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C-3 oxidation of non-reducing sugars by a fungal pyranose dehydrogenase: spectral characterization

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Dedicated to Prof. Joachim Thiem on the occasion of his 60th birthday

Abstract

The quinone-dependent pyranose dehydrogenase (PDH) purified from mycelial extracts of the basidiomycete fungus *Agaricus meleagris* catalyzed oxidation of the non-reducing oligosaccharides sucrose, melezitose and erlose at C-3 of their terminal glucopyranosyl moiety while α,α-trehalose was double oxidized at both C-3 and C-3′. Analogously, using MS and in situ NMR spectroscopy, formation of C-3 carbonyl derivatives was also demonstrated with methyl-α-D-Glc*p*, methyl-β-D-Glc*p* and methyl-α-D-Gal*p*. Yields of conversions performed at room temperature with 10 mM sugars and 1,4-benzoquinone as an electron acceptor in non-buffered systems were ≥90% within 3–20 h, depending on substrate.

Keywords: Dicarbonyl sugars; Keto sugars; Pyranose dehydrogenase; Sucrose; Trehalose

1. Introduction

Enzymes catalyzing oxidation of free (non-phosphorylated) saccharides to carbonyl derivatives are becoming increasingly important as biocatalysts for a variety of sugar transformations into useful products or building blocks for organic synthesis [1,2]. This is based on the fact that certain sugars represent a relatively inexpensive renewable and so far underutilized raw materials which are often available in bulk scale with high purity. Their reactive carbonyl derivatives were suggested as key intermediates in for example biotechnical processes for isomerization aldose → ketose, such as conversion of D-glucose to D-fructose via 2-keto-D-glucose [3,4] and D-galactose to D-tagatose via 2-keto-D-galactose [5,6]. In this two-step processes, the first enzymatic step involving pyranose oxidase (pyranose:oxygen 2-oxidoreductase, EC 1.1.3.10) of some wood degrading basidiomycete fungi is coupled with either chemical or enzymatic C-1 reduction of the intermediate carbonyl compound.

Another enzyme recognized to act at secondary hydroxyl functions of aldopyranose sugars is quinone-dependent pyranose dehydrogenase (PDH) recently

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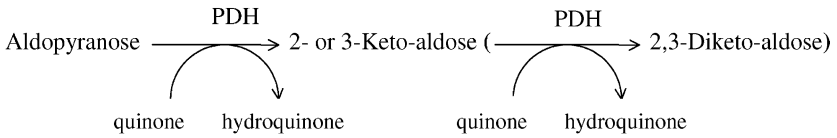

Fig. 1. Catalytic reaction scheme for oxidation of aldoses by PDH of *Agaricus* species.

detected and partially characterized from mycelial cultures of the common mushroom *Agaricus bisporus* [7,8].

Since this sugar oxidoreductase exhibits extremely broad substrate tolerance and regioselectivity (Fig. 1), it was of interest to investigate whether its unique catalytic properties offer convenient access to new keto-sugars unattainable through pyranose oxidase activities. Apart from the ability to act on monosaccharides with axial hydroxyl at C-2 (e.g. D/L-arabinose, D-mannose) and 1,4-glucooligosaccharides, other features distinguishing PDH from P2O are its preferred C-3 attack of D-glucose, high degree of glycosylation and single polypeptide structure, unlike homotetrameric pyranose 2-oxidase. To date, only the reaction products of PDH with of D-glucose [7,8], D-galactose [8] and D-xylose [9] have been identified and shown to be single or double oxidized with variable C-2 and C-3 regioselectivity depending on the substrate.

The aim of the present work was to demonstrate oxidative activities towards non-reducing sugars by PDH purified from *A. meleagris*, which also represents a new source of the enzyme. Structures of the reaction products derived from sucrose, trehalose, melezitose, erlose, methyl-α/β-D-Glc*p* and methyl-α-D-Gal*p* were determined using MS and in situ NMR spectroscopy and shown to possess ketonic carbonyl exclusively at C-3 of their glucopyranosyl moiety. The straightforward NMR and MS identification of the non-reducing sugar derivatives was made feasible due to the relatively simple composition of the reaction mixtures with the PDH/benzoquinone oxidant which, unlike those of reducing oligosaccharides, gave only one reaction product (with exception of trehalose) and did not produce a complex equilibrium mixture of anomeric/cyclic-tautomeric isoforms. *A. meleagris* PDH shows relatively high affinity to oligosaccharides and was selected for the sugar transformation experiments on the basis of extensive screening for the enzyme among basidiomycete fungi [10].

2. Experimental

2.1. Enzyme source and production

A. meleagris Schaeff (strain CCBAS 907) obtained from the Culture Collection of Basidiomycetes (CCBAS), Institute of Microbiology, Prague, Czech Republic, was grown for 40 days at 28 °C in stationary cultures on liquid glucose-corn steep medium [8] (50 ml per 500 ml Roux flask). Mildly homogenized 30-day-old 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 determined by following the D-glucose-dependent reduction of 1,4-benzoquinone (Sigma, Prague, Czech Republic) to hydroquinone at 290 nm for 1 min in the assay mixture (2 ml) containing 100 μmol sodium citrate, pH 4.5, 50 μmol D-glucose, 4.6 μmol 1,4-benzoquinone and PDH sample [10]. One unit (U) of the enzyme activity was defined as the amount of activity that reduced 1 μmol benzoquinone per min at 25 °C in the above assay system ($\varepsilon_{290} = 2.3\,\mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$).

Intracellular PDH was purified using a modified protocol reported earlier for the *A. bisporus* enzyme [8]. Wet mycelium (25 g) was homogenized using an Ultra Turrax (IKA, Staufen, Germany, full speed, 6 min) in 170 ml 20 mM sodium phosphate, pH 7.0, containing 2 mM Pefablock (Boehringer, Mainnheim, Germany). The supernatant resulting from centrifugation at 20,000 × *g* for 30 min was loaded onto a DEAE-Sephacell (Pharmacia, Uppsala, Sweden) 10 cm × 1.5 cm column equilibrated with the same buffer. The bound protein was eluted with 200 mM NaCl in the same buffer and the active PDH eluate (21 ml) supplemented with solid $(NH_4)_2SO_4$ to 1.5 M. Proteins were further fractioned on Phenyl-Superose

HR 5/5 (Pharmacia) using a gradient (20 ml) of 0–90% 20 mM sodium phosphate, pH 7.0, in the equilibration buffer (1.5 M $(NH_4)_2SO_4$ in the buffer). The PDH recovered was transferred by diafiltration into 20 mM bisTris–HCl pH 5.8 (1.5 ml) and applied onto a Mono Q HR 5/5 column (Pharmacia) that was subsequently eluted with 0–35% gradient (22 ml) of 1 M NaCl. Final purification of the recovered enzyme was performed on a Superose 12 HR 10/30 column (Pharmacia) using 50 mM sodium phosphate, pH 7.0, containing 100 mM NaCl.

2.3. Bioconversions

PDH from the final purification step was transferred into 10 mM sodium phosphate, pH 7.0, by diafiltration and concentrated (24 U ml^{-1}) in a microcentrifuge filter unit (YM 10 membrane, Millipore, Bedford, MA) before adding to the reaction mixture. Non-reducing sugars tested were: sucrose, α,α-trehalose, erlose [β-D-Fru*f*-(2 ↔ 1)-α-D-Glc*p*-(4 → 1)-α-D-Glc*p*], melezitose [α-D-Glc*p*-(1 → 3)-β-D-Fru*f*-(2 ↔ 1)-α-D-Glc*p*], methyl-α/β-D-glucopyranoside and methyl-α-D-galactopyranoside, all from Sigma.

In the NMR study of PDH catalysis, sugar transformations were monitored directly in an NMR sample tube (5 mm) containing non-reducing sugar (10 mM), 1,4-benzoquinone (25 mM) and 50 mU (methyl-α-D-Glc*p*, methyl-β-D-Glc*p*, sucrose) or 100 mU PDH (trehalose, erlose, melezitose, methyl-α-D-Gal*p*) in deuterium oxide (0.7 ml).

Sugar transformations for HPLC analysis were carried out under air in the dark at 30 °C under gentle stirring in stoppered 5 ml flasks containing 10 mM sugar substrate, 1,4-benzoquinone (20 mM, 2 × 5.4 mg, the second quantity added after 1 h incubation) and 2 U purified PDH (thoroughly desalted on a YM 30 ultrafiltration membrane) in 5 ml deionized H_2O. Samples of the reaction mixture (60 μl) withdrawn at time intervals were deproteinized by passing through Ultrafree-MC 30,000 NMWL filter units (Millipore) and diluted twice with H_2O prior to HPLC analysis.

2.4. Analytical methods

HPLC of sugar transformation products was performed on a SP 8800 liquid chromatograph (Spectra Physics, San Jose, CA) fitted with a refractive index detector, using 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}).

Protein was determined by the Folin reagent [11] using bovine serum albumin as a standard.

2.5. NMR spectroscopy

NMR spectra were recorded on a Varian INOVA-400 spectrometer (400 MHz for ^1H, 100 MHz for ^{13}C) in D_2O at 30 °C. Chemical shifts were referenced to internal acetone (δ_H 2.030, δ_C 30.50). All two-dimensional NMR experiments (homonuclear two-dimensional *J*-resolved spectroscopy, HOM2DJ; correlated spectroscopy, COSY; total correlation spectroscopy, TOCSY; heteronuclear multiple quantum correlation, HMQC; heteronuclear multiple bond correlation, HMBC) were performed using the manufacturers software. The sequence for one-dimensional TOCSY experiments [12] was obtained through Varian User Library, the sequence for gradient HMBC was obtained from Varian Application Laboratory in Darmstadt (Germany). The in situ transformation reactions in NMR tubes (see above) were monitored by ^1H NMR and short COSY or HMQC experiments. After reaching a complete conversion (3–20 h), the experiments necessary for structure elucidation were performed. Aliquot samples of the final reaction mixtures were used directly for MS analysis.

2.6. Matrix-assisted laser desorption/ionization (MALDI) MS

A mixture of sinapinic and 2,5-dihydroxybenzoic acid (Sigma, 10 mg/ml, 1:1) in aqueous 50% acetonitrile/0.3% TFA was used as the MALDI matrix. A 2 μl of sample and 2 μl of matrix solution were premixed in a tube, 0.5 μl of the mixture was placed on the sample target and allowed to dry at ambient temperature. Positive ion MALDI MS were measured on a Bruker BIFLEX II reflectron time-of-flight mass spectrometer (Bruker-Franzen, Bremen, Germany) equipped with a SCOUT 26 sample inlet, a gridless delayed extraction ion source and a nitrogen laser (337 nm, Laser Science, Cambridge, MA). Ion acceleration voltage was 19 kV and the reflectron voltage was set to 20 kV. Reported spectra were

accumulated from 20 to 40 laser shots. Spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion of sinapinic acid and a peptide standard (angiotensin II, Sigma). Post-source decay (PSD) spectra were typically recorded in 7–12 segments, with each succeeding segment representing a 20% reduction in reflector voltage. About 50 shots were averaged per segment. Segments were pasted, calibrated and smoothed under computer control by Bruker XMASS 5.0 software.

3. Results and discussion

3.1. PDH catalysis

Following the four-step purification procedure, substantially homogeneous 65-fold purified PDH was obtained in 19% yield from mycelial extracts of *A. meleagris*. The enzyme had specific activity of 14.7 U mg^{-1} protein with D-glucose/1,4-benzoquinone as electron donor/acceptor and was stable for at least 14 days during storage at 20 °C. The relative PDH activities with sugars examined, substituting for 25 mM D-glucose (100%) in the standard assay, were: methyl-α-D-Glc*p*, 126%; methyl-β-D-Glc*p*, 20%; methyl-α-D-Gal*p*, 7%; sucrose, 11%; erlose, 9%; melezitose, 7%; trehalose, 5%.

The time course of α,α-trehalose (10 mM) oxidation by PDH/1,4-benzoquinone as followed by HPLC is shown in Fig. 2. Clearly, this sugar was not converted into a single product. The primary product (peak II at retention time of 9.23 min), spectroscopically identified as 3-keto-α,α-trehalose (see below), was subsequently symmetrically oxidized at C-3′ and almost completely converted into a compound giving peak III. In contrast, the remaining sugars tested gave only one peak of a single oxidation product (not shown). When using 10 mM sugar concentrations, conversion yields ≥90% were obtained within 3–20 h depending on the substrate and in accord with the time course NMR studies. These conversion times could be substantially reduced by applying higher PDH activities in the transformation mixtures.

3.2. Structure elucidation

PDH reaction products with all the sugars tested exhibited intense $[M + Na]^+$ ions in conventional MALDI MS. To obtain additional structural information, PSD experiment allowing analysis of fragment ions was conducted. While oxidation products of methyl-α-D-Glc*p*, methyl-β-D-Glc*p* and methyl-α-D-Gal*p* (Fig. 3, compounds **1**, **2a**, **3a**) gave parent ion $[M + Na]^+$ at *m/z* 215 only, the characteristic cleavage of glycosidic bonds was observed for oxidation products of trehalose (Fig. 3, compound **4**, $[M+Na]^+$ at *m/z* 363; Fig. 4, compound **5**, $[M+Na]^+$ at *m/z* 361) and oxidation products of sucrose, erlose and melezitose (Fig. 4, $[M + Na]^+$ at *m/z* 363, 525 and 525 for compounds **6**, **7** and **8**, respectively). The

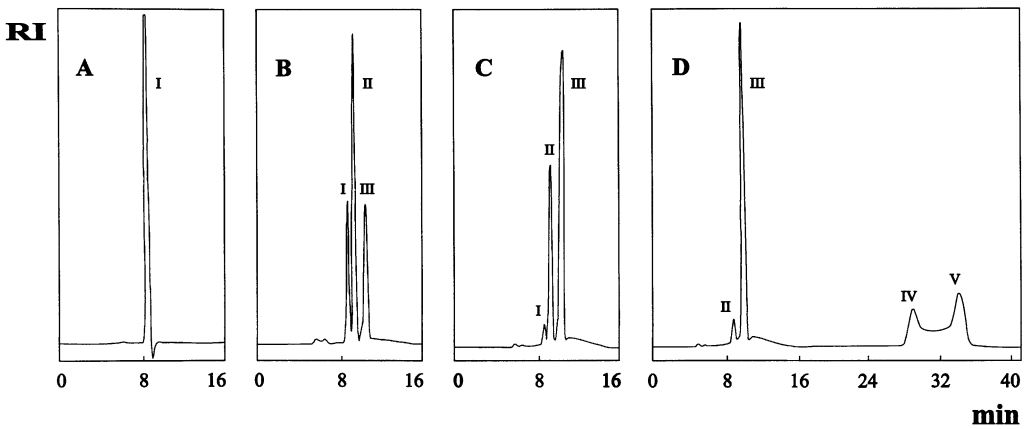

Fig. 2. HPLC monitoring oxidation of α,α-trehalose by PDH from *A. meleagris* at incubation times of 0 (A), 1 (B), 1.5 (C) and 2 h (D), respectively. Peaks: I, trehalose; II, 3-keto-trehalose; III, 3,3′-diketo-trehalose; IV, 1,4-benzoquinone; V, hydroquinone. Reaction conditions are described in Section 2.

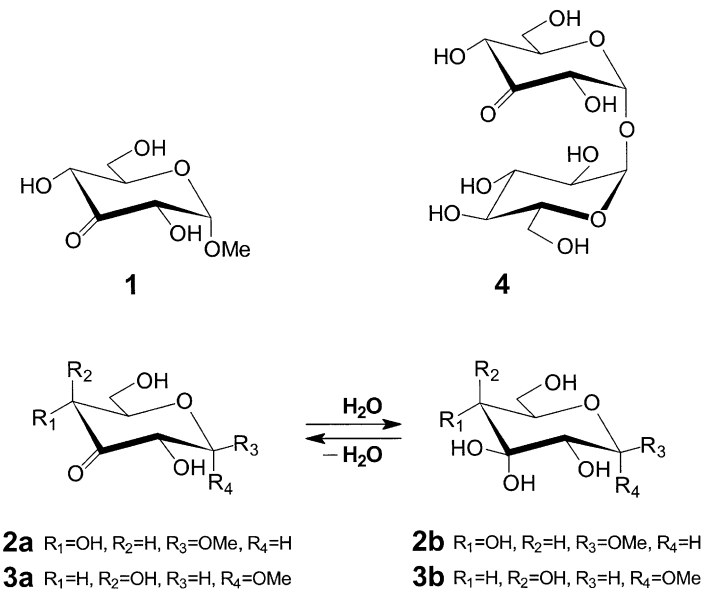

Fig. 3. Equilibrium of C-2 free carbonyl (**a**) and hydrated carbonyl (**b**) molecular forms of methyl-3-keto-β-D-Glc*p* (**2**) and methyl-3-keto-α-D-Gal*p* (**3**). Methyl-3-keto-α-D-Glc*p* (**1**); 3-keto-α,α-trehalose (**4**).

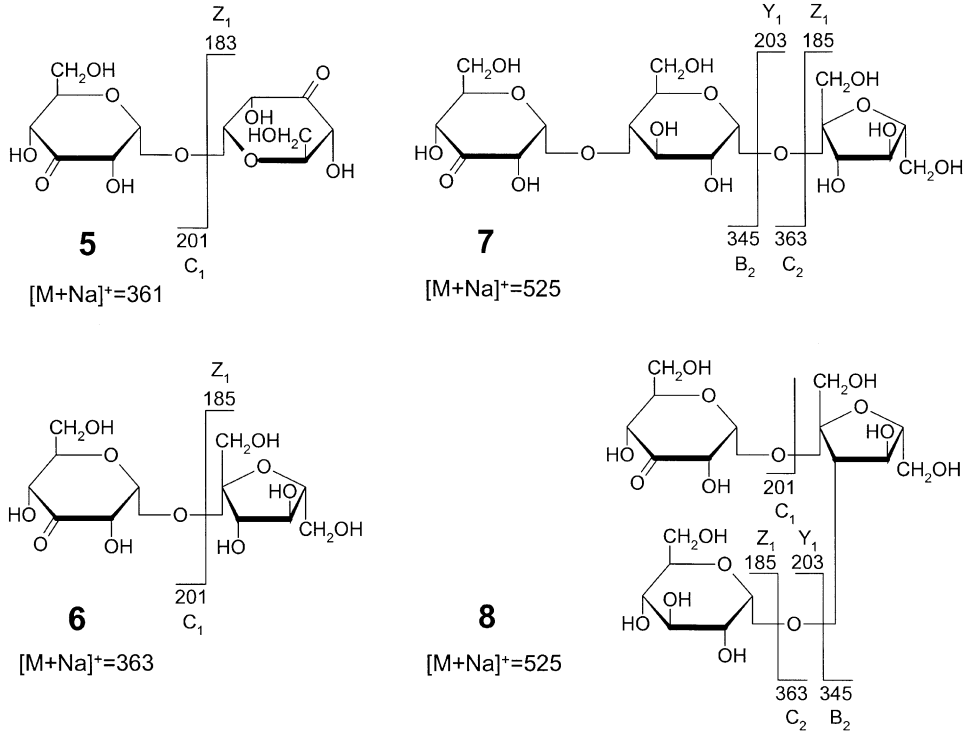

Fig. 4. Structures and PSD-MALDI MS fragmentations of 3-keto-oligosaccharides resulting in PDH/benzoquinone oxidation of selected non-reducing sugars at the α-D-glucopyranosyl moiety (all presented ions are adducts with Na^+). The ion assignment is according to the nomenclature introduced by Domon and Costello [21]: 3,3′-diketo-trehalose (**5**); 3-keto-sucrose (**6**); 3-keto-erlose (**7**); 3-keto-melezitose (**8**).

Table 1
^{1}H NMR data of C-3 oxidized methyl glycosides

Proton ($J_{i,j}$)	Chemical shifts in ppm (coupling constants in Hz)				
	1	**2a**	**2b**	**3a**	**3b**
H-1 ($J_{1,2}$, $J_{1,5}$)	4.980 (4.4, –)	4.286 (8.1, –)	4.231 (8.1, –)	4.626 (4.4, 0.5)	4.986 (4.5, 0.7)
H-2 ($J_{2,4}$)	4.391 (1.6)	4.058 (1.7)	3.114	3.669	4.775
H-4 ($J_{4,5}$)	4.173 (9.9)	4.159 (10.3)	3.258 (10.0)	3.437 (1.3)	3.984 (1.6)
H-5 ($J_{5,6d}$, $J_{5,6u}$)	3.569 (2.2, 4.6)	3.338 (2.2, 5.1)	3.366 (2.3, 5.9)	3.906 (6.2, 6.2)	3.857 (7.4, 4.8)
H-6d ($J_{6d,6u}$)	3.730 (12.5)	3.792 (12.5)	3.693 (12.3)	3.535	3.667 (11.9)
H-6u	3.654	3.634	3.501	3.535	3.584
OMe	3.203	3.413	3.345	3.201	3.201

above data indicated a mono-oxidation except for the final conversion product of trehalose (**5**) corresponding to double oxidation. NMR data (Tables 1–4) unambiguously pointed to C-3 as the site of oxidation: the signal of H-3 was absent, there was a carbonyl signal at 206–208 ppm (Tables 3 and 4), and H-2 exhibited through-the-carbonyl coupling [13] to H-4. The latter feature was common to the carbonyl form of the oxidized (3-keto-α/β-D-glycopyranosyl) moiety of all conversion products. However, this diagnostic coupling was missing in hydrated forms **2b** and **3b** of oxidized methyl-β-D-Glc*p* and α-D-Gal*p* (Fig. 3) that showed a signal of a quaternary carbon of the sp^3 type at 93–94 ppm in ^{13}C NMR spectra, characteristic

Table 2
^{1}H NMR data of α-D-Glc*p* C-3-oxidized oligosaccharides

Proton ($J_{i,j}$)	Chemical shifts in ppm (coupling constants in Hz)				
	4	**5**	**6**	**7**	**8**
β-D-Fru*f*-	–	–	(2 → 1)	(2 → 1)	(2 → 1)
H-1 ($J_{1d,1u}$)	–	–	3.428	3.447	3.512, 3.403 (12.4)
H-4 ($J_{3,4}$)	–	–	3.970 (8.6)	3.977 (8.7)	4.113 (8.2)
H-4 ($J_{4,5}$)	–	–	3.759 (8.4)	3.783 (8.5)	4.041 (8.3)
H-5 ($J_{5,6d}$, $J_{5,6u}$)	–	–	3.672 (3.1, 7.2)	3.661 (3.2, 6.9)	3.688 (3.5, 6.6)
H-6d ($J_{6d,6u}$)	–	–	3.606 (12.2)	3.593 (12.2)	3.614 (12.3)
H-6u	–	–	3.562	3.545	3.575
3-Keto-Glc*p*-	(1 ↔ 1)	(1 ↔ 1)[a]	(1 ↔ 2)	(1 → 4)	(1 ↔ 2)
H-1 ($J_{1,2}$)	5.357 (4.5)	5.361 (4.4)	5.590 (4.6)	5.603 (4.5)	3.536 (4.4)
H-2 ($J_{2,4}$)	4.481 (1.6)	4.438 (1.6)	4.404 (1.6)	4.436 (1.6)	4.446 (1.6)
H-4 ($J_{4,5}$)	4.226 (9.9)	4.204 (9.9)	4.243 (9.8)	4.226 (9.5)	4.236 (9.9)
H-5 ($J_{5,6d}$, $J_{5,6u}$)	3.807 (2.2, 4.6)	3.778 (2.2, 4.5)	3.817 (2.3, 3.5)	3.680 (1.6, 3.3)	3.875 (2.2, 4.5)
H-6d ($J_{6d,6u}$)	3.723 (12.5)	3.714 (12.6)	3.700 (12.7)	3.723 (12.6)	3.754 (12.7)
H-6u	3.652	3.641	3.665	3.656	3.671
Glc*p*-	(1 ↔ 1)	–	–	(1 ↔ 2)	(1 → 3)
H-1 ($J_{1,2}$)	3.976 (3.8)	–	–	5.176 (3.9)	5.179 (3.3)
H-2 ($J_{2,3}$)	3.885 (9.9)	–	–	3.359 (10.0)	3.313 (9.9)
H-3 ($J_{3,4}$)	3.633 (9.0)	–	–	3.768 (8.8)	3.353 (8.2)
H-4 ($J_{4,5}$)	3.210 (9.9)	–	–	3.517 (10.0)	3.174 (9.8)
H-5 ($J_{5,6d}$, $J_{5,6u}$)	ND (ND, 4.9)	–	–	3.694 (2.6, 2.8)	3.500 (2.5, 4.7)
H-6d ($J_{6d,6u}$)	ND (10.7)	–	–	3.548 (ND)	3.618 (12.2)
H-6u	3.545	–	–	3.548	3.547

ND, not determined.
[a] Two equivalent 3-keto-α-D-Glc*p*-(1 ↔ 1) moieties of **5**.

Table 3
^{13}C NMR data of C-3 oxidized methyl glycosides

Carbon	Chemical shifts in ppm				
	1	**2a**	**2b**	**3a**	**3b**
1	101.94	104.64	102.61	99.39	102.15
2	74.47	76.81	74.09	68.84	73.30
3	**207.32**	**206.66**	**94.53**	**196.46**	**93.47**
4	71.96	72.33	70.62	72.30	74.58
5	74.69	76.18	75.23	69.95	72.75
6	60.65	60.96	61.25	61.50	60.30
OMe	55.46	57.58	57.29	55.81	55.56

Chemical shifts at oxidized position are given in bold.

for a gem-diol. The localization of carbonyl group was further supported by heteronuclear couplings of H-1, H-2 and H-4 to C-3 observed in gradient-HMBC experiments [14]. The comparison of our NMR data with those published showed a good agreement for proposed structures of methyl-3-keto-β-D-Glc*p* (methyl-β-D-ribohexopyranosyl-3-ulose) (**2a**) and its hydrated form (**2b**) (Fig. 3) [15] and 3-keto-sucrose (**6**) (Fig. 4) [16]. For the two observed structural forms of **2** and **3** (Fig. 3, **2a**/2b = 2/1; **3a**/3b = 2.4/1), the reported spectroscopic characterization is based on the analysis of mixtures aided by one-dimensional TOCSY, COSY, HMQC and HMBC. The ratio **2a**/**2b** agrees with the literature [15]; for the related methyl-β-D-Gal*p*, consistently only gem-diol form was reported [15].

Time course ^{1}H NMR monitoring of trehalose biotransformation revealed the formation of an intermediate (Fig. 5). The analysis of the reaction mixture within the appropriate time window showed signals due to two different 3-keto-α-D-Glc*p* units and one Glc (different from that of parent compound). The prevailing component was symmetric and identical with the final 3,3′-diketo-derivative (Fig. 4, compound **5**), confirmed by MS and HMBC cross-peak between H-1 and C-1. Thus, the reaction intermediate was identified as 3-keto-trehalose (**4**).

Table 4
^{13}C NMR data of α-D-Glc*p* C-3-oxidized oligosaccharides

Carbon	Chemical shifts in ppm				
	4[a]	**5**	**6**	**7**	**8**
β-D-Fru*f*-	–	–	(2 → 1)	(2 → 1)	(2 → 1)
1	–	–	61.42	61.61	62.53
2	–	–	104.28	103.86	103.81
3	–	–	76.44	76.79	83.82
4	–	–	74.23	74.29	73.27
5	–	–	81.78	81.58	81.52
6	–	–	62.66	62.56	62.23
3-Keto-Glc*p*-	(1 ↔ 1)	(1 ↔ 1)[b]	(1 ↔ 2)	(1 → 4)	(1 ↔ 2)
1	96.4	96.85	95.26	102.46	91.76
2	73.8	73.89	74.12	74.90	74.69
3	**206.7**	**206.77**	**207.58**	**207.53**	**207.25**
4	71.3	71.84	71.63	71.89	72.02
5	75.5	75.56	75.72	75.98	75.74
6	60.6	60.73	60.54	60.79	60.89
Glc*p*-	(1 ↔ 1)	–	–	(1 ↔ 2)	(1 → 3)
1	93.6	–	–	92.15	103.25
2	71.1	–	–	71.03	71.18
3	72.4	–	–	72.94	73.31
4	69.7	–	–	77.07	69.85
5	72.4	–	–	70.94	72.29
6[b]	60.7	–	–	60.22	60.89

Chemical shifts at oxidized position are given in bold.
[a] HMQC and HMBC readouts.
[b] Two equivalent 3-keto-α-D-Glc*p*-(1 ↔ 1) moieties of **5**.

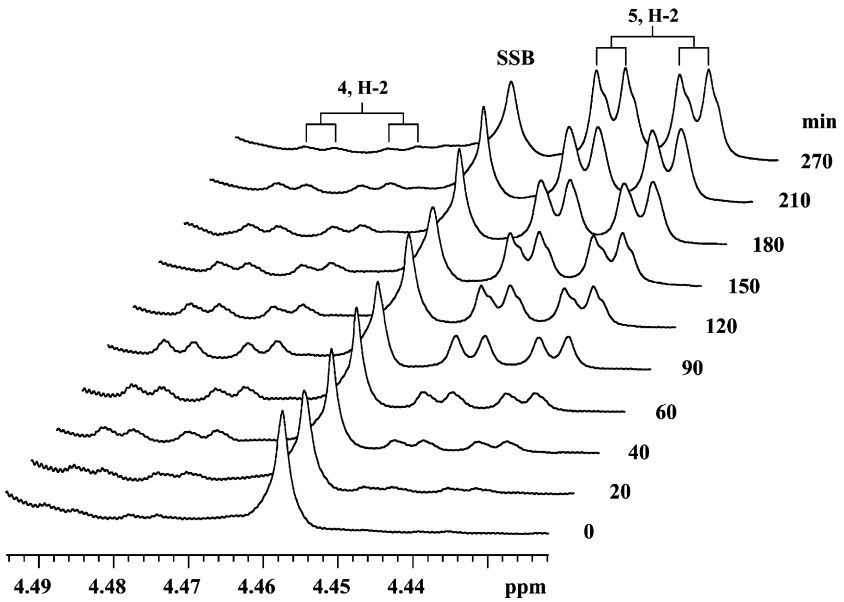

Fig. 5. Time course of trehalose biotransformation monitored by ^{1}H NMR—H-2 region: 3-keto-trehalose intermediate (**4**); 3,3′-diketo-trehalose (**5**). SSB, spinning side band of water.

With erlose and melezitose, containing two non-equivalent Glc*p* units, a problem arose as to which one of these was oxidized. The answer was obtained by HMBC. H-1 of the glucose unit that was oxidized in erlose is coupled to the ketone carbon (C-3) and to C-4 of the unmodified Glc*p*. Furthermore, H-1 of the non-oxidized Glc*p* is coupled to fructose C-2. Therefore, the structure **7** was assigned to the respective oxidation product. Similarly for oxidized melezitose, H-1 of the transformed Glc*p* is coupled to the ketone carbon (C-3) and H-1 of the second Glc*p* is coupled to C-3 of Fru*f* in accord with the structure **8**. MS fragmentation pattern resulting from the PSD experiments (Fig. 4) confirms the above deduction.

3.3. C-3 oxidation of non-reducing sugars by PDH

Based on results of HPLC analyses and the structures of sugar oxidation products deduced from the above NMR and MS data, we demonstrate that PDH oxidized the non-reducing di- and trisaccharides exclusively at C-3 of their terminal α-D-glucopyranosyl moiety to the corresponding glycopyranosyl 3-keto-D-glucopyranosides (glucopyranosid-3-uloses, Fig. 6). Analogously, using 1,4-benzoquinone as an electron acceptor, PDH oxidized methyl-α/β-D-Glc*p* and methyl-α-D-Gal*p* to the corresponding 3-keto-derivatives (methyl-hexopyranosid-3-uloses). Of the two terminal α-D-glucopyranosyl residues of melezitose, the one bound 1 → 3 to β-D-fructofuranosyl unit was unexpectedly unattacked.

High specificity of *A. meleagris* PDH for the C-3 of glycosidically bound D-glucose contrasts with simultaneous C-2 and C-3 oxidation of free D-glucose by this enzyme [10] and PDH from *A. bisporus* [8] and the primary C-2 attack of free D-galactose [8] and D-xylose [9] by *A. bisporus* PDH. Regioselective C-3 oxidation of methyl-β-D-Glc*p* to **3** (in addition to methyl-β-D-Gal*p*, which was not the PDH substrate) was reported for the related enzyme pyranose oxidase of the basidiomycete *Peniophora gigantea*, while this enzyme oxidized free D-glucose at C-2 only [14]. Thus, proximity of the *O*-glycosidic bond apparently prevents (i.e. sterically) C-2 oxidation by pyranose oxidoreductases, making exclusive C-3 attack possible.

3-Keto disaccharides **4** and **6** were first identified as conversion products of α,α-trehalose and sucrose

R	Substrate	Product
α-D-Glc*p*-(1↔1)	Trehalose	3-Keto-trehalose (**4**)
3-Keto-α-D-Glc*p*-(1↔1)	3-Keto-trehalose	3,3'-Diketo-trehalose (**5**)
β-D-Fru*f*-(2↔1)	Sucrose	3-Keto-sucrose (**6**)
β-D-Fru*f*-(2↔1)-α-D-Glc*p*-(4→1)	Erlose	3-Keto-erlose (**7**)
α-D-Glc*p*-(1→3)-β-D-Fru*f*-(2↔1)	Melezitose	3-Keto-melezitose (**8**)

Fig. 6. C-3 oxidation of non-reducing oligosaccharides at terminal α-D-Glc*p* moiety by PDH purified from *A. meleagris*.

by intact cells of *Agrobacterium tumefaciens*; the enzyme responsible for the catalysis, D-glucoside 3-dehydrogenase (hexopyranoside:cytochrome *c* oxidoreductase, EC 1.1.99.13) was subsequently isolated from this plant tumor-inducing bacterium [17,18] and *Flavobacterium saccharophilum* [19], and characterized as an iron–sulfur flavoprotein acting with cytochrome *c* as an electron acceptor. Its reaction product with methyl-α-D-Glc*p* was identified as 3-keto-glycoside **1** by GC–MS [20]. Applied aspects of this enzyme have been studied for **6** which was shown to be an appropriate and versatile synthon for regioselective synthesis [16].

4. Conclusions

PDH catalysis with 1,4-benzoquinone as an oxidant provides biocatalytic sugar chemistry with a new convenient tool for high yield production of the above 3-keto-oligosaccharides (oligosaccharide-3-uloses) and 3-keto-glycosides. To our knowledge, this is the first reported identification of 3-keto- (3-dehydro-) derivatives of methyl-α-D-Gal*p*, erlose and melezitose (compounds **3a**, **7** and **8**, respectively) and double oxidation product of α,α-trehalose (**5**). Considering the extremely broad substrate tolerance and easily available co-factor (1,4-benzoquinone) of quinone-dependent PDH, its good stability and modified properties (substrate selectivity) depending on the fungal source [10], this sugar oxidoreductase offers an attractive possibility to access a variety of non-reducing oligosaccharides and glycosides modified (C-3 carbonyl functionality) at their terminal α-D-glucopyranosyl residue(s).

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References

[1] F.W. Lichtenthaler, S. Himmel, D. Martin, V. Müller, in: G. Descotes (Ed.), Carbohydrates as Organic Raw Materials, Vol. 2, VCH Publishers, Weinheim, 1993, p. 59 (Chapter I.4).
[2] F. Giffhorn, S. Köpper, A. Huwig, S. Freimund, Enzym. Microb. Technol. 27 (2000) 734.
[3] S.L. Neidleman, W.F. Amon, J. Geigert, European Patent 88,103 (1981).
[4] C. Leitner, W. Neuhauser, J. Volc, K.D. Kulbe, B. Nidetzky, D. Haltrich, Biocatal. Biotransform. 16 (1998) 365.
[5] D. Haltrich, C. Leitner, W. Neuhauser, B. Nidetzky, K.D. Kulbe, J. Volc, Ann. N.Y. Acad. Sci. 864 (1998) 296.
[6] S. Freimund, F. Giffhorn, A. Huwig, S. Köpper, J. Carbohydr. Chem. 15 (1996) 115.
[7] J. Volc, E. Kubátová, D. Wood, G. Daniel, Arch. Microbiol. 167 (1997) 119.
[8] J. Volc, P. Sedmera, P. Halada, V. Pikrylová, G. Daniel, Carbohydr. Res. 310 (1998) 151.
[9] J. Volc, P. Sedmera, P. Halada, V. Přikrylová, D. Haltrich, Carbohydr. Res. 329 (2000) 219.
[10] J. Volc, E. Kubátová, G. Daniel, P. Sedmera, D. Haltrich, Arch. Microbiol. 176 (2001) 178.
[11] E.F. Hartree, Anal. Biochem. 48 (1972) 422.
[12] D. Uhrín, P. Barlow, J. Magn. Reson. 126 (1997) 248.

[13] L.M. Jackman, S. Sternhell, Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry, 2nd Edition, Pergamon Press, Oxford, 1969, p. 338.
[14] R.E. Hurd, B.K. John, J. Magn. Reson. 91 (1991) 648.
[15] S. Freimund, A. Huwig, F. Giffhorn, S. Köpper, Chem. Eur. J. 4 (1998) 2442.
[16] M. Pietsch, M. Walter, K. Buchholz, Carbohydr. Res. 254 (1994) 183.
[17] K. Hayano, S. Fukui, J. Biol. Chem. 242 (1967) 3665.
[18] J. Van Beeumen, J. De Ley, Eur. J. Biochem. 6 (1968) 331.
[19] M. Takeuchi, N. Asano, Y. Kameda, K. Matsui, Agric. Biol. Chem. 52 (1988) 1905.
[20] M. Takeuchi, N. Asano, Y. Kameda, K. Matsui, J. Biochem. 99 (1986) 1571.
[21] B. Domon, C. Costello, Glycoconjugate J. 5 (1988) 397.