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Enzymatic Assay of D-Glucuronate Using Uronate Dehydrogenase

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Running title: Enzymatic assay of D-glucuronate

1   **Abstract**

2   D-Glucuronate is a key metabolite in the process of detoxification of xenobiotics

3   and in a recently constructed synthetic pathway to produce D-glucaric acid, a "top-value

4   added chemical" from biomass. A simple and specific assay of D-glucuronate would be

5   useful for studying these processes, but existing assays are either time-consuming or non-

6   specific. Using uronate dehydrogenase cloned from *Agrobacterium tumefaciens*, we

7   developed an assay for D-glucuronate with a detection limit of 5 μM. This method was

8   shown to be more suitable for a system with many interfering compounds than previous

9   methods and was also applied to assays for *myo*-inositol oxygenase activity.

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11   Keywords: enzymatic assay; uronate dehydrogenase; D-glucuronate; *myo*-inositol

12   oxygenase

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1   D-Glucuronate is a metabolite in the synthesis of vitamin C [1] and plays an

2   important role in glucuronidation, a conjugation reaction of D-glucuronate with

3   xenobiotics and endobiotics, allowing for their detoxification and elimination [2]. D-

4   Glucuronate has also been known to be a conversion product of inositol, a constituent of

5   membrane phospholipids and a precursor for cell signaling molecules in mammals [3],

6   and to be converted to D-glucaric acid via a three-step route in mammals or a uronate

7   dehydrogenase-catalyzed step in bacteria [4]. Recently, we constructed a synthetic

8   pathway with D-glucuronate as an intermediate to produce D-glucaric acid from D-

9   glucose and have been interested in developing a simple and specific assay of D-

10   glucuronate [5]. In addition, D-glucuronate units occur in several classes of

11   glycosaminoglycans [6], and assays for the D-glucuronate units have been the standard

12   methods for estimating the polysaccharides containing D-glucuronate [7].

13

14   Colorimetric assays using orcinol [3] or carbazole [8] have been widely used to

15   quantify D-glucuronate, but these methods lack specificity. Pentoses such as 2-deoxy-D-

16   ribose and hexoses such as D-glucose also react with these reagents [9]. The linearity of

17   the orcinol method was assessed using D-glucuronate standard solutions (Alfa Aesar,

18   Ward Hill, MA) and measuring absorbance at 670 nm. A fairly good linear relationship

19   ($y=1.833x+0.007$, $R^2=0.993$) was obtained with a detection limit of 20 µM D-

20   glucuronate, which corresponds to an absorbance change of 0.04 units. However, as

21   expected, D-glucose, a common hexose component in culture media, also gave a strong

22   signal ($y=0.627x-0.012$, $R^2=0.991$). In addition, both colorimetric assays require harsh

23   conditions including the use of concentrated acids with boiling steps. An HPLC assay

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1   has been developed [10], but this approach is usually time consuming and requires the

2   appropriate instrumentation. Moreover, pretreatment of samples with boronic acid

3   affinity gel was reported to be required to separate D-glucuronate from D-glucarate

4   because these two compounds had almost the same retention time [5; 10]. A new assay

5   based on two purified enzymes, uronate isomerase and mannonate dehydrogenase, and

6   oxidation of NADH has also been reported as an alternative [9]. This method is quite

7   sensitive and specific to D-glucuronate, with reduced activity on D-galacturonate.

8   However, a significant amount of work to purify the two enzymes is required.

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10   We have recently cloned, purified, and characterized uronate dehydrogenase

11   (Udh) from *Agrobacterium tumefaciens* str. C58 using a commercially-available protein

12   purification kit (Pro-Bond purification system, Invitrogen Corporation, Carlsbad, CA)

13   [11]. Udh is specific to D-glucuronate and D-galacturonate but does not accept

14   aldohexoses, aldopentoses, polyols, or other uronates as substrates [11; 12]. This enzyme

15   was found to have a high rate constant ($k_{cat} = 1.9 \times 10^2\,\text{s}^{-1}$ on D-glucuronate) with a low

16   K_m (0.37 mM). Using this newly cloned Udh, we constructed a specific and simple

17   method to assay for D-glucuronate (Supplemental Material). The linearity of our newly

18   developed enzymatic assay was assessed using D-glucuronate standard solutions similar

19   to those used to examine the orcinol assay (Fig. 1). In this method, NAD^+ and purified

20   Udh were added to the sample, NADH generation was monitored at 340 nm, and the D-

21   glucuronate concentration was calculated using the extinction coefficient of $6.22\,\text{mM}^{-}$

22   $^1\text{cm}^{-1}$ for NADH. As shown in Fig. 1, a comparable linear relationship was observed,

23   with a detection limit of 5 μM, corresponding to an absorbance change of 0.03 units. The

1   linear range was 5 to 320 μM D-glucuronate, in which the upper limit was determined not

2   by the activity of Udh but by the reliable measurement of the spectrophotometer

3   (maximum reliable absorbance unit of 2). The corresponding calibration curve

4   (absorbance at 340 nm vs. D-glucuronate concentration) has a slope of 6.26, which is 3.4

5   times higher than that of the calibration curve obtained by the orcinol assay, indicating

6   the increased sensitivity of the enzymatic assay. The plot of NADH produced versus D-

7   glucuronate supplied as substrate yields a slope of 1.0 and an intercept of 0.0 (Fig. 1),

8   indicating that the NADH concentration as determined by absorbance readings at 340 nm

9   can be used to directly determine the D-glucuronate concentration.

10

11   The effect of culture broth from two different *E. coli* strains containing D-glucose

12   on the enzymatic assay was also investigated. A standard solution of D-glucuronate (160

13   μM) was added to distilled water or samples of culture supernatant after centrifugation to

14   see whether medium components or cellular metabolites interfere with the enzymatic

15   assay. Almost 100% recovery was achieved, showing no interference by the culture

16   broth of the two typical laboratory *E. coli* strains (D-glucuronate measured (μM,

17   mean±SD): 161±2 for distilled water; 163±2 for DH10B; 162±2 for BL21 Star™ (DE3)).

18   HPLC analysis indicated that the assay samples contained ~0.4 mM D-glucose, which

19   would result in at least 180% recovery with the orcinol assay as calculated from the

20   calibration curves (y=1.833x+0.007 for D-glucuronate; y=0.627x-0.012 for D-glucose).

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22   We applied this enzymatic assay to a system [5] in which D-glucuronate is

23   produced from D-glucose by *E. coli* BL21 Star™ (DE3) containing two recombinant

1 genes encoding *myo*-inositol-1-phosphate synthase and *myo*-inositol oxygenase (BL21

2 Star™ (DE3)(pRSFD-IN-MI)). BL21 Star™ (DE3)(pRSFDuet-1) containing empty

3 plasmid pRSFDuet-1 was used as the negative control. Cultures were grown at 30°C for

4 24 hrs in LB medium supplemented with 10 g/L D-glucose and induced with 0.5 mM

5 IPTG. Culture supernatant was collected after centrifugation, and the D-glucuronate

6 concentration was determined by either the enzymatic method or HPLC analysis as

7 described previously [5]. Three enzymatic or HPLC measurements gave D-glucuronate

8 concentrations of 791±13 or 804±32 μM, respectively, from the culture of BL21 Star™

9 (DE3)(pRSFD-IN-MI). A t-test determined that the difference in the average values is

10 not statistically significant (95% confidence level). As expected, no D-glucuronate was

11 detected from the negative control although ~0.6 mM D-glucose was present in the assay

12 samples, confirming that the enzymatic assay is not affected by this component.

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14   We applied the enzymatic assay to the determination of *myo*-inositol oxygenase

15 (MIOX) activity. Assays for MIOX activity have been performed using lysates or

16 purified enzymes and *myo*-inositol, and the produced D-glucuronate has been determined

17 by the orcinol method [5; 13; 14]. Once the MIOX enzyme is purified, there would be no

18 interfering compounds in the sample, and the orcinol method could be used reliably to

19 determine the MIOX activity. However, MIOX assays using lysates are sometimes

20 preferred because sample preparation does not require extensive enzyme purification

21 steps. Enzyme activities measured in lysates might also estimate more accurately the *in*

22 *vivo* activity compared to that of the purified enzyme. As discussed above, the orcinol

23 method results in a higher level of background signal because many interfering

1   compounds (e.g., pentoses and hexoses) are likely to exist in the lysates. To study the

2   feasibility of our enzymatic method for this application, we determined the conversion

3   rates of *myo*-inositol by MIOX with different lysate amounts by enzymatically measuring

4   the D-glucuronate produced (Fig. 2). A good linearity was observed especially when

5   more than 0.1 mL of the lysate was used ($R^2 = 0.9805$ with only the last four data points).

6   We also performed experiments to determine the specific activity of MIOX using the

7   orcinol method [13; 14] or the enzymatic method. The total protein concentration of

8   lysates was determined using the Bradford method [15], and six measurements gave

9   specific activities of 7.2±1.5 nmol/min/mg or 5.4±0.9 nmol/min/mg, respectively. Net

10   activity is reported for the orcinol assay, in which the background signal measured in the

11   absence of substrate (20 to 80% of the total signal in these samples) has been subtracted.

12   While the difference in activities obtained using these two different methods was found

13   to be statistically significant (95% confidence level of a *t*-test), the high and variable

14   background of the orcinol assay makes it less reliable. The difference in standard

15   deviation between the two data sets was found to be insignificant (at the 95% confidence

16   level).

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18   Our interest in studying a synthetic pathway to produce D-glucuronic and D-

19   glucaric acids [5] triggered the development of a simple and specific assay for D-

20   glucuronate using Udh. The MIOX enzyme step of the biosynthetic pathway was found

21   to be rate limiting, implying that improving its activity and measuring it accurately is

22   important for enhancing the productivity of these acids. We are now applying these

23   enzymatic assays to our systems to determine D-glucuronate titer and MIOX activity.

1 Our methods might be also useful to study other systems, including the biosynthetic

2 pathway for vitamin C, glucuronidation processes, and specific quantification of

3 glycosaminoglycans containing D-glucuronate units in the mixture of polysaccharides.

4 D-Galacturonate is also a substrate of Udh ($k_{cat} = 9.2$ x $10\,\text{s}^{-1}$; $K_m = 0.16$ mM) and its

5 presence would interfere with D-glucuronate measurement [11]. However, D-

6 galacturonate is absent in both our system and many others of interest [5; 9]. The current

7 assay could also prove equally useful as a specific assay for D-galacturonate. The

8 stability in solution (stable at 4°C for more than six months so far) and high activity of

9 Udh as well as its relatively easy purification method provides researchers with an

10 alternative method to study D-glucuronate-related metabolism.

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1 **Figure Captions**

2   **Fig. 1.** Calibration curves for D-glucuronate by the enzymatic method using Udh.

3 The reaction mixture (1ml) contained 0.8 mM NAD^+, 100 mM Tris-Cl (pH 8.0), 20 μl

4 sample containing D-glucuronate, and 0.06 U (1U = 1 μmol/min) Udh prepared as

5 described previously [11] and was incubated at room temperature for 30 min. The

6 absorbance increase was measured at 340 nm using a Beckman DU-800

7 spectrophotometer (Beckman Coulter, Fullerton, CA), and the D-glucuronate

8 concentration was calculated using the extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ for

9 NADH.

10

11   **Fig. 2.** Enzymatic assay of MIOX using Udh. MIOX was expressed from pTrc-

12 MIOX [5] in *E. coli* DH10B grown in LB medium with 0.1 mM IPTG at 30°C for 24 hrs.

13 Lysates were prepared by resuspending cell pellets from 50 mL culture in 8 mL sodium

14 phosphate buffer (100 mM, pH 7) with 1 mg/mL lysozyme. EDTA-free protease

15 inhibitor cocktail tablets (Roche Applied Science, Indianapolis, IN) were added to the

16 resuspension buffer according to the manufacturer's instructions. Cell solutions were

17 lysed by sonication and the resulting solutions were centrifuged at 14,000 rpm at 4°C for

18 15 min to remove insolubles. The reaction buffer consisted of 77 mM sodium phosphate

19 buffer (pH 7), 2 mM L-cysteine, 1 mM $FeSO_4$, and 60 mM *myo*-inositol. Samples were

20 pre-incubated without substrate for 5 minutes at 30°C to activate the MIOX enzyme.

21 Reactions were incubated for 1 hr at 30°C, and the D-glucuronate produced was

22 quantified using Udh and 4 mM NAD^+.