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Short communication

Enzyme assay for chitinase catalyzed hydrolysis of tetra-*N*-acetylchitotetraose by isothermal titration calorimetry

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Abstract

Isothermal titration calorimetry (ITC) has been used to observe the chitinase-catalyzed hydrolysis of tetra-*N*-acetylchitotetraose. Enzymatic hydrolysis of tetra-*N*-acetylchitotetraose by chitinase B from *Serratia marcescens* produces exclusively two molecules of di-*N*-acetylchitobiose allowing for the determination of a single glycosidic bond hydrolysis heat that was used to monitor the rate of the enzymatic reaction. The change in heat rate with respect to time (dQ/dt) was translated to the reaction rate, and the total heat produced was related to substrate concentration throughout the reaction. Reaction rates versus substrates concentration were fit to Michaelis–Menten plots, yielding a k_{cat} of $40.9 \pm 0.5\,s^{-1}$ and a K_m of $54 \pm 2\,\mu M$.

Keywords: Enzymatic assay; Chitinase; ITC

1. Introduction

Chitinases are a widespread group of enzymatic systems that break down chitin, an abundant linear polymer of β-1,4-linked *N*-acetylglucoseamine. Chitin is important in the food and feed industry, pharmacological industry, in water purification systems, and as an antimicrobial additive [1]. The interest in hydrolysis products of chitin (chito-oligosaccharides) is increasing, and efficient production of these compounds requires the use of chitinases. It is therefore of great interest to have a fast, reproducible, and simple method to characterize chitinases that are used in chito-oligosaccharide production. Such a method should preferably use natural substrates. The artificial compound 4-methylumbelliferyl-$(GlcNAc)_2$ that yields the fluorescent 4-methylumbelliferol upon hydrolysis is by far the most frequently used substrate [2–5]. A serious disadvantage with this substrate, besides being artificial, is that it may lead to artifacts caused by substrate inhibition and/or non-natural interactions between enzyme and substrate [2]. Chitin cleavage takes place when the β-1,4 glycosidic bond is hydrolyzed in the sugar polymer. One of the best studied chitinases is chitinase B from the soil bacterium *Serratia marcescens* that primarily produces the dimer di-*N*-acetylchitobiose upon chitin hydrolysis [6–9]. The tetrameric substrate, tetra-*N*-acetylchitotetraose, is exclusively converted to two dimers by chitinase B and many other chitinases, making this substrate the longest natural substrate with one single binding mode and cleavage pattern [6]. This allows for the determination of the reaction heat of a single glycosidic bond hydrolysis that is the result of a single catalytic turnover making tetra-*N*-acetylchitotetraose a promising natural substrate for an ITC-based chitinase assay.

The determination of release or uptake of heat from enzymatically catalyzed chemical reaction has successfully been used to elucidate enzyme mechanisms and kinetics [10–15]. ITC has been used to determine k_{cat} for three chitinases on soluble chitin and activation energies as well as specific activities for the same chitinases on insoluble chitin [16]. However, the use of polymeric substrates prevents the determination of K_m, an essential parameter for the characterization of wild type chitinases and engineered variants [5,17]. Di-*N*-acetylchitobiose, the hydrolysis product, causes little product inhibition. Enzymatic activity of chitinase B has been shown to decrease only with 9% in the presence of 1.2 mM of di-*N*-acetylchitobiose [2]. Kinetic data for degradation of tetra-*N*-acetylchitotetraose by chitinase B or related chitinases have not previously been published.

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2. Materials and methods

2.1. Reagents

Chitinase B from *S. marcescens* was purified as described elsewhere [2,18]. Enzyme purity was verified using SDS/PAGE and was above 95%. Protein concentrations were determined using the Bradford assay kit provided by Bio-Rad (Hercules, CA, USA). Tetra-*N*-acetylchitotetraose was purchased from Sigma–Aldrich.

2.2. ITC experiments

A VP-ITC system (Microcal, USA) was used with control software, VP Viewer. All kinetic measurements were performed in 20 mM potassium phosphate buffer, pH 6.1 at 37 °C, with stirring speed at 260 rpm. The volume of the calorimeter cell was 1.4214 mL. Chitinase B was placed in the syringe at a concentration of 1.2 μM, and 60 μL was injected as a single injection into the calorimeter cell. The cell contained tetra-*N*-acetylchitotetraose at a concentration of 1.0 mM. The reference cell was filled with buffer. After equilibration of the calorimetric cells to 37 °C, the experiment was set to record a baseline for 60 s prior to injection of the enzyme over a time period of 42 s until reaching a final concentration of 0.050 μM. Data used for analysis were taken from 120 s after injection start until the dQ/dt was back to baseline. As a control, tetra-*N*-acetylchitotetraose (23.7 mM and 60 μL) was placed in the syringe and injected as a single injection into the calorimeter cell containing chitinase B (0.050 μM and 1.4214 mL).

2.3. Fitting of theoretical data with experimental data

The use of ITC in enzymatic assays has been described in detail by Todd and Gomez [13]. The following is a brief description of the equations that are used in this work. The power change is given as heat (Q) measured as a function of time (dt):

$$\text{Power change} = \frac{\mathrm{d}Q}{\mathrm{d}t} \tag{1}$$

The amount of heat associated with hydrolysing n moles of the substrate:

$$Q = n \cdot \Delta H_{\text{hydr}} = [S]_{\text{tot}} \cdot V \cdot \Delta H_{\text{hydr}} \tag{2}$$

where ΔH_{hydr} is the experimentally determined hydrolysis enthalpy, $[S]_{\text{tot}}$ the molar concentration of substrate being hydrolyzed, and V is the volume of the cell. ΔH_{hydr} is found by integrating the change of heat with time:

$$\Delta H_{\text{hydr}} = \frac{1}{[S]_{\text{tot}} \cdot V} \cdot \int_{t=0}^{t=\infty} \frac{\mathrm{d}Q}{\mathrm{d}t} \tag{3}$$

Since change in heat with respect to time is related to change in concentration of substrate with time, the following equation yields the rate of catalysis:

$$\text{Rate} = \frac{\mathrm{d}[S]}{\mathrm{d}t} = \frac{1}{V \cdot \Delta H_{\text{hydr}}} \cdot \frac{\mathrm{d}Q}{\mathrm{d}t} \tag{4}$$

Finally, the substrate concentration at any time point (S_t) during the enzymatic catalysis can be calculated from the integral of heat evolved:

$$S_t = S_{\text{tot}} - S_{\text{hydr}} = S_{\text{tot}} - \frac{\int_{t=0}^{t} \mathrm{d}Q/\mathrm{d}t}{\Delta H_{\text{hydr}} \cdot V} \tag{5}$$

where S_{hydr} is the substrate concentration that has been hydrolyzed at any time point during the enzymatic catalysis. By measuring dQ/dt in the calorimeter and by using the above equations, one obtains a continuous set of ([S], rate) data points. k_{cat} and K_{m} for the enzymatic catalysis were calculated from these data points by fitting experimental data to theoretical Michealis–Menten curve using a non-linear curve fit in Origin 7 to the Michaelis–Menten equation:

$$\text{Rate} = \frac{k_{\text{cat}} \cdot [E] \cdot [S]}{K_{\text{m}} + [S]}. \tag{6}$$

3. Results and discussion

Fig. 1 depicts a typical thermogram for the hydrolysis of tetra-*N*-acetylchitotetraose by chitinase B of *S. marcescens*. The thermogram shows that, at 120 s, enzymatic catalysis is at steady state and that the rate of catalysis is at its maximum (V_{max}). Four independent measurements yielded a ΔH_{hydr} of -2.3 ± 0.1 kJ/mol. The measurements yielded a k_{cat}-value of $40.9 \pm 0.5\ \text{s}^{-1}$ and a K_{m}-value, Fig. 2.

Upon completion of the ITC experiment, a second injection of chitinase B was undertaken to record the heat of dilution. This heat of dilution was equal to the heat of dilution when chitinase B was injected into buffer alone. As a control, the substrate was placed in the syringe and injected into the calorimetric cell containing the enzyme to yield the same values within experimental error.

Although tetra-*N*-acetylchitotetraose differs considerably from the most commonly used artificial substrate, 4-

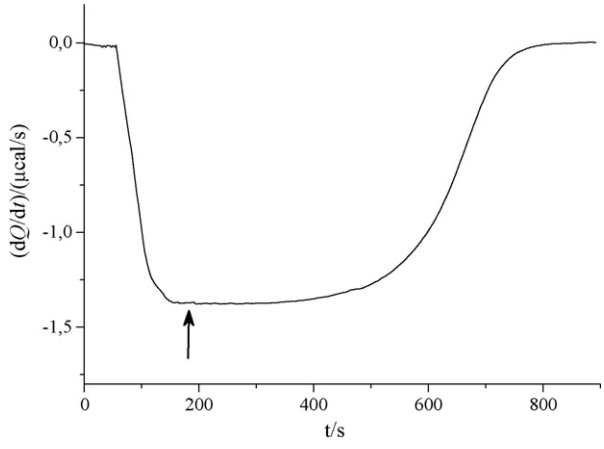

Fig. 1. The thermogram of tetra-*N*-acetylchitotetraose hydrolysis at 37 °C with chitinase B of *Serratia marcescens*. The reaction cell contained 1000 μM of substrate in 20 mM KP_i buffer at pH 6.1. A baseline was recorded for 60 s prior to enzyme addition over a period of 42 s and reached a final concentration of 0.050 μM in the cell. Data used for analysis were taken from 120 s after injection start (marked with an arrow) and until the dQ/dt went back to baseline.

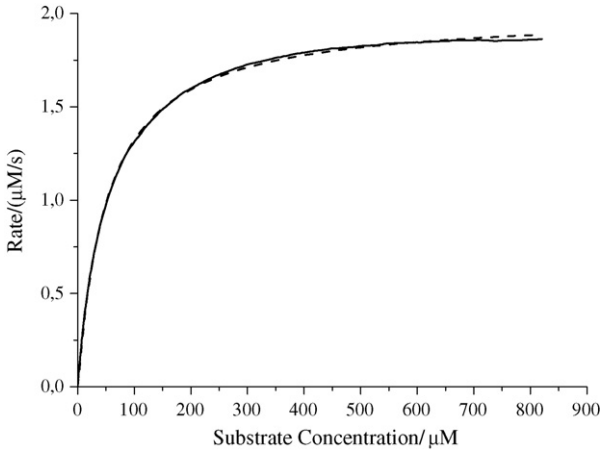

Fig. 2. A Michealis–Menten plot where theoretical data (– – –) are fitted to experimental data (—). The average of four independent experiments yielded a k_{cat}-value of $40.9 \pm 0.5\ s^{-1}$ and a K_m-value of $54 \pm 2\ \mu M$.

methylumbelliferyl-di-*N*-acetylchitobiose, both substrates yield rather similar kinetic parameters at pH 6.1 (for the artificial substrate these parameters are $k_{cat} = 18 \pm 3\ s^{-1}$ and $K_m = 31 \pm 3\ \mu M$ [19]). Clearly, the use of natural substrates should generally be preferred, especially when comparing different natural chitinases or engineered chitinase variants. Other attractive features of the method are the ease and speed of the experiments. Enzyme assays are often laborious and time consuming. With the present method, high quality data are obtained after only 20 min of experimental time, and with minimal prior sample preparations.

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