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Identification in *Agrobacterium tumefaciens* of the D-galacturonic acid dehydrogenase gene

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Abstract There are at least three different pathways for the catabolism of D-galacturonate in microorganisms. In the oxidative pathway, which was described in some prokaryotic species, D-galacturonate is first oxidised to *meso*-galactarate (mucate) by a nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase (EC 1.1.1.203). In the following steps of the pathway mucate is converted to 2-keto-glutarate. The enzyme activities of this catabolic pathway have been described while the corresponding gene sequences are still unidentified. The D-galacturonate dehydrogenase was purified from *Agrobacterium tumefaciens*, and the mass of its tryptic peptides was determined using MALDI-TOF mass spectrometry. This enabled the identification of the corresponding gene *udh*. It codes for a protein with 267 amino acids having homology to the protein family of NAD(P)-binding Rossmann-fold proteins. The open reading frame was functionally expressed in *Saccharomyces cerevisiae*. The N-terminally tagged protein was not compromised in its activity and was used after purification for a kinetic characterization. The enzyme was specific for NAD and accepted D-galacturonic acid and D-glucuronic acid as substrates with similar affinities. NMR analysis showed that in water solution the substrate D-galacturonic acid is predominantly in pyranosic form which is converted by the enzyme to 1,4 lactone of galactaric acid. This lactone seems stable under intracellular conditions and does not spontaneously open to the linear *meso*-galactaric acid.

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Introduction

D-Galacturonate is the primary monomer of pectin and consequently an important carbon source for microorganisms living on decaying plant material. It is also of importance in biotechnology when cheap raw materials such as pectin-rich materials like citrus peel or sugar beet pulp are exploited. Currently, these raw materials are either dumped or used as cattle feed. It is thus desirable to find other applications for these raw materials which are available in vast amounts. It has been suggested to ferment D-galacturonic acid to ethanol using genetically engineered microbes but also other products can be envisaged (Doran-Peterson et al. 2008). It is thus important to have a detailed knowledge of the catabolic pathways and their enzyme properties. Several entirely different pathways for the catabolism of D-galacturonate in microorganisms have been described (for review see (Richard and Hilditch 2009)):

(1) The isomerase pathway is active in *Escherichia coli* and other prokaryotic microorganisms. In this pathway, D-galacturonic acid is converted to D-tagaturonate by an isomerase followed by a conversion through an NADH-dependent reductase to D-altronate. In the next step, a dehydratase forms 3-deoxy-D-*erythro*-hex-2-ulosonate followed by a phosphorylation at C6 by an ATP-requiring kinase. The reaction product is then split by an aldolase to pyruvate and D-glyceraldehyde-3-phosphate. The enzyme activities have been characterised (Ashwell et al. 1960; Cynkin and Ashwell 1960; Hickman and Ashwell 1960; Smiley and Ashwell 1960;

Meloche and Wood 1964) and the corresponding genes identified (Mata-Gilsinger and Ritzenthaler 1983).

(2) The reductive pathway has been described in the eukaryotic microorganisms, filamentous fungi *Hypocrea jecorina* and *Aspergillus nidulans*. In this pathway D-galacturonate is reduced by an NAD(P)H-requiring reductase to L-galactonate and then converted by a dehydratase to 3-deoxy-L-*threo*-hex-2-ulosonate, which is in the next step split by an aldolase to L-glyceraldehyde and pyruvate. L-Glyceraldehyde is oxidised to glycerol in an NADPH-requiring reaction. The enzymes have been characterised and the corresponding genes identified (Kuorelahti et al. 2005, 2006; Liepins et al. 2006; Hilditch et al. 2007; Martens-Uzunova 2008).

(3) The oxidative pathway has been described in some bacteria such as *Agrobacterium tumefaciens* and *Pseudomonas syringae*. In this path, D-galacturonate is first oxidised to *meso*-galactarate (mucate) by an NAD-requiring D-galacturonate dehydrogenase. *meso*-Galactarate is then converted by a dehydratase to 3-deoxy-2-keto-DL-threo-hexarate (2-keto-3-deoxy galactarate). In the following step, this is converted by a 4-deoxy-5-oxoglucarate hydrolase (decarboxylating; EC 4.2.1.41) to α-keto glutarate semialdehyde. In this enzyme reaction, a dehydratation and decarboxylation is completed in a single step (Jeffcoat 1975). The following step is the α-keto glutarate semialdehyde dehydrogenase (EC 1.2.1.26) where the reaction product is α-ketoglutarate (Watanabe et al. 2007; Fig. 1). This route is also sometimes called the α-ketoglutarate pathway.

None of the gene sequences for this pathway in *A. tumefaciens* or *P. syringae* are known; however, gene sequences for bacterial galactarate dehydratase (Hubbard et al. 1998) and α-keto glutaric semialdehyde dehydrogenase (Watanabe et al. 2007) in other pathways have been described. There is a catabolic path for galactaric acid (mucic acid) in *E. coli* where the first enzyme is a galactarate dehydratase of which the gene sequence is known (Hubbard et al. 1998). The following steps of the *E. coli* pathway however are differing from the oxidative D-galacturonate pathway. This pathway is sometimes called the glycerate pathway (Sharma and Blumenthal 1973). A 5-keto-4-deoxy-galactarate (3-deoxy-2-oxo-L-*threo*-hexarate) from the dehydration reaction is split by an aldolase into pyruvate and L-tartronate semialdehyde. L-Tarartronate semialdehyde is then reduced to form D-glycerate which is subsequently phosphorylated to D-glycerate-3-phosphate. The *E. coli* pathway requires a specific galactarate dehydratase. If the enzyme would convert mucic acid to the L- and D-threo form of 3-deoxy-2-keto hexarate, it could

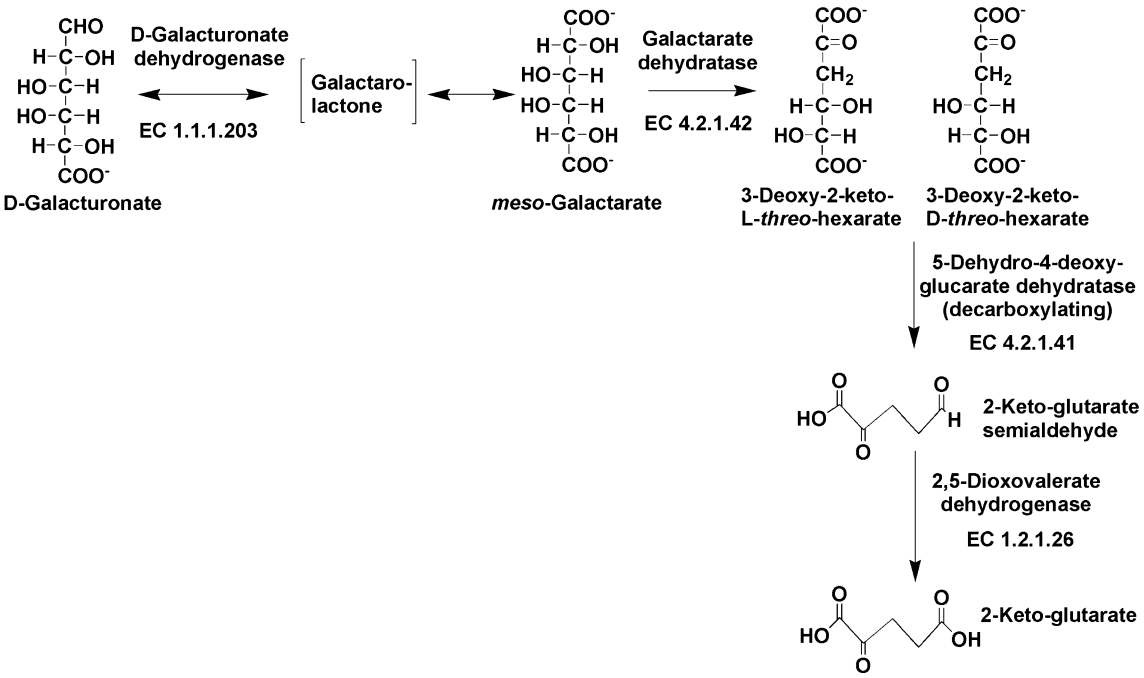

Fig. 1 Pathway for D-galacturonate catabolism in *A. tumefaciens*: D-galacturonate is oxidised by an NAD-dependent dehydrogenase to galactaro-lactone. The galactaro-lactone hydrolyses spontaneously or with the aid of a lactonase to *meso*-galactarate (mucate). A dehydratase is converting the *meso*-galactarate to 2-keto-3-deoxy-galactarate; however, it is not known which stereoisomer is formed. In the following step, a dehydratase-decarboxylase is forming 2-ketoglutarate semialdehyde which is in the last step converted to 2-ketoglutarate by an NAD-dependent dehydrogenase

lead to metabolites with inverse configuration which cannot be metabolised. In the *A. tumefaciens* pathway on the other hand, the product of the pathway, 2-oxo-glutarate, has no chiral centres, accordingly, a more unspecific dehydratase would not be harmful. Indeed the galactarate dehydratase of *A. tumefaciens* does not seem to be very specific. It was suggested to be active with meso-galactaric acid and D-glucaric acid, and from D-glucaric acid the D-erythro and L-threo form of 3-deoxy-2-oxo-hexarate was formed (Chang and Feingold 1970). *E. coli* on the other hand has specific enzymes for the dehydratation of *meso*-galactaric acid or D-glucaric acid.

A D-galacturonate dehydrogenase (EC 1.1.1.203) has been described in *A. tumefaciens* (Chang and Feingold 1969) and in *P. syringae* (Bateman et al. 1970; Wagner and Hollmann 1976b). The enzyme from these organisms had been partially purified and characterised; however, a gene sequence for the corresponding enzyme acitivity has not been identified. The enzyme is specific for NAD as a cofactor but is not specific for the substrate. It can oxidise D-galacturonate and D-glucuronate to meso-galactarate (mucate) and D-glucarate (saccharate), respectively. We present here the purification of the enzyme induced on D-galacturonic acid and the cloning of the corresponding gene from *A. tumefaciens*. The heterologously expressed enzyme was characterised in more detail using ^{1}H nuclear magnetic resonance (NMR).

Materials and methods

Purification and identification of the D-galacturonic acid dehydrogenase

The *A. tumefaciens* (*Rhizobium radiobacter*) strain C58 (ATCC 33970) was obtained from the American Type Culture Collection and grown on the mineral minimal medium containing D-galacturonic acid as a sole carbon source as described by Chang and Feingold (Chang and Feingold 1969). The cells of 1 litre culture (1.1 g wet weight) were resuspended in 12 ml 10 mM Tris-HCl buffer pH 7.5 containing 1 mM EDTA and 1 mM DTT and lysed by sonication. The cell lysate was centrifuged for 30 min at 14,000 rpm at 4°C in an Eppendorf centrifuge, and the protein extract was loaded to a 5 ml DEAE column equilibrated with 10 mM Tris-HCl buffer pH 7.5 containing 1 mM EDTA and 1 mM DTT. It was eluted with a 60-ml linear gradient from 0 to 300 mM of NaCl in the same buffer. The 1 ml fractions collected were tested for D-galacturonic acid dehydrogenase activity using 4 mM D-galacturonic acid and 0.5 mM NAD as substrates (for assay details, see below). The active fractions were concentrated and applied to a non-denaturating PAGE (7.5% acrylamide, BioRad). After separation, the activity-containing bands were identified using a dehydrogenase-specific zymogram staining in essence as described previously (Richard et al. 2001). The gel was stained in a solution containing 10 mM Tris-HCl, pH 7.5, 0.5 mM NAD, 4 mM D-galacturonic acid, 0.25 mM nitroblue tetrazolium and 0.06 mM phenazine methosulfate. The only band that appeared in the staining was cut out and eluted by overnight incubation in 2 ml of 100 mM Tris-HCl, pH 9.0, 0.1% SDS. It was then concentrated to about 80 µl by centrifugation in a Centricon tube (Amicon) and separated on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12% acrylamide). A 32 kDa enzyme observed in the SDS-PAGE gel was in-gel digested with trypsin, and the peptides were extracted essentially according to the method of Rosenfeld et al. (1992).The samples were desalted using a C-18 matrix (Eppendorf Perfect Pure C-18 Tip). The saturated matrix solution was prepared by dissolving recrystallized α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) in a 50% acetonitrile in 0.1% TFA solution. Equal volumes of purified peptide sample or calibration standard (peptide calibration mixture II, Bruker Daltonics) were mixed with the saturated matrix solution. One microlitre of this matrix/sample mixture was applied onto the target (Target plate ground steel T F, Bruker Daltonics) and let to dry at room temperature. The peptide masses were then determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectometry using a Bruker Autoflex II mass spectrometer. FlexAnalysis software (Bruker Daltronics) was used for the data analysis.

Enzyme activity measurements

The uronic acid dehydrogenase activity was measured in a reaction mixture containing NAD, uronic acid and 5 mM $MgCl_2$ in 50 mM Tris-HCl buffer pH 7.5, and the formation of NADH was followed by measuring absorption at 340 nm at 25°C. The concentration of NAD and uronic acid used are stated in the text or in the figures. One unit is the activity that forms 1 µmol of product per minute. Steady-state kinetic parameters are derived from fitting the data to the Michaelis–Menten equation using Microcal™ Origin 6.0 software.

Cloning and heterologous expression

The open reading frame for the *udh* gene was amplified by PCR introducing *Eco*RI and *Bam*HI restrictions sites using genomic DNA isolated from *A. tumefaciens* as a template. The following primers were used, 5′-CACAGCAAAGAC GCAGAATTCGCTTGGAAG-3′ and 5′-GGCTTGGG ATCCCGCTGATCATTCAGCTC-3′. The PCR product was then ligated to a TOPO vector (pCR2.1, Invitrogen)

and cloned in *E. coli*. After sequencing to ensure that the correct sequence was obtained, the *Eco*RI–*Bam*HI fragment was released from the TOPO vector and ligated to the corresponding sites of the yeast expression vector p2159. This expression vector is a multicopy expression vector for *S. cerevisiae* containing the URA3 for selection and the constitutive TPI promoter derived from the pYX212 (R&D Systems). It was modified to remove the ATG and the hemagglutinin-tag from the multiple cloning site and to introduce a *Bam*HI restriction site. The resulting *udh*-containing expression vector was transformed to the *Saccharomyces cerevisiae* strain CEN.PK2-1B and grown on selective medium. The D-galacturonic acid dehydrogenase was also histidine-tagged at the N-terminus by means of PCR. The following primers were used 5′- CCGGAA TTCACCATGCACCACCATCACCATCACATG GCGATGAAACGGCTTCTTG-3′ in sense direction and 5′- GGCTTGGGATCCCGCTGATCATTCAGCTC-3′ in anti-sense. This His_6-tagged gene was expressed using the same expression vector p2159 and expression host as described above. A control strain contained the empty plasmid.

The resulting yeast strains were disintegrated by vortexing with glass beads in a 50-mM Tris-HCl buffer pH 7.5 and Complete protease inhibitor (Roche). The extract was centrifuged in an Eppendorf centrifuge at 4°C at 14,000 rpm and the supernatant analysed for D-galacturonic acid dehydrogenase activity as described above.

Purification of the histidine-tagged D-galacturonic acid dehydrogenase

The His_6-tagged D-galacturonic acid dehydrogenase was purified from the yeast cell lysate as described above in the buffer used for equilibrating the column. A 5 ml Ni-NTA-agarose (Qiagene) equilibrated with 50 mM NaP_i buffer pH 8.0 containing 150 mM NaCl and 1 mM β-mercaptoethanol was loaded with the lysate and the his_6-tagged D-galacturonic acid dehydrogenase was eluted with at linear 40 ml imidazole gradient from 0 to 125 mM. The D-galacturonic acid dehydrogenase containing fractions were pooled and concentrated using an Amincon spin concentrator with a 10 kDa cut-off and analysed using SDS-PAGE electrophoresis.

The protein concentration was measured using the BCA method with BSA as the standard according to the manufacturer's instructions (Pierce).

NMR spectroscopy

All NMR spectra were recorded in D_2O at 25°C on Varian Inova NMR spectrometers operating either at 500 or 600 MHz, using inverse detection room temperature probes. The chemical shifts were referenced to internal 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP, 0.05 wt%). 1D ^1H spectra were recorded with 1 s presaturation of the water signal and typically four or eight scans. For DQFCOSY, TOCSY and HSQC matrices of 2 k×256 complex data points were collected, zero-filled to 2 k×512 complex data points and a cosine weighting function was used in both dimensions prior to the Fourier transformation. The water signal was suppressed by 1 s presaturation in DQFCOSY and TOCSY, the spinlock time (MLEV) in TOCSY was 120 ms and in HSQC the average ^1H–^{13}C coupling constant was estimated to be 140 Hz. For heteronuclear multiple-bond correlation (HMBC) experiment matrices of 2 k×256 complex data points were collected, zero-filled to 2 k×512 and a sine bell weighting function was used in both dimensions. The one bond ^1H–^{13}C coupling constant was estimated to be 140 Hz. For the long range ^1H–^{13}C coupling constants values between 4 and 8 Hz were used.

In order to follow the enzyme reaction in real time by NMR, 10 μmol D-galacturonic acid and 18 μmol NAD were dissolved in 1.1 ml 10 mM Na-phosphate buffer in D_2O. The reaction was first monitored at pH 8 and later on at pH 6.8. After acquiring a 1D ^1H NMR spectrum, purified enzyme was added and the reaction was followed by acquiring a series of ^1H NMR spectra over 1 h (enzyme concentrations of 1–10 μg/ml were studied). For each spectrum four scans were acquired with 1 s relaxation delay, 1 s presaturation of the water signal and 3 s acquisition time. Thus, each spectrum is a sum of four scans recorded during 20 s of time. Before the first spectrum, 16 dummy scans were performed in order to ensure steady state conditions. The fast pulsing relative to the actual relaxation times and the partial saturation of the signals close to the water line distorted the quantitativity of the experiment. Therefore correction coefficients were measured for the integrated signals by acquiring a spectrum of an identical, partially reacted reaction mixture without presaturation and with 20 s relaxation delay. Multiplication of the integral values of the corresponding coefficient resulted in quantitative data on substrate and product concentrations.

Results

D-Galacturonic acid dehydrogenase activity was induced in *A. tumefaciens* during growth on D-galacturonic acid. This enzyme activity was purified from the crude cell extract in a two step procedure. In the first step the crude extract was loaded to a DEAE column and eluted with a salt gradient. The D-galacturonic acid activity eluted as a single peak. In the second step, this partially purified and concentrated activity was separated by a non-denaturating PAGE and the active D-galacturonate dehydrogenase stained by zymogram

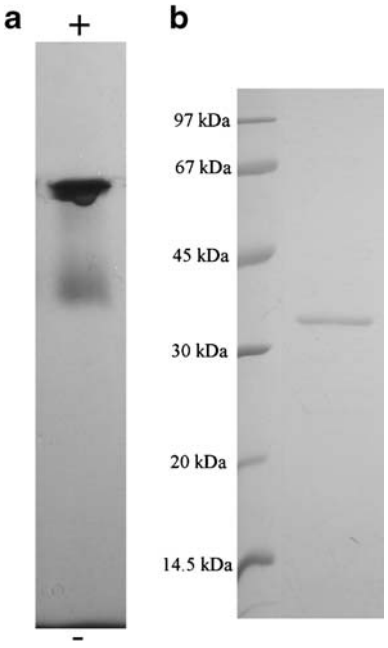

Fig. 2 Identification and expression of the *A. tumefaciens* D-galacturonic acid dehydrogenase (Udh): **a** Zymogram of D-galacturonic acid dehydrogenase activity. The protein that was partially purified by binding to a DEAE column and eluting with a salt gradient was further purified by a non-denaturating PAGE. The active enzyme appears as a *dark blue band*. **b** Coomassie blue-stained SDS-PAGE gel of purified his_6-tagged D-galacturonic acid dehydrogenase. *Left* molecular weight markers proteins. *Right* the purified his_6-tagged D-galacturonic acid dehydrogenase

staining (Fig. 2a). The active fraction was cut out, eluted, and applied to an SDS-PAGE. A 32 kDa protein band was identified as the potential D-galacturonic acid dehydrogenase. The protein band was cut from the gel, digested with trypsin and the resulting peptides were analysed using MALDI-TOF. The following peptide masses were obtained: 665.4160, 966.4224, 1,020.4779, 1,029.5153, 1,407.4625, 1,720.6669, 1,740.7902 and 2,082.9321. These peptide masses were then used to identify the corresponding gene in the *A. tumefaciens* genome (Goodner et al. 2001; Wood et al. 2001). The gene with the locus tag Atu3143 (*udh*) was identified as the coding sequence for the D-galacturonic acid dehydrogenase. After heterologously expressing the *udh* gene in *S. cerevisiae* under the constitutive TPI promoter, 0.12 units D-galacturonic acid dehydrogenase activity per milligram of extracted protein was observed. In the control strain that contained the empty plasmid, no activity could be detected under the same assay conditions.

After conformation of the function of the cloned *udh* gene, an N-terminal his_6-tagged version of the enzyme was expressed in *S. cerevisiae* under the exact same conditions as the non-tagged and the enzyme activity measured in the crude cell extract. The his_6-tagged protein exhibited the same activity as the non-tagged protein in the extract. The his_6-tag made it possible to purify the dehydrogenase in a single step using the Ni-IMAC column, and the purified enzyme was essentially pure as can be seen from the single band observed in the Coomassie stained SDS-PAGE shown in Fig. 2b. The yield was 0.6 mg of enzyme when starting from 14 g yeast cells (wet mass) obtained for a 1-L overnight culture.

The purified enzyme was used to estimate the Michaelis–Menten constants on D-galacturonic and D-glucuronic acid (Fig. 3). For D-galacturonic acid, a Km of 0.5 mM and a Vmax of 124 U/mg and for glucuronic acid a Km of 1.1 mM and a Vmax of 221 U/mg were determined. For NAD a Km of 0.2 mM and a Vmax of 105 U/mg were found. No activity was found with NADPH as a cofactor. The enzyme exhibited activity with the uronic acids D-galacturonic acid and D-glucoronic acid. The aldose or aldonic acid forms of D-glucose and D-galactose were not substrates (Table 1).

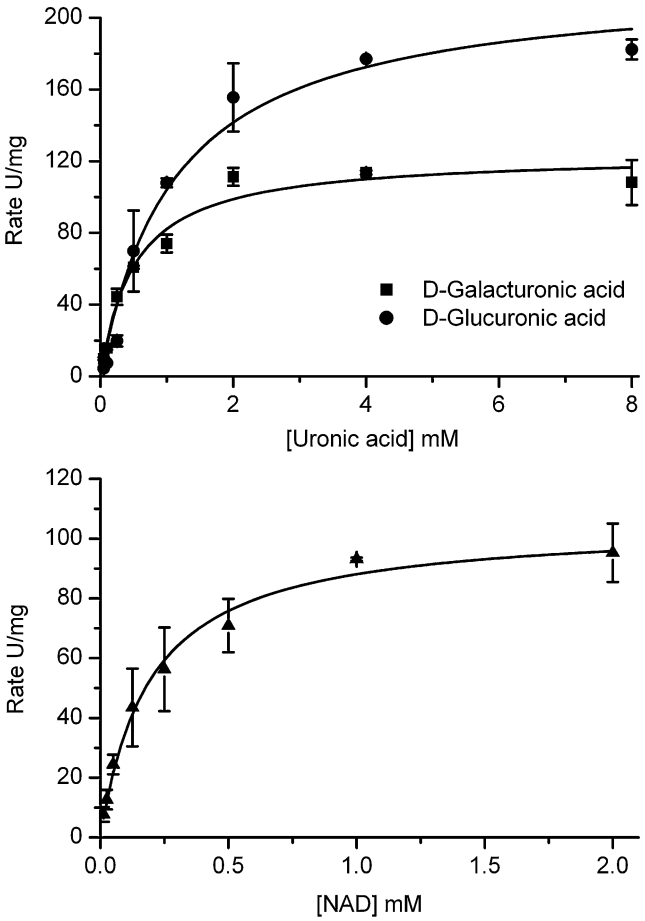

Fig. 3 Kinetic properties of the purified D-galacturonic acid dehydrogenase: (*Top panel*) reaction rate as a function of the uronic acid concentration. The drawn *line* is a fit of the data points to the Michaelis–Menten equation. The determined K_m for D-galacturonic acid was 0.5 mM with a V_{max} of 124 U/mg and for D-glucuronic acid, the K_m of 1.1 mM was with a V_{max} of 221 U/mg. The NAD concentration was 2 mM (*Bottom panel*). Reaction rate as a function of the NAD concentration. The *drawn line* is a fit of the data points to the Michaelis–Menten equation; for NAD, a K_m of 0.2 mM and a V_{max} of 105 U/mg of protein was determined. The D-galacturonic acid concentration was 8 mM

Table 1 Specific activity of the D-galacturonic acid dehydrogenase on different related alduronic acids, aldonic acids and aldoses

Substrate	Specific activity (% of V_{max})
D-galacturonic acd	100%
D-glucuronic acid	178%
D-galactose[a]	Not detectable
D-glucose[a]	Not detectable
D-galactonic acid[a]	Not detectable
D-gluconic acid[a]	Not detectable

[a] Substrate concentrations up to 8 mM were used in the presence of 2-mM NAD

NMR spectroscopic analysis of the substrate D-galacturonic acid revealed that in crystalline form it is predominantly in α-pyranosic form. In the equilibrium in water solution, the α- to β-ratio is about 40% to 60%. The mutarotation, however, is slow and it took more than 10 h to reach the equilibrium in pH 3, 23°C. In water solution, over 90% of the molecules are in pyranose form, as was evident from the $^3J_{H,H}$ coupling constants typical for a galactopyranosic ring (Table 2). In addition to the pyranose forms, at least two other forms could be observed in the NMR spectrum, possibly representing furanosic rings.

D-Galacturonic acid conversion to the product by the purified Udh enzyme was monitored by NMR at pH 8.0. Most of the product (87%) was in a lactone form while the fraction of the linear *meso*-galactaric acid slowly increased when the reaction mixture was incubated for several days. This is probably due to spontaneous opening of the lactone ring in the slightly basic conditions. In order to follow the reaction in more detail, the reaction was repeated with 15 times higher enzyme concentration at neutral pH (6.8) and the reaction was followed in real time by recoding a series of 1D ^{1}H NMR spectra over 1 h with 20 s time steps. From this series of spectra, the signals corresponding to α– and β–D-galaturonic acid, galactaric acid lactone, linear *meso*-galactaric acid and a signal of NADH produced in the reaction (Fig. 4) were integrated and converted to concentrations as described in "Materials and methods". The results (Fig. 5) show that in the beginning of the reaction, the β-pyranosic form of the substrate was consumed faster than the α form, while in the later stage of the reaction the consumption rates were equal. The lactone form of the galactaric acid and NADH were produced with approximately equal rate, but only very small amount of the linear *meso*-galactaric acid was produced (~2% of the total galactaric acid) under this neutral pH.

Both 1,4 and 1,5 lactone of galactaric acid are in principle possible products of the Udh reaction. In order to obtain enough pure material for NMR analysis of the lactone form of galatartic acid, commercial galactaric acid was studied. Only the linear form was present in the commercial galactaric acid, but incubation at pH 1 at 95°C for 5 h resulted in about 90% conversion to a product that in NMR was identical to the lactone produced by the enzyme. The $^3J_{H,H}$ coupling constants between the of pairs ring protons were very similar to each other and clearly different from what would be expected of a 6-membered ring. The carbonyl group in position 1 results in a rigid planar structure of the carbonyl group and the two atoms on both sides of it and only two conformations would be possible for the plausible 5-membered ring, which would result in approximately equal coupling constants. HMBC spectra optimised for different long-range coupling constant could not provide evidence for either of the anticipated lactones. However, further evidence for the 1,4 lactone was obtained from a NOESY spectrum (not shown), where NOEs were observed between all pairs of protons except H2 and H5, which would be in the same side of the ring in 1,5 lactone. Thus, the $^3J_{H,H}$ coupling constants and NOEs prove that the product of the enzyme reaction is galactaric acid 1,4-lactone. In cytosolic pH, the lactone ring is not opened spontaneously with a meaningful rate.

Discussion

We have purified a D-galacturonic acid dehydrogenase activity that was induced to D-galacturonic acid and

Table 2 ^{1}H NMR chemical shifts and coupling constants of D-galacturonic acid and D-galactaric acid in D_2O, pH 6 at 25°C

	Chemical shift (ppm)	$^3J_{H,\ H+1}$ (Hz)
D-galacturonate		
α-H1	5.292	3.7
α-H2	3.824	10.2
α-H3	3.913	3.4
α-H4	4.282	<1
α-H5	4.406	
β-H1	4.570	7.9
β-H2	3.504	9.6
β-H3	3.692	3.2
β-H4	4.215	<1
β-H5	4.055	
D-galatarate 1,4 lactone		
H2	4.646	9.2
H3	4.414	8.4
H4	4.605	2.0
H5	4.244	
meso-D-galactarate		
H2, H5	4.263	
H3, H4	3.956	

The chemical shifts were referenced to internal TSP (0 ppm)

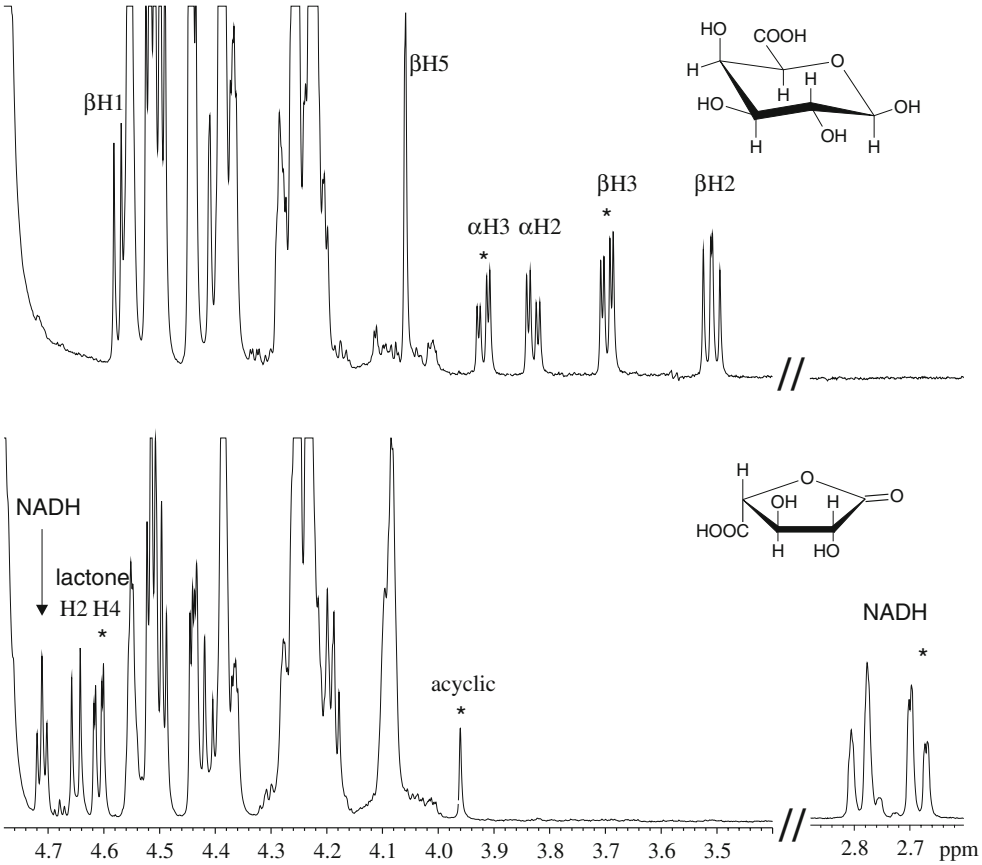

Fig. 4 Expansions of 600 MHz ^{1}H NMR spectra of the reaction mixture of substrates D-galactaric acid and NAD before addition of the enzyme (*upper*) and after full conversion. The signals that were used for quantitative real-time NMR analysis of the time course of the reaction are marked by *asterisks*

identified the corresponding gene. During the purification, we observed only a single peak of activity indicating that a single protein might be responsible for this activity. To confirm that we identified the correct gene coding for a functional enzyme, we expressed it in the heterologous host *S. cerevisiae* and to facilitate the purification, we tagged the protein. Since tagging sometimes compromises the protein's activity, we compared the activity of the tagged and untagged protein. Under identical expression conditions, we found the same activity in the crude cell extract suggesting that this tag has no effect on the enzyme activity. The tagged protein was purified and the kinetic properties analysed. We found a Km of 0.5 mM for D-galacturonic acid and 1.1 mM for D-glucuronic acid. The enzyme is specific for NAD and has a V_{max} with D-galacturonic acid of 105 U/mg. The enzyme is converting the hexuronic acid to the hexarate lactone. The lactone might then hydrolyse spontaneously to the linear hexarate. It was previously described that the reverse reaction with the linear hexarate and NADH as substrates is not occurring (Wagner and Hollmann 1976b). Only at acidic pH, when a small fraction of the hexarate is in the lactone form, a weak reverse reaction was observed (Wagner and Hollmann 1976b).

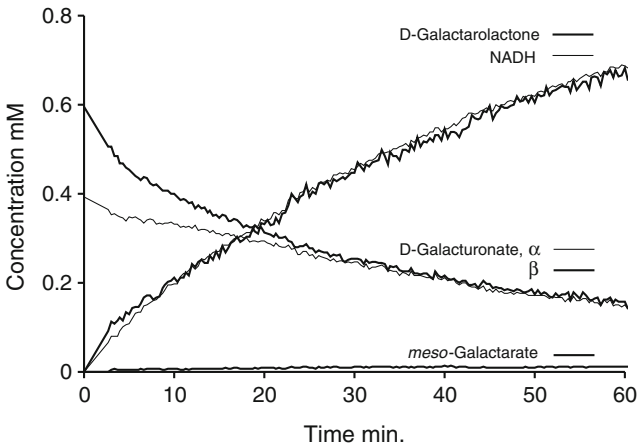

Fig. 5 Real-time NMR analysis of the time course of the reaction. A 500 MHz ^{1}H NMR spectra of the reaction mixture at pH 6.8 were recorded with 20 s time steps and signals indicated in Fig. 4 were integrated and corrected for the effects of incomplete relaxation and water presaturation. The results suggest that both pyranosic anomers of D-galacturonic acid are accepted as subtrates and converted to galactaro-1,4-lactone. The lactone is stable under neutral or acidic pH as is evident by the very low concentration of linear *meso*-galactaric acid

The NMR experiments showed that over 90% of the substrate D-galacturonic acid exists in the pyranosic form, with α-/β-ration of 40/60%. When the enzyme reaction was followed in real time by NMR, the β-pyranosic form of D-galacturonic acid was first consumed faster than α, but at the latter stage the consumption rates converged. This probably reflects the concentration differences of the two

anomers in the beginning of the reaction and suggests that both anomers are accepted as a substrate. Further evidence for this is provided by the slow mutarotation rate observed for freshly dissolved crystalline D-galactaric acid. Structural analysis of the lactone product by NMR showed that the product is galactaric acid 1,4-lactone. In slightly basic pH, the lactone is very slowly (during several days) opening to the linear form, while in neutral or slightly acid pH the lactone is very stable. Thus, our results suggest that the enzyme accepts both pyranosic anomers of D-galacturonic acid as substrates and converts them to a five membered lactone ring of galactaric acid, which does not open spontaneously under intracellular conditions. Formation of a 1,4 lactone has been also reported for the D-galactose dehydrogenases from *Pseudomonas saccharophila* and *Pseudomonas fluorescence* (Ueberschär et al. 1974). For these dehydrogenases producing an aldonic acid, rapid kinetic techniques showed that β−D-galactopyranose is primarily converted to D-galactono-1,5-lactone and rearranges non-enzymatically to the corresponding D-galactono-1,4-lactone. A similar catalytic mechanism could be active for the D-galacturonic acid dehydrogenase, but to prove this rapid quenching of the reaction would be required to trap the primary 6-membered ring reaction intermediate.

The D-galacturonic acid dehydrogenase is part of the oxidative pathway for D-galacturonic acid catabolism. The enzyme activity had been described previously in *A. tumefaciens*, where activities with D-galacturonic, D-glucuronic and D-mannuronic acid were observed in the crude extract (Zajic 1959). Also, the partially purified enzyme exhibited activity with D-galacturonic as well as with D-glucuronic acid. The affinities of the protein purified from D-galacturonate grown cells toward the substrates D-glucuronate and D-galacturonate were similar to the affinities of the enzyme described in this communication (Chang and Feingold 1969). Recently, Yoon et al. (2008) identified a gene from *A. tumefaciens* coding for a D-glucuronate dehydrogenase. In their approach, a genomic library was screened in an *E. coli* mutant that had a deletion in the *uxaC* gene coding for the uronate isomerase converting D-glucuronate to D-fructuronate. This mutant was unable to grow on D-glucuronate but regained this ability when a D-glucuronate dehydrogenase was expressed converting D-glucuronic acid to D-glucaric acid. *E. coli* can use D-glucaric acid as a carbon source for growth. The gene, *udh*, that was identified in this screen for a D-glucuronic acid dehydrogenase turned out to be identical to the D-galacturonate dehydrogenase identified in this communication. This might suggest that *A. tumefaciens* has only the one gene coding for hexuronic acid dehydrogenase. Another interpretation would be that there is another D-glucuronate dehydrogenase that is still to be identified. According to Chang and Feingold (Chang and Feingold 1969) different hexuronic acid dehydrogenases are expressed depending on whether the growth substrate is D-glucuronate or D-galacturonate. The biggest difference between the two enzymes was the affinity toward D-glucuronate which was 0.25 mM for enzyme from D-glucuronate grown cells, as compared to 2.4 mM for enzyme from D-galacturonate grown cells. Also the ratio of activity with D-galacturonate versus the activity with D-glucuronate was different for the different enzymes that were induced during growth on D-galacturonate or D-glucuronate.

The *udh* gene coding for the hexuronic acid dehydrogenase might find applications in the metabolic engineering (Moon et al. 2009). Also the purified protein is potentially useful. It might be used in an assay for the enzyme kinetic quantification of hexuronic acid as suggested previously (Wagner and Hollmann 1976a).

In bacteria the genes of a catabolic pathway are often organised in an operon. In the case of the *udh* gene however, it does not seem that the genes of the remaining pathway are in the same operon (Yoon et al. 2008). It might be that the genes necessary for growth on mucic acid are regulated in a different way than the *udh* and are consequently in a different operon. In an attempt to discriminate whether the D-galacturonate dehydrogenase was induced by the same mechanism as the remaining pathway, Chang and Feingold (Chang and Feingold 1969) tested the oxygen uptake of resting cells grown D-galacturonate and galactarate. Cells grown on D-galacturonate immediately oxidised both, D-galacturonate and galactarate whereas, cells grown on galactarate immediately oxidised galactarate but not D-galacturonate. It was concluded that the difference is due to a lacking D-galacturonate transporter since in the crude extract D-galacturonate activity was found in D-galacturonate and galactarate grown cells. This observation suggested already that not all the genes of the D-galacturonate catabolic pathway are organised in a single operon.

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