *Pichia stipitis* and has been shown to exhibit a dual coenzyme specificity for NADH and NADPH [2]. In most other yeasts, which do not or only slowly ferment xylose anaerobically, xylose reductase activity is NADPH-linked [3,4]. In these yeasts NADH-linked xylose reductase activity is absent or very low [1].

During a comparative study on xylose metabolism in yeasts, it was noticed that *P. tannophilus* CBS4044 contained low but significant NADH-dependent xylose reductase activity [1]. Further enzymatic analysis revealed that the ratio of NADPH- to NADH-linked xylose reductase activity in this yeast varied with growth conditions. This contrasts with the situation in *Pichia stipitis* strains in which the ratio of the two activities is constant and due to a single enzyme [1,2]. Furthermore, since it has been reported that the xylose reductase of *P. tannophilus* is strictly dependent on NADPH [5], it was decided to reinvestigate the characteristics of xylose reductase in this yeast.

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3. MATERIALS AND METHODS

3.1. Microorganism and growth conditions

P. tannophilus CBS4044 was maintained on malt agar slopes. Purity of cultures was checked microscopically and by streaking onto malt agar plates. The identity of the colonies was checked using standard taxonomic procedures [6]. The organism was grown in fermenter or shake-flask cultures at 30°C on a mineral medium [7] or 1% Difco yeast extract, each containing 2.5% xylose. Cells pregrown in shake-flask cultures on 1% yeast extract plus 2% glucose were used as inoculum. Fermenter cultivation was performed as described previously [4] at a constant pH value of 5.0. Shake-flask cultures were incubated on a rotatory shaker at 150 rev./min and contained 30 ml medium in 100-ml Erlenmeyer flasks; the initial pH value was 6.0. Where mentioned, the term aerobic cultivation indicates growth in fermenters at a dissolved oxygen tension not lower than 50% air saturation.

3.2. Preparation of cell-free extracts

Cells were harvested by centrifugation and washed twice with cold 50 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA, and stored as pellets at −40°C until used (within 2 months). Except for the enzyme purification, cell-free extracts were prepared by sonication at 4°C in 100 mM potassium phosphate buffer, pH 7.0, containing 3 mM 2-mercaptoethanol. Whole cells and debris were removed by centrifugation for 20 min at 50000 × *g*. The resulting supernatant was used as the cell-free extract.

3.3. Enzyme assay

Xylose reductase (EC 1.1.1.21) was assayed at 30°C as described previously (2). Enzyme units are given as μmol NAD(P)H oxidized per min. Specific activity is expressed as $U \cdot (\text{mg protein})^{-1}$.

3.4. Detection of the reaction product

For the detection of the product of xylose reduction, one unit of xylose reductase was allowed to react for 60 min under the conditions mentioned above. In this case the reaction mixture (1 ml) contained in addition 10 mM glucose 6-phosphate and 20 U of glucose 6-phosphate dehydrogenase as an NADPH-regenerating system. This allowed the accumulation of xylitol up to 10 mM. After completion of the reaction, the mixture was deproteinized by heat precipitation and centrifugation. In the supernatant, xylitol was determined by HPLC as described below. Incubation of a blank without xylose reductase, included to check for non-enzymatic xylose conversion, did not lead to detectable formation of xylitol.

3.5. Enzyme separation

The yeast was grown aerobically in a fermenter on a mineral medium supplemented with xylose as described above. When the culture reached a density of approx. 5 g dry wt. per l, the air supply and stirrer speed were adjusted to give a dissolved oxygen tension between 0–2% of air saturation.

After 24 h of slow growth under oxygen limitation, cells were harvested. The preparation of the cell-free extract and its treatment with streptomycin sulphate were as described for *Pichia stipitis* [2]. These and subsequent operations were performed at 4°C unless mentioned otherwise. The supernatant of the streptomycin sulphate step was applied to a column (10 × 1 cm) of Cibacron Yellow/Sephacryl S-200, equilibrated in 75 mM potassium phosphate buffer, pH 7.0, containing 1 mM 2-mercaptoethanol (standard buffer). Elution was carried out with the same buffer at a flow rate of $25 \text{ ml} \cdot \text{h}^{-1}$. Fractions (3 ml) containing xylose reductase activity were pooled and stored at −40°C.

Xylose reductase activity absorbed in the column was eluted with a linear gradient of NaCl (0–2 M) in standard buffer. Fractions containing xylose reductase activity were pooled and concentrated to 4 ml with an Amicon PTGC CX-10 filter. The concentrate was desalted by two cycles of 10-fold dilution in standard buffer, followed by reconcentration. As freezing and thawing of this preparation caused considerable loss of activity the enzyme was sterilized by filtration through a Millipore filter (pore size 0.2 μm) and stored at 4°C. Approx. 10% of the activity was lost in one week.

3.6. Determination of M_r

The M_r of the partially purified enzymes was

determined via HPLC on a Serva Si-200 polyol column (9.1 × 500 mm, 5 μm pore size) with a Waters M6000A solvent delivery system. Elution was carried out at room temperature with 100 mM potassium phosphate buffer, pH 7.0, at a flow rate of 0.5 ml · min^{-1}. Methanol dehydrogenase (M_r 130 000), bovine serum albumin (M_r 67 000), lactalbumin (M_r 40 000) and carbonic anhydrase (M_r 30 000) were used for calibration.

3.7. Analytical methods

Xylose and xylitol were determined by HPLC on a BioRad HPX-78C column according to the instructions of the manufacturer. Protein was determined by the method of Bradford [8] with bovine serum albumin as a standard.

3.8. Chemicals

The affinity dye Cibacron Brilliant Yellow GE (Ciba-Geigy, Switzerland) was coupled to Sephacryl S-200 (Pharmacia, Sweden) as described previously [2]. D-Xylose was from Merck (Darmstadt). Other biochemicals were purchased from Boehringer, F.R.G.

4. RESULTS

4.1. Induction of NADH-linked xylose reductase

Extracts of xylose-grown *P. tannophilus* contained NADPH-linked xylose reductase activities of 0.27 ± 0.05 U · mg^{-1} in both mineral and complex media. The dissolved oxygen tension of cultures had no significant effect on the level of this activity. Apart from NADPH-linked xylose reductase also an NADH-linked activity was detected. The NADH-linked xylose reductase activity and thus the ratio of NADH- to NADPH-dependent activities varied in aerobic fermenter cultures. On yeast extract-containing media this ratio was 0.09 ± 0.02, in agreement with results reported previously [1]. However, in mineral media this ratio was 0.23 ± 0.02. After introduction of oxygen limitation in such cultures the ratio increased to 0.39 ± 0.04, both in mineral and complex media. Also growth in shake-flask cultures, which is intrinsically oxygen-limited, resulted in similar high ratios of NADH- to NADPH-linked xylose reductase activities. Therefore an attempt was made to separate two activities in extracts of the yeast, grown under limited oxygen supply.

4.2. Enzyme separation and characterization

Affinity chromatography of streptomycin sulphate-treated cell-free extracts of *P. tannophilus* resulted in the separation of two xylose reductase activities (Fig. 1). The enzyme which was not absorbed on the column (enzyme A) exhibited activity with both NADPH and NADH. Heat treatment, ion-exchange chromatography and high-performance gel chromatography did not separate the NADPH- and NADH-linked activities. Furthermore, when enzyme A was assayed in the presence of both NADPH and NADH there was no summation of the reaction rates obtained with the individual coenzymes. These findings suggest that the activity of enzyme A is due to a single enzyme with a dual cofactor specificity, similar to the enzyme purified from *Pichia stipitis* [2]. Similar to the latter enzyme, enzyme A from *P. tannophilus* had a twofold higher affinity for NADPH than for NADH. However, the enzymes differ in many respects such as K_m values for xylose, NADPH and NADH, and M_r (Table 1). In addition, the peculiar inhibition pattern of NADP$^+$

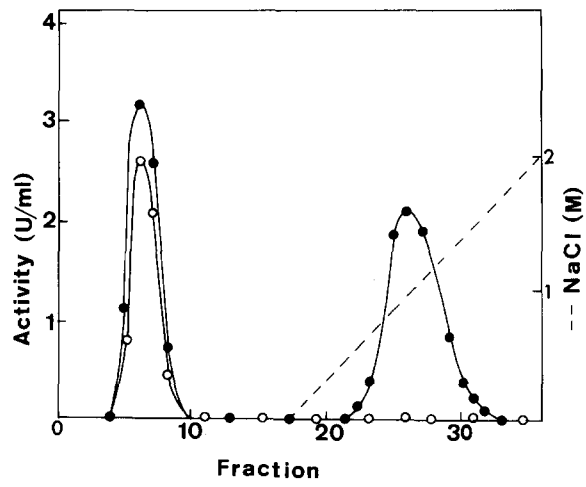

Fig. 1. Separation of two xylose reductase activities from cell-free extracts of *P. tannophilus* CBS4044 by affinity chromatography on Cibacron Yellow/Sephacryl. ●, NADPH-linked xylose reductase; ○, NADH-linked xylose reductase.

Table 1

Properties of partially purified xylose reductase from *P. tannophilus* CBS4044

For comparison, data on the purified enzymes of *Pichia stipitis* CBS5773 [2] and *P. tannophilus* NRRLY-2460 [5] are included. Specific activities are expressed as U·(mg protein)$^{-1}$

	P. tannophilus			*Pichia stipitis*
	[This study]		[5]	[2]
	Enzyme A	Enzyme B		
Specific activity with NADH	0.44	0.07	0.08	16.7
Specific activity with NADPH	0.53	4.6	16.0	23.2
Ratio NADH/NADPH	0.83	0.015	0.005	0.72
K_m for xylose with NADH (mM)	12.5	–	–	42
K_m for xylose with NADPH (mM)	14.0	180	160	42
K_m for NADH (μM)	40	–	–	21
K_m for NADPH (μM)	21	–	57	9
M_r	41000	37000	38000	65000

on the enzyme of *Pichia stipitis* [2] was not observed with enzyme A of *P. tannophilus*.

Part of the NADPH-dependent xylose reductase activity in extracts of *P. tannophilus* absorbed to the affinity column and could be eluted with NaCl (Fig. 1). This activity (enzyme B) was specific for NADPH and had properties similar to the NADPH-linked xylose reductase purified from *P. tannophilus* by Ditzelmüller et al. [5]. Enzyme B had a much lower affinity for xylose than enzyme A (Table 1). The enzymes A and B both had an M_r much lower than usually observed for xylose reductases of yeasts (60000–70000) (Table 1; [9–11]). For both enzymes it was shown that the product of xylose reduction was xylitol.

5. DISCUSSION

Xylose reductase (EC 1.1.1.21) is a key enzyme in the metabolism of xylose by yeasts, which proceeds via xylitol and xylulose as intermediates [1,4,11,12]. The observation that the NADPH-linked xylose reductase in cell-free extracts of *P. tannophilus* resolved into two different enzymes (Fig. 1, Table 1) adds further to the complexity of xylose metabolism in this organism. The physiological basis for the variation in the relative amounts of the two enzymes as a function of growth conditions is at present unclear. It is relevant, however, that the presence of the NADH-linked activity (i.e., enzyme A) explains the, albeit slow, alcoholic fermentation of xylose by *P. tannophilus* under anaerobic conditions [1]. As pointed out by Bruinenberg et al. [4], anaerobic fermentation of xylose requires the presence of NADH-linked xylose reductase. Anaerobic metabolism of xylose via NADPH-linked reductase leads to an imbalance of the NAD^+/NADH redox system [1,4]. Therefore it is plausible that oxygen limitation introduces the need for NADH-linked xylose reductase in order to avoid accumulation of NADH.

NADH-linked xylose reductase activity was very low in aerobic cultures of *P. tannophilus* growing in xylose-containing complex medium. Hence, the main activity of the NADPH-linked xylose reductase in these cells must be ascribed to enzyme B (Fig. 1, Table 1). Since under these growth conditions *P. tannophilus* grew with doubling times of less than 3 h, it is peculiar that enzyme B had a very low (180 mM, Table 1) affinity for xylose. Either the in vivo K_m of the enzyme for xylose is strongly dependent on the NADPH concentration, or the estimated K_m value results from an artefact of the in vitro assay conditions. A K_m value of 180 mM (Table 1) and specific activities in cell-free extracts of 0.27 U · (mg protein)$^{-1}$ seem incompatible with the observed growth rate under conditions where over 90% of the in vitro xylose reductase activities is due to enzyme B.

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REFERENCES

[1] Bruinenberg, P.M., de Bot, P.H.M., van Dijken, J.P. and Scheffers, W.A. (1984) Appl. Microbiol. Biotechnol. 19, 256–260.
[2] Verduyn, C., van Kleef, R., Frank, J., Schreuder, H., van Dijken, J.P. and Scheffers, W.A. (1985) Biochem. J. 226, 669–677.
[3] Scher, B.M. and Horecker, B.L. (1966) In Methods in Enzymology (Wood, W.A., Ed.), vol. 9, pp. 166–170. Academic Press, New York.
[4] Bruinenberg, P.M., de Bot, P.H.M., van Dijken, J.P. and Scheffers, W.A. (1983) Eur. J. Appl. Microbiol. Biotechnol. 18, 287–292.
[5] Ditzelmüller, G., Kubicek, C.P., Wöhrer, W. and Röhr, M. (1984) Can. J. Microbiol. 30, 1330–1336.
[6] Barnett, J.A., Payne, R.W. and Yarrow, D. (1979) A Guide to Identifying and Classifying Yeasts. Cambridge University Press, London.
[7] Bruinenberg, P.M., van Dijken, J.P. and Scheffers, W.A. (1983) J. Gen. Microbiol. 129, 965–971.
[8] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[9] Scher, B.M. and Horecker, B.L. (1966). Arch. Biochem. Biophys. 116, 117–128.
[10] Sheys, G.H., Arnold, W.J., Watson, J.A., Hayashi, J.A. and Doughty, C.C. (1971) J. Biol. Chem. 246, 3824–3827.
[11] Chakravorty, M., Veiga, L.A., Bacila, M. and Horecker, B.L. (1962) J. Biol. Chem. 237, 1014–1020.
[12] Horecker, B.L. (1962) Pentose Metabolism in Bacteria. Wiley, New York and London.