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Pyranose 2-dehydrogenase, a novel sugar oxidoreductase from the basidiomycete fungus *Agaricus bisporus*

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Abstract A novel C-2-specific sugar oxidoreductase, tentatively designated as pyranose 2-dehydrogenase, was purified 68-fold to apparent homogeneity (16.4 U/mg protein) from the mycelia of *Agaricus bisporus*, which expressed maximum activity of the enzyme during idiophasic growth in liquid media. Using 1,4-benzoquinone as an electron acceptor, pyranose 2-dehydrogenase oxidized D-glucose to D-*arabino*-2-hexosulose (2-dehydroglucose, 2-ketoglucose), which was identified spectroscopically through its *N*,*N*-diphenylhydrazone. The enzyme is highly nonspecific. D-,L-Arabinose, D-ribose, D-xylose, D-galactose, and several oligosaccharides and glycopyranosides were all converted to the corresponding 2-aldoketoses (aldosuloses) as indicated by TLC. D-Glucono-1,5-lactone, D-*arabino*-2-hexosulose, and L-sorbose were also oxidized at significant rates. UV/VIS spectrum of the native enzyme (λ_{max} 274, 362, and 465 nm) was consistent with a flavin prosthetic group. In contrast to oligomeric intracellular pyranose 2-oxidase (EC 1.1.3.10), pyranose 2-dehydrogenase is a monomeric glycoprotein (pI 4.2) incapable of reducing O_2 to H_2O_2 ($> 5 \times 10^4$-fold lower rate using a standard pyranose oxidase assay); pyranose 2-dehydrogenase is actively secreted into the extracellular fluid (up to 0.5 U/ml culture filtrate). The dehydrogenase has a native molecular mass of ~79 kDa as determined by gel filtration; its subunit molecular mass is ~75 kDa as estimated by SDS-PAGE. Two pH optima of the enzyme were found, one alkaline at pH 9 (phosphate buffer) and the other acidic at pH 4 (acetate buffer). Ag^+, Hg^{2+}, Cu^{2+}, and CN^- (10 mM) were inhibitory, while 50 mM acetate had an activating effect.

Key words Pyranose 2-dehydrogenase · Pyranose 2-oxidase · D-*arabino*-2-Hexosulose · 2-Dehydroglucose · 2-Ketoglucose · Aldoketoses · *Agaricus bisporus* · Lignocellulose · Biodegradation

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Introduction

Oxidoreductases of free (nonphosphorylated) sugars participate in the carbohydrate metabolism of a wide range of higher fungi. While glucose 1-oxidase (EC 1.1.3.4) is typical for numerous Ascomycetes and fungi imperfecti, its C-2-specific counterpart, widespread among basidiomycete fungi, is the flavoprotein pyranose 2-oxidase (EC 1.1.3.10). The latter enzyme catalyzes oxidation of D-glucose, D-xylose, and usually D-galactose to the corresponding 2-aldoketoses; furthermore, it has been proposed that it plays a role in the ligninolytic system(s) of some white-rot fungi by providing H_2O_2, the cosubstrate of ligninolytic peroxidases (Daniel et al. 1994). Another enzyme, the heme/FAD cellobiose dehydrogenase (previously known as cellobiose oxidase, EC 1.1.99.18) has been proposed to be involved in cellulose breakdown through Fenton's reaction mechanisms (Kremer and Wood 1992).

Laccase (EC 1.10.3.2), a copper-containing *p*-diphenol (polyphenol) oxidase catalyzing reduction of O_2 to water during the one-electron oxidation of a phenolic substrate, is also considered to be a fundamental component of oxidative ligninolyic system(s) of white-rot wood-degrading fungi that secrete this enzyme (Thurston 1994). One of the well-studied laccases is the enzyme of the litter-decomposing basidiomycete *Agaricus bisporus*, which is secreted and accumulated at high levels during vegetative lignolytic growth of the fungus. On the other hand, to our knowledge, there is no information available on the syn-

thesis and molecular-catalytic properties of the above sugar oxidoreductases from the fungus. Such knowledge would be of value considering that these enyzmes have been postulated to function in concert with laccase to maintain a sugar:quinone oxidoreductase cycle in order to prevent repolymerization of phenoxyradical and quinone degradation products formed during ligninolysis (Green 1977; Szklarz and Leonowicz 1986; Samejima and Eriksson 1992).

Here we present the production, purification, and preliminary characterization of a novel quinone-dependent sugar oxidoreductase, pyranose 2-dehydrogenase, revealed during the search for oxidative carbohydrate-metabolizing enzymes in mycelial extracts of *Agaricus bisporus* grown in glucose liquid media.

Materials and methods

Culture conditions

Agaricus bisporus (Lange) Imbach, hybrid strain U3, obtained from the culture collection at Horticulture Research International (Wellesbourne, UK), was grown at 27° C in stationary cultures on liquid complex medium [pH 6.8; 2% glucose, 1.5% corn steep, 0.3% $MgSO_4 \cdot 7H_2O$ in tap water (50 ml per 500-ml flask)] or in agitated cultures on synthetic medium [Fremor and Wood 1981; basal salts plus 2% glucose, 0.3% glutamic acid (80 ml per flask)]. Mildly homogenized cultures (10 days old) derived from malt-agar stock cultures showing sufficient growth were used for inoculations (8%).

Purification of pyranose 2-dehydrogenase

Enzymes were extracted from washed 17-day-old mycelia (40 g wet wt., static cultures) in 140 ml of 20 mM Tris-HCl (pH 7) plus 2 mM Pefabloc SC (Boehringer, Mannheim, Germany) using an Ultra-Turrax homogenizer T25 (IKA-Labortechnik, Staufen, Germany). The supernatant obtained after centrifugation (25,000 × *g*, 30 min) was directly loaded onto the column (20 ml) of DEAE-Sepharose CL-6B (Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer. After washing the column with 50 mM NaCl in the buffer, pyranose 2-dehydrogenase activity was recovered in the protein fraction eluted with 0.2 M NaCl (80 ml), while most of the dark pigment remained bound to the gel matrix. In the second step, the enzyme solution was supplemented with solid $(NH_4)_2SO_4$ to 1.5 M and applied via a 50-ml loop onto a phenyl Superose HR 10/10 column (Pharmacia) equilibrated with 1.5 M $(NH_4)_2SO_4$ in 20 mM Na phosphate (pH 6.5). The bound protein was fractionated using a linear gradient from 0 to 70% (150 ml, 1 ml/min) of 20 mM Na phosphate (pH 6.5) in the starting buffer. The pyranose 2-dehydrogenase peak, which was substantially free of laccase activity, was collected, concentrated to 3.6 ml (YM 10 membrane; Amicon, Danvers, Mass., USA), and transferred to 20 mM Bistris-HCl (pH 6), a starting buffer for the next step on Mono Q HR 5/5 (Pharmacia). The whole sample volume was loaded onto the column, and a linear gradient of NaCl (0–0.35 M in 24 ml, 1 ml/min) was applied. The dehydrogenase recovered from the active fractions was then rechromatographed on Mono Q at pH 5.5 (Fig. 2) using 0–0.35 M NaCl (36 ml). Final purification [gel filtration on Superose 12 HR 10/30 (Pharmacia)] was performed in 50 mM Na phosphate (pH 7) plus 0.1 M NaCl (sample volume 0.1 ml, 7.4 U per run, flow rate 0.2 ml/min).

Enzyme assays

Pyranose 2-dehydrogenase activity was assayed by monitoring between 30 and 90 s the increase in absorbance at 290 nm corresponding to reduction of 1,4-benzoquinone to hydroquinone (ε_{290} = 2.3 mM^{-1} cm^{-1}; Ciucu and Patroescu 1984) by D-glucose. The standard assay mixture (2 ml) consisted of 100 µmol Na phosphate (pH 6.5), 50 µmol D-glucose, 4.6 µmol 1,4-benzoquinone, and dehydrogenase to be assayed. One unit of the enzyme activity (U) was defined as the amount of activity effecting production of 1 µmol hydroquinone per min at 25° C in the above system. Substrate specificity assays were performed in the standard mixture containing 4.6 µg pyranose 2-dehydrogenase and 25 mM sugar. In tests for effectors, pyranose 2-dehydrogenase (26 µg) was preincubated for 30 min at 22° C with 1 or 10 mM reagent in 20 µl 20 mM Na malonate (pH 6.5) and was subsequently assayed for remaining activity in Na malonate buffer that replaced phosphate in the standard assay. The laccase assay mixture (2 ml) contained 100 µmol Na lactate (pH 5.6), 24 nmol syringaldazine [*N*,*N*′-bis(3,5-dimethoxy-4-hydroxybenzylidene hydrazine)], and enzyme sample; the increase in absorbance was followed at 525 nm (ε_{525} = 65 mM^{-1} cm^{-1}; Harkin and Obst 1973).

Molecular mass and isoelectric point determination

The native molecular mass of pyranose 2-dehydrogenase was estimated by gel filtration on a Superose 12 HR 10/30 column run as above in the purification protocol except that 0.2 mg (3.3 U) of the enzyme protein was chromatographed. The standards used were: ferritin (440 kDa), catalase (232 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). Molecular mass under denaturing conditions was determined by SDS-PAGE according to Laemmli (1970) as described in the legend to Fig. 3. Glycoproteins in the gels were detected by the dansyl hydrazine method of Eckhard et al. (1976). Analytical isoelectric focusing (IEF) of the purified enzyme (2 µg) was performed in 1.5% agarose gels (0.5 mm) containing 7.8% Servalyt (pH 3–6; Serva Feinbiochemica, Heidelberg, Germany) using the Pharmacia Multiphor II apparatus and the Low pI Calibration Kit.

Identification of the reaction products

The reaction mixtures (1 ml) used for TLC analysis of pyranose 2-dehydrogenase reaction products contained 50 µmol sugar substrate, 55 µmol 1,4-benzoquinone (Sigma, St. Louis, Mo., USA), 10 µmol Na phosphate (pH 6.5), and 3.5 U of the purified enzyme. Samples obtained after incubation for 1–5 h at 25° C were analyzed on SigmaCell Type 100 cellulose-coated foils (Sigma) using diphenylamine-aniline-phosphoric acid detection reagent as described previously (Volc et al. 1995).

D-Glucose oxidation product(s) used after derivatization for spectroscopic identification were prepared in the above reaction mixture (14 ml) containing 0.7 mmol D-glucose incubated under N_2 atmosphere at 30° C for 1 h, after which time the maximum accumulation of a dicarbonyl product cochromatographing with standard D-*arabino*-2-hexosulose (TLC monitoring) was observed. (Hydro)quinone components of the vacuum concentrate were removed by extraction in ethylacetate, and residual product in 15 ml H_2O reacted with 1.5 mmol *N*,*N*-diphenylhydrazine (Koch-Light, Haverhill, UK) as in Volc et al. (1995). After being transferred to 1 ml $CHCl_3$, the hydrazone products were subjected to preparative TLC on 20 Silufol silica gel foils (Kavalier, Votice, Czech Republic) with 32:1 $CHCl_3$-ethanol. Yellow-to-orange streaks of hydrazone derivatives were cut out, combined separately, and extracted in ethanol. The main component (19 mg) was further purified by rechromatography, then peracetylated as in Volc et al. (1980), crystallized from aqueous ethanol (m.p. 128–129° C), and subsequently analyzed by ^1H and ^{13}C NMR. The spectra (399.95 and 100.577 MHz, respectively) were recorded on a Varian VXR-400 spectrometer in $CDCl_3$ at 25° C:

1H NMR: δ 1.929 (3 H, s, Ac), 2.030 (3 H, s, Ac), 2.202 (3 H, s, Ac), 2.207 (3 H, s, Ac), 4.203 (1 H, dd, *J* = 12.6, 4.1 Hz, H-6u), 4.427 (1 H, dd, *J* = 12.6, 2.3 Hz, H-6d), 5.404 (1 H, ddd, *J* = 9.5,

4.1, 2.3 Hz, H-5), 6.018 (1 H, dd, *J* = 9.5, 1.7 Hz, H-4), 6.330 (1 H, d, *J* = 1.7 Hz, H-3), 6.573 (1 H, s, H-1), 7.223–7.624 (10 H, m, 2 × Ph).^{13}C NMR: δ 20.52 q (2 C), 20.58 q, 20.85 q, 61.58 t (C-6), 68.61 d (C-5), 69.58 d (C-4), 72.36 d (C-3), 130.57 d (C-1), [Ph carbon signals are seriously broadened], 169.44 s, 169.52 s, 170.19 s, 170.59 s, 190.18 s (C-2).

Results and discussion

Production and purification of pyranose 2-dehydrogenase

Agaricus bisporus has been the subject of extensive biochemical studies directed to the properties and metabolic role of the enzyme laccase expressed in liquid cultures in the laboratory and in solid-state commercial cultures. Correlation of intracellular laccase activity with that of pyranose 2-dehydrogenase during the course of stationary cultivation on glucose complex medium is shown in Fig. 1. Quinone production (i.e., via laccase) and utilization (via pyranose 2-dehydrogenase) may form a link between the two enzymes especially during idiophasic growth, when pyranose 2-dehydrogense reaches its maximum (40 U/g mycelium dry wt.). The enzyme was also actively secreted and accumulated in the culture medium (0.5 U/ml culture filtrate in 17-day-old culture on the synthetic medium). Using the purification scheme shown in Table 1, pyranose 2-dehydrogenase was purified 68-fold from 40 g (wet wt.) of 17-day-old mycelia to apparent homogeneity as demonstrated by SDS-PAGE (Fig. 2) and analytical isoelectric focusing (not shown), with an overall yield of ~32%.

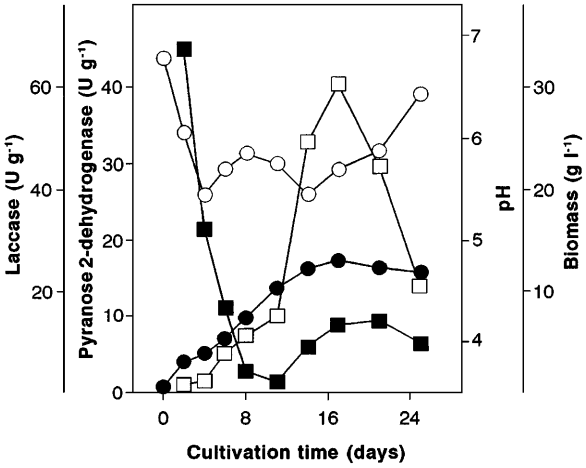

Fig. 1 Time course of pyranose 2-dehydrogenase and laccase activities in crude mycelia extracts of *Agaricus bisporus* grown in stationary cultures on glucose-corn steep medium. □ Dehydrogenase activity (U/g mycelium dry wt.) was assayed by measuring (A_{290} corresponding to reduction of 1,4-benzoquinone to hydroquinone; ■ laccase activity was monitored by measuring ΔA_{525} for oxidation of syringaldazine. ● Growth, ○ pH

Table 1 Purification of pyranose 2-dehydrogenase from *Agaricus bisporus*. Total protein and total activity were measured after desalting/buffer exchange (PD-10 column, Pharmacia) for 20 mM Na phosphate (pH 6.5). Protein was determined by the Folin reagent (Hartree 1972) using bovine serum albumin as a standard; for assay of the enzyme activity, see Materials and methods

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Recovery (%)	Purification (-fold)
Crude extract	324.1	77	0.24	100	1
DEAE-Sepharose	63.7	73.3	1.2	95	5
Phenyl Superose	6.3	68.6	10.9	89	45.4
Mono Q (pH 6)	3.5	55.2	15.8	72	65.8
Mono Q (pH 5.5)	2.3	37.4	16.3	49	67.9
Superose 12	1.8	29.9	16.4	39	68.3

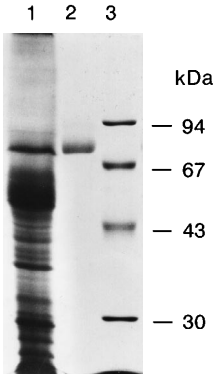

Fig. 2 SDS-PAGE analysis of pyranose 2-dehydrogenase from *Agaricus bisporus*. Electrophoresis was performed with 3% stacking and 10% running standard gel. *1* Sample of the desalted mycelial crude extract, *2* sample (7 µg) of the enzyme from the final purification step, *3* molecular-mass marker proteins (Pharmacia LMW Calibration Kit). Gel was stained with Coomassie blue R 250

Some properties of pyranose 2-dehydrogenase

Purified pyranose 2-dehydrogenase solutions had a yellow color, exhibited absorption spectra with λ_{max} 274, 362, and 465 nm (A_{274}/A_{465} = 12.4), and showed no heme absorption. Addition of glucose substrate resulted in elimination of the maximum at 465 nm (Fig. 3). These spectral properties are characteristic of flavoproteins. The carbohydrate moiety of the enzyme was demonstrated in SDS polyacrylamide gels by positive dansyl hydrazine detection giving a strong UV-fluorescent band that was not observed with the control enzyme sample untreated with HIO_4 (Fig. 4). It is, however, unclear whether high microheterogeneity of pyranose 2-dehydrogenase apparent from Mono Q (Fig. 5) and phenyl Superose elution profiles (very broad peaks) is due to a high degree of glycosylation or another post-translational modification.

The molecular mass of the native enzyme was approximately 79 kDa as estimated by gel filtration on a Superose 12 HR 10/30 column. This value is considerably lower than the molecular mass (approximately 300 kDa) of pyranose 2-oxidases isolated from different sources that are also shown to consist of four subunits of identical size (Volc et al. 1991; Danneel et al. 1993; Shin et al. 1993). SDS-PAGE of the pyranose 2-dehydrogenase sam-

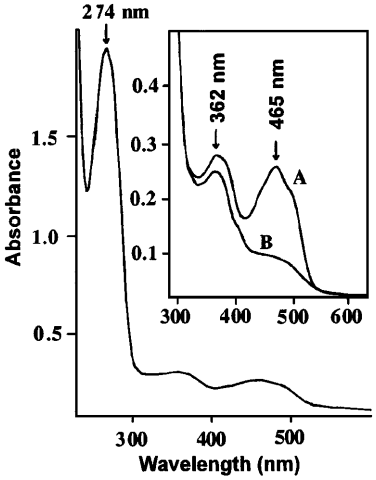

Fig. 3 Absorption spectrum of the purified pyranose 2-dehydrogenase from *Agaricus bisporus*. *Insert A* native enzyme, *B* the reduced enzyme after the addition of 50 mM glucose (Perkin-Elmer spectrophotometer Lambda 3, 60 nm min^{-1}). The protein was 1.8 mg ml^{-1} in 20 mM Na phosphate (pH 5)

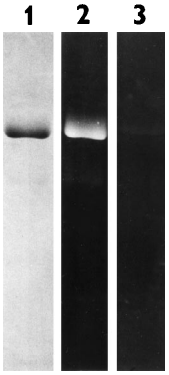

Fig. 4 Staining for carbohydrates bound to protein after SDS-PAGE of purified pyranose 2-dehydrogenase from *Agaricus bisporus*. The enzyme (2 µg) was stained *1* for protein with Coomassie blue and *2* for carbohydrate by the dansyl hydrazine method as described in the text. The control sample (*3*) was processed in the same way as the sample in *2* except that the treatment with periodic acid was omitted

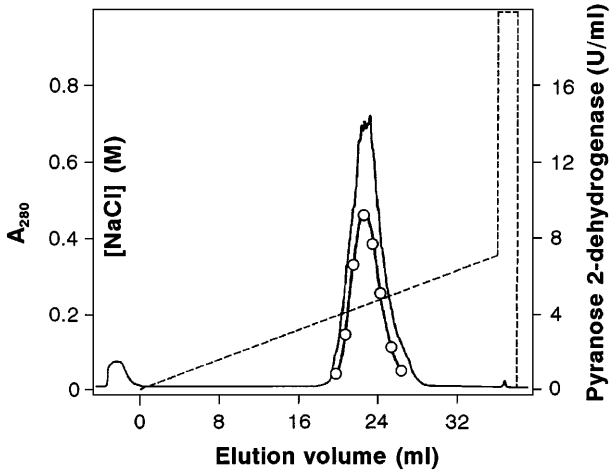

Fig. 5 Mono Q (pH 5.5) elution profile of protein (*solid line*) and activity of pyranose 2-dehydrogenase (○) prepurified from the extract of *Agaricus bisporus* by means of DEAE-Sepharose, phenyl-Superose, and Mono Q (pH 6) chromatography as described in Materials and methods. The enzyme was eluted as a broad peak at 0.2–0.27 M NaCl, showing apparent microheterogeneity (*broken line*, NaCl gradient)

ple resulted in one polypeptide band of ~75 kDa (Fig. 2). The purified enzyme exhibited a pI value of 4.2 as determined by analytical isoelectric focusing (not shown).

Substrate specificity

In addition to 1,4-benzoquinone, also 3,5-di-*tert*-butyl-1,2-benzoquinone, 2,6-dichlorophenol-indophenol, and ferricyanide served as pyranose 2-dehydrogenase electron acceptors, substituting for benzoquinone in the standard reaction mixture (Table 2). 1,4-Benzoquinone was preferred to 2,6-dichlorophenol-indophenol in standard assays due to the relative resistance of its reduced form to reoxidation by interfering laccase. The rate of glucose oxidation with O_2 as the electron acceptor, determined by assaying production of H_2O_2 (see legend to Table 2) was not measurable (120 µg enzyme protein, 1 h incubation, $\Delta A = 0$), i.e., it was at least four orders of magnitude lower [< 0.5 nmol min^{-1} (mg enzyme protein)$^{-1}$ based on the sensitivity of the method] as compared to the specific activities of pyranose 2-oxidases purified from different basidiomycete fungi (Machida and Nakanishi 1984; Volc et al. 1991; Danneel et al. 1993; Shin et al. 1993). The blue, oxidized chromogen from the above coupled peroxidase H_2O_2 assay (prepared using pyranose oxidase) did not serve as an electron acceptor for pyranose 2-dehydrogenase in the presence of 25 mM D-glucose. In the opposite case, i.e., if the blue peroxidase reaction product were reduced by the dehydrogenase, the reactivity of the latter enzyme with O_2 could have been masked. Negative TLC detection of any glucose oxidation product during aerobic incubations with the pure enzyme in the absence of benzoquinone indicated that the reduction of O_2 to other reduced oxygen species (e.g., superoxide) rather than H_2O_2 did not occur. Cytochrome *c*, NAD(P), and nitroblue tetrazolium were not utilized by *A. bisporus* pyranose 2-oxidase under the assay conditions used. 1,4-Benzoquinone was also shown to serve as an electron acceptor for pyranose 2-oxidase of *Pleurotus ostreatus*; however, it exerted a high affinity for O_2 (Shin et al. 1993).

Pyranose 2-dehydrogenase exhibited extremely broad substrate specificity with various sugars (Table 3). A number of monosaccharides, oligosaccharides, and glycosides served as substrates. None of the sugars oxidized was a prominently preferred substrate. The highest well-comparable activities were obtained with nearly all main sugar components (building units) of wood polysaccharides: D-glucose, D-galactose, D-xylose, L-arabinose, cellobiose, and, interestingly, with D-glucono-1,5-lactone, the product of reactions catalyzed by fungal cellobiose oxidase/dehydrogenase and β-glucosidase (Eriksson 1978).

Table 2 Relative efficiencies of some electron acceptors in the pyranose 2-dehydrogenase reaction. The initial reduction rate of each acceptor, substituting at the given concentration 1,4-benzoquinone in the standard assay mixture (see Materials and methods), was determined by following the absorbance changes at the wavelength indicated by the absorption coefficient (ε) of the corresponding reduced/oxidized acceptor form. The value obtained was referred to mg enzyme protein and expressed as a percentage of that of benzoquinone. The standard pyranose oxidase-peroxidase-chromogen assay (Volc et al. 1991) was used for measuring H_2O_2 production with O_2 as an only electron acceptor, D-glucose (25 mM) as a donor, and pyranose 2-dehydrogenase of *Agaricus bisporus* (120 μg) substituting for pyranose 2-oxidase

Electron acceptor	Absorption coefficient ($mM^{-1}cm^{-1}$)	Concentration (μM)	Initial rate (μmol min^{-1} mg^{-1})	Relative activity (%)
1,4-Benzoquinone	ε_{290} = 2.31	575	16.4	100
3,5-Di-*t*-butyl-1,2-benzoquinone	ε_{420} = 1.75	250	71.0	433
2,6-Dichlorophenol-indophenol	ε_{600} = 11.8	75	10.8	65.9
Ferricyanide	ε_{420} = 1.04	500	1.7	10.4
O_2	ε_{590} = 32.9[a]	250[b]	< 0.0005	0
Nitroblue tetrazolium	ε_{540} = 7.20	100	0	0
Cytochrome *c*	ε_{550} = 28.0	50	0	0
NAD(P)	ε_{340} = 6.22	100	0	0

[a] The absorption coefficient of oxidized chromogen (3-methyl-2-benzothiazolinone hydrazone + 3-dimethylaminobenzoic acid) used in the H_2O_2 assay

[b] The saturation concentration of O_2 at 25°C (Truesdale and Downing 1954)

Table 3 Substrate specificity of pyranose 2-dehydrogenase. Enzyme reactions were performed in the standard assay mixture (see Materials and methods) containing 4.6 μg of the enzyme and 50 μmol of the listed substrate

Substrate	Relative activity (%)	Substrate	Relative activity (%)
D-Glucose	100	Cellobiose	94
D-Xylose	116	Maltose	93
L-Arabinose	113	Maltotriose	70
D-Galactose	88	Sucrose	63
L-Glucose	61	Cellotriose	56
D-Ribose	34	Trehalose	54
D-Allose	30	Lactose	21
D-Arabinose	18	Methyl-α-D-glucopyranoside	117
L-Sorbose	10	Methyl-β-D-glucopyranoside	69
D-*arabino*-2-Hexosulose	59	Methyl-α-D-galactopyranoside	23
D-Glucono-1,5-lactone	118		

With reference to the activity with D-glucose, less than 10% relative activity was recorded with D-altrose, D-fructose, D-lyxose, D-mannose, D-arabinitol, D-glucitol, D-mannitol, or erythritol.

pH Dependence of the dehydrogenase activity and inhibitors

The enzyme was found to be most active at pH 4 (acetate buffer), 4.5 (lactate buffer), and 5 (malonate buffer) on the acidic side, and at pH 9 (phosphate buffer) on the alkaline side (100 mM buffer each; Fig. 6). Activity values in the pH range 3–9.5 were higher than 50% of the reaction rate at acidic optimum (Na acetate). Measurements at pH > 8 were, however, complicated by high blank readings caused by the instability of the benzoquinone and aldoketose reaction products.

Pyranose 2-dehydrogenase activity was not significantly influenced by chelating, reducing, and thiol reagents and by metal ions at 1 mM. Only Ag^+ (100% inhibition), $HgCl_2$ (100%), Cu^{2+} (90%), and CN^- (83%) were inhibitory at 10 mM concentration, while no significant

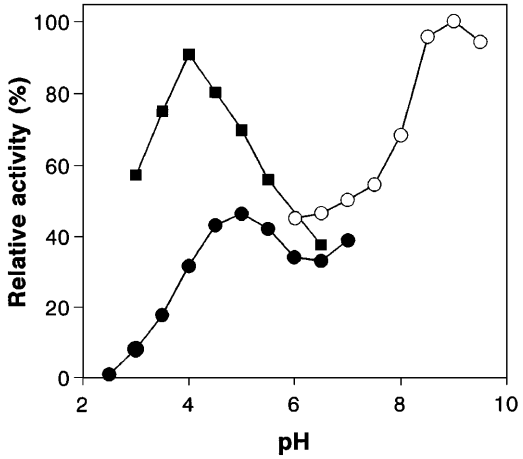

Fig. 6 Effect of pH on the activity of pyranose 2-dehydrogenase. Relative activity was assayed by measuring the reduction of 1,4-benzoquinone under standard reaction conditions (see Materials and methods) except that 100 mM Na phosphate buffer (○) was replaced by Na malonate (●) or Na acetate (■) buffer (100 mM) of varying pH at acidic side

effect was observed after 30-min preincubations with Ca^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, NH_4, Zn^{2+}, F^-, EDTA, NaN_3, dithiothreitol, phenanthroline, and H_2O_2 (10 mM each). 100 mM Na acetate (pH 4), substituting for phosphate in the standard assay, had an activating effect (~100% increase in activity), while activities in the presence of 100 mM malonate (Fig. 6) and 100 mM lactate buffers at their activity optima (pH 5 and 4.5, respectively) were comparable to that of the standard assay (pH 6.5). The purified enzyme (0.6 mg/ml) retained its full activity after storage in 100 mM Na phosphate (pH 6,7,8, and 9) at 4° C for 100 days. Its freezing in 50 mM Na phosphate (2.4 mg/ml) resulted in a 90% loss of activity.

Characterization of reaction products

The oxidation products of D-glucose, D-galactose and D-xylose exhibited the same R_f values (0.25, 0.27, and 0.31, respectively) on TLC as the corresponding authentic 2-aldoketoses (2-aldosuloses) prepared enzymatically using pyranose 2-oxidase purified from *Oudemansiella mucida* (Volc et al. 1995); they also yielded the blue or violet (D-*lyxo*-2-hexosulose, 2-dehydrogalactose) color with diphenylamine-aniline-phosphoric acid reagent that is characteristic of 1,2-dicarbonyl sugars. Similar color detections were also obtained with products of D- and L-arabinose, D-ribose, methyl-α- and β-D-glucopyranoside, and cellobiose, which are not pyranose 2-oxidase substrates, thereby indicating formation of aldoketose structures.

1H NMR spectral data of the peracetylated hydrazone derived from the D-glucose oxidation product (see Materials and methods) were in a good agreement with those previously published for 3,4,5,6-tetra-O-acetyl-1-(diphenylhydrazono)-D-*arabino*-2-hexosulose, a well-defined derivative used for identification of D-*arabino*-2-hexosulose (2-dehydroglucose, 2-ketoglucose) produced by *O. mucida* (Volc et al. 1980). ^{13}C NMR data (not measured previously) were also consistent with the above hydrazone structure. Consequently, the oxidoreductase purified from *A. bisporus* acts as glucose 2-dehydrogenase. Time course TLC analyses of D-glucose transformation showed that accumulation of D-*arabino*-2-hexosulose is transient, this pyranose 2-dehydrogenase product being further oxidized by the same enzyme. Accordingly, the pure enzyme was demonstrated to exhibit high activity with authentic D-*arabino*-2-hexosulose as substrate (59% relative to D-glucose). TLC analyses also revealed concomitant formation of another oxidation product of D-glucose of R_f 0.36 (greyish-yellow spot, not observed with D-galactose). Structural determination of the hydrazone corresponding to this product is under way.

The characteristics presented above suggest that pyranose 2-dehydrogenase from *A. bisporus* acts preferentially at the C-2 of a number of saccharides to produce the corresponding 2-aldoketoses and that it utilizes some quinones as electron acceptors. Another enzyme oxidizing cello-oligosaccharides and practically incapable of utilizing O_2 has been detected in the cellulolytic fungus *Sporotrichum thermophile* (Canevascini et al. 1991). Unlike pyranose 2-dehydrogenase, this enzyme (isofunctional with cellobiose dehydrogenase/oxidase of some white-rot fungi; Ander et al. 1994) has specificity for C-1 (formation of aldonic acids) and is not active against monosaccharides and most disaccharides. Interesting is a comparison with bacterial D-glucoside 3-dehydrogenase (EC 1.1.99.13). This flavo iron-sulfur enzyme acts at C-3 and converts D-glucose and a number of glucosides and oligosaccharides to the corresponding 3-aldoketoses [see Takeuchi et al. (1988)].

Because of the studied enzyme's very broad substrate specificity and the fact that the enzyme oxidizes glycopyranosides and D-glucono-1,4-lactone (assuming conformation similar to sugar pyranose ring structures) and not D-fructose (furanose ring) and acyclic sugar alcohols, we tentatively designated this enzyme by analogy to pyranose 2-oxidase as pyranose 2-dehydrogenase (pyranose : quinone acceptor 2-oxidoreductase):

$$\text{aldopyranose} + \text{quinone} \xrightarrow[\text{2-dehydrogenase}]{\text{pyranose}} \text{2 - aldoketose} + \text{hydroquinone}$$

Common structural features of the substrates and the identity of the physiological sugar-oxidizing agent (electron acceptor) are so far unknown. As an alternative to cellobiose dehydrogenase in some white-rot fungi (Ander 1994), pyranose 2-dehydrogenase may play an important role in fungal lignocellulose decomposition by interconnecting ligninolysis with degradation of cell-wall polysaccharide components. Similar to fungal 1,4-benzoquinone reductase (Brock et al. 1995), these quinone-reducing enzymes are likely to be involved in further breakdown of toxic monomeric quinone intermediates generated during extracellular peroxidative oxidation of lignin. The metabolic fate of a number of the dicarbonyl sugars produced during reactions catalyzed by pyranose 2-dehydrogenase presents an intriguing problem for carbohydrate biochemists.

In conclusion, we have purified and partially characterized a pyranose 2-dehydrogenase from *Agaricus bisporus*. Further studies are planned to elucidate in more detail the regulation, molecular properties, and kinetic mechanism of the enzyme.

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