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Fingerprinting of oligosaccharide-hydrolyzing enzymes that catalyze branched reaction schemes

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ABSTRACT

We present three case studies, based on literature data, in which the "fingerprinting" method of determining the relative specificities of an enzyme is applied to branched reaction schemes. The first case study involves the hydrolysis of maltoheptaose by a β-amylase and shows that the fingerprinting method can be applied to schemes involving processivity. The analysis reveals that the native β-amylase has a 1.26-fold preference for attacking maltoheptaose by the processive route over the non-processive route, but that for a mutant enzyme this preference is 0.18-fold. The second case study involves the hydrolysis of β-1,6-*N*-acetylglucosamine oligomers by DispersinB. Our set of relative specificity constants is more consistent with the results of initial rate experiments than is the set that the authors obtained by fitting a pseudo-first order model to their data. The third case study involves the hydrolysis of galacturonic acid oligomers by an endopolygalacturonase. This enzyme can catalyze a total of 11 different reactions with a mixture of tri-, tetra-, penta-, hexa- and heptagalacturonates. We determined the relative specificity constants for these 11 reactions. The fingerprinting method has advantages over the methods that have been previously used to determine specificity constants for branched reaction schemes, being able to use a single experimental reaction profile for determination of all relative specificity constants.

1. Introduction

The enzymatic saccharification of polysaccharides will become of increasing importance as biorefineries are developed to take advantage of biomass [1]. Many of the enzymes that are used in the saccharification of polysaccharides are capable not only of catalyzing different reactions with the same substrate but also of catalyzing subsequent reactions with the products of the initial reactions, giving rise to branched reaction schemes. In order to select suitable enzymes for use in saccharification processes, or even to change the properties of native enzymes in order to make them more suitable for such processes, it is important to know the relative specificities that the enzyme has for the various different reactions that it is capable of catalyzing.

With polymers, the specificity of endoenzymes and exoenzymes tends to be relatively insensitive to chain length [2]. However, near the end of a saccharification process, there will be many oligosaccharides present and, in this case, the specificities of endoenzymes and exoenzymes can be significantly affected by the number of residues in the oligosaccharide [3–11]. It is interesting, then, to characterize the specificities that exoenzymes and endoenzymes have for the various reactions that they can catalyze with oligosaccharides.

The methods that have been applied to date for estimating relative specificities of enzymes in branched reaction schemes are often experimentally cumbersome, requiring numerous assays. Additionally, they provide estimates of only some of the specificity constants. For example, some give global specificity constants for the various substrates, failing to characterize the specificity constants for different reactions that the same substrate can suffer [3–12]. Other methods give specificity constants for different reactions with the same substrate, but are incapable of characterizing specificities of the enzyme for different substrates [13–17].

These limitations are avoided in the so-called "fingerprinting" approach of Mitchell et al. [18–20]. This approach can use data from a single time course experiment to determine the relative specificity of the enzyme for all the reactions that it can catalyze starting from the initial substrate. It has the additional advantage of using the fractional reaction extent, rather than time, as the independent variable; this means that complicating phenomena, such as enzyme denaturation and substrate or product inhibition, do not interfere

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with the analysis. This approach has been well-developed for linear reaction schemes [18], but not for branched reaction schemes, in which the enzyme may catalyze more than one reaction with a given species and both products of a reaction may suffer further cleavage. To date, it has only been applied to schemes with two simple branches [19,21].

The aim of this work is, therefore, to extend the fingerprinting method to the analysis of reaction schemes with various branches. It does this by considering three case studies of increasing complexity, namely: (i) hydrolysis of maltoheptaose by a β-amylase in a scheme that can follow either a processive or non-processive route, using the data of Ishikawa et al. [22], (ii) hydrolysis of β-1,6-*N*-acetylglucosamine oligomers by DispersinB, using the data of Fazekas et al. [12], and (iii) hydrolysis of galacturonic acid oligomers by an endopolygalacturonase from *Fusarium moniliforme*, using the data of Bonnin et al. [6].

2. Mathematical methods

2.1. Analysis of the consistency of the data set

The consistency of the data was checked for each case study. In case studies 1 and 2 (Sections 3.1 and 3.2), the remnants of the "backbones" of the original oligomers were identifiable amongst the reaction products. In this case, the consistency analysis checked whether the number of "backbone remnants" detected at a sampling time was equal to the original number of oligomers. In case study 3 (Section 3.3), the experimental analysis provided data for the concentrations of all reaction species. In this case, the consistency analysis checked whether the number of monomeric residues detected at a sampling time was equal to the initial number of residues in the reaction mixture. Details are given in the individual case studies. In case studies 1 and 2, the data sets passed the consistency test and were used as extracted from the source. In case study 3, the data sets extracted from the original source failed the test, as too many of the residues (>5% of the original number) disappeared during the time course of the hydrolysis reaction. In this case, the data were corrected to a set of data in which the total number of residues was conserved throughout the reaction. The correction method is given in the case study.

2.2. Calculation of relative concentrations and the fractional reaction extent

The concentrations of all species were expressed relative to the concentration of the initial substrate:

$$X\# = \frac{[\mathrm{X}\#]}{[\mathrm{X}n]_0} \tag{1}$$

where $X\#$ is the relative concentration of the oligomer of length # and $[\mathrm{X}n]_0$ is the initial concentration of the original substrate, which is an oligomer of length n.

In those cases in which the original data were not presented in terms of fractional reaction extent (represented here by the symbol F), but rather in terms of time, the data were converted to F as the independent variable. Mitchell et al. [18] calculated F for linear reaction schemes in terms of the number of attackable bonds that had been hydrolyzed. This calculation was possible since, in the schemes that they analyzed, one mole of final product was formed per mole of hydrolyzed bonds. In branched reaction schemes, the same product can potentially be generated by different routes that involve different numbers of hydrolysis reactions and which, therefore, make different contributions to the advance of F. In the current work, the value of F was therefore calculated by subtracting the "unhydrolyzed attackable bonds" from the total initial number of attackable bonds. When an enzyme can potentially attack all the bonds between the residues of the original substrate, F is given by:

$$F = 1 - \left(\frac{n[\mathrm{X}_n] + (n-1)[\mathrm{X}_{n-1}] + \cdots + [\mathrm{X}_2]}{n[\mathrm{X}_n]_0}\right) = 1 - \left(\frac{nX_n + (n-1)X_{n-1} + \cdots + X_2}{n}\right) \tag{2}$$

In this equation, n is the number of residues in the initial substrate. The various $[\mathrm{X}_\#]$ terms represent the concentrations of oligomers with the number of residues indicated by the subscript # and $[\mathrm{X}_\mathrm{n}]_0$ is the initial concentration of X_n. The various $X_\#$ terms represent the relative concentrations of the oligomers with # units. The individual case studies show variations of this equation for specific situations: case study 1 (Section 3.1) gives the equation for the release of disaccharides from a heptamer; case study 2 (Section 3.2) gives the equation for when the reducing end of the molecule is marked and only the hydrolysis of marked oligomers is taken into account; and case study 3 (Section 3.3) gives the equation for when the initial substrate is contaminated with another hydrolysable species.

2.3. Determination of relative specificity constants

Mitchell et al. [18] described the general method for obtaining the set of differential equations that describes the hydrolysis of the initial substrate and the formation and consumption of the various intermediates generated during the reaction. Although they described the methodology in the context of linear reaction schemes, the same considerations apply to branched schemes. The equations deduced in the current work are shown in the individual case studies. Two of the case studies involve reaction profiles generated in different experiments, each experiment starting with a different oligomer. In these cases, the equation sets are shown here only for the hydrolysis of the longest oligomer. The equation sets for the shorter oligomers are shown in the Supplementary Material.

Each model is a system of differential equations describing the relative concentrations of the various species involved in the reaction, with the fractional reaction extent as the independent variable. The parameters of these equations are the relative specificities of the enzyme for the various reactions that it can catalyze with the various species. The models were solved numerically using the function *ode45* of MATLAB®, which is based on an explicit Runge-Kutta (4,5) algorithm. The values of the parameters of the model were obtained by using the non-linear optimization function *fminsearch* of MATLAB® to adjust the parameters to minimize the following objective function:

$$F_{obj} = \sum_{i=1}^{j}\left(d^2\right) = \sum_{i=1}^{j}\left(\sqrt{\left(x_{iexp} - x_{icalc}\right)^2 + \left(y_{iexp} - y_{icalc}\right)^2}\right)^2 = \sum_{i=1}^{j}\left[\left(x_{iexp} - x_{icalc}\right)^2 + \left(y_{iexp} - y_{icalc}\right)^2\right] \tag{3}$$

where x_{exp} and x_{calc} are the experimental and predicted values of the fractional reaction extent, y_{exp} and y_{calc} are the experimental and predicted values of the relative concentrations of the species and j is the total number of experimental data points.

This objective function was chosen because the experimental error in the dependent variables (the relative concentrations) introduces experimental error into the calculated independent variable (the fractional reaction extent). It minimizes the sum of the squares of the smallest distances between the predicted curve and the experimental points [23]: it does not restrict these distances to be calculated "on the vertical" (i.e. at the same value of