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Conversion of lactose to β-D-galactopyranosyl-(1 → 4)-D-*arabino*-hexos-2-ulose-(2-dehydrolactose) and lactobiono-1,5-lactone by fungal pyranose dehydrogenase

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Abstract

The quinone-dependent pyranose dehydrogenase (PDH) purified from culture media of the basidiomycete fungus *Agaricus xanthoderma* catalyzed the simultaneous oxidation of lactose to β-D-galactopyranosyl-(1 → 4)-D-*arabino*-hexos-2-ulose (2-dehydrolactose) and lactobiono-1,5-lactone, with the latter spontaneously hydrolyzing to lactobionic acid. These products were identified by MS and in situ NMR spectroscopy. C-2 oxidation at the reducing moiety of lactose was confirmed by analysis of *N*,*N*-diphenylhydrazone derivatives of the reaction products. The proportions of C-1 to C-2 oxidation varied according to the source of PDH, ranging from almost exclusive formation of lactobionic acid to 2-dehydrolactose as a major product. The highest selectivity for C-2 was exhibited by PDH from *A. xanthoderma* and *A. meleagris*. Yields of 2-dehydrolactose in laboratory transformations monitored by HPLC approached 75%. This dicarbonyl derivative may serve as a key intermediate in a potential process for adding value to lactose by its (chemo)enzymatic isomerization to lactulose. A minor product in the PDH–lactose reaction mixtures formed on prolonged incubations with excess of oxidant was identified through its hydrazone derivative as β-D-galactopyranosyl-(1 → 4)-D-*threo*-hexos-2,3-diulose (2,3-didehydrolactose).

Keywords: 2-Dehydrolactose; 2-Ketolactose; Lactobionic acid; Lactose; Pyranose dehydrogenase

1. Introduction

Pyranose dehydrogenase (PDH), an extracellular oxidoreductase of some litter-decomposing basidiomycete fungi, catalyzes the quinone-dependent (di)oxidation of free (nonphosphorylated) sugars in their pyranose form to di- or tricarbonyl derivatives, aldoketoses or aldodiketoses [1,2]. This enzyme exhibits an extremely broad substrate tolerance and variable regioselectivity for the oxidation of both mono- and oligosaccharides. Therefore, variety of interesting novel sugar redox transformations are possible. Biological function of this ∼66 kDa single polypeptide flavoglycoprotein is largely unknown and the gene encoding it has not been cloned so far. PDH selectivity for secondary alcoholic group(s) at C-2, C-3, or C-2 + C-3 is sugar substrate-dependent and changes also with the PDH source. Thus, D-galactose was exclusively oxidized at C-2 (*A. bisporus*) [3], glucose (*Macrolepiota rhacodes*) [2] and the D-glucosyl moiety of non-reducing oligosaccharides (*A. meleagris*) [4] at C-3, whereas final C-2, C-3 double oxidation to the corresponding aldodiketose was demonstrated for D-glucose conversion by *A. bisporus* [3] and D-xylose by *A. meleagris* [5] PDH.

A catalytically related enzyme is the well-understood homotetrameric ca. 300 kDa FAD-protein pyranose oxidase (EC 1.1.3.10) of numerous white-rot wood degrading fungi. It shows higher substrate specificity and C-2 regioselectivity for oxidation of some aldoses [6,7]. This enzyme, in

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contrast to PDH, has already been suggested for biotechnological applications such as isomerization of D-glucose and D-galactose via the corresponding aldoketoses to D-fructose [8] and D-tagatose [9], respectively. However, 1 → 4 glucooligosaccharides do not serve as substrates of this enzyme.

Considering the recognized significant activity of PDH towards lactose and the above application potential of the transformation of D-galactose (a component of lactose) to D-*lyxo*-hexos-2-ulose (2-dehydrogalactose) in a process for production of D-tagatose [9], we have now examined other possibilities of lactose valorization by its conversion into a useful synthon(s) using PDH. Here we show for the first time that this easily available disaccharide can be conveniently enzymatically oxidized at C-2 of its reducing moiety to 2-dehydrolactose, along with oxidation at C-1 yielding lactobionic acid in variable proportion depending on the PDH source. Emphasis is put on the detailed structure identification of the new enzyme reaction products.

2. Experimental

2.1. Enzyme source and production

Mycelial cultures of litter-degrading mushrooms (Table 1) employed in the screening for expression of PDH with optimum catalytic characteristics for the conversion lactose → 2-dehydrolactose were obtained from the Culture Collection of Basidiomycetes (CCBAS), Institute of Microbiology, Prague, Czech Republic. The fungi were grown at 28 °C under stationary conditions on liquid glucose–corn steep medium [4] (50 ml per 500 ml Roux flask) for 4–6 weeks until a mycelial mat was formed. Mildly aseptically homogenized cultures derived from malt-agar stock cultures, showing sufficient growth on the same liquid medium, were used for inoculations (10%). Production cultures of *Agaricus xanthoderma* Gen. (CCBAS 225) used for isolation of pure PDH were grown on glucose–casein liquid medium [2] for 6 weeks.

2.2. Enzyme assay and purification

PDH was assayed by following the D-glucose-dependent reduction of the ferricenium ion to ferrocene at 300 nm ($\varepsilon = 4.3\,mM^{-1}\,cm^{-1}$) for 1 min in the standard reaction mixture (2 ml) containing 100 μmol Na phosphate pH 7.5, 50 μmol D-glucose, 0.4 μmol ferricenium hexafluorophosphate ($Fc^+PF_6^-$, Sigma–Aldrich, Prague, Czech Republic), and PDH sample [2]. One unit (U) of PDH activity was defined as the amount of activity that reduced 2 μmol of Fc^+ per min at 25 °C in the above assay system.

In the screening experiment, extracellular PDH of individual fungi was partially purified on a DEAE-Sephacel (Pharmacia Biotech, Uppsala, Sweden) column (1 cm × 2 cm) equilibrated with 20 mM Na phosphate pH 7.5. Five to 30 ml aliquots (depending on the PDH activity) of a culture liquid were directly applied, the column was washed with the same buffer and the enzyme activity eluted by a stepwise gradient of 0–250 mM NaCl in the buffer. Proteins were transferred into deionized H_2O using diafiltration on YM-10 membrane (Millipore, Bedford, MA, USA), and the volume was reduced (200 μl). PDH of the optimal producer (*A. xanthoderma*) was purified to homogeneity using the procedure described previously [2], including hydrophobic interaction, ion exchange chromatography and gel filtration. For the use in lactose transformations monitored by NMR or HPLC, the purified enzyme (59 U mg protein^{-1}) was transferred into distilled H_2O (2.5 mg ml^{-1}).

2.3. PDH catalyzed transformations of lactose

The reaction mixture (500 μl) for lactose transformations in the screening experiment contained lactose monohydrate (1.8 mg, 10 mM), 1,4-benzoquinone (1.7 mg, 15 mM, Sigma–Aldrich) and a particular PDH enzyme preparation (0.2–2 U depending on the enzyme source) in distilled H_2O. Conversions performed at 25 °C for 16 to 48 h were monitored by HPLC analysis of samples (60 μl) withdrawn at appropriate time intervals, deproteinized by passing through

Table 1
Relative simultaneous conversion of lactose to 2-dehydrolactose and lactobionic acid by PDH from various *Agaricus* species

Agaricus sp.	C-2/C-1[a]	Relat. act.[b]	*Agaricus* sp.	C-2/C-1[a]
A. xanthoderma	1.70	14	*A. radicatus*	0.12
A. meleagris	1.10	12	*A. arvensis*	0.20
A. subperonatus	0.87	12	*A. augustus*	0.08
A. bisp. avellaneus	0.84	13	*A. maleolens*	0.06
A. perdicinus	0.81	8	*A. abruptibulbus*	<0.05
A. bitorquis	0.48	9	*A. campestris*	<0.05
A. bisporus	0.46	14	*A. comtulus*	<0.05
A. nivescens	0.26	4	*A. essetei*	<0.05
A. silvicola	0.26	3	*A. excellens*	<0.05
A. vaporarius	0.13	10	*A. silvaticus*	<0.05

[a] Ratio of HPLC peak areas of lactose oxidation products: 2-dehydrolactose/lactobionic acid.
[b] Relative activity of PDH with lactose (%) compared to D-glucose (100%) using standard ferricenium assay (see Section 2).

Ultrafree-MC 30,000 NMWL Filter Units (Millipore) and diluted twice with H_2O before analysis.

For HPLC analysis, the transformations were carried out with pure PDH in the dark at 25 °C under gentle stirring in stoppered 2 ml Eppendorf vials containing lactose monohydrate (18 mg, 50 mM), 1,4-benzoquinone (5.4 mg, 50 mM) and purified PDH (5 U, *A. xanthoderma*) in deionized H_2O (1 ml). Samples of the reaction mixture (60 μl) processed as above were diluted ten times with H_2O prior to HPLC analysis. In the NMR study, the transformations were monitored directly in a 5 mm NMR sample tube (solution volume 0.7 ml) containing lactose (10 mM), 1,4-benzoquinone (10 mM) and 1 U PDH in deuterium oxide (1 ml). In semipreparative transformations, used also for derivatization of lactose oxidation products, 50 mM lactose was oxidized with 75 mM 1,4-benzoquinone (added in eight 25 mg portions at 1 h intervals) and PDH (60 U, *A. xanthoderma*) in 25 mM Na acetate, pH 4.5 (25 ml) at 30 °C for 12 h in darkness. Drop of pH in the reaction mixture was compensated by $NaHCO_3$ (0.5 M).

2.4. Derivatization of lactose oxidation products

In semipreparative runs, the acidity of the transformation mixture was adjusted to pH 2 with 2 M HCl. Hydroquinone and residual benzoquinone were removed by extraction with $CHCl_3$ (10 ml). The remaining dark components (derived nonenzymatically from benzoquinone) present in the aqueous layer were adsorbed on charcoal and filtered off. Following concentration to 7 ml, the solution was supplemented successively with the same volume of ethanol and freshly distilled *N*,*N*-diphenylhydrazine (350 μl, Koch-Light Lab., Colnbrook, UK), intensively stirred for 2 h and left for 24 h at room temperature. The solvent was then removed by vacuum evaporation and the residue was dissolved in methanol (1 ml).

Hydrazones were fractionated using TLC on Silica Gel 60 F_{254} aluminium sheets 20 cm × 20 cm (Merck, Darmstadt, Germany) developed with $CHCl_3$–MeOH, 5:1. Two major hydrazone derivatives giving orange streaks at $R_f = 0.32$ (Fig. 5, compound **6**, 254 mg) and $R_f = 0.59$ (Fig. 5, compound **7**, 10 mg) were excised, combined separately and extracted with MeOH. Further purification was achieved by rechromatography in the same solvent system.

2.5. Analytical methods

HPLC of lactose transformation products was performed on a SP 8800 liquid chromatograph (Spectra Physics, San Jose, CA, USA) fitted with a refractive index detector, using an Ostion LG KS 0800 Na^+ column 250 mm × 8 mm (Watrex, Prague, Czech Republic) eluted at 80 °C with deionized water (0.5 ml min^{-1}).

MALDI mass spectrometry: Positive-ion mass spectra were measured in reflectron mode on a Bruker BIFLEX II time-of-flight mass spectrometer (Bruker, Bremen, Germany) equipped with a SCOUT 26 sample inlet, a gridless delayed extraction ion source and a 337 nm nitrogen laser. Solutions of ferulic acid (analysis of free sugars) or α-cyano-4-hydroxycinnamic acid (hydrazone derivatives) in aqueous 40% acetonitrile/0.2% TFA (10 mg/ml) were used as MALDI matrices. Spectra were calibrated externally using the monoisotopic $[M + H]^+$ ions of the matrix and a peptide standard angiotensin.

NMR spectroscopy: In situ spectra were recorded on a Varian INOVA-400 spectrometer (400 MHz for ^1H, 100 MHz for ^{13}C) in D_2O at 30 °C; the diphenylhydrazone derivatives were measured in CD_3OD. Chemical shifts were referenced to internal acetone ($\delta_H = 2.030$, $\delta_C = 30.50$) or residual methanol signal ($\delta_H = 3.33$, $\delta_C = 49.3$). All 2D NMR experiments (HOM2DJ, homonuclear two-dimensional J-resolved spectroscopy; COSY, correlated spectroscopy; TOCSY, total correlation spectroscopy; HMQC, heteronuclear multiple quantum correlation; HMBC, heteronuclear multiple bond correlation) were performed using the manufacturers software. The sequence for 1D-TOCSY experiments [10] was obtained through Varian User Library, the sequence for gradient HMBC was obtained from Varian Application Laboratory in Darmstadt (Germany).

3. Results and discussion

3.1. Selection of PDH source for lactose oxidation

Preliminary TLC analyzes of reaction product(s) of PDH (*A. bisporus*) with lactose and benzoquinone indicated C-2 oxidation of the sugar substrate (typical blue staining of the derivative with diphenylamine–aniline–phosphoric acid reagent diagnostic for 2-aldoketoses [2]) and simultaneous C-1 oxidation to lactobionolactone (not shown). The observed differences in substrate specificity among PDHs from related fungi [2] and production of both the lactose oxidation products in different ratios depending on the enzyme source prompted us to search for a fungal strain expressing PDH with relatively high and C-2 specific activity towards this disaccharide or PDH providing the 2-ketoderivative as a major product. Results of this screening based on HPLC analysis (see Section 2) of lactose reaction products by individual PDHs are summarized in Table 1.

Out of the fungi tested, only PDH of several *Agaricus* species showed significant activity towards lactose. Whereas a number of these PDHs had nearly exclusive selectivity for C-1, none of the fungal strains tested expressed PDH that provides the C-2 derivative as the only product. The highest ratios of the C-2 to C-1 oxidation products in this experiment were found with the *A. xanthoderma* (1.7) and *A. meleagris* (1.1) enzymes. Under experimental conditions used, lactose was not a significant substrate (<2% of the activity with D-glucose) for the enzyme from other genera of the PDH-positive litter-decomposing fungi [2] tested

(*Macrolepiota procera*, *M. puelaris*, *M. rhacodes*, *Calvatia utriformis*, *Coprinus sterquilinus*, *Leucoagaricus naucinus*, *Leucocoprinus birnbaumii*, *Lycoperdon echinatum*, and *L. pyriforme*).

3.2. Lactose transformations by purified PDH

The relative activity of purified PDH (*A. xanthoderma*, 49 U mg protein^{-1}) with lactose under otherwise standard assay conditions (25 mM substrate, ferricenium) and relative v_{max} were 14% and 25% (D-glucose, 100%). K_m values determined for D-glucose, lactose and ferricenium were 0.97, 82 and 0.192 mM, respectively. Significantly higher relative activity (32%) was found for lactose when 1,4-benzoquinone ($K_m = 2.2$ mM) was used as an electron acceptor at pH 4.5. Accordingly, lactose biotransformations were conveniently performed at higher concentration of the substrate and relatively low concentration of benzoquinone. This co-substrate was added portionwise during the time course due to its instability in solution under aerobic conditions. The quinone was preferred as stoichiometric electron acceptor also because of its much higher solubility compared to ferricenium that allows, on the other hand, high sensitivity of the PDH assay [2].

A typical time course of lactose transformation by the enzyme (*A. xanthoderma*) as monitored by HPLC is shown in Fig. 1. Because of gradual acidification of the reaction mixture due to spontaneous hydrolysis of the co-produced lactobionolactone to lactobionic acid, the transformations significantly slowed down with some lactose left (ca. 10%) at the end (pH ~2.8). Buffering was omitted because of interference of salts with HPLC analysis of the standard lactobiono-1,5-lactone (both eluting at the column void volume) under conditions when the second product (presumed 2-dehydrolactose) was separated from lactose.

Based on HPLC, yields of 2-dehydrolactose in semipreparative transformations using purified *A. xanthoderma* PDH, 50 mM lactose and buffering at pH 4.5 (optimal for 1,4-benzoquinone) were approaching 75% with <2% lactose unreacted at the end of conversion. Compared with non-buffered systems, these results and other observations indicated that the ratio of C-2 and C-1 oxidation products, and thereby yields of 2-dehydrolactose, can be improved by modifying some reaction parameters such as pH, temperature, sugar/enzyme concentration and the nature of the electron acceptor used.

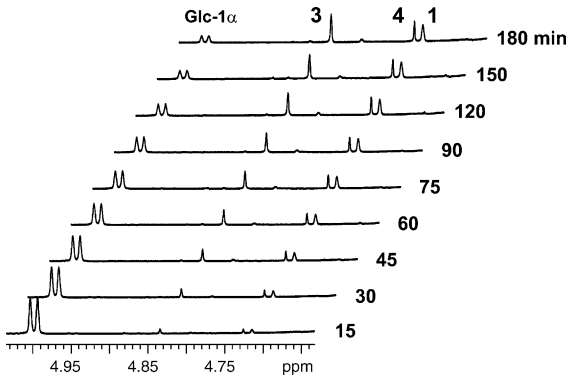

Fig. 2. Time course of lactose biotransformation monitored by ^{1}H NMR (part of anomeric region, anomeric protons in 2-dehydrolactose forms **1**, **3** and **4**, Fig. 4).

3.3. Product identification

To obtain a better insight into the reaction mechanism and outcome, the lactose biotransformations were carried out directly in an NMR sample tube. The enzyme was added to the fresh solution of lactose and benzoquinone. Therefore, no equilibrium was established and the mixture consisted of the substrate α- and β-pyranose forms with the former prevailing. Proton spectra were used to monitor the reaction course (Fig. 2). As soon as the concentration of some products was sufficient for their detection, "snapshots" were taken using 2D NMR techniques (COSY, TOCSY,

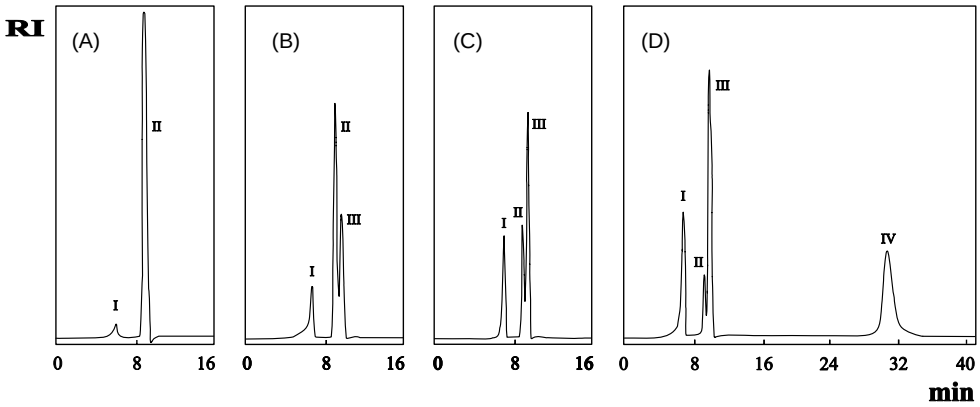

Fig. 1. (A)–(D), HPLC monitoring of lactose oxidation by pyranose dehydrogenase from *A. xanthoderma* and benzoquinone at incubation times of 0, 2, 6 and 14 h, respectively. Peaks—I: lactobionic acid (hydrolysis product of lactobiono-1,5-lactone); II: lactose; III: β-D-galactopyranosyl-(1 → 4)-D-*arabino*-hexos-2-ulose (2-dehydrolactose); IV: 1,4-benzoquinone/hydroquinone; the peak I at (A) corresponds to the residual salt originating from the enzyme preparations added to the reaction mixture.

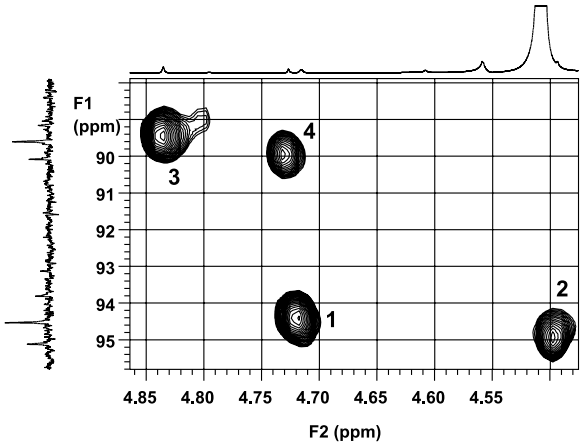

Fig. 3. Heteronuclear multiple quantum correlation (HMQC) of anomeric protons and carbons in 2-dehydrolactose (forms **1**–**4**, Fig. 4).

HOM2DJ, HMQC) or their selective one-dimensional variants (1D-TOCSY, 1D-NOE). When all substrate was consumed (judging from the disappearance of distinct H-1α and H-2β signals, the complete analysis (including ^{13}C NMR and HMBC) was undertaken.

New signals are appearing in the 3.8–4.9 ppm range, together with decaying signals of anomeric protons of glucose (Fig. 2). H-1′ of galactose unit resonate as five distinct doublets (J = 7.7–7.8 Hz). The corresponding H-2′ to H-4′ were found by 1D-TOCSY. Singlets of anomeric protons (4.872, 4.836, 4.804, 4.727, 4.716, 4.492, and 4.308 ppm) indicate that C-2 of glucose carries no protons. Correspondingly, MALDI-MS of the lactose oxidation product(s) gave $[M + H]^+$ ion at m/z 358.2. These data are consistent with structure of β-D-galactopyranosyl-(1 → 4)-D-*arabino*-hexos-2-ulose-(2-dehydrolactose, Fig. 6) and, therefore, C-2 oxidation of lactose by PDH.

As there were no carbonyl signals in ^{13}C NMR and no correlations to this region in HMBC, all carbonyl groups formed have to be hydrated. The comparison of δ(H-1), δ(C-1) (Fig. 3), and δ(C-2) with published data for glucosone [11] support the presence in water solution of 2-dehydrolactose forms **1**–**5** (Table 2, Fig. 4). Our assignment is supported by measurement of diagnostic direct couplings [12] J(C-1, H-1) in coupled HMQC (174.0 Hz for **1**, 164.2 for **2**, intermediate values around 166 Hz for **3**–**5**). Complete agreement for all carbon data cannot be expected as C-4 should experience a downfield glycosidation shift accompanied by slight upfield shift of C-3 and C-5. Several signals were only found for the form **1**—^{1}H NMR: 3.652 (d, J = 9.1, H-3), 3.505 (dd, J = 10.5, 9.1, H-4); ^{13}C NMR: 72.36 (C-3), 78.18 (C-4); whereas complete sets were obtained for the forms **4** and **5** (an epimer of **3**); results for form **4**—^{1}H NMR: 4.115 (d, J = 7.2, H-3), 4.008 (dd, J = 7.2, 7.1, H-4), 3.790 (ddd, J = 7.1, 5.6, 3.6, H-5), 3.595 (dd, J = 12.3, 3.6, H-6a), 3.495 (dd, J = 12.3, 5.6, H-6b); ^{13}C NMR: 75.16 (C-3), 84.26 (C-4), 70.85 (C-5), 62.24 (C-6); for form **5**—^{1}H NMR: 4.308 (s, H-1), 3.818 (d, J = 10.1, H-3), 3.898 (dd, J = 10.1, 3.2, H-4), 3.972 (m, H-5), 3.600 (dd, J = 10.3, 2.0, H-6a), 3.542 (dd, J = 10.3, 2.0, H-6b); ^{13}C NMR: 92.22 (C-1), 66.50 (C-3), 77.74 (C-4), 66.95 (C-5), 62.43 (C-6).

Signals δ_C = 86.7, δ_H = 4.132 (d, J = 3.2 Hz) evidently represent a C-4 in a product of 2,3-dioxidation that might account for one of the two unassigned singlets of anomeric protons.

A six-proton spin system, attributable to *gluco* part of lactobionic acid was also found by 1D-TOCSY: 4.081 (d, J = 3.0, H-2), 3.920 (dd, J = 5.1, 3.0, H-3), 3.791 (dd, J = 5.8, 5.1, H-4), 3.745 (m, H-5), 3.635 (dd, J = 11.9, 3.2, H-6a), 3.532 (dd, J = 11.9, 6.7, H-6b). Running HMBC with 50 mM transformation product provided the final proof: a crosspeak of H-2 doublet with a carbonyl at 178.5 ppm. Hence, PDH catalyzes simultaneous oxidation of lactose at C-1. The proposed primary reaction product, lactobiono-1,5-lactone (Fig. 6), is rapidly hydrolyzed to lactobionic acid, which was confirmed by a comparative experiment with the commercial lactone (Sigma–Aldrich).

C-2 oxidation of lactose was further confirmed by analysis of the *N*,*N*-diphenylhydrazone (DPH) derivatives of the PDH reaction products. The main hydrazone isolated ($[M + Na]^+$ = 695.4) was a bis-DPH derivative according to ^{1}H and ^{13}C NMR spectra. It contained an intact β-Gal*p* moiety (Table 3), a singlet of H-1, and a five-spin system –CH(OH)CH(OH)CH(OH)CH$_2$OH. The heteronuclear coupling pattern (Figs. 5 and 6) leads to the structure of 1,2-bis-DPH derivative of 2-dehydrolactose.

The derivatization provided conclusive proof for partial dioxidation of lactose. The minor hydrazone component ($[M + Na]^+$ = 859.5), detectable in derivatized mixtures of prolonged transformations, was identified as a Tris–DPH derivative. COSY and 1D-TOCSY established again the β-Gal*p* unit. Protons of the derivatized glucose part resonated as a singlet (H-1) and a four-spin system

Table 2
Comparison of selected chemical shifts for different forms of 2-dehydroglucose [11] and 2-dehydrolactose (Fig. 4, forms **1**–**4**)

Form	δ(H-1)		δ(C-1)		δ(C-2)	
	DehydroGlc	DehydroLac	DehydroGlc	DehydroLac	DehydroGlc	DehydroLac
1	4.749	4.716	94.82	94.54	93.79	93.81
2	4.499	4.492	95.26	95.12	93.23	93.13
3	4.844	4.836	89.60	89.61	97.54	97.58
4	4.742	4.727	90.23	90.09	101.00	101.04

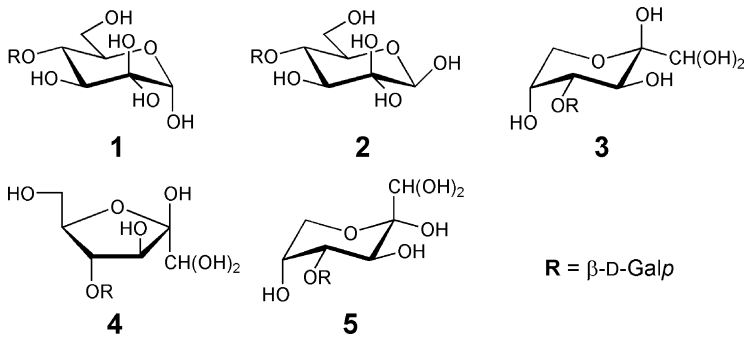

Fig. 4. Structures of detected forms of 2-dehydrolactose.

Table 3
NMR data of diphenylhydrazones isolated from the biotransformation of lactose (399.89 and 100.55 MHz, CD_3OD, 30 °C)

C/H-	**6**		**7**	
	δ_C (ppm)	δ_H m (*J* (Hz))	δ_C (ppm)	δ_H m (*J* (Hz))
1	129.48	7.007 s	129.85	6.147 s
2	160.46	–	159.32	–
3	71.87	5.435 d (4.5)	148.14	–
4	84.40	4.440 dd (5.5, 4.5)	84.65	5.195 d (5.8)
5	73.57	4.046 ddd (5.8, 5.5, 4.0)	75.14	4.308 ddd (5.8, 5.7, 3.6)
6	64.28	3.941 dd (11.5, 4.0)	64.42	4.077 dd (11.6, 3.6)
		3.905 dd (11.5, 5.8)		4.007 dd (11.6, 5.7)
1′	105.84	4.521 d (7.8)	105.49	4.750 d (7.8)
2′	73.28	3.710 dd (9.8, 7.8)	73.35	3.790 dd (9.7, 7.8)
3′	75.23	3.563 dd (9.8, 3.4)	75.34	3.499 dd (9.7, 3.4)
4′	70.11	3.881 dd (3.4, 1.1)	70.18	3.863 dd (3.4, 1.1)
5′	76.78	3.486 ddd (5.9, 5.2, 1.1)	76.88	3.487 ddd (7.1, 5.8, 1.1)
6′	62.10	3.619 dd (13.6, 5.2)	62.21	3.734 dd (10.8, 7.1)
		3.520 dd (13.6, 5.9)		3.645 dd (10.8, 5.8)

Additional signals.
^{1}H NMR—**6**: 6.904 (4H, m, 4 × *ortho*-Ph_1), 6.921 (4H, m, 4 × *ortho*-Ph_2), 7.032 (2H, m, 2 × *para*-Ph_2), 7.212 (2H, m, 2 × *para*-Ph_1), 7.248 (4H, m, 4 × *meta*-Ph_2), 7.372 (4H, m, 4 × *meta*-Ph_1); **7**: 6.714 (4H, 4 × *ortho*-Ph_2), 6.721 (4H, m, 4 × *ortho*-Ph_1), 7.031 (2H, m, 2 × *para*-Ph_2), 7.053 (2H, m, 2 × *para*-Ph_3), 7.059 (4H, m, 4 × *ortho*-Ph_3), 7.155 (2H, m, 2 × *para*-Ph_1), 7.201 (4H, m, 4 × *meta*-Ph_2), 7.214 (4H, m, 4 × *meta*-Ph_3), 7.277 (4H, m, 4 × *meta*-Ph_1). ^{13}C NMR—**6**: 123.33 (4C, 4 × *ortho*-Ph_2), 123.78 (4C, 4 × *ortho*-Ph_1), 124.82 (2C, 2 × *para*-Ph_2), 126.88 (2C, 2 × *para*-Ph_1), 130.56 (4C, 4 × *meta*-Ph_2), 131.34 (4C, 4 × *meta*-Ph_1), 143.97 (2C, 2 × *ipso*-Ph_1), 150.41 (2C, 2 × *ipso*-Ph_2); **7**: 123.00 (4C, 4 × *ortho*-Ph_3), 123.71 (4C, 4 × *ortho*-Ph_2), 124.14 (4C, 4 × *ortho*-Ph_1), 125.08 (2C, 2 × *para*-Ph_2), 125.86 (2C, 2 × *para*-Ph_3), 126.63 (2C, 2 × *para*-Ph_1), 130.22 (4C, 4 × *meta*-Ph_2), 130.64 (4C, 4 × *meta*-Ph_3), 131.11 (4C, 4 × *meta*-Ph_1), 143.96 (2C, 2 × *ipso*-Ph_1), 148.42 (2C, 2 × *ipso*-Ph_3), 148.90 (2C, 2 × *ipso*-Ph_2).

–CH(OH)CH(OH)CH_2OH. The first member of the latter system was H-4, according to the downfield shift of the corresponding carbon (84.65 ppm) and its heteronuclear coupling to H-1′ (and vice versa). The reported assignment (Table 3) is based on the HMBC results (Fig. 5, **7**). Thus, the corresponding structure is the evidence for dioxidation of lactose to β-D-galactopyranosyl-(1 → 4)-D-*erythro*-hexos-2,3-diulose (2,3-didehydrolactose, Fig. 6).

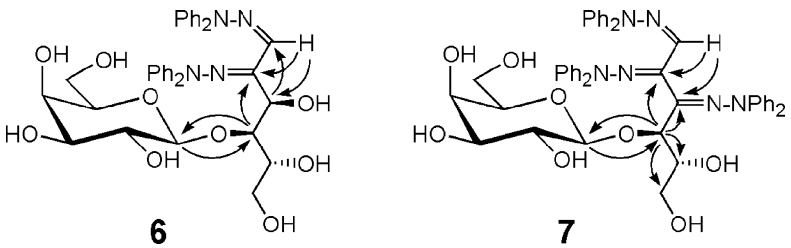

Fig. 5. Structures with pertinent heteronuclear couplings of *N,N*-diphenylhydrazone derivatives **6** and **7** used for NMR identification of the oxidation products of lactose by pyranose dehydrogenase.

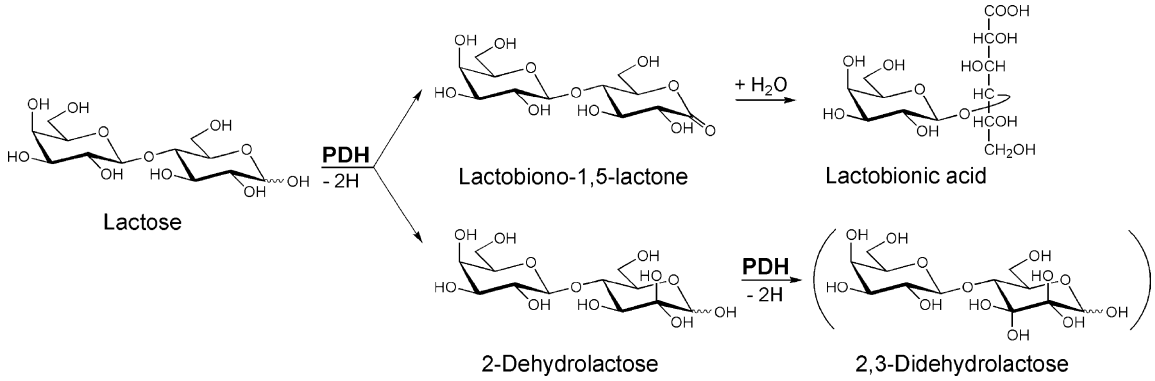

Fig. 6. Reaction scheme for (di)oxidation of lactose by pyranose dehydrogenase from *A. xanthoderma*. Secondary oxidation at C-3 to 2,3-didehydrolactose (β-D-galactopyranosyl-(1 → 4)-D-*threo*-hexos-2,3-diulose) takes place in prolonged incubations with excess of oxidant when oxidation at C-2 is close to completion. For simplicity, only one of the possible equilibrium cyclic forms is shown for the dehydroderivatives; carbonyl hydration also takes place.

3.4. PDH regioselectivity and related enzyme activities

Characteristic feature of pyranose dehydrogenase from a number of litter-decomposing fungi is variable C-2/C-3 regioselectivity of its attack and extremely broad substrate tolerance for oxidations of various mono- and oligosacchrides [2], some of which are not attainable with other known sugar oxidoreductases. Here we report for the first time the enzymatic C-2 and C-2,3-(di)oxidation of the disaccharide lactose at its reducing moiety. The search of Chemical Abstracts database for chemical C-2 oxidation of lactose was unsuccessful. In contrast to PDH, a catalytically related and well understood C-2 sugar oxidoreductase, pyranose oxidase (EC 1.1.3.10) [6] does not accept 1 → 4 disaccharides, including lactose.

C-1 oxidation of lactose to lactobionic acid, shown here to take place to variable extent simultaneously to C-2 oxidation, has been earlier reported to be specifically catalyzed by other enzymes: fungal cellobiose dehydrogenase (EC 1.1.99.18) [13], seaweed or plant hexose oxidase (EC 1.1.3.5) [14] and bacterial glucose–fructose oxidoreductase (EC 1.1.99.28) [15]. Lactobionolactone or its hydrolytic product, lactobionic acid, present also a higher-value lactose derivative that has commercial applications in pharmaceutical preparations and laundry detergents.

Oxidation of lactose at C-3 by PDH was in this work observed only in prolonged transformations (∼24 h, ca. 15% yield) using an excess of benzoquinone (semipreparative conversions) after the starting substrate was almost quantitatively oxidized at C-2 or C-1 of the glucose moiety. Interestingly, free D-glucose was oxidized by PDH simultaneously at C-2 and C-3 (finally at C-2 + C-3), but not at C-1 [3]. Because non-reducing disaccharides, such as trehalose and sucrose, were exclusively C-3 oxidized [4], lactose was attacked preferentially at C-1/C-2 apparently due to the fact that 1 → 4 linkage of D-glucose significantly prevented PDHs C-3 regioselectivity. Specific C-3 oxidation of lactose is, on the other hand, known to be catalyzed by D-glucoside 3-dehydrogenase (hexopyranoside:cytochrome *c* oxidoreductase, EC 1.1.99.13) purified from plant tumor-inducing bacterium *Agrobacterium tumefaciens* [16,17] and *Flavobacterium saccharophilum* [18], and characterized as an iron–sulfur flavoprotein acting with cytochrome *c* as an electron acceptor.

4. Conclusions

The present study extends our earlier findings [3–5] demonstrating the high potential of PDH for production of reactive di- and tricarbonyl sugars that may be useful in synthetic carbohydrate chemistry. Lactose is accumulated in large quantities as a by-product of dairy industry. Thus, the reported C-2 oxidative functionalization of this strongly under-utilized renewable resource may offer a route to increasing its value by providing access to novel conversions to desired products such as isomerization of lactose to lactulose (a dietetically beneficial prebiotic sugar) via 2-dehydrolactose, a key intermediate which is in the second step (bio)chemically reduced at C-1. Work on PDH sequencing and heterologous expression is currently underway in order to make the enzyme easily available.

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