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Properties of the NAD(P)H-dependent xylose reductase from the xylose-fermenting yeast *Pichia stipitis*

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Xylose reductase from the xylose-fermenting yeast *Pichia stipitis* was purified to electrophoretic and spectral homogeneity via ion-exchange, affinity and high-performance gel chromatography. The enzyme was active with various aldose substrates, such as DL-glyceraldehyde, L-arabinose, D-xylose, D-ribose, D-galactose and D-glucose. Hence the xylose reductase of *Pichia stipitis* is an aldose reductase (EC 1.1.1.21). Unlike all aldose reductases characterized so far, the enzyme from this yeast was active with both NADPH and NADH as coenzyme. The activity with NADH was approx. 70% of that with NADPH for the various aldose substrates. $NADP^+$ was a potent inhibitor of both the NADPH- and NADH-linked xylose reduction, whereas NAD^+ showed strong inhibition only with the NADH-linked reaction. These results are discussed in the context of the possible use of *Pichia stipitis* and similar yeasts for the anaerobic conversion of xylose into ethanol.

Whereas in most bacteria metabolism of D-xylose proceeds via direct isomerization to D-xylulose, catalysed by xylose isomerase (EC 5.3.1.5), in yeasts this conversion is catalysed by the sequential action of two oxidoreductases (eqn. 1):

$$\text{Xylose} \xrightarrow[\text{NADPH}\ \curvearrowright\ \text{NADP}^+]{\text{Xylose reductase}} \text{xylitol} \xrightarrow[\text{NAD}^+\ \curvearrowright\ \text{NADH}]{\text{Xylitol dehydrogenase}} \text{xylulose} \qquad (1)$$

[Both enzymes are also known as polyol dehydrogenases. For convenience the names xylose reductase and xylitol dehydrogenase are used throughout the present paper, in line with the fact that the conversion of xylose into xylitol is hardly reversible.] In *Candida utilis* and most other xylose-assimilating yeasts the xylose reductase (EC 1.1.1.21) is specific for NADPH whereas the xylulose-producing xylitol dehydrogenase (EC 1.1.1.9) is NAD^+-specific (Jeffries, 1983). It has been pointed out by Bruinenberg *et al.* (1983*a*) that this mode of xylose metabolism is incompatible with anaerobic utilization of this sugar, since it leads to a net production of NADH in the overall conversion of xylose into ethanol. Recently a number of yeasts have been found that, in contrast with *Candida utilis*, are capable of anaerobic alcoholic fermentation of xylose (Toivola *et al.*, 1984). The key to the observed differences among yeasts with respect to anaerobic xylose fermentation is the coenzyme specificity of the first reaction (i.e. the reduction of D-xylose to D-xylitol). *Pichia stipitis* and the few other yeasts that showed significant anaerobic xylose fermentation possess, in addition to NADPH-linked xylose reductase, an NADH-

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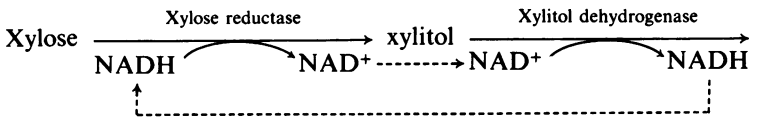
linked xylose reductase activity (Bruinenberg *et al.*, 1984) (eqn. 2):



(2)

This mode of xylose metabolism results in a closed redox balance under anaerobic conditions since the redox equivalents produced during xylitol oxidation can be used for xylose reduction (eqn. 2).

Since metabolism of xylose via the NADPH-linked reductase (eqn. 1) along with metabolism via the NADH-linked reaction (eqn. 2) is an obstacle for efficient anaerobic metabolism, removal of the NADPH-linked reductase activity by mutation could improve the efficiency and rate of anaerobic ethanol production with yeasts such as *Pichia stipitis*. It was therefore decided to investigate whether NADH-linked and NADPH-linked xylose reductase activities in this yeast are due to different enzymes.

Materials and methods

Micro-organisms and cultivation

Pichia stipitis C.B.S. 5773 was obtained from the Centraalbureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands. The organism was grown in batch culture on a mineral medium (Bruinenberg *et al.*, 1983*b*) containing 2% xylose in a 2-litre fermenter. The partial pressure of dissolved O_2, temperature and pH were automatically controlled at 50% of air saturation, 30°C and 5.4 respectively. Cells (approx. 30g wet wt./litre of culture) were harvested towards the end of the exponential growth phase, washed twice with cold 50mM-potassium phosphate buffer, pH7.5, containing 1mM-EDTA and stored as pellets at −40°C until used (within 2 months).

Enzyme assays

Enzymes were assayed spectrophotometrically at 30°C. In the standard assays described below the reaction rates were linearly proportional to the amount of enzyme added. Reaction rates were corrected for endogenous NAD(P)H consumption or production (occurring in crude extracts only). Unless mentioned otherwise, enzyme activities refer to the standard xylose reductase assay with NADPH as coenzyme.

Xylose reductase (forward reaction) (EC 1.1.1.21). The assay mixture (1ml) contained 50mM-potassium phosphate buffer, pH6.0, 0.15mM-NADPH or -NADH, and an appropriate amount of enzyme. After temperature equilibration the reaction was started by the addition of D-xylose to a final concentration of 0.2M.

Xylose reductase (reverse reaction). The assay mixture (1ml) contained 50mM-Tris/HCl buffer, pH8.2, 0.4mM-NAD^+ or -$NADP^+$, and an appropriate amount of enzyme. After temperature equilibration the reaction was started by the addition of xylitol to a final concentration of 0.2M.

Xylitol dehydrogenase (EC 1.1.1.9). The assay mixture (1ml) contained 50mM-Tris/HCl buffer, pH8.2, 5mM-$MgCl_2$, 0.4mM-NAD^+ or -$NADP^+$, and an appropriate amount of enzyme. After temperature equilibration the reaction was started by the addition of xylitol to a final concentration of 0.2M.

Enzyme units

Enzyme units are defined as μmol of nicotinamide nucleotide reduced or oxidized/min. Specific activities are expressed as units/mg of protein.

Preparation of affinity gel

Triazine dyes, bound to a polymer matrix, have been found to be suitable tools for the separation of a variety of yeast enzymes (Johansson *et al.*, 1984). In comparison with the well-known Cibacron Blue (Thompson *et al.*, 1975), we found Cibacron Brilliant Yellow GE more effective for the isolation of the xylose reductase from *Pichia stipitis*. The dye was immobilized as follows.

A jacketed glass column (1.5cm × 25cm) was filled with Sephacryl S-200. After settling of the gel the column was heated to 60°C and then washed with 500ml of a solution containing 40g of $Na_2CO_3,10H_2O$ and 25g of NaCl per litre of distilled water (coupling buffer) of the same temperature. Then 100ml of dye solution (at 60°C) containing 1mg of Cibacron Brilliant Yellow GE/ml in coupling buffer was passed through the column at a flow rate of 1ml/min. Continuous circulation of the eluate was performed for 12–16h, during which time the column and dye solution were kept at 60°C. Thereafter the column was washed with coupling buffer (at 60°C) until the eluate was colourless. The column was then cooled to room temperature via washing with coupling buffer at 20°C, and after a washing with 250ml of the required starting buffer it was ready for use.

The immobilized dye thus constructed exhibited excellent flow rates, mechanical stability and

protein-binding capacity (at least 0.25mg of protein/ml of gel). It could be used many times, during which leakage of dye was not observed. The procedure described here is applicable to the coupling of a wide variety of textile dyes to a polymer matrix.

Purification of xylose reductase

All steps were performed at 4°C unless mentioned otherwise. Where indicated, protein concentration was performed with Amicon PTGC CX-10 filters. Enzyme from each step of the purification procedure could be stored at −40°C without significant loss of activity, provided that the protein concentration was higher than 0.5mg/ml. When storage of enzyme in between two purification steps was required the enzyme was therefore kept frozen.

Preparation of cell-free extract. Frozen cells (3.5g wet wt.) were resuspended in 6.5ml of 75mM-potassium phosphate buffer, pH7.0, containing 1mM-2-mercaptoethanol (standard buffer) and disrupted by four successive passages through a French pressure cell (Amicon) at 110MPa (16000lbf/in^2). Whole cells and debris were removed in two centrifugation steps (2×10min at 50000*g*). The clear supernatant, containing 10–14mg of protein/ml, was used as cell-free extract.

Precipitation of nucleic acids and associated protein. To the cell-free extract a 15% solution of streptomycin sulphate in standard buffer was added to give a streptomycin sulphate/protein ratio (w/w) of 2:1. After stirring for 40min the precipitate was removed by centrifugation (15min at 50000*g*).

Ion-exchange chromatography. The supernatant of the previous step was applied to a column (1.5cm×25cm) of DEAE-Sephacel equilibrated in standard buffer. Xylose reductase did not bind to the column and was eluted with standard buffer at a flow rate of 15ml/h. Fractions (3ml) containing xylose reductase activity were pooled and concentrated to 4ml by ultrafiltration.

Affinity chromatography. The concentrated eluate was applied to a column (1.2cm×10cm) of Cibacron–Sephacryl (prepared as specified above) that had been equilibrated in standard buffer. The column was then washed with standard buffer at a flow rate of 18ml/h until the absorbance of the eluate at 280nm was less than 0.01. Adsorbed proteins were eluted with a linear gradient of NaCl (0–1.2M) in standard buffer (total volume 90ml). Fractions of 2.5ml volume were collected. Those that contained xylose reductase activity (eluted between 0.40M- and 0.70M-NaCl) were pooled and concentrated to 0.5mg of protein/ml. As prolonged dialysis at this stage of the purification markedly decreased activity, the concentrate was desalted by three cycles of 5-fold dilution (with 10mM-potassium phosphate buffer, pH7.0, containing 1mM-2-mercaptoethanol) and re-concentration. The preparation was stored at −40°C.

Freezing and thawing. The frozen protein from the previous step was thawed and centrifuged for 15min at 50000*g*. This resulted in an additional 60% purification. The supernatant was either frozen again or used for high-performance gel chromatography. Unless mentioned otherwise, enzyme from this stage of purification was used in the experiments described below.

High-performance gel chromatography. As further purification with conventional hydrostatic columns turned out to be very difficult, final purification was performed on a small scale by means of high-performance gel-permeation chromatography on a Serva Si-200 polyol column (9.1mm×500mm, 5μm pore size) with a Waters M 6000 A solvent-delivery system and a Waters WISP automatic injector. Elution was carried out at room temperature with 100mM-potassium phosphate buffer, pH7.0, at a flow rate of 0.5ml/min. Detection and on-line spectral analysis were performed with a Hewlett–Packard HP 1040A photodiode array detector. Full spectra (190–390nm) were recorded at a frequency of 10min^{-1}.

Electrophoresis

Purity of the fractions containing xylose reductase activity was checked by polyacrylamide-gel electrophoresis by the procedure of Laemmli & Favre (1973). Protein was detected by using the silver stain of Wray *et al.* (1981).

Determination of M_r

The M_r of the enzyme was determined via Sephadex G-100 chromatography in standard buffer containing 0.1M-NaCl. Conalbumin (M_r 86000), albumin (M_r 67000), ovalbumin (M_r 45000), peroxidase (M_r 40000), chymotrypsinogen (M_r 25000) and cytochrome *c* (M_r 12500) were used for calibration. The subunit M_r was determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by the procedure of Laemmli & Favre (1973).

Analytical methods

Protein was determined by the method of Bradford (1976), with bovine serum albumin as a standard (with correction for moisture content). NADH was determined with lactate dehydrogenase, and NADPH with glutamate dehydrogenase, by the method of Klingenberg (1974).

Chemicals

DEAE-Sephacel, Sephadex G-100 and Sephacryl S-200 were obtained from Pharmacia, Uppsala, Sweden. Streptomycin sulphate, Red Agarose,

bovine serum albumin and DL-glyceraldehyde were from Sigma Chemical Co., St. Louis, MO, U.S.A. Other biochemicals and enzymes were from Boehringer, Mannheim, Germany. The dye of the affinity gel, Cibacron Brilliant Yellow GE, was obtained from Ciba-Geigy, Basel, Switzerland.

Results

Enzyme purification

During initial attempts to purify NADH-linked and NADPH-linked xylose reductase from xylose-grown *Pichia stipitis* it was noticed that both activities behaved identically with respect to various treatments. Among others, heating of enzyme preparations at 50°C for 2 min resulted in a 90% loss of both activities. Both activities also declined to the same extent upon storage at 4°C, and stabilization was obtained to the same degree by including 1 mM-2-mercaptoethanol in the purification buffer. In addition, both activities bound irreversibly to Red Agarose and could not be eluted with xylose, NAD^+ or $NADP^+$ in the presence or absence of 10% poly(ethylene glycol), or with 2 M-NaCl. The results of the purification procedure, summarized in Table 1, further confirm that NADH-linked and NADPH-linked xylose reductase activities are due to the same enzyme. The ratio of NADH- to NADPH-linked activity remained almost constant during purification. The slightly higher ratio in the initial stages of purification must probably be ascribed to the presence of another enzyme, performing a low, non-specific, reduction of xylose with NADH. After high-performance gel chromatography the enzyme was electrophoretically pure (Fig. 1 inset). In addition, spectral analysis of the fractions eluted between 40 and 42 min (Fig. 1), which showed a constant ratio of NADH- to NADPH-linked activity, revealed the presence of only one protein. A plot of the normalized spectra (245–300 nm) of these fractions did not reveal spectral differences (results not shown). Furthermore the ratio of the absorbancies at 205 and 280 nm was nearly constant over the peak, yielding values between 29.2 and 29.9. From these data and an M_r value of 65000 a molar absorption coefficient ε_{280} of $7.0 \times 10^5 M^{-1} \cdot cm^{-1}$ of the pure xylose reductase was calculated by using the method of Scopes (1974). The turnover number of the NADPH-linked activity at the standard assay conditions was $1400 min^{-1}$. The M_r of the enzyme as determined by Sephadex-gel filtration was 65000 ± 4000. A value of 63000 ± 3000 was obtained by high-performance gel filtration. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis yielded a value of 34000 ± 2000. These results

Table 1. *Purification of xylose reductase from Pichia stipitis*
For experimental details see the text.

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)		NADH/-NADPH-linked activity ratio	Specific activity (units/mg of protein)		Yield* (%)	Purification factor*
			NADH-linked	NADPH-linked		NADH-linked	NADPH-linked		
Cell-free extract	7.0	87	25.5	33	0.77	0.29	0.38	100	1
Streptomycin sulphate treatment	6.5	59	27.5	37	0.74	0.47	0.63	110	1.6
Ion-exchange chromatography (after concentration)	6.2	11	23	29.5	0.78	2.1	2.7	89	7.1
Affinity chromatography (after concentration)	4.6	2.2	20	26	0.77	9.1	11.8	78	31
Freezing and thawing	4.4	1.2	17	23.5	0.72	14.1	19.6	69	50
High-performance gel chromatography†	–	–	–	–	0.72	16.7	23.2	–	59

* Average for NADH and NADPH.
† Performed on a small scale only (see the text).

indicate that the enzyme consists of two, probably identical, subunits.

Effect of pH and temperature on enzyme activity

Xylose reductase exhibited a nearly symmetrical pH–activity curve (Fig. 2). The optimum pH under standard assay conditions was 6.0. The ratio of activities with NADH and NADPH was approximately constant between pH 5 and 8. An Arrhenius plot of NADPH-linked xylose reductase activity yielded a straight line between 20 and 38°C (results not shown). The calculated activation energy E_A of the reaction was 35.1 kJ/mol (8.4×10^3 kcal/mol).

Kinetics of xylose reduction

The enzyme exhibited Michaelis–Menten kinetics with respect to its substrates: substrate inhibition (tested up to 0.5M-xylose and 0.5mM-NADH or -NADPH) was not observed. The ratio of activities with NADH and NADPH was independent of the xylose concentration but varied with the concentration of the coenzymes. Product inhibition by xylitol (tested up to 0.5M) was not observed. A summary of the kinetic parameters of the enzyme is listed in Table 2. The $K_m^{app.}$ for xylose was 42mM with either NADH or NADPH as coenzyme. The enzyme had a lower affinity for

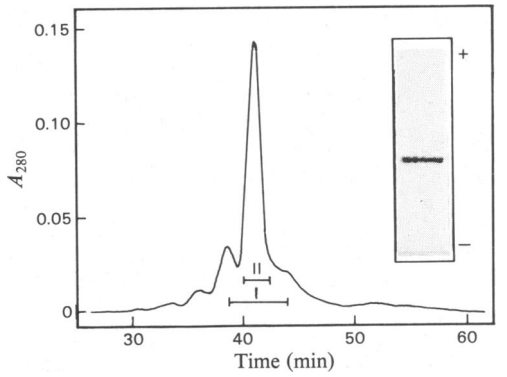


Fig. 1. *Protein elution profile during high-performance gel chromatography of a 50-fold-purified xylose reductase preparation*

Activity of xylose reductase (bar I) coincided with the major protein peak. Bar II indicates the fractions used for polyacrylamide-gel electrophoresis (inset). Migration was from bottom (negative pole) to top (positive pole).

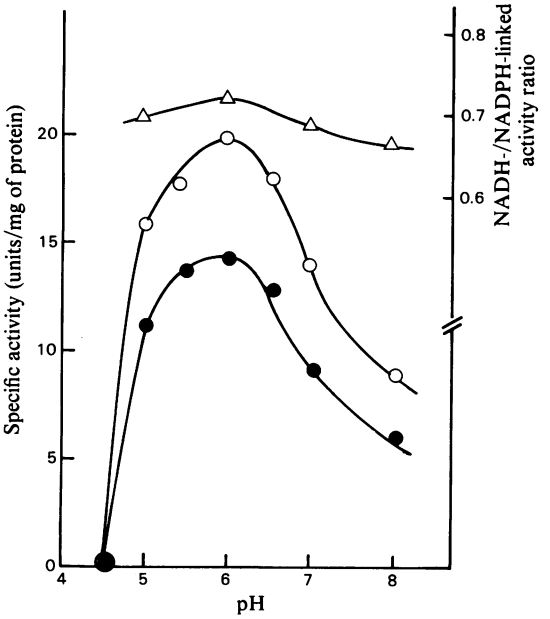


Fig. 2. *Effect of pH on xylose reductase activity*

Both NADPH-linked (○) and NADH-linked (●) xylose reduction showed an optimum at pH 6.0 under standard assay conditions. The ratio of NADH-linked to NADPH-linked activity (△) is also shown.

Table 2. *Kinetic parameters of xylose reduction by purified xylose reductase*

Reaction	Varied-concentration substrate	$K_m^{app.}$ (mM)	Inhibitor	$K_i^{app.}$ (mM)
Xylose + NADH	Xylose	42	–	–
	NADH	0.021	–	–
	Xylose	–	NAD^+	0.65
	NADH	–	NAD^+	0.13
	NADH	–	$NADP^+$	0.006
Xylose + NADPH	Xylose	42	–	–
	NADPH	0.009	–	–
	Xylose	–	$NADP^+$	0.17
	NADPH	–	$NADP^+$	0.030
	NADPH	–	NAD^+	>1.4

NADH than for NADPH (values of 0.021 and 0.009 mM respectively). NAD^+ was a non-competitive inhibitor with respect to xylose (Fig. 3*a*) in the NADH-linked xylose reduction and a competitive inhibitor with respect to NADH. Similar results were obtained with $NADP^+$: competition with NADPH (Fig. 3*b*) and non-competitive inhibition with xylose in the NADPH-linked xylose reduction. $NADP^+$ also competed with NADH (Fig. 3*c*), whereas NAD^+ did not significantly inhibit the NADPH-linked reaction (Table 2). This latter phenomenon probably contributes to the observation that under non-equilibrium conditions NADPH can successfully compete with NADH when both coenzymes are present in equimolar amounts (Table 3). The $NADP^+$ generated during NADPH-linked xylose reduction inhibited the NADH-linked activity much more strongly than the NADPH-linked activity (Table 2). This resulted in a preferential utilization of NADPH over NADH for xylose reduction upon incubation of enzyme with both NADH and NADPH (Table 3).

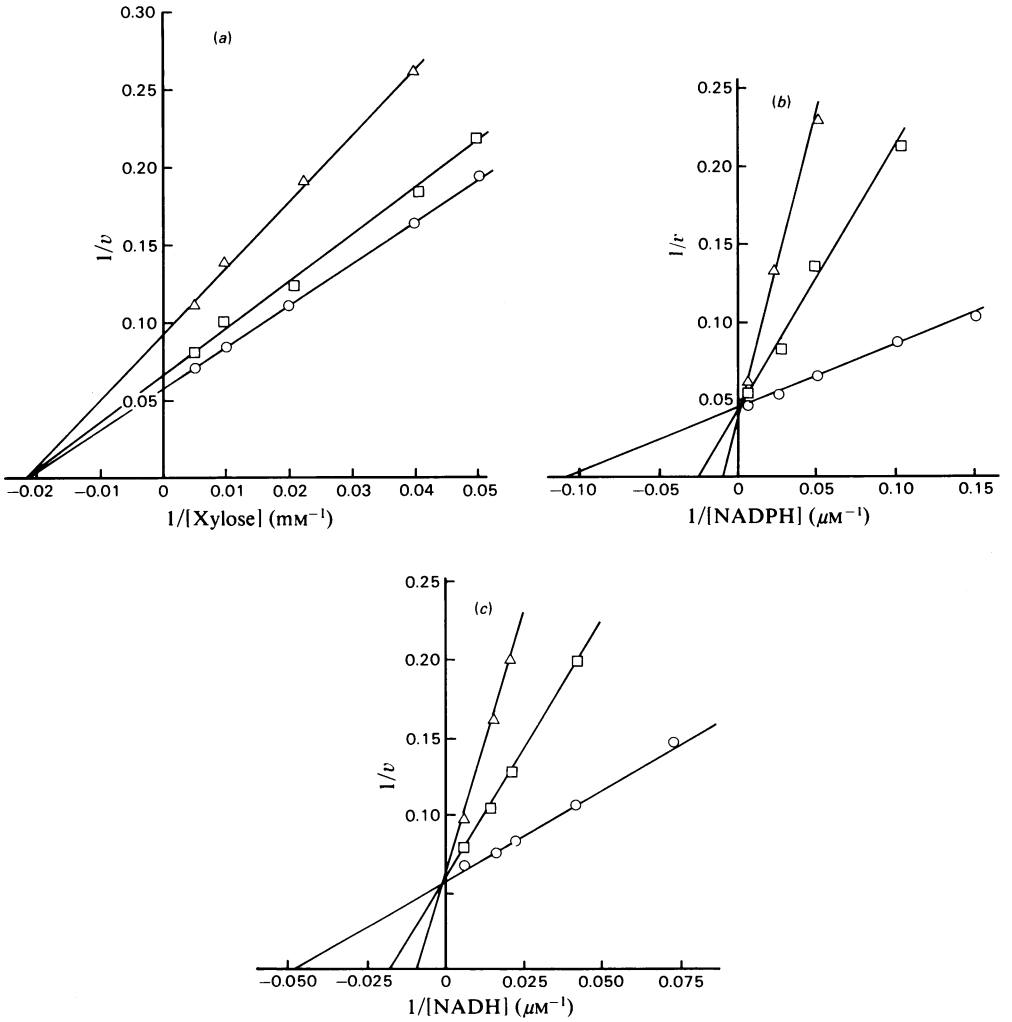


Fig. 3. *Inhibition by NAD^+ and $NADP^+$ of xylose reduction by xylose reductase*
(*a*) Non-competitive inhibition by NAD^+ in the NADH-linked activity with respect to xylose: ○, no NAD^+; □, +0.1 mM-NAD^+; △, +0.4 mM-NAD^+. (*b*) Competitive inhibition by $NADP^+$ with respect to NADPH: ○, no $NADP^+$; □, +100 μM-$NADP^+$; △, +300 μM-$NADP^+$. (*c*) Competitive inhibition by $NADP^+$ with respect to NADH: ○, no $NADP^+$; □, +10 μM-$NADP^+$; △, +25 μM-$NADP^+$. The velocity (*v*) is expressed as units/mg of protein.

Table 3. *Preferential utilization of NADPH by purified xylose reductase in a reaction mixture containing NADPH and NADH*

The standard assay mixture (1 ml final volume) was used. At 40 min after the reaction was started the control reactions with the separate nucleotides had finished and the reaction in the mixture containing both NADPH and NADH proceeded at a rate of less than 10% of the initial velocity. NADPH and NADH were then determined enzymically.

Initial amount of NAD(P)H (nmol)		Initial reaction rate [nmol of NAD(P)H/min]	Amount of NAD(P)H consumed (nmol)	Residual amount of NAD(P)H (nmol)	
NADH	NADPH			NADH	NADPH
150	0	9.3	144	8	–
0	150	12.9	140	–	6
150	150	12.0	179	104	8

Table 4. *Kinetic parameters for reduction of various aldoses by xylose reductase with either NADH or NADPH as coenzyme at a fixed concentration (0.15 mM)*

Substrate	$K_m^{app.}$ (mM)	$V_{max.}$ (units/mg of protein)		$\frac{V_{max.}}{K_m^{app.}}$		$\frac{V_{max.}\ (NADH)}{V_{max.}\ (NADPH)}$
		NADH	NADPH	NADH	NADPH	
DL-Glyceraldehyde	18	40.0	51.8	2.22	2.88	0.77
L-Arabinose	40	26.2	34.5	0.66	0.88	0.76
D-Xylose	42	16.7	23.2	0.41	0.55	0.72
D-Ribose	310	15.6	23.3	0.050	0.075	0.67
D-Galactose	140	15.4	23.3	0.11	0.17	0.66
D-Glucose	420	11.8	17.5	0.028	0.042	0.67

Substrate specificity

Various substrates were tested at a concentration of 0.2 M. Those that gave a reaction velocity of 10% or more of that for xylose were studied in more detail. The K_m value and specificity (expressed as $V_{max.}/K_m$) for these substrates as estimated from Lineweaver–Burk plots are presented in Table 4. The affinity and specificity decreased with increasing chain length.

Of the poorer substrates, D-lyxose exhibited 5% of the activity with D-xylose (with either NADH or NADPH as coenzyme); with L-xylose and D-arabinose the activity was 1%. Of the ketoses tested, only dihydroxyacetone showed some activity (5% of that with xylose). Activities with D-xylulose, D-fructose and D-sorbose were negligible (less than 1%). Uronic acids, which are good substrates for some mammalian aldose reductases (Hayman & Kinoshita, 1965), were inactive. Thus it may be concluded that, similarly to the enzymes from *Pichia quercuum* (Suzuki & Onishi, 1975) and *Candida utilis* (Scher & Horecker, 1966; Horitsu *et al.*, 1968), the enzyme has a preference for aldoses with the hydroxy group attached to C-2 in the D-*glycero* configuration.

The reverse reaction, dehydrogenation of polyols, was studied with xylitol, arabinitol and glycerol as substrate. At pH 6.0 polyol oxidation was not observed, but between pH 8 and 9 the enzyme oxidized these polyols both with NAD^+ and with $NADP^+$ as coenzyme. However, with xylitol the rates were only 4% and 5% of the forward reactions (specific activities of 0.83 and 0.98 unit/mg of protein respectively). Low reaction rates were also observed with arabinitol and glycerol.

Effect of various inhibitors

A requirement for bivalent cations was not observed; nor was the enzyme activity affected by chelating agents such as EDTA (tested at 5 mM). The enzyme was strongly susceptible to thiol-blocking reagents: incubation with 1 μM-$HgCl_2$ or -*p*-chloromercuribenzoate for 2 min resulted in a complete loss of activity. Sulphate (tested up to 500 mM), which inhibits xylose reductase from *Rhodotorula* sp. (Sheys *et al.*, 1971) and stimulates calf lens aldose reductase (Hayman & Kinoshita, 1965), had no effect on enzyme activity. 2-Mercaptoethanol (5 mM) also had no effect, but 1 mM-dithiothreitol decreased the activity by 25%.

Discussion

The xylose reductase of *Pichia stipitis* is active with various aldose substrates and hence is an aldose reductase (EC 1.1.1.21).

A comparison of the properties of xylose reductases of various yeasts (Table 5) reveals a strong similarity with respect to M_r, pH optimum, substrate specificity and a low rate of the reverse reaction. However, unlike the enzymes from *Candida utilis, Pichia quercuum* and *Rhodotorula* sp. as well as xylose reductases from other sources that are specific for NADPH (Jeffries, 1983), the enzyme from *Pichia stipitis* reduced aldoses with both NADPH and NADH. Dual coenzyme specificity seems to be uncommon among nicotinamide nucleotide-dependent enzymes. Only 14% of these (40 in total; Dixon & Webb, 1979) show activities exceeding 10% of the rate of that observed with the other coenzyme. The only yeast enzyme reported to exhibit this behaviour is homoserine dehydrogenase (EC 1.1.1.3) (Black & Wright, 1955). The ratio of activities of our aldose reductase with NADH and NADPH was similar for a variety of substrates (Table 4), and unlike the situation with homoserine dehydrogenase this ratio was not affected by pH (Fig. 2).

The dual coenzyme specificity of the xylose reductase from *Pichia stipitis* has important physiological consequences. NADH-linked xylose reduction is a prerequisite for anaerobic alcoholic fermentation of this wood sugar by yeasts (Bruinenberg *et al.*, 1983*a*, 1984). For such a process the use of yeasts such as *P. stipitis* is presently considered (Alfa-Laval, 1983). Other facultatively fermentative xylose-assimilating yeasts such as *Pachysolen tannophilus* or *Candida utilis* show negligible or no fermentation of xylose at all under anaerobic conditions, in line with the low or zero NADH-linked xylose reductase activity in these yeasts (Bruinenberg *et al.*, 1984).

Although *in vivo* under anaerobic conditions xylose fermentation by *Pichia stipitis* must proceed via NADH-linked xylose reduction, kinetic studies indicate that *in vitro* NADPH is the preferred coenzyme. The K_m value of the xylose reductase for NADPH is twice that for NADH, and also the $V_{max.}$ is slightly higher (Tables 2 and 4). Other important parameters for NADH- or NADPH-linked xylose reduction by the enzyme are, however, the concentrations of the oxidized coenzymes that strongly inhibit enzyme activity (Tables 2 and 3 and Fig. 3). Hence it is evident that *in vivo*, not only the relative concentrations of NADPH and NADH, but also those of $NADP^+$ and NAD^+, are decisive for the choice between NADPH- or NADH-linked xylose reduction.

The dual coenzyme specificity of the aldose reductase of *Pichia stipitis* also has an important bearing on our attempts to improve the kinetics and efficiency of xylose fermentation by this yeast. Since NADPH-linked xylose reduction by *Pichia stipitis* under anaerobic conditions, as in other yeasts, results in the formation and excretion of xylitol (M. R. Wijsman, unpublished work), it was expected that the ethanol yield from xylose may be improved by elimination of the NADPH-linked activity via genetic manipulation. However, both NADPH- and NADH-linked activities are due to a single enzyme. Hence alteration of the relative flows of NADH- and NADPH-linked xylose reduction *in vivo* probably must await further developments in the field of 'enzyme engineering'. An alternative approach could be the expression in yeasts of the bacterial gene coding for xylose isomerase, thereby circumventing the necessity of

Table 5. *Properties of xylose reductases from various yeasts*

A comparison of the properties of xylose reductases from the yeasts *Pichia stipitis* (present study), *Pichia quercuum* (Suzuki & Onishi, 1975), *Candida utilis* (Scher & Horecker, 1966) and *Rhodotorula* sp. (Sheys *et al.*, 1971). All strains were grown on xylose, except for *Rhodotorula*, which was grown on glucose. Abbreviations: N.A., not applicable; N.D., not determined.

	P. stipitis	*P. quercuum*	*C. utilis*	*Rhodotorula* sp.
M_r (gel filtration)	65000	61000	70000	62000
pH optimum	6.0	6.2	5.5–7.6	N.D.
Activity with NADH (relative to NADPH)	70%	0	0	0
$K_m^{app.}$ (xylose) (mM)	42	78	28	N.D.
$K_m^{app.}$ (NADPH) (μM)	9	8	16	25*
$K_m^{app.}$ (NADH) (μM)	21	N.A.	N.A.	N.A.
Activity with DL-glyceraldehyde	+	+	+	+
Inhibition by thiol-blocking reagents	+	+	N.D.	+*
Reverse reaction	+	+	+	N.D.
(% of forward reaction)	(4–5%)	(5–8%)	(1%)	

* Glyceraldehyde as substrate.

nicotinamide nucleotide-dependent conversion of xylose into xylulose. However, attempts in this direction so far have not been successful (Ho *et al.*, 1983).

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