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Biochemical characterization of uronate dehydrogenases from three Pseudomonads, *Chromohalobacter salixigens*, and *Polaromonas naphthalenivorans*

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ABSTRACT

Enzyme catalysts will be vital in the development of synthetic biology approaches for converting pectinic monosaccharides from citrus and beet processing waste streams to value-added materials. We describe here the biophysical and mechanistic characterization of uronate dehydrogenases from a wide variety of bacterial sources that convert galacturonic acid, the predominate building block of pectin from these plant sources, and glucuronic acid to their corresponding dicarboxylic acids galactarate and glucarate, the latter being a DOE top value biochemical from biomass. The enzymes from *Pseudomonas syringae* and *Polaromonas naphthalenivorans* were found to have the highest reported k_{cat}(glucuronic acid) values, on the order of 220–270 s^{-1}. The thermal stability of this enzyme type is described for the first time here, where it was found that the $K_t^{(0.5)}$ value range was >20 °C, and the enzyme from *Chromohalobacter* was moderately thermostable with $K_t^{(0.5)}$ = 62.2 °C. The binding mechanism for these bi-substrate enzymes was also investigated in initial rate experiments, where a predominately steady-state ordered binding pattern was indicated.

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1. Introduction

Pectins from citrus waste processing and sugar beet refining represent a significant untapped biomass resource since the annual U.S. citrus production is ~14×10^6 metric tons/year (http://apps.fas.usda.gov/psdonline/circulars/citrus.pdf), and the sugar beet production in the U.S. is ~30×10^6 metric tons/year (http://usda01.library.cornell.edu/usda/current/CropProdSu/CropProdSu-01-10-2014.pdf). Pectins are currently used primarily as animal feed or discarded; the use as livestock feed involves drying the pulp to preclude spoilage, with an attendant energy requirement 30–40% of the total energy input for beet processing [1,2], while disposal incurs added expense, and it is thus becoming economically and environmentally desirable to develop value-added products from these bio-resources. Structurally, pectins comprise a family of heterogeneous polysaccharides from plant primary cell walls wherein D-galacturonic acid (GalUA) occurs in abundance, and they can be grouped as homogalacturonan (HG), xylogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II [3]. HG consists of α-1,4-linked GalUA that is highly methyl esterified, with degree of esterification of 60–90%, and acetyl esters may also be present [4]. The pectin extracted from citrus peel cell wall material, which constitutes about 3/4 by weight of dried citrus peel, contains about 1/3 by weight GalUA-rich HG [5]. GalUA can be catabolized to pyruvate and glyceraldehyde [6], and is a precursor of ascorbate [7] and the aldaric acid meso-galactarate [8]. Aldaric acids are of commercial interest with glucaric acid being listed by the DOE as a top value chemicals from biomass [9] (http://www1.eere.energy.gov/bioenergy/pdfs/35523.pdf) due to potential use in synthetic polyhydroxypolyamides (nylons) [10], and as chelators [11] in, e.g. the detergent surfactant market. The mineral acid chemical synthesis route to the aldarics from the corresponding sugars is reportedly low-yielding (~40% for glucaric acid) [12]; other challenges going forward for the inorganic catalysis route include removal of non-specific oxidation side products [13], and potential capital and environmental expense from employing nitric acid. Thus complementary development of an enzymatic route to aldaric acids is of interest owing to its potential to overcome some or all of these limitations, an analogous example being amylase superseding acid-catalyzed depolymerization of starch.

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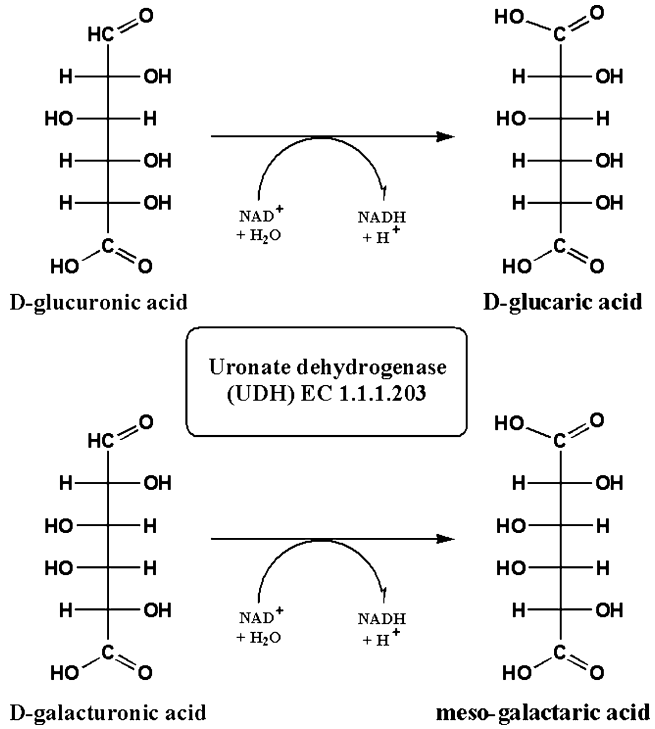

Fig. 1. Reactions catalyzed by uronate dehydrogenase.

An enzymatic route to aldaric acids proceeds through the activity of uronate dehydrogenase (EC 1.1.1.203), which oxidizes uronic acids such as GalUA and D-glucuronic acid (GluUA) to an initial 1,4-lactone product that opens to the linear form under basic conditions [14] such that the conversion is irreversible (Fig. 1). Induction, discovery, purification and characterization of UDH enzyme of unknown amino acid sequence from *Pseudomonas syringae* was first reported in 1959 and intermittently thereafter [8,15,16], while recombinant UDH from this organism, identical to that reported on here, has previously been partially characterized [17]. X-ray crystal structures have been solved for UDH from both *Chromohalobacter salixigens* (pdb code 3AY3) [1,18] and *Agrobacterium tumefaciens* (pdb code 3RFT) [19]. Reported applications of UDH include development of spectrophotometric single- and coupled-enzyme assays for the quantitation of uronate glycosides and uronic acids [20,21], metabolic engineering of the fungi *Hypocrea jecorina* and *Aspergillus niger* [22] and *Saccharomyces cerevisiae* [23] for conversion of GalUA to galactaric acid, and creation of a synthetic metabolic pathway not observed in nature for the (*in vivo*) conversion of glucose to glucaric acid in *E. coli* [24].

We characterized UDH from *Pseudomonas syringae* (PsUDH, NCBI accession EU377538), and extend here characterized UDH enzymes to include two additional *Pseudomonas* species (*Pseudomonas mendocina*, PmUDH; NCBI accession ABP86052.1; *Pseudomonas fluorescens* Pf0-1, PfUDH, NCBI accession ABA75190.1), and two additional bacterial genera (*Polaromonas naphthalenivorans* CJ2, PnUDH, NCBI accession ABM36905; *Chromohalobacter salixigens*, CsUDH, PDB ID 3AY3). We describe for the first time the thermal stability of these enzymes and the binding mechanism of PnUDH and PfUDH.

2. Materials and methods

2.1. Cloning, expression, and purification of enzymes

An internal *Xho*I site in *PsUDH* was removed, and the DNA sequences of *PnUDH*, *PmUDH*, *PfUDH*, and *CsUDH* were each optimized by inspection for maximal identity to that of *PsUDH* for use in on-going enzyme engineering efforts, with the caveat that native amino acid sequences be maintained and internal *Nde*I and *Xho*I restriction sites be avoided (Fig. S1). DNA was synthesized with 5′ *Nde*I and 3′ *Xho*I restriction sites (Genscript USA Inc., Piscataway, NJ) that were employed for subcloning into pET29b(+) (EMD Chemicals, Inc., Gibbstown, NJ) such that the expressed native peptide C-termini residues were followed by Leu-Glu-$(His)_6$. *Escherichia coli* BL21(DE3) codon plus cells (Agilent Technologies, Inc., Santa Clara, CA) were employed for expression. A glycerol stock stab was used to inoculate 100 ml Terrific Broth containing 30 μg/ml kanamycin (TB_{kan}) that was incubated overnight (37 °C, 250 rpm). One liter of TB_{kan} was then seeded and grown to $OD_{600\,nm}$ ~2 before inducing protein expression with 1 mM IPTG, and protein expression occurred overnight at 30 °C. Cells were lysed using ~13 ml/g cell pellet lysis solution: 65 ml CelLytic B reagent, 0.5 mg/ml hen egg-white lysozyme, 2 mM β-mercaptoethanol, 5 U/ml Benzonase® (all from Sigma-Aldrich, St. Louis, MO), and 1 μL/ml CalBiochem protease inhibitor cocktail III (EMD Biosciences). The His-tagged peptides were partially purified using Ni-NTA resin (Qiagen, Valencia, CA), followed by size-exclusion chromatography on a Superdex 200 16/60 column (GE Life Sciences), with protein concentrations measured using absorbance at 280 nm.

2.2. Enzyme kinetic parameters

D-Glucuronic acid (GluUA), D-galacturonic acid (GalUA), D-glucaric acid, NAD^+, and NADH were obtained from Sigma-Aldrich (St. Louis, MO). Water was purified by a Milli-Q Academic A10 unit (Millipore; Billerica, MA). Apparent kinetic parameters in Table 1 for all enzymes were determined using method A where [GluUA] was fixed at 20 mM (>15 × $K_{m(app)}$) for measuring $K_{m(NAD^+)(app)}$, otherwise [NAD^+] was fixed at 4 mM (>10 × $K_{m(app)}$). In method A, varying concentrations of either NAD^+, GluUA, or GalUA were used in 0.2 ml reactions performed in 96-well microplates in triplicate containing 100 mM sodium phosphate, pH 8.0, 1 mg/ml BSA, 4 mM NAD^+, 25 °C. UV/VIS absorbances at 340 nm were determined using a Spectramax plate reader (Molecular Devices, Sunnyvale, CA), and data fitted to Eq. (9).

Kinetic parameters for PfUDH (Table 1, Figs. S11 and S12) and PnUDH (Table 1, Figs. 4–6 and S2–S10) were also obtained using method B, where 1 ml reactions were performed at 25 °C in 100 mM Tris, pH 8.0, ionic strength 0.5 M, and the concentrations of GluUA, GalUA, D-glucaric acid, NADH, and NAD^+ were varied. UV/VIS absorbances were determined using a thermostated Cary 50 Bio UV–Visible spectrophotometer (Agilent; Santa Clara, CA). An upgraded model SX.18MV-R stopped-flow (Applied Photophysics; Leatherhead, UK), with a thermostated compartment for syringes and reaction chamber and a 2 or 10 mm path length for fluorescence measurements, was used for rapid fluorescence monitoring.

2.3. $K_t^{(0.5)}$ determination

An MJ Research thermocycler (Bio-Rad, Hercules, CA) was used to establish a temperature gradient of 20–25 °C depending on the enzyme being tested, and enzyme was incubated at various temperatures for 1 h in assay buffer consisting of 100 mM sodium phosphate, pH 8.0, 1 mg/ml bovine serum albumin (BSA), and 40 mM NAD. After thermal challenge, the temperature was lowered to 0–4 °C for 10 min, then allowed to refold at 30 °C for at least 10 min. Aliquots of treated-enzyme mixture were tested for residual activity; 20 mM GluUA, 4 mM NAD, 100 mM sodium phosphate, pH 8, 1 mg/ml BSA, 25 °C. $K_t^{(0.5)}$ was calculated by fitting the data to Eq. (10) (see equations), where a is an exponential term, t is the temperature, $K_t^{(0.5)}$ is the temperature where p is half of P.

2.4. Activity versus pH profile

The effect of pH on V_{max} for GluUA oxidation was determined using 100 mM sodium succinate for pH 3–7, 100 mM sodium phosphate for pH 6.5–8, 100 mM sodium pyrophosphate for pH 7.5–9, and 100 mM AMPSO for pH 8.5–10. Assays were performed in microplates in quadruplicate using 2.5 nM enzyme, 2 mM NAD^+, 15 mM GluUA, and incubated at 25 °C with spectrophotometric monitoring at 340 nm. Enzyme rates were normalized to 100% in Fig. 3.

2.5. Size exclusion chromatography oligomerization state

Oligomerization state was determined using size exclusion chromatography performed with running buffer 50 mM sodium phosphate, pH 8.0, 150 mM NaCl, and 5 mM DTT using Superdex 200 10/300 GL and Superdex 75 10/300 columns and low and high molecular weight standards (GE Life Sciences); apparent molecular weight was calculated according to the manufacturer's instructions.

2.6. Equations

Data were fit to the following equations where ν is the observed initial (steady-state) rate of catalysis, E_T is the total enzyme concentration, k_{cat} is the turnover number of catalysis, K_m is the Michaelis–Menten constant, K_i is the dissociation constant for I from EI, A is one substrate concentration, B is one substrate concentration, K_{ia} is the binding constant for substrate A to the free enzyme, K_{ma} is the Michaelis constant for substrate A, K_{mb} is the Michaelis constant for substrate B, I is the inhibitor concentration, K_{is} is the inhibition constant of the slope, K_{ii} is the inhibition constant of the intercept, A_0 is the initial value, k is the first-order rate