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In situ proton NMR of glycosidase catalyzed hydrolysis and reverse hydrolysis

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Abstract

In situ proton NMR is used to investigate sweet almond (*Prunus dulcis*) glycosidase catalyzed hydrolysis and reverse hydrolysis. Time courses of six disaccharide hydrolyses indicate that almond protein extract contains fairly high amounts of β-glucosidase and β-galactosidase, while α-glucosidase activity is negligibly low. For the first time the Michaelis Menten constant (K_m) and maximal reaction rate (V_{max}) of a biotransformation are determined by in situ proton NMR. The high stability index of the regression line in Lineweaver Burk plot shows the general applicability of this analytical method in kinetic investigations. Glycosidase catalyzed reverse hydrolyses are performed using glucose and galactose as donors as well as several acceptors with one, two, or several hydroxyl groups. Structures of the generated glycosides are determined directly from the reaction mixture using sel-TOCSY and NOESY measurements. The initial reaction rates and final product yields of the reverse hydrolyses are determined by in situ proton NMR. Thermodynamic equilibria of all investigated reverse hydrolyses cause final yields below 7% of predominantly formed primary glycosides.

Keywords: Almond glycosidases; In situ proton NMR; Kinetics; Reverse hydrolysis; Thermodynamic equilibrium

1. Introduction

Glycosidases (EC 3.2.1.1–151) are widely distributed in microorganisms, plants, and animals [1–3]. They selectively hydrolyze glycosidic bonds and possess decisive roles in many crucial biological pathways like catabolism and cellular signaling [1–4]. As a result these enzymes are widely used in various contexts, like determination of fruit ripeness degree and analysis of polysaccharides from various sources [1,5,6]. Furthermore selective inhibition of defined glycosidases for therapeutic targets is actually in the focus of investigations [7].

As hydrolyses are balanced reactions, glycosidases also catalyze accordingly the reverse hydrolyses and thus, are used in synthetic chemistry to generate di- and oligosaccharides as well as glycosides with an aglycon moiety [3,8,9]. However, thermodynamically controlled glycosidase catalyzed reverse hydrolyses lead to small yields and are reported to show low selectivity between different acceptors [8–10]. Therefore, kinetically controlled transglycosylations using activated glycosides as substrates are generally applied to give better yields [3,11]. These activated glycosides, however, must be prepared as they have to carry an aglycon moiety with good leaving group properties [8,9,11].

The reverse hydrolysis is, however, the preferable reaction to investigate regio- and stereoselectivity as well as kinetic properties of unaffected glycosidase catalysis. Natural free carbohydrates and alcohols can be used as donors and acceptors, respectively, and the reactions are not influenced by unnatural byproducts. The products are often difficult to analyze by classical methods, as metabolic rates are fairly low due to unpropitious thermodynamic equilibrium positions. In situ proton NMR, however, is a valuable technique to analyze biotransformations even with small conversion rates [12–14]. This modern analytical method was used to provide information about the time course and kinetics of various reactions and can easily be adopted on carbohydrate biotransformations as shown in some examples [15–17]. Beyond the online analysis NOESY and sel-TOCSY allow the identification and structural assignment of products directly from the reaction mixture after the thermodynamic

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equilibrium is reached [18]. Furthermore glycoside hydrolyses can also be investigated in detail by in situ proton NMR as shown earlier by Wajda et al. [19].

In this study we apply in situ proton NMR to investigate β-glucosidase (EC 3.2.1.21) and β-galactosidase (EC 3.2.1.23) activities from sweet almond (*Prunus dulcis* var. *dulcis*) protein extract. These two major sweet almond glycosidases are easy to obtain by standard isolation processes and quite stable over longer time periods [4,20,21]. The catalytic activities occur in a four to one ratio and can be adopted directly from protein extract without any further purification of the enzymes [20]. Hence they have been intensively investigated earlier by other analytical methods and were used in different contexts [3,4,21–23].

We now report the determination of K_m and V_{max} of β-glucosidase catalyzed cellobiose hydrolysis by in situ proton NMR. Further on we describe the NMR based online analysis of glycoside formation during reverse hydrolyses. Thereby we applied sel-TOCSY to confirm that the generated products were glycosides. NOESY spectra were used to determine the nature of the glycosidic linkage in reverse hydrolysis experiments with acceptors, which possessed more than one potential acceptor site.

2. Experimental

2.1. Chemicals

All chemicals were purchased from Sigma–Aldrich Chemical Co., St Louis, USA and Acros Organics, Beel, Belgium in the highest available purity and used without further purification.

2.2. Isolation of glycosidases from sweet almonds (*Prunus dulcis*)

Commercial almond meal gained from Italian and Spanish almonds was obtained from a local Viennese supermarket. The almond meal (200 g dry weight) was successively washed with 600 ml acetone and 200 ml cyclohexane to remove hydrophobic components. Insoluble material was filtered off and dried under vacuum at 40 °C to give 125 g (62%) dry weight. Dry almond meal (10 g) was extracted with 100 ml sodium acetate buffer (20 mM, pH 5.0) and filtered off. Proteins were separated by ammonium sulfate precipitation (70% saturation) and centrifugation. The precipitate was suspended in 10 ml sodium acetate buffer (20 mM, pH 5.0) and intensively dialyzed five times against 2.0 l of the same buffer. A small amount of residual precipitate in the dialyzed suspension was separated by centrifugation and the supernatant was lyophilized to obtain 90 mg of dialyzed proteins. The dry proteins were dissolved in 6.5 ml D_2O and stored at −20 °C for several weeks without any significant loss of β-glucosidase and β-galactosidase activity [20].

2.3. Nuclear magnetic resonance spectroscopy

The proton NMR spectra were recorded on a DRX-400 WB AVANCE and on a DRX-600 AVANCE spectrometer (Bruker, Rheinstetten, Germany), equipped with a *z*- and an triple resonance *xyz*-gradient inverse probe, respectively. Proton irradiation and measurement frequencies were 400.13 and 600.13 MHz, respectively, and the sample temperature was 298 K. All spectra were processed with the XWINNMR 3.1 software. 1D proton, sel-TOCSY [24], and sel-NOESY [25] spectra were recorded with an acquisition of 16,384 data points, a relaxation delay of 1.0 s, and between 16 and 128 scans. For selective excitation in sel-TOCSY and sel-NOESY Gaussian pulses with a duration of 80 ms were used. After zero filling to 32,768 data points the free induction decays were directly Fourier transformed to spectra with ranges between 1800 and 6000 Hz. For 2D NOESY spectra 1024 data points in the t_2-dimension and 256 to 384 experiments in t_1-dimension were recorded. After appropriate sinusoidal multiplication a Fourier transformation led to spectra with ranges between 200 and 4800 Hz. Mixing times of sel-TOCSY varied between 20 and 100 ms and the mixing time in sel-NOESY and 2D NOESY spectra was 800 ms. All spectra were recorded in D_2O and referenced on external acetone (δ ^{1}H: 2.225 ppm). The overwhelming water signal was suppressed by presaturation [26].

2.4. NMR spectroscopic assays of hydrolysis and reverse hydrolysis

All biotransformations were performed directly in the NMR sample tube (0.65–0.70 ml) at 298 K using acetate buffer in D_2O (20 mM, pD 5.0), which contained 1.0 mM 4-hydroxybenzoic acid ethyl ester to inhibit microbial growth. Reactions which took less than 24 h were performed in the magnet and between 16 and 64 proton spectra were directly taken from the sample. When the reaction time exceeded 24 h, the biotransformation was performed in a temperature-controlled water bath whereby six to ten proton spectra were recorded during the reaction time.

Glycosidase catalyzed hydrolyses of sucrose, maltose, methyl α-D-glucopyranosid, lactose, cellobiose, and gentiobiose were analyzed online from 200 mM solutions that contained 70 μl almond meal extract in a total volume of 700 μl. To determine the enzyme stability first fresh enzyme extract was added to the reaction mixtures after some days and further transformation was checked. Additionaly, 600 μl solutions containing 40 mM cellobiose and 50 μl almond meal extract were allowed to react and stored for 20, 116, and 215 h at 300 K. Afterwards cellobiose was added to get again a concentration of 40 mM. Then kinetics of the cellobiose hydrolyses were measured.

The Michaelis Menten constant (K_m) and maximal reaction rate (v_{max}) of cellobiose hydrolysis were determined by 15 experiments with varying substrate concentrations (4.6–184 mM) and a constant amount of 50 μl protein extract

in a total volume of 650 μl. All 15 reactions were monitored online with sixteen proton NMR measurements during time periods of 80–150 min. Between 5 and 10 proton NMR measurements of each biotransformation were recorded during the steady state phase.

β-Glycosidase catalyzed reverse hydrolyses were performed using glucose and galactose as donors in 100 mM concentration and glycol, glycerol, 1-propanol, 2-propanol, glucose, galactose, mannose, and methyl α-D-glucopyranoside as acceptors in 750 mM concentration. Volumes of 12.5 and 50 μl almond meal extract were added to the reactions with the donors glucose and galactose, respectively. The total volume of each reaction was 700 μl. The product formations were monitored by in situ proton NMR over about 200 h and after the reaction was completed sel-TOCSY as well as 2D NOESY and confirming sel-NOESY were recorded to identify the products.

3. Results and discussion

3.1. Hydrolytic activities of almond meal protein extracts

We obtained protein extract from sweet almonds according to Singh et al. [20] and applied in situ proton NMR to investigate its β-glucosidase and β-galactosidase activities towards natural substrates. As a result hydrolyses of cellobiose, gentiobiose, and lactose were monitored over 17 h. Additionally the not yet examined α-glucosidase activity was monitored using sucrose, maltose, and methyl α-D-glucopyranoside as substrates. Integrals over anomeric proton signals of the glycosides and monosaccharides provide the hydrolysis time courses. Cellobiose, gentiobiose, and lactose were efficiently hydrolyzed, whereas reaction rates of α-glucosidase catalyzed hydrolyses were very low (Fig. 1). No further significant biotransformation of the reactants and the products was observed in the experiments.

We checked the hydrolytic activity of the investigated glycosidases in reaction mixtures stored for 20, 116, and 215 h at 300 K to demonstrate that the enzymes still work with an appropriate reaction rate after 200 h. Therefore, additional cellobiose was added and its subsequent hydrolysis was monitored. The reaction rates were 101, 97, and 94 μM min^{-1}, respectively. The loss of β-glucosidase activity is negligible, since the transformation rates only decreased to an amount of 93% during 215 h.

The pronounced and persistent β-glucosidase and β-galactosidase activities confirm that the enzyme extracts are suitable for the examination of β-glucoside and

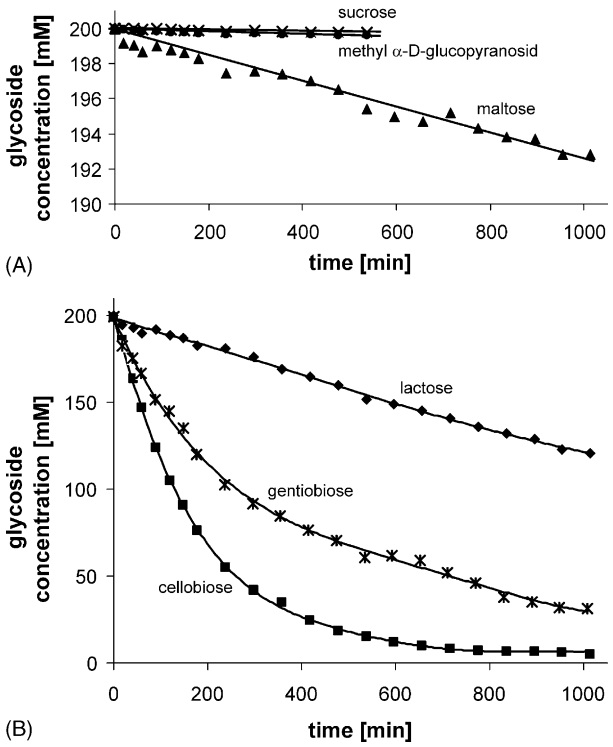
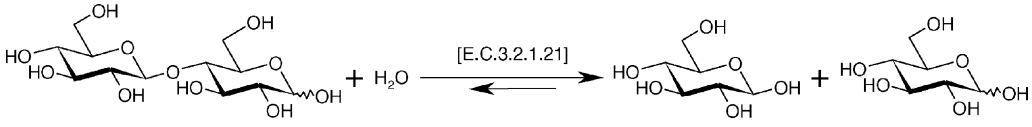


Fig. 1. Time courses of sweet almond meal protein extract catalyzed glycoside hydrolyses. Shown are the time courses of the substrate consumption determined by in situ proton NMR. All transformations are performed with 1.49 mg ml^{-1} protein extract. (A) The α-glucosidase catalyzed hydrolyses of sucrose (cross), maltose (triangle), and methyl α-D-glucopyranosid (circle) show low initial reaction rates of 7.2, 0.4, and 0.7 μM min^{-1}, respectively. (B) The β-glucosidase catalyzed hydrolysis of cellobiose (square) and gentiobiose (star) as well as the β-galactosidase catalyzed hydrolysis of lactose (rhomb) show initial reaction rates of 850, 540, and 80 μM min^{-1}, respectively.

β-galactoside hydrolyses and reverse hydrolyses. Furthermore, it has been shown that α-glucosides are not hydrolyzed in appreciable amounts and hence are applicable as acceptors in reverse hydrolysis.

3.2. In situ proton NMR monitored kinetics of cellobiose hydrolysis

Cellobiose is a natural substrate of sweet almond β-glucosidase and hence rapidly hydrolyzed (Fig. 1B) [4]. The reaction scheme of this biotransformation is shown in Scheme 1. However, the Michaelis Menten constant (K_m) of this reaction has been reported only once and was given as 117 mM [4]. The fairly high K_m value and the difficulties in monitoring cellobiose and glucose concentrations



Scheme 1. β-Glucosidase catalyzed cellobiose hydrolysis.

by UV-spectroscopy might account for the paucity of K_m determinations on this system [27].

In situ proton NMR makes online observation of time courses especially from higher concentrated solutions possible. Hence, we used this analytical technique to investigate K_m and V_{max} of sweet almond β-glucosidase catalyzed cellobiose hydrolysis. This investigation is the first reported determination of a Michaelis Menten constant (K_m) using in situ proton NMR. To accomplish the analysis we monitored the almond β-glucosidase catalyzed cellobiose hydrolysis using 15 different initial substrate concentrations varying between 4.6 and 184 mM. In all these reactions 1.06 mg ml^{-1} protein extract was applied. Between five and ten proton NMR measurements were recorded during the steady state phase of the reactions. Integrals over substrate and product anomeric signals in these spectra made the assignment of initial reaction rates possible as shown in Fig. 2A for three examples. The determined reaction rates are used to ascertain the kinetic profile by the method of Lineweaver and Burk, whereby small portions of reverse hydrolysis are neglected. This analysis gave K_m = 106 mM and V_{max} = 12.75 μM s^{-1} for the β-glucosidase catalyzed cellobiose hydrolysis (Fig. 2B).

The K_m value of 106 mM determined by in situ proton NMR is in fairly good agreement with the K_m value, which was earlier reported by Woodward and Wiseman [4]. The method they used for K_m determination was not clearly described. The stability index of the regression line in Fig. 2B is R^2 = 0.9968 pointing out the accuracy of the results gained from in situ proton NMR. Hence, this technique appears to be suitable for the determination of K_m and V_{max} of enzyme catalyzed reactions, in particular for reactions with fairly high K_m values.

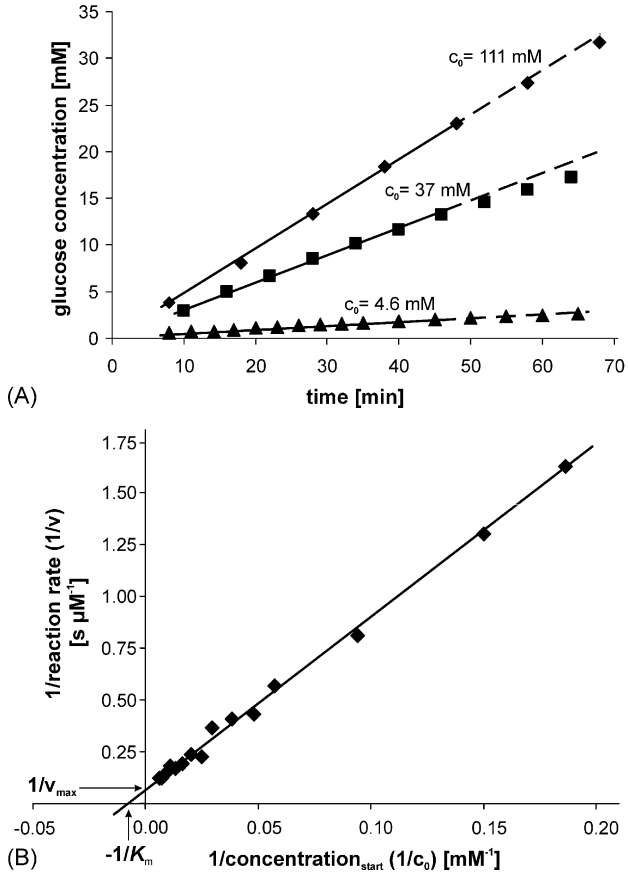
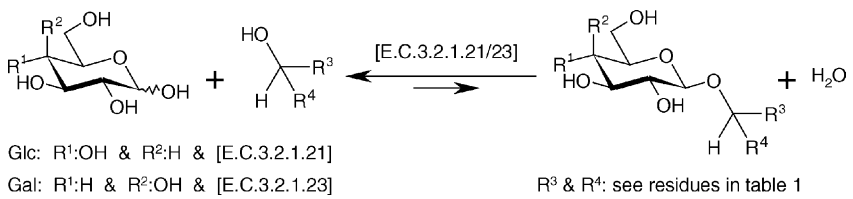


Fig. 2. Determination of kinetic constants of the cellobiose hydrolysis using 1.06 mg ml^{-1} almond protein extract. (A) Time courses of three online monitored cellobiose hydrolyses with cellobiose starting concentration c_0 = 111 mM (rhomb), c_0 = 37 mM (square), and c_0 = 4.6 mM (triangle). Shown are the increasing glucose concentrations. The steady state phases are indicated with persistent regression lines. (B) Graphical determination of reciprocal K_m and V_{max} by the method of Lineweaver and Burk. The regression line shows a stability index of R^2 = 0.9968. This small variance indicates the accuracy of in situ proton NMR in determining kinetic data of an enzyme catalyzed reaction.

3.3. Reverse hydrolysis

Apart from the kinetic investigations of the cellobiose hydrolysis we performed sweet almond β-glucosidase and β-galactosidase catalyzed reverse hydrolyses. For this purpose donors and acceptors were used in 100 and 750 mM concentration, respectively. Protein extract was added to a final concentration of 266 μg ml^{-1} and 1.06 mg ml^{-1} for the β-glucosidase and β-galactosidase catalyzed reactions, respectively. A general reaction scheme of β-glucosidase and β-galactosidase catalyzed reverse hydrolyses is shown in Scheme 2.

Even such relative high substrate concentrations allowed proton NMR spectra with adequate spectral resolution. The anomeric proton signals of glycosides formed appeared in a distinct spectral region and were not overlapped by signals of reactants or products. Hence selective excitation of these anomeric protons was possible. This, in turn, made it possible to obtain sel-TOCSY and sel-NOESY as well as expedient 2D NOESY spectra directly from the reaction mixtures.



Scheme 2. β-Glucosidase and β-galactosidase catalyzed reverse hydrolysis.

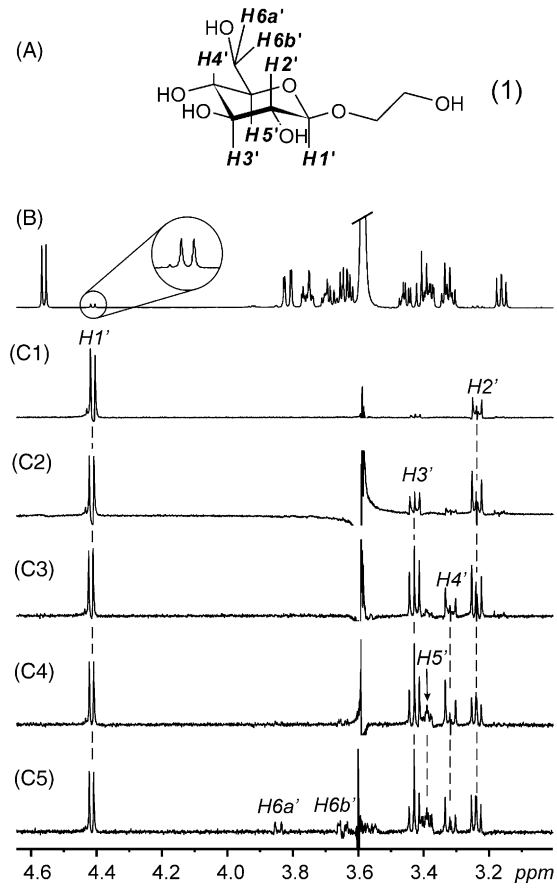


Fig. 3. Sel-TOCSY measurements of the glucose unit in 2-hydroxyethyl β-D-glucopyranoside (**1**) taken directly from the reaction mixture. (A) Structure of the product (**1**). All protons in the glucose unit are indicated. (B) Proton NMR spectrum of the reaction mixture in thermodynamic equilibrium. The β-anomeric proton signal of the glycoside (**1**; H1′; 4.42 ppm) is enlarged. All more intense signals belong to the substrates glucose and glycol. (C1–C5) Sel-TOCSY measurements with irradiation of the β-anomeric proton (**1**; H1′; 4.42 ppm). Mixing times are 20, 40, 60, 80, and 100 ms, respectively. Proton signals of the glucose unit are indicated along with their appearance in the spectra. A residue of the large glycerol signal at 3.60 ppm is present in all sel-TOCSY measurements.

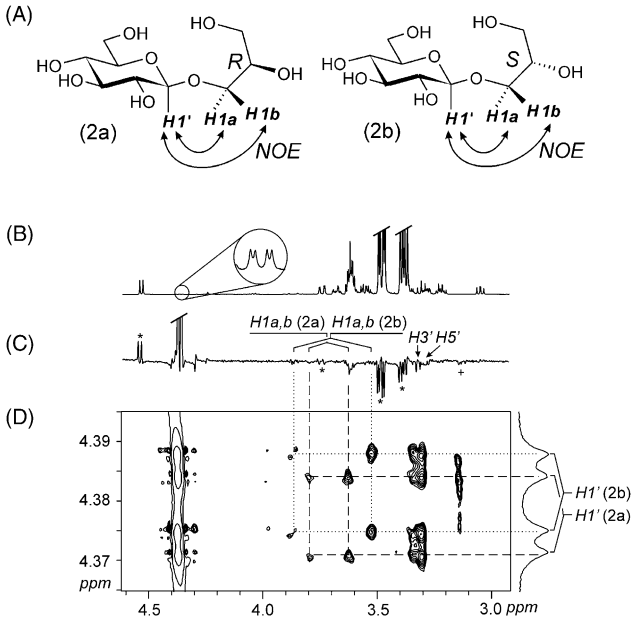


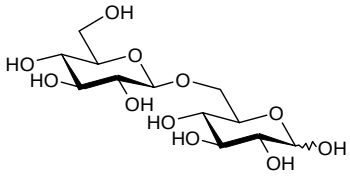
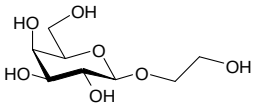
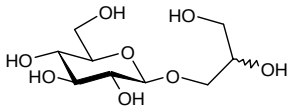
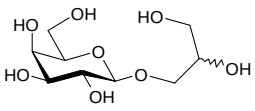
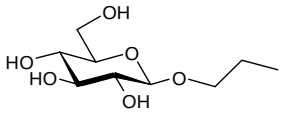
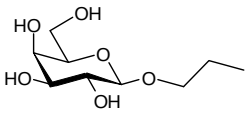
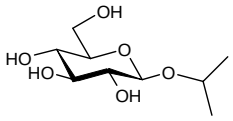
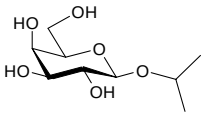
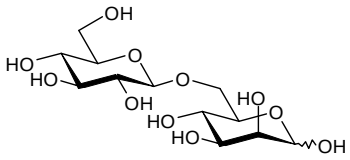
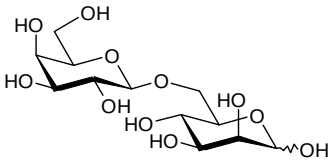
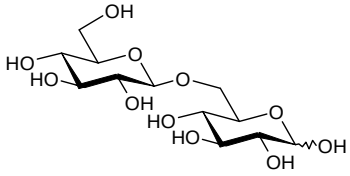
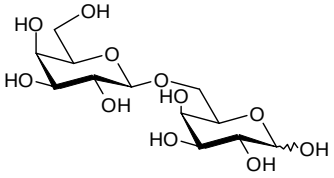
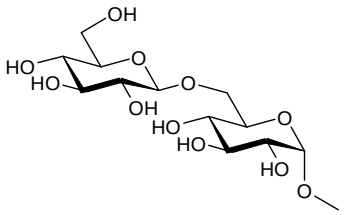
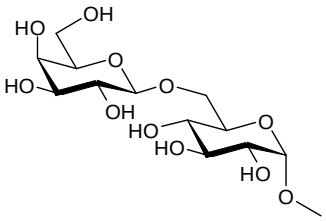
Fig. 4. NOEs to determine the position of the glycosidic linkage in [2*R*]- and [2*S*]-2,3-dihydroxy-1-propyl β-D-glucopyranosides (**2a** and **b**). The spectra were recorded directly from the reaction mixture. (A) Structures of the two diastereomeric glycosides (**2a** and **b**). (B) Proton NMR spectrum with enlarged anomeric proton signals of both glycosides (H1′ (**2a**) and H1′ (**2b**)). All more intense signals belong to the substrates glucose and glycerol. (C) Sel-NOESY with irradiation of H1′ (**2a**) and H1′ (**2b**) at 4.38 ppm and a mixing time of 800 ms. Both anomeric protons show NOEs to H3′, H5′, H1a, and H1b in the respective diastereomer. Signals caused by spin diffusion from H1′ to their neighbored H2′ are indicated with a cross (+) and residual signals of substrates are marked with an asterisk (*). (D) 2D NOESY of the reaction mixture recorded with 800 ms mixing time shows NOEs from H1′ to H1a and H1b in both diastereomers. Dashed and dotted lines indicate the assignment of the signals to glycoside (**2a**) and (**2b**), respectively.

These spectra led to the identification of the glycosides produced, although their concentrations were much lower than the concentrations of the substrates. In detail sel-TOCSY measurements with different mixing times [24] enabled the determination of all proton shifts in the non reducing saccharides. Fig. 3 shows, as an example, the identification of glucose in 2-hydroxyethyl β-D-glucopyranoside (**1**) directly from the reaction mixture.

Furthermore, the anomeric proton of the non-reducing saccharide is spatially close to some protons of the aglycon moiety. Hence, between these protons there is a nuclear Overhauser effect (NOE), which can be measured by 2D NOESY and confirmed by sel-NOESY [25]. This NOE can be used to determine the regio- and stereoselectivity of glycoside formation and consequently makes determination of the glycosidic structure possible. As an example sel-NOESY and 2D NOESY of the two diastereomeric [2*R*]- and [2*S*]-2,3-dihydroxy-1-propyl β-D-glucopyranosides (**2a** and **b**) are shown in Fig. 4. The spectra obtained from the 2D NOESY experiments are more convincing, since they do not include disturbing residual signals from the starting material, which are present in the sel-NOESY. These glycosides were generated in equal amounts from glucose as donor and glycerol as acceptor. It is remarkable that neither of the two diastereomers was preferentially formed and that no detectable amounts of a secondary glycoside was generated. The structures of all glycosides formed in the reverse hydrolyses are given in Table 1. Characteristic proton NMR shifts of these compounds are listed in Table 2.

Anomeric proton signals of the generated glycosides were further on used for in situ proton NMR analysis of reverse hydrolysis time courses. In this way, reaction rates were determined from the increase of the glycoside concentration over a time period of about 200 h. In the majority of cases the initial reaction rates lay between 0.1 and 1.0 μM min^{-1}. Sole exceptions were the formation of gentiobiose (**6a** and **b**) and β-D-Glc*p*-(1 → 6)-α-D-Glc*p*-OMe (**7**), which possessed initial reaction rates of 16.6 and 5.5 μM min^{-1}, respectively. The final product yields were identified directly

Table 1
Structures of the β-glucosides and β-galactosides that were generated in the investigated reverse hydrolyses[a]

Acceptor (750 mM)	Formed β-glucoside [Donor: glucose (100 mM)]		Formed β-galactoside [Donor: galactose (100 mM)]	
Glycol		(**1**)		(**8**)
Glycerol		(**2a** and **b**)		(**9a** and **b**)
1-Propanol		(**3**)		(**10**)
2-Propanol		(**4**)		(**11**)
Mannose		(**5a** and **b**)		(**12a** and **b**)
Glucose		(**6a** and **b**)	–	
Galactose	–			(**13a** and **b**)
Methyl-α-D-gluco-pyranosid		(**7**)		(**14**)

[a] Glycosides are nominated in Table 2.

from the reaction mixture after about 8 days of incubation time when no further change in the glycoside concentration was observed. These yields are equilibrium yields, because during 200 h the glycosidases did not loose an appreciable amount of their activity, as shown before. The observed reaction rates and product yields are also given in Table 2.

3.4. Selectivity of glycoside formation

All detected products of the investigated reverse hydrolyses are β-glycosides confirming that β-glucosidase and β-galactosidase effectively catalyze reverse hydrolysis. If the acceptor contains primary and secondary hydroxyl groups, the glycosidic bond is predominately formed with

Table 2
Initial reaction rates and conversions of the reverse hydrolyses as well as indicative proton NMR shifts of the formed glycosides

Glycoside	Initial reaction rate[a] (μM min^{-1})	Conversion[b] (%)	Proton NMR shifts (ppm)										
			H1′	H2′	H3′	H4′	H5′	H6a′	H6b′	Ha[c]	Hb[c]	Position	References[d]
2-Hydroxyethyl β-D-glucopyranoside (**1**)	1.7	6.4	4.42	3.24	3.43	3.25	3.39	3.84	3.64	3.92	3.70	(H1a,b)	
[2*R*]-2,3-dihydroxy-1-propyl-β-D-glucopyranosides (**2a**)	1.0	2.2	4.38	3.22	3.40	3.29	3.37	3.82	3.64	3.88	3.70	(H1a,b)	[28]
[2*S*]-2,3-Dihydroxy-1-propyl-β-D-glucopyranosides (**2b**)	1.0	2.2	4.39	3.22	3.40	3.29	3.37	3.82	3.64	3.95	3.61	(H1a,b)	[28]
1-Propyl-β-D-glucopyranoside (**3**)	0.4	2.6	4.38	3.19	3.41	3.30	3.37	3.84	3.65	3.56	3.56	(H1a,b)	[29]
2-Propyl-β-D-glucopyranoside (**4**)	0.1	2.4	4.45	3.14	3.40	3.29	3.37	3.83	3.64	4.02		(H2)	[29]
β-D-Glc*p*-(1 → 6)-α-D-Man*p* (**5a**)	} 0.1 {	0.9	4.40	3.15	3.38	3.29	3.37	3.82	3.62	3.79	nd[e]	(H6a,b)	[30]
β-D-Glc*p*-(1 → 6)-β-D-Man*p* (**5b**)		0.6	4.41	3.22	3.40	3.29	3.37	3.77	3.62	4.04	3.98	(H6a,b)	[30]
β-D-Glc*p*-(1 → 6)-α-D-Glc*p* (**6a**)	} 16.6 {	1.0	4.40	3.21	3.39	3.29	3.36	3.81	3.62	4.08	3.74	(H6a,b)	[19]
β-D-Glc*p*-(1 → 6)-β-D-Glc*p* (**6b**)		1.5	4.39	3.14	3.38	3.29	3.36	3.79	3.62	4.03	3.77	(H6a,b)	[19]
β-D-Glc*p*-(1 → 6)-α-D-Glc*p*-O-Me (**7**)	5.5	1.5	4.41	3.20	3.42	3.33	3.38	3.83	3.64	4.02	3.81	(H6a,b)	[31]
2-Hydroxyethyl-β-D-galactopyranoside (**8**)	0.7	3.7	4.36	3.42	3.53	3.82	3.64	3.70	3.70	3.90	3.68	(H1a,b)	
[2*R*]-2,3-Dihydroxy-1-propyl-β-D-glucopyranosides (**9a**)	0.3	0.3	4.32	3.40	3.53	3.82	nd[e]	nd[e]	nd[e]	3.82	3.54	(H1a,b)	
[2*S*]-2,3-Dihydroxy-1-propyl β-D-glucopyranosides (**9b**)	0.3	0.3	4.34	3.40	3.53	3.82	nd[e]	nd[e]	nd[e]	3.95	3.69	(H1a,b)	
1-Propyl-β-D-galactopyranoside (**10**)	0.3	3.2	4.30	3.42	3.52	3.81	3.63	3.68	3.68	3.78	3.78	(H1a,b)	
2-Propyl-β-D-galactopyranoside (**11**)	0.6	1.8	4.35	3.35	3.52	3.80	3.62	3.70	3.70	4.00		(H2)	[32]
β-D-Gal*p*-(1 → 6)-α-D-Man*p* (**12a**)	} 0.1 {	<0.2	4.33	3.40	3.53	3.81	nd[e]	nd[e]	nd[e]	3.83	nd[e]	(H6a,b)	
β-D-Gal*p*-(1 → 6)-β-D-Man*p* (**12b**)		<0.2	4.34	3.40	3.53	3.81	nd[e]	nd[e]	nd[e]	3.99	3.95	(H6a,b)	
β-D-Gal*p*-(1 → 6)-α-D-Gal*p* (**13a**)	} 0.1	$\sum$0.9 {	4.35	3.43	3.55	3.82	3.64	3.70	3.63	3.78	3.76	(H6a,b)	[33]
β-D-Gal*p*-(1 → 6)-β-D-Gal*p* (**13b**)			4.35	3.39	3.53	3.82	3.64	3.70	3.63	3.78	3.76	(H6a,b)	[33]
β-D-Gal*p*-(1 → 6)-α-D-Glc*p*-O-Me (**14**)	0.2	0.8	4.33	3.45	3.55	3.82	3.63	3.69	3.67	4.08	3.79	(H6a,b)	

[a] Initial reaction rates for the formation of the respective glycoside using 266 μg ml^{-1} and 1.06 mg ml^{-1} protein extract for β-glucosidase and β-galactosidase catalyzed reactions, respectively.
[b] Conversions refer to the initial donor concentration.
[c] Proton(s) geminal to glycosidic linkage.
[d] Reference of comparable NMR shifts.
[e] Not determined due to low signal to noise ratio.

the primary hydroxyl group, resulting in a primary glycoside. In particular when a monosaccharide acts as acceptor, its primary hydroxyl group in position 6 is the favored point of attack and a (1 → 6) linked disaccharide is generated. A primary hydroxyl group in the acceptor is, however, not a prerequisite for sweet almond β-glucosidase catalyzed reverse hydrolysis, as 2-propanol was also a successful acceptor for the formation of 2-propyl β-D-glucopyranoside (**4**) and 2-propyl β-D-galactopyranoside (**11**).

Such preferred formation of primary glycosides in reverse hydrolysis has been reported earlier and is caused by the thermodynamic reaction control in many cases [3,34,35]. An example is the difference in the yield of the two glucobioses gentiobiose (**6a** and **b**) and cellobiose, when glucose is used as donor and as acceptor. These two disaccharides are hydrolyzed by sweet almond β-glucosidase with high reaction rates, confirming that both sugars are natural substrates of β-glucosidase (Fig. 1B). In reverse hydrolysis, the initial reaction rate of gentiobiose formation is up to 2 orders of magnitude higher than the reaction rates of the investigated reverse hydrolyses with acceptors other than glucose. Cellobiose, however, was not detected as a product of the reverse hydrolysis. This pronounced difference in product yields is mainly caused by the thermodynamic equilibria, because the thermodynamic equilibrium constant (K) of gentiobiose hydrolysis is $K =\sim 18$, while cellobiose hydrolysis has a $K > 155$ [35].

On the molecular level, differences in the nucleophilicity of primary and secondary hydroxyl groups in acceptors appear to exert only a minor influence on their reactivities as de Roode et al. [36] have shown in a computational approach. Rather the differences in steric interactions in primary and secondary alcohols likely causes the frequent preference of primary hydroxyl groups as point of attack in the investigated glycosylations.

4. Conclusions

In situ proton NMR is a valuable method for the determination of Michaelis Menten constants (K_m) and maximal reaction rates (V_{max}) of enzyme catalyzed reactions. An appropriate K_m value of a biotransformation, which enables in situ NMR measurement with initial substrate concentrations above 0.1 mM, is the sole prerequisite for successful implementation of this analytical method. Furthermore, the structures of products, which are formed in low yields, can be determined directly from the reaction mixture by proton NMR. For this purpose 2D NOESY were measured and single protons of the products were selectively irradiated to perform sel-TOCSY and sel-NOESY measurements. Usually, these spectra allow a clear determination of the product structure, especially when the substrate structure and the reaction catalyzed by the applied enzyme are known. Both determination of kinetic constants and identification of products formed in low yields should be possible for a wide variety of enzyme catalyzed reactions, using these methods. They thus extend the scope of in situ proton NMR as an analytical tool in biocatalytic studies.

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References

[1] Y. Bhatia, S. Mishra, V.S. Bisaria, Crit. Rev. Biotechnol. 22 (2002) 375–407.
[2] K. Yamamoto, S.-C. Li, Y.-T. Li, in: B. Ernst, G. W. Hart, P. Sinay (Eds.), Carbohydrates in Chemistry and Biology, Wiley-VCH, Weinheim, 2000, pp. 497–511.
[3] F. van Rantwijk, M. Woudenberg-van Oosterom, R.A. Sheldon, J. Mol. Catal. B: Enzymatic 6 (1999) 511–532.
[4] J. Woodward, A. Wiseman, Enzyme Microb. Technol. 4 (1982) 73–79.
[5] J.R. Whitaker, Principles of Enzymology for the Food Sciences, Food Science and Technology, second ed., vol. 61, Marcel Dekker, New York, 1994 (Chapter 15).
[6] D.L. Bouranis, C.A. Niavis, Plant Cell Physiol. 33 (1992) 999–1008.
[7] N. Asano, Glycobiology 13 (2003) 93R–104R.
[8] K. Ajisaka, Y. Yamamoto, Trends Glycosci. Glyc. 14 (2002) 1–11.
[9] E.J. Toone, E.S. Simon, M.D. Bednarski, G.M. Whitesides, Tetrahedron 45 (1989) 5365–5422.
[10] M. Scigelova, S. Singh, D.H.C. Crout, J. Mol. Catal. B: Enzymatic 6 (1999) 483–494.
[11] K.G.I. Nilsson, in: L.G. Copping, R.E. Martin, J.A. Pickett, C. Bucke, A.W. Bunch (Eds.), Opportunities in Biotransformation, Elsevier, London, 1990, pp. 131–139.
[12] J.N. Barbotin, J.C. Portais, NMR in Microbiology. Theory and Applications, Horizon Scientific Press, Wymondham, Norfolk, 2000.
[13] L. Brecker, D.W. Ribbons, Trends Biotechnol. 18 (2000) 197–202.
[14] H.-J. Weber, L. Brecker, Curr. Opin. Biotechnol. 11 (2000) 572–578.
[15] J. Volc, P. Sedmera, P. Halada, G. Daniel, V. Přikrylová, D. Haltrich, Mol. Catal. B: Enzymatic 17 (2002) 91–100.
[16] P. Spangenberg, V. Chiffoleau-Giraud, C. André, M. Dion, C. Rabiller, Tetrahedron: Asymmetry 10 (1999) 2905–2912.
[17] P. Spangenberg, C. André, M. Dion, C. Rabiller, R. Mattes, Carbohydr. Res. 329 (2000) 65–73.
[18] H.-J. Weber, L. Brecker, D. DeSouza, H. Griengl, D.W. Ribbons, H.K. Weber, J. Mol. Catal. B: Enzymatic 19–20 (2002) 149–157.
[19] R. Wajda, M. König, H. Ludwig, S. Mronga, H. Friebolin, Carbohydr. Res. 235 (1992) 259–267.
[20] S. Singh, M. Scigelova, D.H.G. Crout, Tetrahedron: Asymmetry 11 (2000) 223–229.
[21] M.P. Dale, H.E. Ensley, K. Kern, K.A.R. Sastry, L.D. Byers, Biochemistry 24 (1985) 3530–3539.
[22] T. Hansson, P. Adlercreutz, Biotechnol. Lett. 24 (2002) 1465–1471.
[23] S.U. Hansen, I.W. Plesner, M. Bols, Chem. Biol. Chem. 1 (2000) 177–180.
[24] T. Fäcke, S. Berger, J. Magn. Reson. Ser. A 113 (1995) 257–259.
[25] J. Stonehouse, P. Adel, J. Keeler, A.J. Shaka, J. Am. Chem. Soc. 116 (1994) 6037–6038.
[26] M. Guéron, P. Plateau, M. Decorps, Prog. NMR Spectrosc. 23 (1991) 135–209.

[27] M. Lewin, I. Ziderman, N. Weiss, A. Basch, A. Ettin, Carbohydr. Res. 62 (1978) 393–398.
[28] F.M. Albini, C. Murelli, G. Patritti, M. Rovati, Synth. Commun. 24 (1994) 1651–1661.
[29] B.W. Sigurskjold, I. Haunstrup, K. Bock, Acta Chem. Scand. 46 (1992) 451–458.
[30] T. Tanase, K. Ishida, T. Watanabe, M. Komiyama, K. Koumoto, S. Yano, M. Hidai, S. Yoshikawa, Chem. Lett. (1988) 327–330.
[31] P.-E. Jansson, L. Kenne, I. Kolare, Carbohydr. Res. 257 (1994) 163–174.
[32] Y. Ooi, T. Hashimoto, N. Mitsuo, T. Satoh, Chem. Pharm. Bull. 33 (1985) 1808–1814.
[33] A.K. Bhattacharjee, E. Zissis, C.P.J. Glaudemans, Carbohydr. Res. 89 (1981) 249–254.
[34] K. Kurashima, M. Fujii, Y. Ida, H. Akita, J. Mol. Catal. B: Enzymatic 26 (2003) 87–89.
[35] Y.B. Tewari, R.N. Goldberg, J. Biol. Chem. 264 (1989) 3966–3971.
[36] B.M. de Roode, H. Zuilhof, M.C.R. Franssen, A. van der Padt, A. de Groot, J. Chem. Soc., Perkin Trans. 2 (2000) 2217–2224.