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Technical note

A general correction to catalytic rates determined for nonprocessive exo-depolymerases acting on both substrate and product in the initial-rate measurement

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ABSTRACT

We recently reported on the kinetics of the polygalacturonase TtGH28 acting on trimer and dimer substrates. When the starting substrate for hydrolysis is the trimer, the product dimer is also subject to hydrolysis, resulting in discrepancies when either the concentration of dimer or monomer product is used for analysis of trimer hydrolysis. Here, we derive a method for determining catalytic rates of exo-hydrolases acting on trimer (and higher order) substrates when products may also be substrates for hydrolysis and show how this correction may be applied for TtGH28.

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Enzymatic depolymerization of renewable lignocellulosic and food processing waste biomass to constituent monomeric components for direct use or as platform chemicals is an important bioprocessing approach [1]. Glycoside hydrolases (GH) comprise a large group of enzymes for deconstruction of biomass, currently comprised of 135 families defined by amino acid sequence similarity [2]. For the depolymerization of homogalacturonan derived from pectin to its constituent monomer galacturonic acid, a precursor of galactaric acid [3] as well as ascorbate [4], exo-polygalacturonases have been characterized from both eukaryotes [5–11] and prokaryotes [12–18]. In the course of our studies of the hyperthermostable bacterial polygalacturonase TtGH28 from *Thermotoga thermophilus*, which is a non-processive exo-glycoside hydrolase, we had occasion to determine kinetic constants of the enzyme operating on the trimer substrate trigalacturonic acid (triGalUA) and the dimer substrate digalacturonic acid (diGalUA) [19]. It is desirable to have accurate kinetic constants to help predict enzyme performance under industrial depolymerization conditions. Detection of the enzyme activity was limited to a discontinuous assay method requiring the separation of products from reactant and subsequent detection using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) employing a Dionex DX500 HPLC and PA-100 (4 × 250 mm) anion exchange column coupled with an ED40 PAD, and data analysis was performed using Chromeleon software (Dionex, Sunnyvale, CA). The column was equilibrated with 0.1N NaOH, followed by duplicate injections of sample (25 μL) and elution using a 5-min linear gradient (0.05–0.45 M sodium acetate in 0.1N NaOH). The analytes were baseline resolved using this method, and the retention times were 5.5 min for monoGalUA, 6.4 min for diGalUA, and 7.2 min for triGalUA. Standard curves were fitted to linear regression using several concentrations between 0.005 and 0.080 mM of the analytes of interest, with the limit of detection being 0.001 mM. In doing so, we immediately noticed a discrepancy between the measured concentrations of the dimer product diGalUA and the monomer product galacturonic acid (monoGalUA) when the enzyme acted on the trimer: unlike the reaction stoichiometry, which indicates equality, the concentration of monoGalUA was greater than that of diGalUA, with more noticeable differences at low concentrations of the starting

Abbreviations: TtGH28, exo-polygalacturonase from *Thermotoga thermophilus*; triGalUA, trigalacturonic acid; diGalUA, digalacturonic acid; monoGalUA, galacturonic acid.

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substrate triGalUA, which indicated strongly the need for a correction to the data to estimate trimer hydrolyzed. Additionally, the initial rate data were fit to the following equation using Grafit [20] to determine kinetic values and standard errors (Fig. 1, wherein duplicate data points obtained for each substrate concentration are shown):

$$\frac{v}{E_t} = \frac{k_{cat}S}{K_m + S\left(1 + \frac{S}{K_{si}}\right)}, \quad (1)$$

where K_{si} is the substrate inhibition constant. Fig. 1 illustrates the problem that results: for TtGH28 acting on the starting substrate triGalUA, the quantification of monoGalUA produced gives kinetic parameters of k_{cat} 9.62 $s^{-1}$and K_m 3.2 μM whereas quantification of diGalUA produced gives k_{cat} 9.60 s^{-1} and K_m 26.7 μM. The large discrepancy in K_m values determined is purported to result from the action of TtGH28 on the product diGalUA as well as the starting substrate triGalUA. We derived a method to correct the data to account only for the hydrolysis of triGalUA (shown in hollow circles), giving kinetic parameters of k_{cat} 9.51 s^{-1} and K_m 15.8 μM. In the following we show how the correction was accomplished. In doing so, we will discuss a more general case for discontinuous assays of an enzyme acting non-processively to hydrolyze a substrate to a product that is also hydrolyzed by the enzyme, as is the case for TtGH28.

When an exo-hydrolase acts on a trimer substrate, hydrolysis results in two products: a monomer and dimer. In a simple case where further hydrolysis of product dimer by the enzyme does not occur to a significant extent, the monomer and dimer concentrations produced by hydrolysis are equivalent to each other and to the concentration of trimer hydrolyzed – and thus the catalytic rate of the enzyme acting on the trimer – from the concentration of monomer or dimer produced by discontinuous assay method. However, when hydrolysis of the dimer product also occurs to a significant extent, the concentration of monomer product will be greater than (and the concentration of dimer product will be less than) the concentration of trimer hydrolyzed, resulting in difficulty in accurately determining the turnover of trimer from measuring either monomer or dimer concentrations. Since measuring the disappearance of substrate trimer in a discontinuous assay (a small difference in large numbers) is not as accurate as measuring the appearance of newly formed products, it is advantageous to have a method using the measured concentrations of both products to determine the turnover of trimer hydrolysis without the contribution from dimer hydrolysis. Thus, it is desirable to find a mathematical approach to account for dimer hydrolysis when determining trimer hydrolysis.

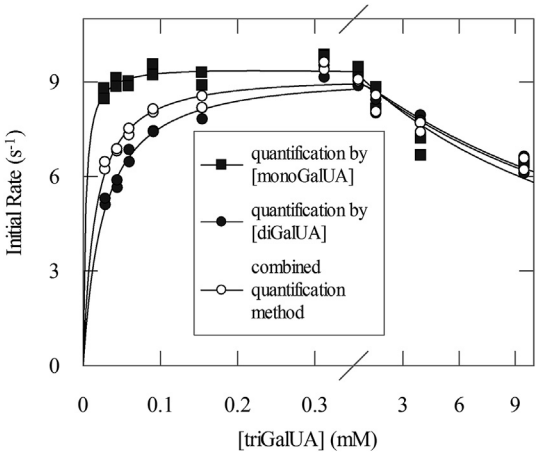

Fig. 1. Steady state kinetics of TtGH28 acting on triGalUA at pH 6.0 and 40 °C with three different quantification methods for initial rates. Data were collected in duplicate and are fit to Eq. (1). The fitted values obtained for TtGH28 acting on triGalUA are k_{cat} 9.62 ± 0.19 s^{-1}, k_{cat}/K_m 3020 ± 1120 $s^{-1}mM^{-1}$, K_m 3.2 ± 1.2 μM, K_{si} 15.3 ± 2.1 mM for quantification by monoGalUA concentration; k_{cat} 9.60 ± 0.19 s^{-1}, k_{cat}/K_m 360 ± 27 $s^{-1}mM^{-1}$, K_m 26.7 ± 2.4 μM, and K_{si} 17.9 ± 2.3 mM for quantification by diGalUA concentration; and k_{cat} 9.51 ± 0.17 s^{-1}, k_{cat}/K_m 603 ± 54 $s^{-1}mM^{-1}$, K_m 15.8 ± 1.6 μM, and K_{si} 17.7 ± 2.1 mM for the combined quantification method using Eq. (6).

In the trimer hydrolysis reaction, when hydrolysis of the product dimer occurs to a large extent, the measured molar concentration of monomer (X_1) is dependent on the molar concentration of trimer hydrolyzed (ΔX_3), with one monomer released per trimer hydrolyzed, and the molar concentration of dimer hydrolyzed (ΔX_2), with two monomers released per dimer hydrolyzed. Thus, one can state the equation

$$X_1 = \Delta X_3 + 2\Delta X_2. \quad (2)$$

Additionally, the measured concentration of dimer (X_2) is dependent on the concentration of trimer hydrolyzed (ΔX_3), with one dimer released per trimer hydrolyzed, and the concentration of dimer hydrolyzed (ΔX_2), which decreases the concentration of dimer. Thus, one can similarly state another equation,

$$X_2 = \Delta X_3 - \Delta X_2, \quad (3)$$

which can be rearranged to yield

$$\Delta X_2 = \Delta X_3 - X_2. \quad (4)$$

Substituting Eq. (4) for ΔX_2 into Eq. (2) yields

$$X_1 = \Delta X_3 + 2(\Delta X_3 - X_2) = 3\Delta X_3 - 2X_2. \quad (5)$$

Solving for the concentration of trimer hydrolyzed, ΔX_3, gives the following result:

$$\Delta X_3 = 2X_2/3 + X_1/3. \quad (6)$$

Thus, the concentration of trimer hydrolyzed can be quantified from the measured concentrations of the dimer and monomer products using Eq. (6).

This method for quantification of the hydrolysis of a starting substrate by an enzyme when the products of the hydrolysis are also substrates for the enzyme can be expanded for starting substrates of any length. For example, it is easily derived for a tetramer starting substrate that the concentration of tetramer hydrolyzed (ΔX_4) is

$$\Delta X_4 = 3X_3/4 + X_2/2 + X_1/4. \quad (7)$$

Similarly, the concentration of starting substrate pentamer hydrolyzed is given by

$$\Delta X_5 = 4X_4/5 + 3X_3/5 + 2X_2/5 + X_1/5. \quad (8)$$

A more general equation for the concentration of the starting substrate hydrolyzed (ΔX_n) for a starting substrate of any length n with product molecules of length i can be written as the summation

$$\Delta X_n = \sum_{i=1}^{n-1} \frac{iX_i}{n}, \quad (9)$$

where X_i is the measured concentration of product of length i. The length of starting substrate analyzed using Eq. (9) is mathematically unlimited; however, the practical consideration of the need