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New biotransformations of some reducing sugars to the corresponding (di)dehydro(glycosyl) aldoses or aldonic acids using fungal pyranose dehydrogenase

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Abstract

The fungal enzyme pyranose dehydrogenase (PDH) (EC 1.1.99.29) purified to apparent homogeneity from culture media of *Agaricus meleagris* catalyzed the substrate-dependent C-1, C-2, C-3, C-1,3′ or C-2,3(′) (di)oxidation of a number of mono- and disaccharides with 1,4-benzoquinone as an electron acceptor. D-Ribose, D-allose, D-gulose and D-talose were oxidized to the corresponding aldonic acids. L-Arabinose was converted exclusively to 2-dehydro-L-arabinose (L-*erythro*-pentos-2-ulose) whereas D-xylose underwent competing C-2 and C-3 oxidation followed by dioxidation to 2,3-didehydro-D-arabinose (D-*glycero*-pentos-2,3-diulose). The major final oxidation products of maltose and cellobiose were the novel compounds 2,3′-didehydromaltose and 2,3′-didehydrocellobiose (α- and β-D-*ribo*-hexos-3-ulopyranosyl-(1 → 4)-D-*arabino*-hexos-2-ulose), formed via 2- and 3′-monooxidation intermediates. Minor concomitant (di)oxidation at C-1,(3′) to the corresponding bionic acids also took place. Maltotriose was preferentially oxidized at C-3″ of the terminal glucopyranosyl unit and at C-1 of the reducing moiety. The structures of these sugar oxidation products were established by in situ 1D and 2D NMR spectroscopy and ESI–MS. Based on HPLC analysis, conversions of (glycosyl)aldoses in non-buffered systems were nearly quantitative within 3–24 h, depending on the substrate. As the enzyme allows an easy access to highly reactive di- or tricarbonyl sugars, it might become a useful catalyst in carbohydrate chemistry.

Keywords: Aldonic acids; Biotransformations; 2-Dehydro-L-arabinose; Dicarbonyl sugars; Pyranose dehydrogenase

1. Introduction

Application of enzymes in synthetic carbohydrate chemistry has been receiving growing attention, mainly due to far greater regio/enantiospecificities displayed in enzyme catalysis compared to more conventional chemical procedures requiring complex protection strategies. A new powerful tool for redox biotransformations of sugars to derivatives featuring multiple reactive carbonyl groups is the enzyme pyranose dehydrogenase (PDH, EC 1.1.99.29, pyranose:acceptor oxidoreductase). PDH, produced by some basidiomycete fungi (*Agaricales*), was recently characterized as a quinone-dependent monomeric extracellular flavoglycoprotein [1] exhibiting broad substrate tolerance with variable catalytic regioselectivity for C-2, C-3, C-2,3 and C-3,4 (di)oxidation of a wide variety of saccharides including aldoses [1–3], non-reducing disaccharides [4] and alkyl/arylglycosides [4,5] in their pyranose cyclic form:

(1) pyranose + acceptor → 2-dehydropyranose (or 3-dehydropyranose or 2,3-didehydropyranose) + reduced acceptor
(2) pyranoside + acceptor → 3-dehydropyranoside (or 3,4-didehydropyranoside) + reduced acceptor

Regioselectivity of PDH depends on both the sugar substrate and the PDH source. For example, while *Macrolepiota rhacodes* PDH oxidized D-glucose exclusively at C-3 [1], the enzyme purified from *A. bisporus* catalyzed quantitative 2,3-dioxidation of

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this substrate [2]. To date, the metabolic function of this enzyme is still unknown.

Oxidative transformations of aldoses to their corresponding di- and tricarbonyl derivatives by PDH have applied potential in developing procedures for redox aldose → ketose isomerizations. Products of C-2 oxidation, that is aldoketoses (aldosuloses), were already suggested as key intermediates in biotechnologically interesting (chemo)enzymatic isomerizations of D-glucose and D-galactose to D-fructose and D-tagatose by another fungal enzyme, O_2-dependent pyranose oxidase (EC 1.1.3.10) [6]. Oxidation by PDH presents an alternative giving, for example, far better yields of the 2-dehydroderivative with D-galactose as substrate [2]. In addition, we have recently demonstrated the C-2 oxidation of lactose at its reducing end yielding an analogous 2-dehydroproduct [7] that could serve as possible intermediate in a two-step conversion of lactose to dietetically valuable lactulose.

The versatility of the above indicated sugar transformations by PDH prompted us to extend our knowledge on the applied potential of PDH for sugar redox conversions. Based on the established broad spectrum of sugars accepted by the enzyme purified from *Agaricus meleagris*, the reaction products of several new major substrates, (glucosyl)aldoses, were analyzed using in situ NMR spectroscopy. Some of the (di)dehydroderivatives thus produced have so far not been accessible either enzymatically or chemically. Furthermore, the demonstration of C-1 specificity for the oxidation of several aldoses by PDH was also unique, as this reaction is not feasible with pyranose oxidase. Emphasis is put on more detailed assessment of biocatalysis by PDH through structural elucidation of its new reaction products, di- and tricarbonyl sugars, presented in solution in their multiple equilibrium (cyclic tautomeric, anomeric and hydratation) forms.

2. Materials and methods

2.1. Enzyme source

The mycelial culture of the PDH-producing litter-decomposing mushroom *A. meleagris* SCHAEFF (strain CCBAS 907) was obtained from the Culture Collection of Basidiomycetes (CCBAS), Institute of Microbiology, Prague, Czech Republic. The fungus was grown for 4 weeks at 26 °C in stationary cultures on glucose–casein liquid medium [1] (50 ml per 500-ml Roux flask). Mildly homogenized cultures derived from malt-agar stock cultures, showing sufficient growth on the same liquid medium, were used for inoculations (10%).

2.2. Enzyme assay and purification

PDH activity was assayed spectrophotometrically by monitoring for 1 min the reduction of the ferricenium ion (Fc^+) at 300 nm ($\varepsilon = 4.3\ mM^{-1}\ cm^{-1}$) and 25 °C with D-glucose as an electron donor. The standard reaction mixture (2 ml) contained 100 μmol Na phosphate pH 7.5, 50 μmol D-glucose, 0.4 μmol $Fc^+PF_6^-$ (Sigma–Aldrich, Prague, Czech Republic), and PDH sample. One unit (U) of PDH was defined as the amount of enzyme activity effecting the consumption of 1 μmol D-glucose per minute under standard assay conditions. Two molecules of Fc^+ are reduced per molecule of sugar oxidized. In sugar biotransformation experiments, Fc^+ salt was replaced by the more soluble 1,4-benzoquinone as an electron acceptor.

PDH was purified from the culture supernatant of mycelial cultures of *A. meleagris* to apparent homogeneity using a procedure described previously based on DEAE-Sephacell anion exchange chromatography and FPLC on Phenyl-Superose HR 5/5 and Mono Q HR 5/5 (all media from Amersham Biosciences, Vienna, Austria) [1]. The purified enzyme used in NMR and HPLC studies was transferred to distilled H_2O (2.5 mg ml^{-1}) by diafiltration through a YM10 membrane (Millipore, Prague, Czech Republic).

2.3. PDH-catalyzed sugar transformations

Sugar conversions monitored by HPLC analysis were carried out in the dark at 25 °C under gentle stirring in stoppered 2-ml Eppendorf vials containing 10 mM sugar, 25 mM (15 mM for L-arabinose) 1,4-benzoquinone and purified PDH (1 U, 8 μl of the enzyme preparation; 0.5 U for L-arabinose and D-xylose) in deionized H_2O (1 ml). Buffering was omitted since salts interfere with HPLC analysis of lactones/aldonic acids (eluting at the column void volume) under conditions optimal for the separation of the other reaction components. Samples of the reaction mixture (60 μl) were withdrawn at intervals, deproteinized by passing through Ultrafree-MC 30,000 NMWL Filter Units (Millipore), and diluted two times with H_2O prior to HPLC analysis. For the in situ NMR analysis, transformations were performed at 30 °C in 5-mm NMR sample tubes (solution volume 0.7 ml) containing the same reaction mixture as above except that deionized water was replaced by deuterium oxide (>99% D_2O).

In semipreparative transformations used for derivatization of maltose oxidation products, 20 mM maltose was converted in the reaction mixture (50 ml) containing 40 mM 1,4-benzoquinone (added in three equal portions in 1-h intervals) and PDH (25 U) in H_2O. The incubation proceeded under gentle stirring at 30 °C for 5 h in darkness. Following substantially the procedure described previously for oxidation products of lactose [7], the final reaction mixture was prepurified, concentrated to 20 ml and supplemented with the same volume of ethanol. On adding freshly distilled *N*,*N*-diphenylhydrazine (0.36 ml; Koch-Light Lab., Colnbrook, UK), the mixture was left standing overnight at 22 °C. Following the vacuum evaporation of solvent, the solid obtained was extracted into 2 ml methanol and subjected to chromatographic (TLC) fractionation of hydrazone components.

2.4. Analytical methods

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

Protein was assayed using the bicinchoninic acid method (Sigma procedure No. TPRO 562) with γ-globulin as a standard.

TLC analysis and isolation of hydrazone derivatives of maltose oxidation products were performed on Silica gel 60 F_{254} aluminium sheets using 12:1 $CHCl_3$–MeOH and UV/vis detection [7]. Two main hydrazone compounds (Fig. 6, **10**, 39 mg and **11**, 21 mg) isolated were dissolved in deuteromethanol prior to NMR analysis.

2.5. Product identification

NMR spectra were measured on a Varian INOVA-400 spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C) in D_2O or CD_3OD at 30 °C. Chemical shifts were referenced to internal acetone (δ_H 2.030, δ_C 30.50) or the residual solvent signal (δ_H 3.33, δ_C 49.30). Standard software was used for all 2D NMR experiments; the pulse sequence for 1D-total correlation spectroscopy (TOCSY) [8] was obtained through Varian User Library.

Sugar transformations in the NMR tube were monitored by ^{1}H NMR. COSY, heteronuclear multiple quantum correlation (HMQC), and 1D-TOCSY experiments were performed when the concentration of some products surpassed the detection limit. Complete set of experiments, including also TOCSY ([9]), rotating frame nuclear overhauser and exchange spectroscopy (ROESY [10]), coupled-HMQC ([11]), heteronuclear multiple bond correlation (HMBC [12]), and directly observed ^{13}C NMR, were done upon completing the conversion or when ∼2/3 of a substrate was consumed to examine possible intermediate compounds. The structure determination is based upon the extracted coupling constants (including $^1J_{C,H}$), chemical shifts, and NOE's. The comparison with published data was made whenever possible.

The positive-ion mass spectra were recorded on a LCQ Deca (Finnigan, San Jose, CA) ion trap mass spectrometer equipped with a nanospray ion source. Samples dissolved in 30% aqueous acetonitrile were sprayed directly from EconoTip™ emitters (New Objective, Woburn, MA). Spray voltage was 1.2 kV and the heated capillary was kept at 150 °C. Full scan spectra were recorded over the mass range of 100–1000 Da. Only the final reaction mixtures (with one single or one prevailing product) were studied.

3. Results and discussion

3.1. Substrate selectivity of purified PDH

Out of several known PDH sources [1], *A. meleagris* was chosen in this study because of good growth characteristics of this fungus (not shown), extremely broad substrate tolerance of its PDH, and the stable expression of the enzyme (in contrast to PDH from *A. bisporus* partially characterized earlier) [2,3].

Purification of extracellular PDH from mycelial cultures of the fungus (350 ml culture liquid) resulted in a substantially homogeneous enzyme preparation (6.8 mg, 19% yield) that had specific activity of 52 U/mg protein with Fc^+ and 22 U/mg protein with 1,4-benzoquinone as an oxidant. Standard SDS-PAGE analysis (Coomassie Blue detection) revealed a single protein band at 67 kDa (not shown), a value somewhat lower than that of PDH purified from *M. rhacodes* (78 kDa) [1].

Table 1
Substrate selectivity of pyranose dehydrogenase (PDH) from *A. meleagris*

Substrate	RA (%)	Oxidation site (C-)
D-Xylose	113	2 + 3 + 2,3 [3]
L-Arabinose	100	2
D-Glucose	100	2 + 3 + 2,3 [1,2]
D-Galactose	99	2 [2]
L-Glucose	42	2 + 3 + 2,3[a]
D-Mannose	24	nd[b]
D-Ribose	19	1
D-Allose	15	1
D-Talose	10	1
D-Gulose	7	1
6-Deoxy-D-Glc	105	nd
2-Deoxy-D-Gal	31	nd
2-Deoxy-D-Glc	27	nd
L-Fucose	7	nd
D-Tagatose	67	nd
D-Ribulose	17	nd
L-Sorbose	5	nd
D-Glucono-1,5-lactone	42	nd
Palatinose	89	nd
Cellobiitol	77	nd
Cellobiose	70	1 + 2 + 3′ + 1,3′ + 2,3′
Maltose	61	1 + 2 + 3′ + 1,3′ + 2,3′
Melibiose	48	nd
Sucrose	46	3 (Glc*p*) [4]
Trehalose	27	3 + 3,3′ [4]
Lactose	15	1 + 2 + 2,3′ [7]
Cellotetraose	14	nd
Melezitose	13	3″ (Glc*p*) [4]
Maltotriose	12	1 + 3″ + 2,3″
Maltotetraose	9	nd
Methyl-α-D-Glc*p*	107	3 [4]
Salicin	78	3 + 3,4 [5]
Methyl-β-D-Glc*p*	58	3 [4]
Methyl-α-D-Gal*p*	17	3 [4]
Methyl-α-D-Man*p*	5	nd

[a] Unpublished results.
[b] Not determined.

Table 1 shows that PDH from *A. meleagris* is capable of oxidizing a broad variety of sugar substrates. This table also summarizes the regioselectivity of the enzyme in biotransformations so far studied, including this work and conversions studied previously using PDHs from other sources (*A. bisporus*, *A. xanthoderma*, *M. rhacodes*). Both numerous monosaccharides (aldoses, ketoses and deoxysugars) and oligosaccharides/heteroglycosides could be oxidized at variable positions, either at the anomeric or more frequently at the secondary carbon atom. Reaction products of eight additional PDH substrates (reducing sugars) are here examined for the first time, out of these maltose, cellobiose, D-xylose and L-arabinose present cheap sugar resources that can be of interest for large scale conversions.

Enzyme reactions were performed in the standard assay mixture containing PDH, a listed substrate (25 mM) and fericenium (Fc^+) as an electron acceptor (see Section 2). Relative activities (RA) of the enzyme towards D-glucose (100%,

52 U/mg protein) are given. In addition to the substrates shown, D-altrose, D-arabinose, D-lyxose, D-fructose, 2-deoxy-D-ribose, methyl-β-D-Gal*p*, L-arabinitol, dulcitol, mannitol, erythritol and *N*-acetyl-D-glucosamine were tested and could not serve as PDH electron donors. The site(s) of oxidation given for a number of substrates compile both present data and results of other biotransformations so far studied with PDHs of different origin (see the references).

Direct NMR analysis of aldose (di)oxidation derivatives that are transformed at secondary C-atom(s) is complicated because of their presence in solution in multiple equilibrium forms of variable proportions, corresponding to possible pyranose/furanose ring closures through any of the carbonyl groups in the molecule. Hydration of the remaining free carbonyl group(s) also occurs. Thus, structures of a number of individual components (anomers, isomers and hydration products) in the complex reaction mixture are to be determined to reveal the regioselectivity of PDH for each substrate. We tried to account for the majority of signals observed both in ^{1}H and ^{13}C NMR spectra. A previous indirect approach to the analysis, eliminating cyclic isoforms by carbonyl derivatization with *N*,*N*-diphenylhydrazine [2,3], was abandoned because of inconveniences related to the hydrazone separation and purification. The exception was analysis of the maltose oxidation products where both the approaches were used.

Four types of oxidation with the PDH substrates studied were evidenced, depending on the site of oxidation (C-1, C-2, C-3, and C-2,3) and nature of the substrate.

C-1: In this case, the contiguous spin system of aldose or the oligosaccharide reducing end residue is one proton shorter since the signal of the anomeric proton is missing. The anomeric carbon is replaced by a carbonyl to which the corresponding H-2 proton is coupled. The ring size of the resulting lactone is then deduced from the HMBC coupling pattern, magnitudes of the H,H vicinal coupling constants and the downfield shift of carbons involved in the lactone formation. The coupling constants not compatible with the six- or five-membered ring indicate a linear acid form.

C-2: Anomeric protons resonating as singlets are typical for the products of C-2 oxidation. The well documented equilibrium compositions of some 2-dehydroaldoses in aqueous solutions [13–15] could be used for reliable identification of this kind of products, based on a simultaneous comparison of ^{1}H and ^{13}C NMR data through HMQC. The chemical shifts of anomeric carbons and the HMBC coupling pattern determine the nature of the oxidized sites (keto group versus geminal diol).

C-3: The anomeric protons in 3-oxidized products are doublets; the multiplicity of H-2 depends on the C-3 functionality – a doublet for the hydrated keto group (a geminal diol), doublet of doublets for a keto group (a result of the through-the-carbonyl coupling [16], typical for 3-dehydropyranoses [4,17]). Again, the use of ^{13}C dimension is needed for the determination of the nature of keto groups.

C-2,3: The presence of 2,3-dioxidized compounds in the biotransformation mixture is manifested by singlet H-1 resonances having chemical shifts different from those of 2-dehydrosugars. The couplings of these protons to the quaternary carbons C-2 and C-3 (HMBC) help to find out the rest of the molecule (the protons that are coupled to these carbons, too).

3.2. *Conversion of* L-*arabinose*

The time course of L-arabinose oxidation by PDH, studied using HPLC analysis (Fig. 1) revealed the rapid substrate conversion to one product at almost quantitative yield (up to 95%, Fig. 2). Formation of a dicarbonyl sugar by-product was not detected. The small peak at the column void volume may represent a minor decomposition product of the benzoquinone cosubstrate (peak III).

The transformation of L-arabinose in the NMR-monitored experiment was completed within 3 h. A pseudomolecular ion $[M+Na]^{+}$ at *m*/*z* 171.1 in the ESI MS indicated a monooxidation. Analysis of the NMR data obtained for this reaction (Supplementary Table 1) revealed a C-2 type of oxidation. Seven equilibrium components (Fig. 3, **1a**–**1g**) out of the eight reported [14] for 2-dehydro-D-arabinose were positively identified. No resonances of free carbonyl groups were observed, probably because of low occurrence of the **1c** and **1d** species. Thus, PDH oxidizes L-arabinose exclusively at the position C-2 to 2-dehydro-L-arabinose (**1**, L-*erythro*-pentos-2-ulose).

Chemical methods for production of **1** (hydrolysis of L-arabinose osazone or its treatment with benzaldehyde) are

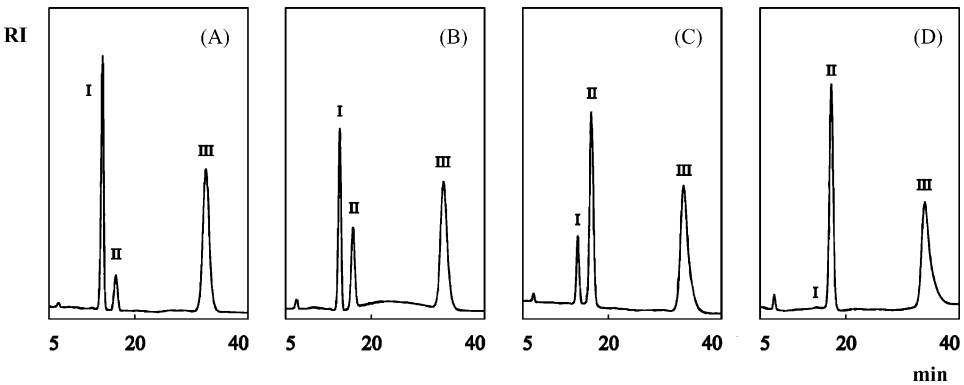


Fig. 1. HPLC monitoring of L-arabinose oxidation by pyranose dehydrogenase from *A. meleagris* at incubation times of 0.25 h (A), 0.5 h (B), 1.5 h (C) and 3 h (D), respectively. Peaks: I, L-arabinose; II, 2-dehydro-L-arabinose (**1**); III, benzoquinone/hydroquinone.

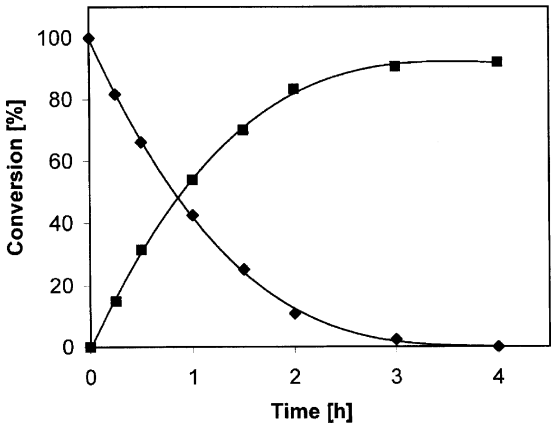


Fig. 2. Conversion of L-arabinose into 2-dehydro-L-arabinose using pyranose dehydrogenase (PDH). Conditions: 10 mM L-arabinose; 15 mM 1,4-benzoquinone; 0.5 U PDH per 1 ml reaction mixture in deionized H_2O. (♦), L-Arabinose; (■), 2-dehydro-L-arabinose.

known for more than a century and are marked by poor yields. Reichstein et al. [18] suggested **1** as an intermediate in a procedure for the synthesis of L-araboascorbic acid (L-*erythro*-hex-2-enono-1,4-lactone). To our best knowledge, the enzymatic procedure for conversion of L-arabinose to **1** has not yet been reported to date. In this context, our unique enzymatic access to **1** in yields approaching 95% offers new possibilities for its biotechnological applications. No significant double oxidation of L-arabinose to the 2,3-dihydroderivative was observed, contrary to the reaction of PDH with D-xylose.

3.3. Conversion of D-xylose

The transformation of D-xylose with PDH was analyzed at the stage of 70% conversion. The observed NMR spectra (Supplementary Table 2) corresponded to the mixture of products of C-2, C-3 and C-2,3 (di)oxidation. Both ^{1}H and ^{13}C data of the products **2a** and **2b** (Fig. 4) were in agreement with those published before for 2-dehydro-D-lyxose (**2**, D-*threo*-pentos-2-ulose) [15]. The localization of the keto group at C-3 (Fig. 4, **3a**, **3b**) corresponded to the structure of 3-dehydro-D-ribose (**3**, D-*erythro*-pentos-3-ulose). The last two reaction mixture components were assigned the structures **4a** and **4b** of a tricarbonyl sugar derivative, 2,3-didehydro-D-arabinose (**4**, D-*glycero*-pentos-2,3-diulose). The $[M+Na]^+$ ion at *m/z* 169.1 supported this conclusion. These findings indicate a competitive oxidation of D-xylose by PDH at either C-2 or C-3 followed by a second step oxidation to the final C-2,3 dioxidized product **4**.

Both chemical (cupric acetate method) [19] and enzymatic (pyranose oxidase) [17,20] preparation of **2** were described. Double oxidation of D-xylose to **4** (>90% yields) via **2** or **3** has

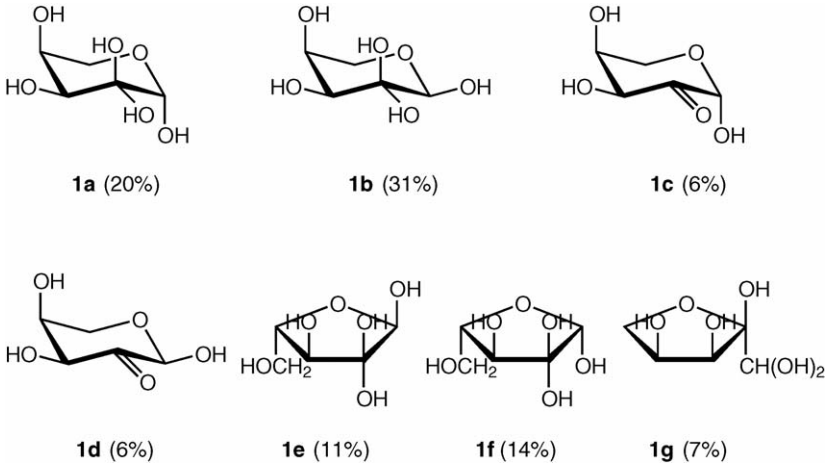


Fig. 3. Structures of detected forms of the L-arabinose oxidation product, 2-dehydro-L-arabinose (**1**).

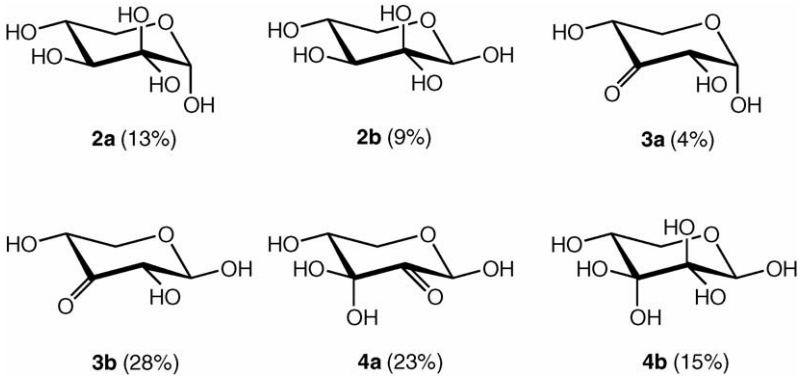


Fig. 4. Structures of detected isomeric/hydration forms of the oxidation products of D-xylose, 2-/3-dehydro-D-lyxose/ribose (**2/3**) and 2,3-didehydro-D-arabinose (**4**).

only been reported recently for PDH purified from *A. bisporus* [3]; in this work, structures of the intermediates and final product were, however, determined indirectly through the isolated *N*,*N*-diphenylhydrazone derivatives. Time course HPLC patterns of D-xylose dioxidation by *A. bisporus* and *A. meleagris* PDHs (not shown) were substantially identical reflecting the same sequence of multiple reaction products and related catalytic activities.

3.4. Conversion of maltose and cellobiose (α-and β-D-Glcp-(1 → 4)-D-Glcp)

A typical time course of maltose oxidation by the system 1,4-benzoquinone–PDH in a non-buffered solution as monitored by HPLC is shown in Fig. 5(a). Formation of intermediate compound(s) (peak III) and final accumulation of a major (peak IV) and a minor (I) end-product is obvious.

The spectral data obtained for biotransformation of maltose (Supplementary Table 3) and analyzed using the above NMR approach evidence a multiple oxidation pattern of the substrate. The major reaction product corresponds to C-2,3 dioxidation to 2,3′-didehydromaltose (**5**, α-D-*ribo*-hexopyranosyl-3-ulose-(1 → 4)-D-*arabino*-hexos-2-ulose) detected in its four major equilibrium forms **5a**–**5d** (Fig. 6). The $[M+Na]^+$ ion at *m*/*z* 361.3 in ESI MS is a further evidence for this double oxidation. Minor signals were assigned to maltose C-3′ monooxidation to intermediate 3′-dehydromaltose (Fig. 6, **6a**, **6b**, α-D-*ribo*-hexopyranosyl-3-ulose-(1 → 4)-D-glucose), and C-2 monooxidation to intermediate 2-dehydromaltose (**7**, α-D-glucopyranosyl-(1 → 4)-D-*arabino*-hexos-2-ulose). Products of maltose C-1 monooxidation (maltobionic acid, **9**) and C-1,3′ dioxidation, 3′-dehydromaltobionic acid (**8**, α-D-*ribo*-hexopyranosyl-3-ulose-(1 → 4)-D-gluconic acid) were also detected (see Supplement for details).

Obviously, the reaction mixture analyzed at the stage when ∼2/3 of the initial substrate was consumed exhibited high complexity, with five products (in multiple isomeric forms) detected. As seen from Fig. 5(a), the composition of the reaction mixture was substantially simplified at the end of the conversion with products of double oxidation **5** and **8** being accumulated. Products of maltose C-2 and C-3′ monooxidations were apparently unresolved, as were the products of C-1 mono- and C-1,3′ double oxidation. C-1, C-2 and C-3′ mono oxidations proceeded simultaneously, with the C-2/C-3′ attack taking also place on the corresponding product of the primary oxidation. Conversion was completed in 8 h, yielding 86% of **5** and 14% of **8**.

To obtain additional evidence, the maltose reaction mixture from the semipreparative transformation was treated with *N*,*N*-diphenyl hydrazine (DPH) and the main hydrazone products were isolated. Structure **10** (Fig. 6) was elucidated for the prevailing compound. The second hydrazone obtained contained

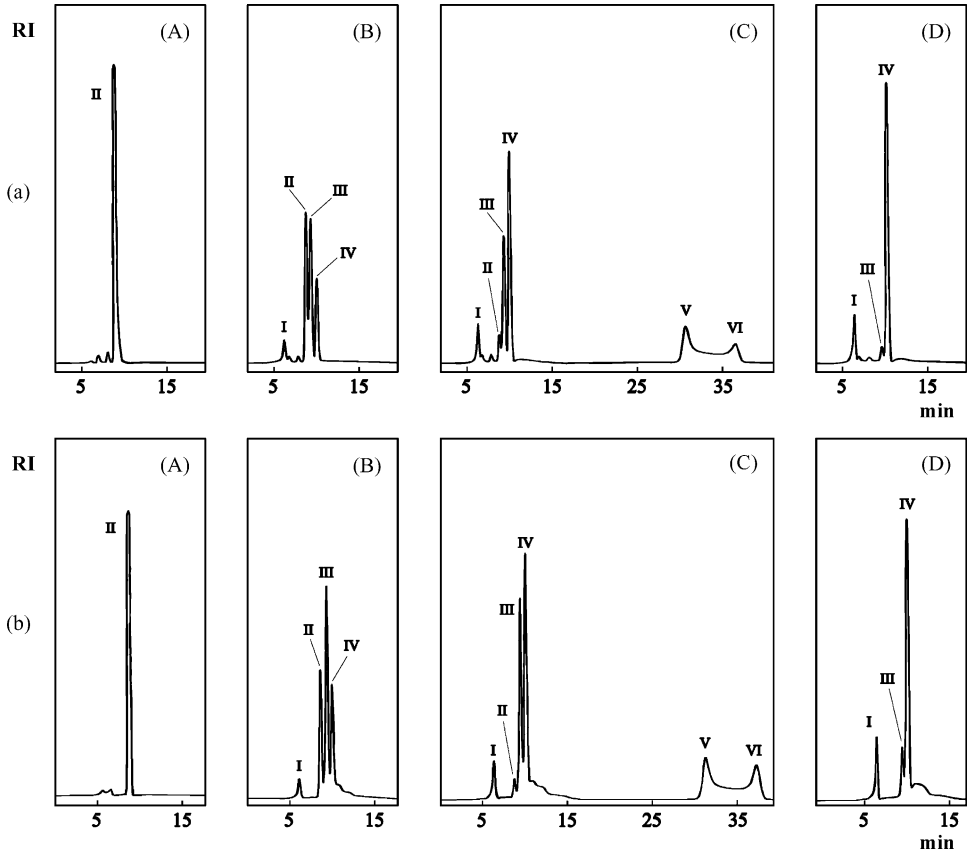


Fig. 5. HPLC monitoring of maltose (a) and cellobiose (b) oxidation by pyranose dehydrogenase from *A. meleagris* at incubation times of (a): 0 h (A), 0.5 h (B), 1.5 h (C) and 8 h (D); (b): 0 h (A), 0.5 h (B), 1 h (C) and 5 h (D), respectively. Peaks: I, (3′-dehydro)-maltobionic/cellobionic acid (**8/9/13**, hydrolysis products of the corresponding lactones); II, maltose/cellobiose; III, 3′-/2-dehydromaltose (**6/7**); 3′-/2-dehydrocellobiose (structures not shown); IV, 2,3′-didehydro-maltose/cellobiose (**5/12**); V, benzoquinone; VI, hydroquinone.

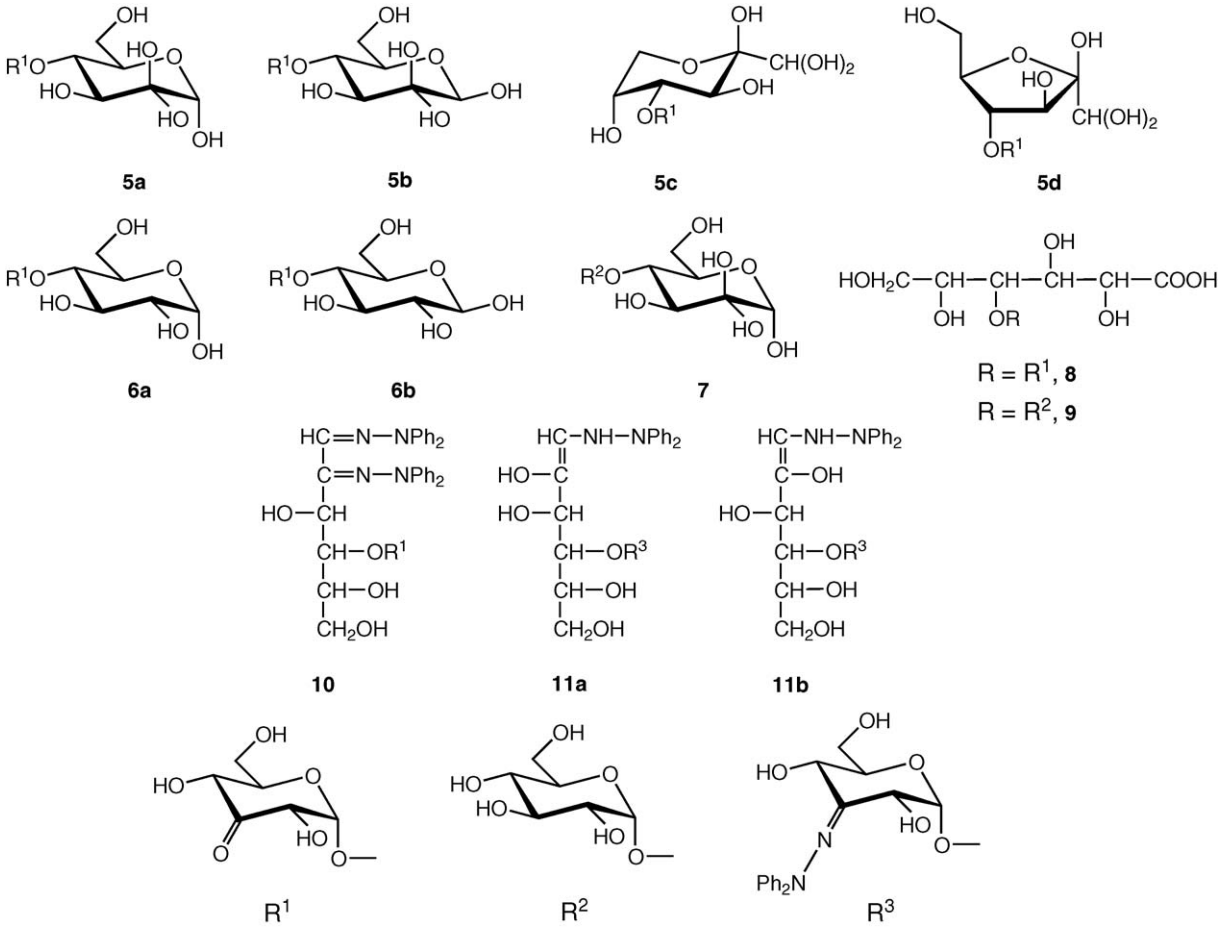


Fig. 6. Structures of detected forms of maltose oxidation products: 2,3′-didehydromaltose (**5**), 3′-/2-dehydromaltose (**6/7**), 3′-dehydromaltobionic acid (**8**), maltobionic acid (**9**). **10** and **11** (in two *cis*/*trans* isomers) illustrate structures of the isolated major hydrazone derivatives of **5**.

isomeric components **11a** and **11b**. For the NMR data and the spectra interpretation, see Supplementary Table 4. The demonstrated structures of the DPH-derivatives thus independently proved the prevailing oxidation of maltose at C-2 of the reducing end and C-3′ of the glucosyl moiety.

HPLC monitoring of cellobiose oxidation by PDH (Fig. 5(b)) gave substantially the same spectrum and relative proportions of the corresponding transient and final products as that with maltose (Fig. 5(a)). Consequently, the type of 1 → 4 glycosidic linkage (α/β) has no effect on what position of the disaccharide is oxidized. The corresponding final dioxidation products were obtained in yields of 83% and 17% in the 8-h reaction mixture. Also the NMR picture of cellobiose transformation is similar to that of maltose. Simultaneous C-2 and C-3′ oxidations followed by C-2,3 dioxidation result in the structure of 2,3′-didehydrocellobiose (Fig. 7, **12a**–**h**, β-D-*ribo*-hexopyranosyl-3-ulose-(1 → 4)-D-*arabino*-hexos-2-ulose; spectral data not shown) of the major final product. The mass spectrum, especially the $[M + Na]^+$ ion at *m*/*z* 361.3, is also consistent with this deduction. A HMBC evidence for the presence of C-1,3′ dioxidation (**13**, 3′-dehydrocellobionic acid) was also found. Despite the lower initial concentration of α-cellobiose, its H-1α signal persisted in the ^{1}H NMR spectrum hours after that of H-1β had disappeared. Therefore, *A. meleagris* PDH exhibits similar anomeric preference to β-cellobiose as cellobiose dehydrogenase from *Phanerochaete chrysosporium* [21].

Bioconversions of various disaccharides including maltose and cellobiose to 3′-dehydro derivatives with the carbonyl at the glycosyl moiety have been reported for D-glucoside 3-dehydrogenase (EC 1.1.99.13) from *Agrobacterium tumefaciens* [22,23] and a marine bacterium of *Halomonas* sp. [24]. C-2 oxidation at the reducing terminus of disaccharides has only been reported for some 1 → 6 hexose dimers (gentiobiose, isomaltose and melibiose) as substrates of pyranose oxidase [25], and for oxidation of lactose by PDH [7]. C-2,3′ dioxidation of 1 → 4 glucose dimers by PDH seems unique because pyranose oxidase does not use them as substrates [6] and glucoside 3-dehydrogenase was not reported to catalyze oxidation at the reducing end of disaccharides. Also, a search in the Chemical Abstract database did not reveal 2-dehydroderivatives of these compounds at the reducing end as well as 2,3′-didehydroderivatives with C-3′ carbonyl at the glucosyl moiety.

Introducing specific sites for chemical synthesis (carbonyl functions) into these disaccharides, which are easily available from natural resources, presents a challenge for their valorisation as new building blocks in carbohydrate chemistry. This may be, for example, the synthesis of surfactants and polymers as has been suggested before for 3-dehydrosucrose

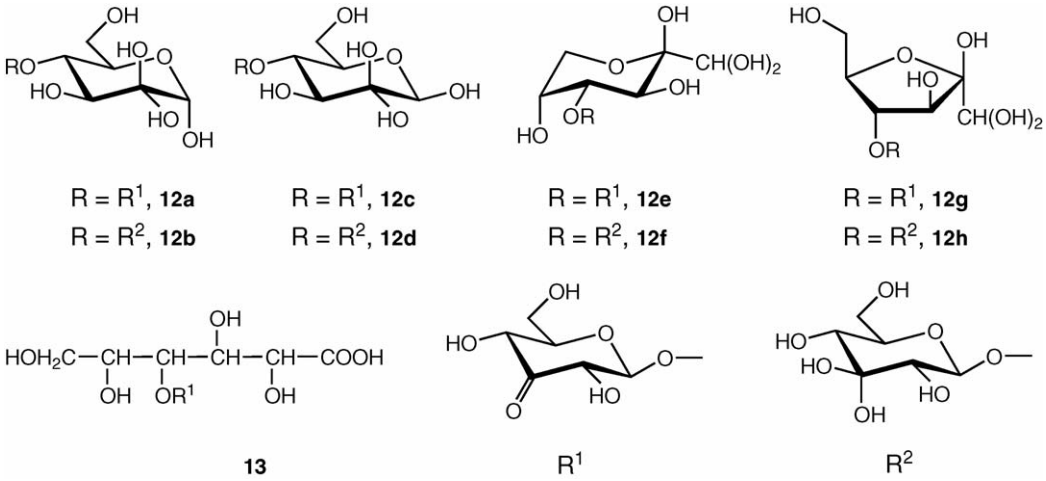


Fig. 7. Structures of detected forms of cellobiose oxidation products: 2,3′-didehydrocellobiose (**12**), 3′-dehydro-cellobionic acid (**13**).

(β-D-fructofuranosyl 3-dehydro-α-D-allopyranoside) and other related compounds [26,27].

3.5. Conversion of maltotriose

HPLC analysis was not performed in this case. The changes found by NMR (Supplementary Table 5) during the biotransformation of maltotriose were explained by competing C-3″ and C-1 oxidations producing compounds **14a**, **14b** and **15** (Fig. 8). The middle sugar unit was unchanged, according to COSY and 1D-TOCSY. This deduction is supported by the $[M+Na]^+$ ion at *m/z* 525.4 in ESI mass spectrum. Low intensity proton singlets might be attributed to the trace product of C-2,3″ dioxidation. With the exception of the compound **15**, our search in the literature for the above maltotriose oxidation products was unsuccessful.

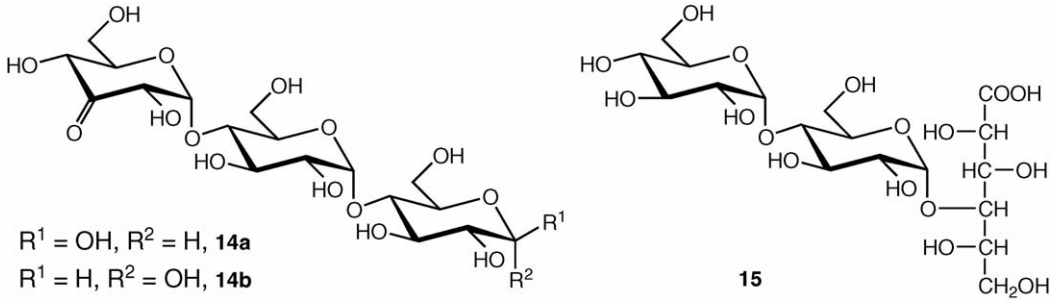


Fig. 8. Structures of detected forms of maltotriose oxidation products: 3″-dehydromaltotriose (**14**), maltotrionic acid (**15**).

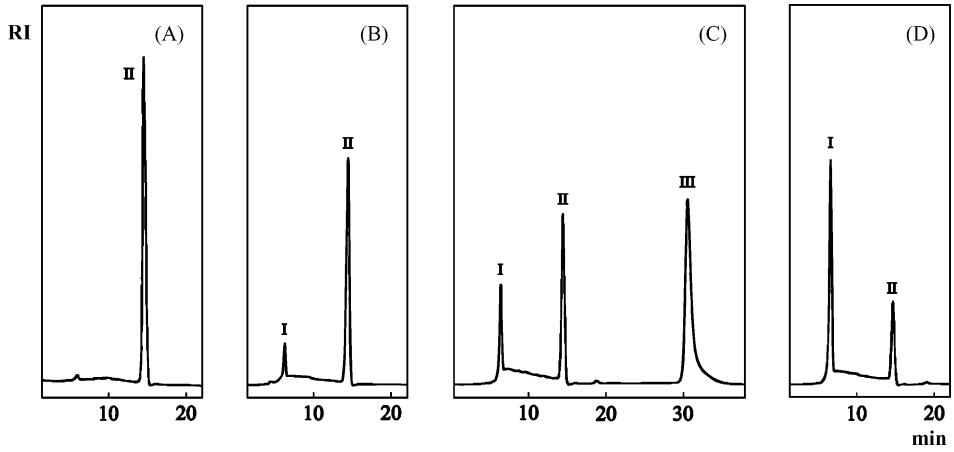


Fig. 9. HPLC monitoring of D-ribose oxidation using pyranose dehydrogenase from *A. meleagris* at incubation times of 0 h (A), 0.5 h (B), 3 h (C) and 18 h (D), respectively. Peaks: I, D-ribonic acid; II, D-ribose; III, benzoquinone/hydroquinone.

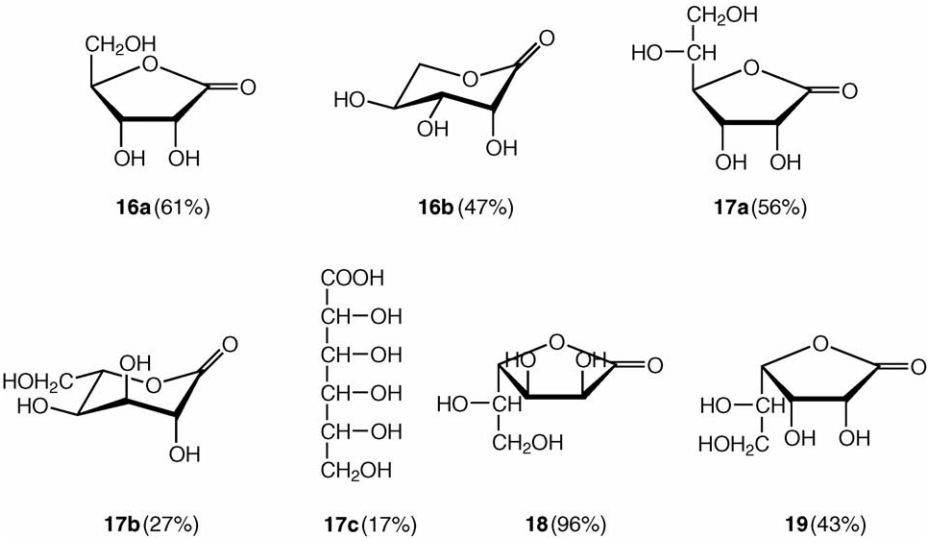
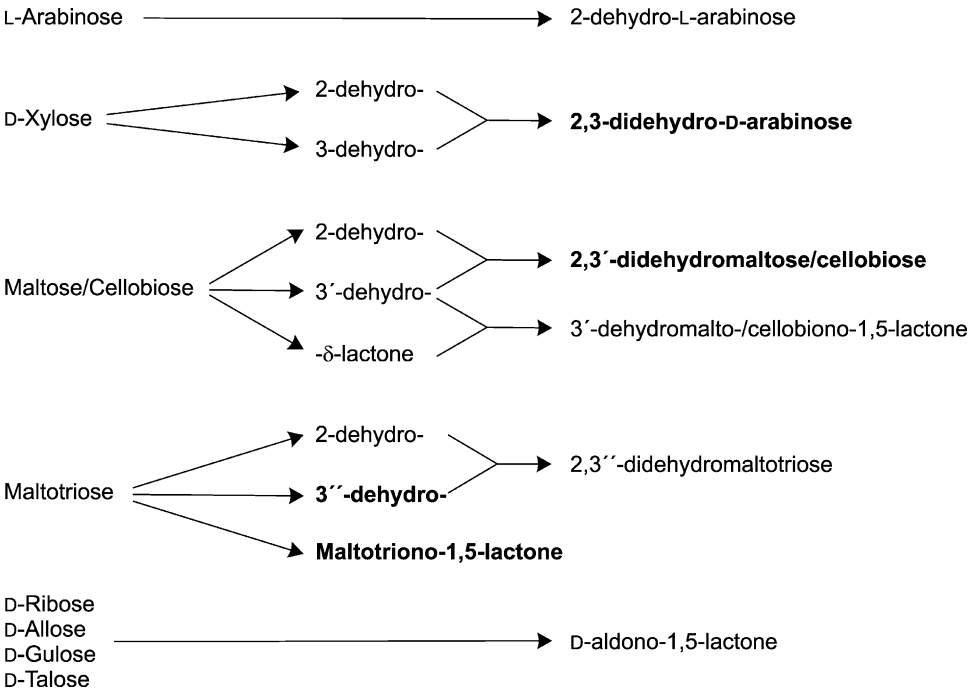


Fig. 10. Structures of detected forms of oxidation products of D-ribose, D-allose, D-talose and D-gulose, aldonolactones **16**–**19**, respectively.

3.6. *Conversions of* D-*ribose,* D-*allose,* D-*gulose, and* D-*talose*

Chromatograms taken during the time course of the transformation of 10 mM D-ribose by PDH (Fig. 9) indicated formation of one reaction product. Analogous chromatographic patterns were obtained with D-allose, D-gulose and D-talose transformed. The reaction rate of unbuffered transformations decreased with time due to gradual acidification of the reaction mixture (pH ∼2.5 at the end) as the spontaneous hydrolysis of the produced lactones took place.

The $[M+Na]^+$ ions observed in the mass spectra at *m/z* 180.1 (ribose) and *m/z* 201.1 (allose, talose, gulose) indicated a monooxidation. Analysis of the ^1H and ^{13}C NMR spectral data (Supplementary Table 6) of the biotransformation products provided a clear evidence of the C-1 oxidation; one to three equilibrium forms (1,4- , 1,5-lactone, aldonic acid) were detected with individual products. In 24-h incubations, substrate conversions reached up to 90% (43% for D-gulose). 1,4-Lactones prevailed among the products. Percentage content of the major product isoforms is given in Fig. 10 (**16**, D-ribonolactone; **17**, D-allonolactone/allonic acid; **18**, D-talonolactone; **19**, D-gulonolactone).



Scheme 1. Summary of the analyzed biotransformations of selected reducing sugars by pyranose dehydrogenase from *Agaricus meleagris*.

Compared to dicarbonyl sugar-producing carbohydrate oxidoreductases (PDH, pyranose oxidase), enzymes catalyzing the C-1 oxidation of free aldoses are more common in microbial metabolism. While glucose oxidase (EC 1.1.3.4, β-D-glucose:O_2 1-oxidoreductase) is specific for D-glucose, carbohydrate oxidase (*Microdochium nivale*) shows a broader substrate tolerance towards aldoses [28]. Similarly, NAD(P)-dependent aldose dehydrogenases (EC 1.1.1.113–EC 1.1.1.122), and glucose oxidoreductases EC 1.1.5.2 (ubiquinone) and EC 1.1.99.10 (acceptor), all catalyze exclusively the C-1 oxidation of aldoses, and show variable substrate specificity for reducing sugars (D-allose, D-galactose, D-glucose, L-arabinose, D-xylose, cellobiose and maltose) that were so far identified as substrates for PDH (Table 1). We could not identify any precedent in the literature for product identification of the enzymatic C-1 oxidation of non-derivatized D-talose and D-gulose. The above mentioned conversion of cellobiose to cellobionolactone → cellobionic acid, the minor intermediate product of PDH activity towards the substrate, is also known to be catalyzed by other enzymes, cellobiose dehydrogenase (EC 1.1.99.18, cellobiose:acceptor 1-oxidoreductase) from a number of fungi [29], carbohydrate oxidase from *M. nivale* [28] and glucooligosaccharide oxidase from *Acremonium strictum* acting also on maltooligosaccharides [30].

The newly reported above biotransformations by PDH with benzoquinone as an oxidant are summarized in Scheme 1 (main reaction products are marked in bold).

Our preliminary experiments indicate that in the case when multiple reaction products are formed, relative proportions of the reaction components can be substantially controlled by modifying reaction conditions such as pH, substrate concentration, temperature and reaction time. This is subject of our further study aiming at elaborating optimized biotransformation protocols for individual reaction products of interest.

4. Conclusions

This study enhances our knowledge of the biotransformation potential of the fungal enzyme pyranose dehydrogenase and provides access to new interesting carbohydrate structures displaying reactive vicinal carbonyl functionalities (glycosuloses). Specifically, this work offers for the first time enzymatic procedures for conversion of L-arabinose to its 2-dehydroderivative **1**, and maltose or cellobiose to the corresponding 2,3′-didehydroderivatives **5** or **13** in high yields. Products of C-2,3 (D-xylose) and C-1 (D-ribose, D-allose, D-gulose, D-talose) (di)oxidations are also easily available. New di- and tricarbonyl sugar derivatives may present useful synthons for a variety of chemical procedures.

Our previous findings together with the new data (Table 1, Scheme 1) show that the broad substrate tolerance of PDHs is accompanied with variable regioselectivity, i.e. oxidation at C-1 through C-4 is possible depending on the saccharide structure. Moreover, some substrates are sequentially double oxidized at C-2,3 or C-3,4. Consequently, product(s) of each new PDH substrate should be examined individually, before minimum structural requirements for substrates and a substrate model can be established.

In situ NMR spectroscopy used here for monitoring sugar redox conversions provides straightforward structural information of a mixture of multiple reaction components that are formed during the enzymatic reaction without need of their chemical processing (such as derivatization to hydrazones) and subsequent separation prior to analysis.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2006.04.004.

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