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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

the independent variable), but rather calculates these distances "on the diagonal". In order to implement this method, the differential model was solved at intervals of 0.001 of the fractional reaction extent. A matrix was then calculated in which each column corresponded to a particular experimental data point. The values in the column of the matrix gave the distances of each predicted point on the curve from that particular experimental data point. The function *min* of MATLAB® was then used to generate a row vector whose entries represented the lowest values in each of the columns of the matrix (i.e. a row vector of *j* "minimum distances").

In case studies 2 and 3, the authors undertook several different experiments with oligomers of different lengths. In each of these case studies, a hydrolysis model was developed for each experiment and these models were solved and optimized simultaneously. In this manner, a single set of optimized parameter values was obtained and not one set for each experiment.

3. Results

3.1. Case study 1: hydrolysis of maltoheptaose by a processive β-amylase

The first case study was selected to demonstrate two points about the fingerprinting method: First, that it can quantify specificity changes that result from enzyme engineering programs and, second, that it can be used to characterize enzymes that show the phenomenon of processivity. This particular case study involves β-amylase, which can be used to produce maltose-rich hydrolysates from starch. It uses data obtained by Ishikawa et al. [22] for the removal of maltose units from the non-reducing end of maltoheptaose, an oligomer containing seven units of α-1,4-glucopyranosyl, by the β-amylase of soybeans. The final products of this reaction are two molecules of maltose and one molecule of maltotriose (Fig. 1).

Initially, Ishikawa et al. [22] undertook studies with the native β-amylase, which can catalyze this reaction following two possible routes: a non-processive route and a processive route. The non-processive route consists of two separate steps: in the first step, the enzyme binds to maltoheptaose and liberates maltose and maltopentaose into the medium; in the second step, the enzyme binds to the maltopentaose and liberates maltose and maltotriose into the medium. In the processive route, the enzyme binds to maltoheptaose and liberates the first maltose without releasing the maltopentaose; the enzyme then slides along the maltopentaose and liberates maltose and maltotriose into the medium. The enzyme does not hydrolyze maltotriose. Ishikawa et al. [22] then produced two mutant enzymes. The first of these was D53A, in which the tendency to follow the processive route was diminished in relation to the native enzyme; the second was W55R, in which the processive route was eliminated.

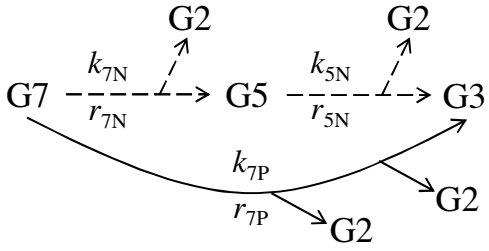

Fig. 1. Reaction scheme for the hydrolysis of maltoheptaose by a soybean β-amylase. The dashed arrows represent the non-processive route while the continuous arrows represent the processive route. Key: G7 is maltoheptaose, G5 is maltopentaose, G3 is maltotriose and G2 is maltose; k_{7N}, k_{5N} and k_{7P} represent the specificity constants of the enzyme for each of the reactions.

Although Mitchell et al. [18] applied the fingerprinting analysis to the native enzyme, they only allowed for the non-processive route in their model, and therefore the model was not able to fit well to the data obtained by Ishikawa et al. [22] for this enzyme. On the other hand, the model did fit well to the data obtained with the non-processive mutant enzyme, W55R. The current case study demonstrates that it is possible to apply the fingerprinting analysis when the reaction scheme has both processive and non-processive branches.

In this analysis, [G7], [G5] and [G3] are the concentrations of maltoheptaose, maltopentaose and maltotriose, respectively. Similarly, *G7*, *G5* and *G3* are the relative concentrations of maltoheptaose, maltopentaose and maltotriose, respectively (i.e. normalized with respect to the initial concentration of maltoheptaose, $[G7]_0$).

The consistency index (*CI*) of the data was calculated at each sampling time. It expresses the recovery of "remnants of the backbones of the original G7":

$$CI_m = \frac{[G7]_m + [G5]_m + [G3]_m}{[G7]_0} = G7_m + G5_m + G3_m \quad (4)$$

where the subscript "*m*" denotes the *m*th sampling time. For all sampling times, $0.95 \leq CI_m \leq 1.05$, and therefore the data set was used without correction.

For the reaction scheme given in Fig. 1, the fractional reaction extent is defined as:

$$F = 1 - \left(\frac{2[G7] + [G5]}{2[G7]_0}\right) = 1 - \left(\frac{2G7 + G5}{2}\right) \quad (5)$$

Application of the approach described by Mitchell et al. [18] leads to the following set of differential equations, expressed in terms of fractional reaction extent:

$$\frac{dG3}{dF} = 2\frac{(R_{7P}G7 + R_{5N}G5)}{(R_{7N}G7 + 2R_{7P}G7 + R_{5N}G5)} \quad (6)$$

$$\frac{dG5}{dF} = 2\frac{(R_{7N}G7 - R_{5N}G5)}{(R_{7N}G7 + 2R_{7P}G7 + R_{5N}G5)} \quad (7)$$

$$\frac{dG7}{dF} = 2\frac{-(R_{7N}G7 + R_{7P}G7)}{(R_{7N}G7 + 2R_{7P}G7 + R_{5N}G5)} \quad (8)$$

where the *R*-parameters represent relative specificity constants (i.e. normalized with respect to k_{7N}). By definition, $R_{7N} = 1$. The step-by-step deduction of the model is given in the Supplementary Material.

This set of equations was able to fit well to the profiles reported by Ishikawa et al. [22] for the two β-amylases with processive action, namely the native enzyme and the mutant enzyme D53A (Fig. 2). The values of the relative specificity constants are shown in Fig. 3.

The value of R_{7P} ($= k_{7P}/k_{7N}$) of 1.26 shows that the native soybean β-amylase has a slight preference for attacking maltoheptaose by the processive route, compared to the non-processive route. The ratio $R_{5N}/(R_{7N} + R_{7P})$ can be calculated from the relative specificities in Fig. 3; this ratio represents the specificity that the free enzyme has for attacking maltopentaose, divided by the specificity for attacking maltoheptaose by either route. This ratio is 0.89 for D53A, showing that when this enzyme is free, it has a reasonably similar specificity for attacking maltoheptaose (by either route) and maltopentaose. On the other hand, the value of R_{7P} of 0.18 shows that when this mutant enzyme binds to maltoheptaose, it is 5 times as likely to follow the non-processive route than the processive route.

This analysis shows that it is, indeed, possible to apply the fingerprinting analysis when an enzyme can follow either a processive or non-processive route. It is simply necessary to allow for both routes

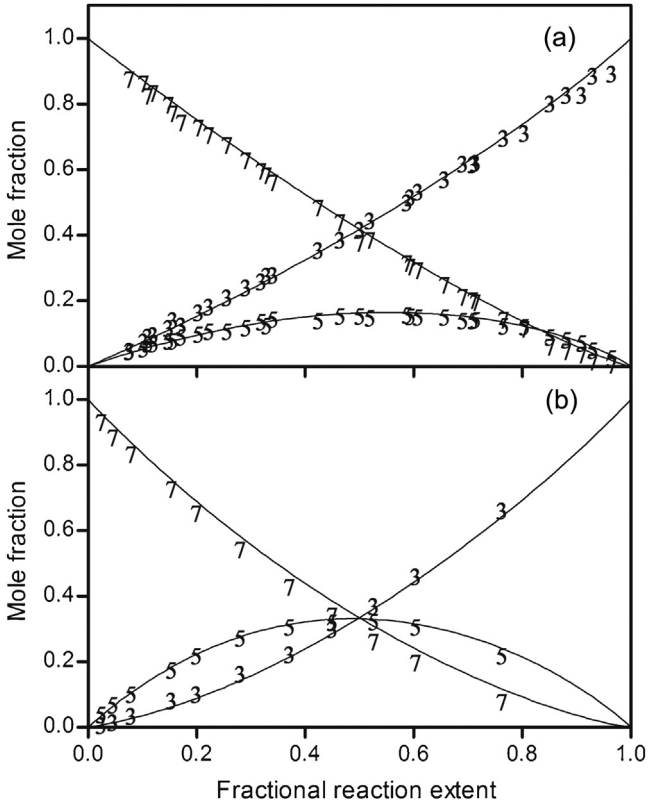

Fig. 2. Fitting of the model to the data of Ishikawa et al. [22] for the action of soybean β-amylase on maltoheptaose. (a) Native β-amylase; (b) mutant D53A. Key: (3) Maltotriose; (5) Maltopentaose; (7) Maltoheptaose; (—) best fit of the model.

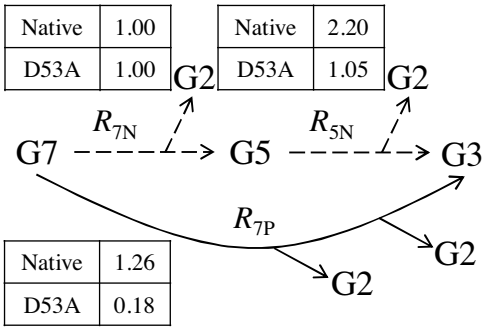

Fig. 3. Values of relative specificities for native soybean β-amylase and the mutant enzyme D53A. The dashed arrows represent the non-processive route while the continuous arrows represent the processive route. Key: G7 is maltoheptaose, G5 is maltopentaose, G3 is maltotriose and G2 is maltose; *R*-values represent relative specificity constants normalized with respect to k_{7N} for each of the reactions; k_{7N} is the specificity constant for the non-processive cleavage of maltoheptaose.

in the reaction scheme, which Mitchell et al. [18] did not do in their analysis.

3.2. Case study 2: hydrolysis of β-1,6-N-acetylglucosamine oligomers by DispersinB

The second case study was selected to demonstrate two additional points about the fingerprinting method: First, that it can be used with reaction schemes involving more than two branches and, second, that it has advantages over the method of fitting a pseudo first-order mathematical model. This case study uses data obtained by Fazekas et al. [12] for the hydrolysis of β-1,6-*N*-acetylglucosamine thiophenylglycosides, containing from 3 to 5 residues, by DispersinB, which is an *N*-acetyl-glucosaminidase that

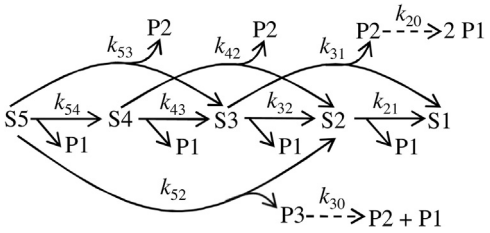

Fig. 4. Reaction scheme given by Fazekas et al. [12] for the hydrolysis of thiophenyl *N*-acetyl-β-(1,6)-pentaglucosamine oligomers by DispersinB. The solid lines represent reactions that are visible to the analytical method that was used (i.e. reactions for which one of the products contains a thiophenyl group on the reducing end). The dashed lines represent reactions that consume species that do not contain this functional group. Key: S*n* represents a thiophenyl β-1,6-*N*-acetylglucosamine oligomer with *n* units; P*n* represents a β-1,6-*N*-acetylglucosamine oligomer with *n* units; S1 is thiophenyl *N*-acetyl-β-D-glucosamine; P1 is *N*-acetyl-D-glucosamine; and the parameters represented by *k* are the specificity constants of the enzyme for each of the reactions.

is specific for β-(1,6) glycosidic bonds. This enzyme has potential for use in the prevention or eradication of biofilms on biomedical devices destined for implantation [12].

Fazekas et al. [12] proposed the scheme shown in Fig. 4. They undertook three different experiments, starting with β-(1,6)-linked *N*-acetylglucosamine thiophenyl glycosides with degrees of polymerization of 3, 4 and 5. We applied the fingerprinting analysis simultaneously to these three reaction profiles. We determined the relative specificities of DispersinB for bonds within oligomers marked on the reducing end with a thiophenyl group, as these were the only species for which Fazekas et al. [12] provided data.

Application of the approach described by Mitchell et al. [18] leads to three sets of differential equations, one set for each experiment (i.e. starting with the triglucosamine, the tetraglucosamine and the pentaglucosamine). Each set is expressed in terms of the appropriate fractional reaction extent as the independent variable. Below, we present the equations for reactions initiated with the pentaglucosamine. In these equations, [S*n*] represents the concentration of the thiophenyl glycoside with *n* *N*-acetylglucosamine residues, while *Sn* represents the relative concentration of this species (i.e. normalized with respect to the initial concentration of the pentaglucosamine, $[S5]_0$). The corresponding equations for the reactions initiated with the triglucosamine and the tetraglucosamine are given in the Supplementary Material.

The consistency index (*CI*) of the data was calculated at each sampling time. It expresses the recovery of "marked reducing ends". For the reaction started with the pentaglucosamine, this index is given by:

$$CI_m = \frac{[S5]_m + [S4]_m + [S3]_m + [S2]_m + [S1]_m}{[S5]_0} = S5_m + S4_m + S3_m + S2_m + S1_m \quad (9)$$

where the subscript "*m*" denotes the *m*th sampling time. For all sampling times of the three experiments of Fazekas et al. [12], $0.95 \leq CI_m \leq 1.05$. The data were therefore used without correction.

The fractional reaction extent for the reaction started with the pentaglucosamine is defined as:

$$F_5 = 1 - \left(\frac{4[S5] + 3[S4] + 2[S3] + [S2]}{4[S5]_0}\right) = 1 - \left(\frac{4S5 + 3S4 + 2S3 + S2}{4}\right) \quad (10)$$

The set of differential equations for the pentaglucosamine (S5) as initial substrate is:

$$\frac{dS1}{dF_5} = 4\frac{R_{31}S3 + R_{21}S2}{(R_{54} + 2R_{53} + 3R_{52})S5 + (R_{43} + 2R_{42})S4 + (R_{32} + 2R_{31})S3 + R_{21}S2} \quad (11)$$

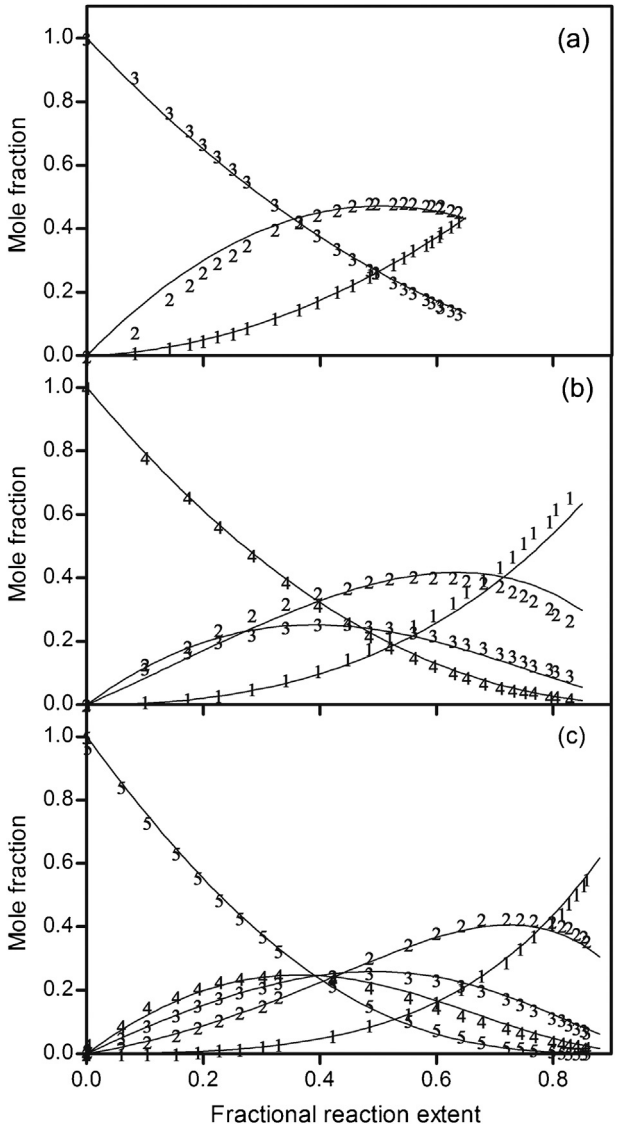

Fig. 5. Fitting of the model to the data of Fazekas et al. [12] for the hydrolysis of oligomers of thiophenyl β-1,6-*N*-acetylglucosamine by DispersinB. Fazekas et al. [12] performed the hydrolysis with different initial substrates, namely (a) the triglucosamine, (b) the tetraglucosamine and (c) the pentaglucosamine. The experimental data points are represented by numbers that reflect that number of residues in the oligomer. The continuous lines give the fit of the model.

$$\frac{dS2}{dF_5} = 4\frac{R_{52}S5 + R_{42}S4 + R_{32}S3 - R_{21}S2}{(R_{54}+2R_{53}+3R_{52})S5 + (R_{43}+2R_{42})S4 + (R_{32}+2R_{31})S3 + R_{21}S2} \quad (12)$$

$$\frac{dS3}{dF_5} = 4\frac{R_{53}S5 + R_{43}S4 - R_{32}S3 - R_{31}S3}{(R_{54}+2R_{53}+3R_{52})S5 + (R_{43}+2R_{42})S4 + (R_{32}+2R_{31})S3 + R_{21}S2} \quad (13)$$

$$\frac{dS4}{dF_5} = 4\frac{R_{54}S5 - R_{43}S4 - R_{42}S4}{(R_{54}+2R_{53}+3R_{52})S5 + (R_{43}+2R_{42})S4 + (R_{32}+2R_{31})S3 + R_{21}S2} \quad (14)$$

$$\frac{dS5}{dF_5} = 4\frac{-R_{54}S5 - R_{53}S5 - R_{52}S5}{(R_{54}+2R_{53}+3R_{52})S5 + (R_{43}+2R_{42})S4 + (R_{32}+2R_{31})S3 + R_{21}S2} \quad (15)$$

In these equations, the *R*-parameters are relative specificity constants, based on k_{21} (i.e. they are defined as $R_{xy} = k_{xy}/k_{21}$). By definition, $R_{21} = 1$.

The three sets of equations fit well to the three data sets that Fazekas et al. [12] obtained with the different initial substrates, namely S3, S4 and S5 (Fig. 5). The set of relative specificity constants obtained through the fitting procedure (Fig. 6) has been referred to as a "fingerprint" of the enzyme [19]. These relative specificity constants can be analyzed to show the preferences of DispersinB for the various bonds within the various substrates that it can attack. In the reaction that removes a single residue from the non-reducing end of an oligomer marked on the reducing end with a thiophenyl group, DispersinB prefers to attack S3 compared to S2 ($R_{32} = k_{32}/k_{21} = 1.81$). Of the two possible forms of attack on S3, the enzyme has a significant preference for attacking the glycosidic bond at the non-reducing end ($R_{32}/R_{31} = k_{32}/k_{31} = 1.81/0.06 = 30$). Of the two possible forms of attack on S4, the enzyme has a 1.6-fold preference for attacking the glycosidic bond next to the non-reducing end, over attack on the central glycosidic bond ($R_{43}/R_{42} = k_{43}/k_{42} = 1.41/0.84$). Amongst the three possible forms of attack on S5, the preference of the enzyme falls almost linearly with the distance of the glycosidic bond from the non-reducing end of the molecule ($R_{54}/R_{53}/R_{52} = k_{54}/k_{53}/k_{52} = 1.98/1.25/0.45$).

Fazekas et al. [12] used "pseudo first-order kinetic constants" to characterize the preference of DispersinB for attacking the various glycosidic bonds in the various oligomers. These constants were obtained by fitting a set of pseudo first-order differential equations to temporal profiles of the reaction species (their full equation set is provided in the Supplementary Material). The relative values of their pseudo first-order kinetic constants (obtained by dividing the values of their constants by the value of the constant for the hydrolysis of the marked dimer) are larger than the relative specificity constants that we obtained by applying our fingerprinting analysis to their data, with the degree of discrepancy being greater with increasing oligomer size (Fig. 6). Our estimates are preferable, since pseudo first-order kinetic constants are not true constants, but rather complex expressions that involve the concentrations of the various species present in the reaction medium (a detailed analysis is provided in the Supplementary Material). In fact, this explains why Fazekas et al. [12] obtained different estimates for these constants when they fitted their equation set individually to the temporal profiles obtained in three different reactions (i.e. with the three different initial substrates): the concentrations of the various species in the reaction mixture are different in the three reactions. Since the constants of the equations used in the fingerprinting method are true constants (specificity constants), they are not affected by the variations in the concentrations of reaction species, neither during a particular hydrolysis reaction nor between reactions carried out with different substrates.

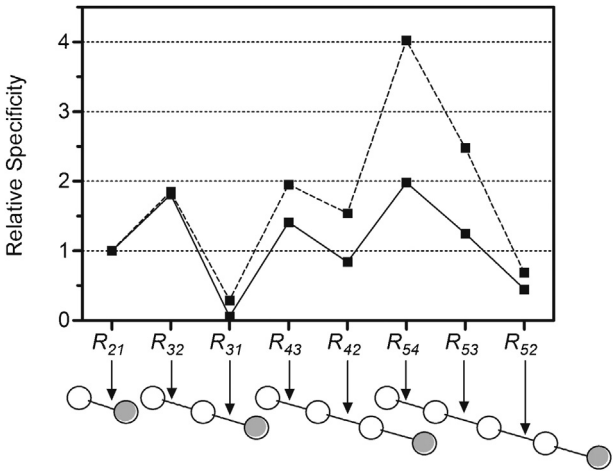

Fig. 6. The "fingerprint" of relative specificity constants for the attack of DispersinB on oligomers of thiophenyl β-1,6-*N*-acetylglucosamine. Key: R_{xy} represents the ratio of the specificity constant for the attack of the enzyme on the glycosidic bond indicated by the arrow, relative to the specificity constant for hydrolysis of the glycosidic bond of the disaccharide; (■—■) results of the fingerprinting analysis performed in the current work; (■---■) relative values of the pseudo first-order constants obtained by Fazekas et al. [12], normalized with respect to the pseudo first-order constant for hydrolysis of the glycosidic bond of the disaccharide. In the diagrams of the various oligomers shown below the abscissa, the grey circles indicate the marked residues at the reducing ends of the oligomers.

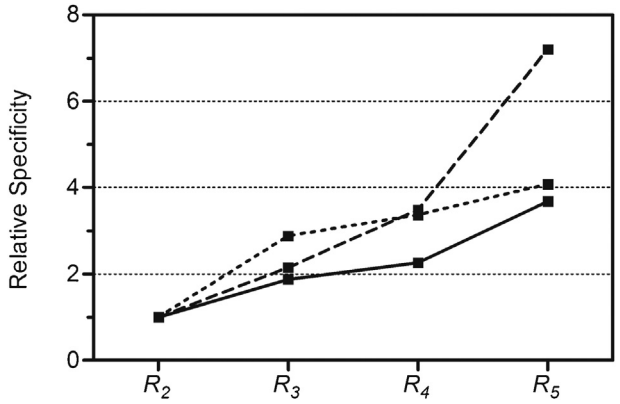

Fig. 7. Values of the relative global specificity constants of DispersinB for each oligomer of thiophenyl β-1,6-*N*-acetylglucosamine (i.e. normalized relative to k_{21}). Key: (■....■) values determined experimentally by Fazekas et al. [12] using initial rate experiments; (■—■) results of the fingerprinting analysis performed in the current work; (■---■) results of the analysis performed by Fazekas et al. [12] based on pseudo first-order constants.

Fazekas et al. [12] also obtained direct independent estimates of the specificity constants for each of the oligomers in experiments based on measurements of the initial rates of consumption of each of the oligomers from S2 to S5. Since the "global specificity constant" for an oligomer is given by the sum of the specificity constants for the various reactions that the enzyme can catalyze with that oligomer, it is possible for us to estimate the relative values of the global specificity constants for each oligomer by summing our relative specificity constants for the various reactions of that oligomer. Similar "relative global pseudo first-order constants" for each oligomer can be calculated by summing the appropriate relative pseudo first-order constants obtained by Fazekas et al. [12]. As shown by Fig. 7, the relative value of the experimentally determined global specificity constant (i.e. normalized relative to k_{21}) increases approximately linearly with oligomer size from the diglucosamine to the pentaglucosamine. This figure also shows that the relative global specificity constants that we obtained through the fingerprinting analysis are closer to the directly measured experimental values of Fazekas et al. [12] than are the values of the relative global pseudo first-order constants that they themselves obtained.

3.3. Case study 3: hydrolysis of galacturonic acid oligomers by an endopolygalacturonase from Fusarium moniliforme

The third case study was selected to show the importance of checking the consistency of the data and to demonstrate possible strategies for data correction when the consistency is not adequate. Additionally, it is the most complex branched reaction scheme for which all the species (i.e. reaction substrates, intermediates and final products) were quantified, involving eleven reactions and seven species. This case study uses data obtained by Bonnin et al. [6] for the hydrolysis, by an endopolygalacturonase of *Fusarium moniliforme*, of α-(1,4) glycosidic bonds between residues of D-galacturonic acid within oligogalacturonates. Endopolygalacturonases are an essential component of pectinase preparations that have the potential to be used for the saccharification of pectin in biorefineries [24].

Within the text, the oligogalacturonates will be represented by "Q*n*", where *n* represents the number of residues in the oligomer. Bonnin et al. [6] undertook experiments starting with the tetragalacturonate (Q4), the pentagalacturonate (Q5) the hexagalacturonate (Q6) and the heptagalacturonate (Q7). We applied the fingerprinting analysis simultaneously to these four reaction profiles.

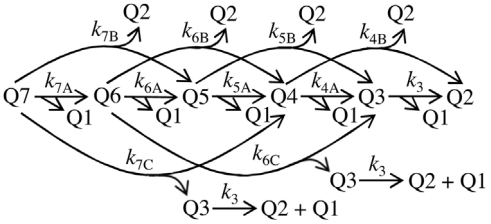

Fig. 8. Reaction scheme for the hydrolysis of oligomers of D-galacturonic acid by the endopolygalacturonase of *Fusarium moniliforme*. Key: Q*n* represents an oligogalacturonate with *n* units; the parameters represented by *k* are the specificity constants of the enzyme for each of the reactions.

Here, we present the equations for reactions initiated with Q7. In these equations, [Q*n*] represents the concentration of Q*n*, while *Qn* represents the relative concentration of this species (i.e. normalized with respect to the initial concentration of the heptagalacturonate, $[Q7]_0$). The corresponding equations for the reactions initiated with Q4, Q5 and Q6 are given in the Supplementary Material.

Fig. 8 shows the reaction scheme that was represented by our model. In this scheme, the enzyme cannot hydrolyze Q2, as demonstrated experimentally by Bonnin et al. [6]. It is important to note that when two different reactions give the same two products from the same substrate, they are treated as a single reaction. For example, Q7 can give rise to Q2 and Q5 in two manners: by hydrolysis of the second glycosidic bond from the non-reducing end or by hydrolysis of the second glycosidic bond from the reducing end. This means that the value of the specificity constant for the cleavage of Q7 into Q2 and Q5 represents the sum of the specificities for these two reactions. The fingerprinting method cannot differentiate these two reactions because the reducing ends of the original substrates were not marked in the experiments of Bonnin et al. [6].

The consistency index (*CI*) of the data was calculated at each sampling time. In this case study, it expresses the recovery of the D-galacturonic acid residues contained in the original substrate mixture. In the reaction carried out with Q7, there was a small but non-negligible amount of contaminating Q6 at zero time, such that the *CI* is given by:

$$CI_m = \frac{7[Q7]_m + 6[Q6]_m + 5[Q5]_m + 4[Q4]_m + 3[Q3]_m + 2[Q2]_m + [Q1]_m}{7[Q7]_0 + 6[Q6]_0}$$

$$= \frac{7Q7_m + 6Q6_m + 5Q5_m + 4Q4_m + 3Q3_m + 2Q2_m + Q1_m}{7 + 6Q6_0} \quad (16)$$

where the subscript "*m*" denotes the *m*th sampling time, $[Q6]_0$ is the zero-time concentration of the contaminating Q6, while $Q6_0$ is the relative initial concentration of this contaminating Q6 (i.e. normalized with respect to $[Q7]_0$). For all experiments with the various initial substrates, the value of CI_m fell significantly below 0.95. For example, it reached a value of 0.64 in the experiment done with Q7 as the initial substrate.

The "relative concentration of missing residues" at each sampling time (Δ_m) was calculated. For the experiment done with Q7, this is given by:

$$\Delta_m = 7Q7_0 + 6Q6_0 - \sum_{i=1}^{7} iQi_m \quad (17)$$

where Qi_m represents the relative concentration of Qi at the *m*th sampling time.

The data were corrected by applying the following equation:

$$Qn(corrected)_m = Qn(experimental)_m + \frac{1}{n}\frac{\Delta_m}{w} \quad (18)$$

where $Qn(corrected)_m$ and $Qn(experimental)_m$ are, respectively, the corrected and the experimental values of the relative concentration of the oligomer with *n* residues at the *m*th sampling time. The

number of different species present in the reaction mixture at the mth sampling time is given by w, so Δ_m/w residues are attributed equally to each of the species present at that time. The correction in the concentration of an oligomer is then Δ_m/w residues divided by the length, n, of the oligomer. The Supplementary Material shows the raw data taken from Fig. 4 of Bonnin et al. [6], the results of the consistency analysis (as described above in Eq. (16)) and the corrected values obtained by applying Eq. (18).

Eq. (18) was applied because it ensured that the corrected values passed a second consistency test that required that at least two moles of products were formed for each mole of initial substrate consumed. It should be noted that although hydrolysis of the initial substrate generates only two products, if these products suffer further reaction, this generates more than two moles of products per mole of initial substrate consumed. For example, applying this second consistency test to the data for the experiment with Q7, the following condition must apply at each of the m sampling times:

$$\sum_{i=1}^{6} Qi_m \geq 2\,(Q7_0 - Q7_m) \tag{19}$$

The fractional reaction extent in the experiment started with Q7 is given by:

$$F_7 = 1 - \left(\frac{6\,[Q7] + 5\,[Q6] + 4\,[Q5] + 3\,[Q4] + 2\,[Q3] + [Q2]}{6[Q7]_0 + 5[Q6]_0}\right) = 1 - \left(\frac{6Q7 + 5Q6 + 4Q5 + 3Q4 + 2Q3 + Q2}{6 + 5Q6_0}\right) \tag{20}$$

The differential equation set for hydrolysis of Q7 as the initial substrate is as follows:

$$\frac{dQ1}{dF_7} = \frac{(6 + 5Q6_0)(R_{7A}Q7 + R_{6A}Q6 + R_{5A}Q5 + R_{4A}Q4 + R_3Q3)}{(R_{7A} + R_{7B} + R_{7C})Q7 + (R_{6A} + R_{6B} + R_{6C})Q6 + (R_{5A} + R_{5B})Q5 + (R_{4A} + R_{4B})Q4 + R_3Q3} \tag{21}$$

$$\frac{dQ2}{dF_7} = \frac{(6 + 5Q6_0)(R_{7B}Q7 + R_{6B}Q6 + R_{5B}Q5 + 2R_{4B}Q4 + R_3Q3)}{(R_{7A} + R_{7B} + R_{7C})Q7 + (R_{6A} + R_{6B} + R_{6C})Q6 + (R_{5A} + R_{5B})Q5 + (R_{4A} + R_{4B})Q4 + R_3Q3} \tag{22}$$

$$\frac{dQ3}{dF_7} = \frac{(6 + 5Q6_0)(R_{7C}Q7 + 2R_{6C}Q6 + R_{5B}Q5 + R_{4A}Q4 - R_3Q3)}{(R_{7A} + R_{7B} + R_{7C})Q7 + (R_{6A} + R_{6B} + R_{6C})Q6 + (R_{5A} + R_{5B})Q5 + (R_{4A} + R_{4B})Q4 + R_3Q3} \tag{23}$$

$$\frac{dQ4}{dF_7} = \frac{(6 + 5Q6_0)(R_{7C}Q7 + R_{6B}Q6 + R_{5A}Q5 - R_{4A}Q4 - R_{4B}Q4)}{(R_{7A} + R_{7B} + R_{7C})Q7 + (R_{6A} + R_{6B} + R_{6C})Q6 + (R_{5A} + R_{5B})Q5 + (R_{4A} + R_{4B})Q4 + R_3Q3} \tag{24}$$

$$\frac{dQ5}{dF_7} = \frac{(6 + 5Q6_0)(R_{7B}Q7 + R_{6A}Q6 - R_{5A}Q5 - R_{5B}Q5)}{(R_{7A} + R_{7B} + R_{7C})Q7 + (R_{6A} + R_{6B} + R_{6C})Q6 + (R_{5A} + R_{5B})Q5 + (R_{4A} + R_{4B})Q4 + R_3Q3} \tag{25}$$

$$\frac{dQ6}{dF_7} = \frac{(6 + 5Q6_0)(R_{7A}Q7 - R_{6A}Q6 - R_{6B}Q6 - R_{6C}Q6)}{(R_{7A} + R_{7B} + R_{7C})Q7 + (R_{6A} + R_{6B} + R_{6C})Q6 + (R_{5A} + R_{5B})Q5 + (R_{4A} + R_{4B})Q4 + R_3Q3} \tag{26}$$

$$\frac{dQ7}{dF_7} = \frac{(6 + 5Q6_0)(-R_{7A}Q7 - R_{7B}Q7 - R_{7C}Q7)}{(R_{7A} + R_{7B} + R_{7C})Q7 + (R_{6A} + R_{6B} + R_{6C})Q6 + (R_{5A} + R_{5B})Q5 + (R_{4A} + R_{4B})Q4 + R_3Q3} \tag{27}$$

The R-parameters represent relative specificity constants, normalized with respect to k_{4A}. By definition, $R_{4A} = 1$.

The four sets of differential equations were fitted simultaneously to the four corrected data sets of Bonnin et al. [6] (Fig. 9). The fit is quite reasonable in all cases. The greatest discrepancy between fitted and experimental values occurs for Q4 as an intermediate. However, even in this case, the model does describe the initial increase and later decrease of Q4, the discrepancy being that the predicted curve descends later than the experimental results do.

Fig. 10 shows the values of the relative specificity constants obtained from the fingerprinting analysis. Crucially, Bonnin et al. [6] concluded that the "Q1 + Q5" cleavage of Q6 and the "Q1 + Q6" cleavage of Q7 do not occur, but our analysis suggests that they do. Indeed, with R_{6A} (i.e. for "Q1 + Q5") and R_{7A} (i.e. for "Q1 + Q6") both fixed at 0, the model did not adjust well to the data (results not shown). A good fit was obtained with R_{6A} equal to 3.1, which is 29% of the sum of the relative specificity constants for the three possible reactions with Q6 ($R_{6A} + R_{6B} + R_{6C} = 10.8$), and R_{7A} equal to 3.5, which is 25% of the sum of the relative specificity constants for the three possible reactions with Q7 ($R_{7A} + R_{7B} + R_{7C} = 13.8$). The conclusion of Bonnin et al. [6] was based on a visual inspection of their reaction profiles. However, any visual analysis must take into account the fact that many of the reaction species are simultaneously being consumed by more than one reaction and produced by more than one reaction. It must also take into account the contaminants present at zero time (for hydrolysis of Q6, $Q5_0 = 0.15$, while for hydrolysis of Q7, $Q6_0 = 0.04$), since the hydrolysis of these contaminants produces products that are also produced by the hydrolysis of the main substrate. The advantage of the fingerprinting method is that it takes the complex network of reactions into account in a quantitative manner and also explicitly recognizes the presence of contaminants at the beginning of the reaction.

4. General discussion

Although various authors have attempted to determine the preferences that various carbohydrate-hydrolyzing enzymes have for the various reactions that they can catalyze in branched reaction schemes, reliable quantitative estimates of relative specificities are rare. There are two aspects that need to be considered in determining relative specificities. First, the mathematical approach not only must be correct, but it also must be convenient to apply. Second, once the appropriate mathematical approach has been chosen, it must be possible to produce good quality experimental data and analyze it appropriately. The first aspect is discussed below, while guidelines for producing good quality data are provided at the end of the Supplementary Material.

Approaches that have been used previously in attempts to determine the relative specificity of a carbohydrate-hydrolyzing enzyme for the various substrates and intermediates with which it can react include: (1) experiments in which various substrates are tested individually, with k_{cat} (or V_{max}) and K_M being estimated from the initial rates of substrate disappearance [3–12]; (2) visual analysis of reaction profiles [6,8,25–30]; (3) fitting of a pseudo first-order model to time course profiles [12]; and (4) determination of bond cleavage frequency [13–17].

Our fingerprinting analysis is more convenient than these other methods since all the relative specificity constants can be determined on the basis of a single, well-characterized reaction profile. Multiple experiments with the same substrate are not necessary. In

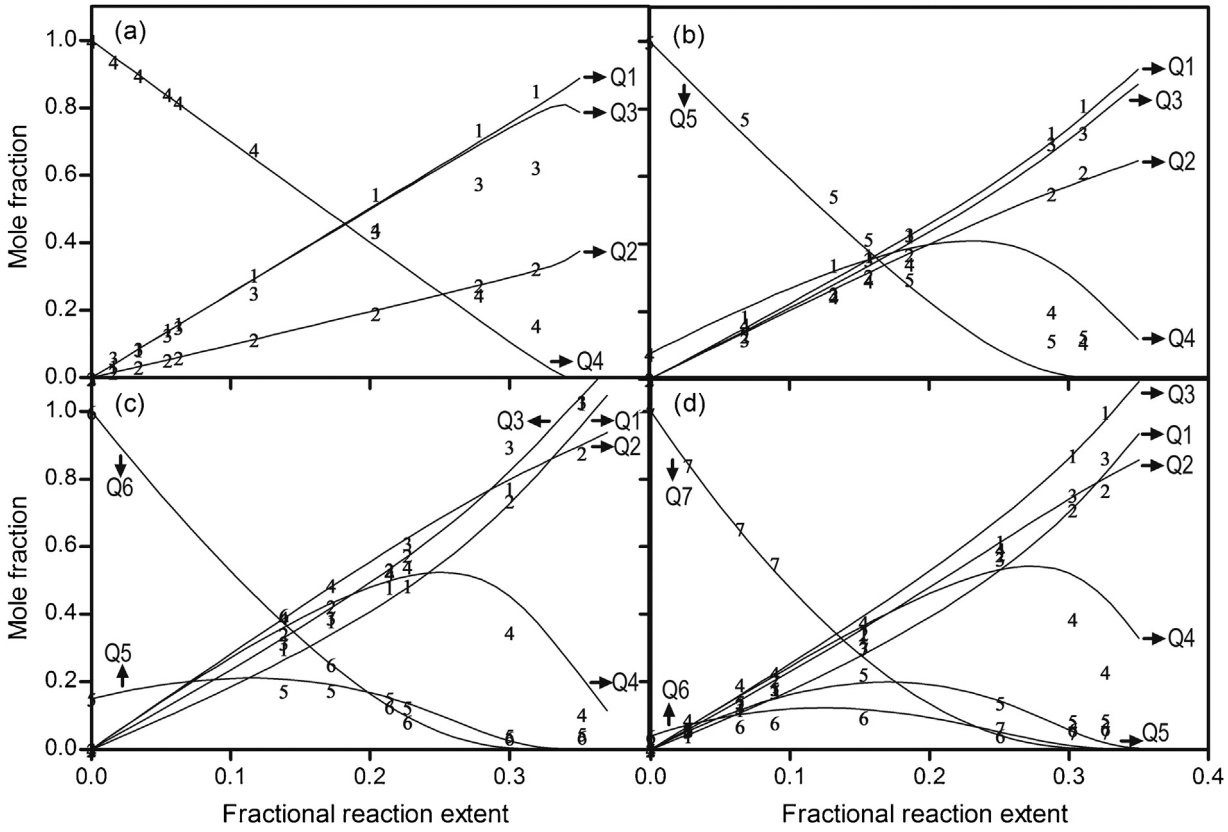

Fig. 9. Fitting of the model to the corrected data of Bonnin et al. [6] for the hydrolysis of oligomers of D-galacturonic acid by the endopolygalacturonase of *Fusarium moniliforme*. Bonnin et al. [6] performed the hydrolysis with different initial substrates, namely (a) the tetragalacturonate, (b) the pentagalacturonate (which was contaminated by the tetragalacturonate), (c) the hexagalacturonate (which was contaminated by the pentagalacturonate), and (d) the heptagalacturonate (which was contaminated by the hexagalacturonate). The experimental data points are represented by numbers that reflect the number of residues in the oligomer. The lines give the fit of the model.

fact, it may not be necessary to do more than one experiment. For example, it would be possible to extract estimates of all the relative specificity constants of the endopolygalacturonase of *Fusarium moniliforme* from the data for the hydrolysis of the heptagalacturonate (i.e. from Fig. 9(d)). Likewise, it would be possible to obtain estimates of all the relative specificity constants of DispersinB from the data for the hydrolysis of the pentaglucosamine (i.e. from Fig. 5(c)). On the contrary, the determination of k_{cat} and K_M requires various initial rate assays with several different initial substrate concentrations for each of the substrates [3–12]. It has the further disadvantage of providing only the global specificity constant for each substrate, not giving any information about the specificities of the various different reactions that are possible with each substrate. Likewise, for the bond cleavage frequency method, it is necessary to conduct at least one experiment for each different substrate and intermediate. Additionally, this method only provides information about the preferences of the enzyme for catalyzing different reactions with the same substrate; it does not provide information about the relative preferences of the enzyme for catalyzing reactions with different substrates.

The fingerprinting method has a further advantage over the bond cleavage frequency method, which cannot be used when the enzyme is capable of processivity [17]. In contrast, as our first case study shows, the fingerprinting method can characterize the relative preference of an enzyme for the processive route, as long as this route is included in the model.

Although the fingerprinting method has advantages over other methods that have been used to characterize relative specificity constants, as discussed above, it is limited to providing only this information. As such, it is useful for investigating how changes in

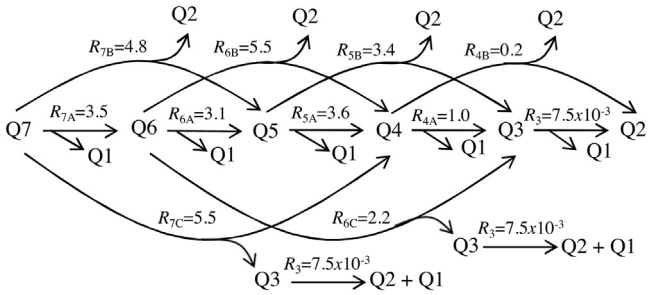

Fig. 10. Values of relative specificities for the endopolygalacturonase of *Fusarium moniliforme* obtained from the corrected experimental data of Bonnin et al. [6]. Key: Q*n* represents an oligogalacturonate with *n* units; *R* values represent relative specificity constants normalized with respect to k_{4A}; k_{4A} is the specificity constant for the cleavage of Q4 into Q1 and Q3.

the reaction conditions or in the enzyme itself affect the specificity of the enzyme. If the aim is to obtain parameters for a kinetic model, then the fingerprinting method can provide specificity constants, but other parameters that are necessary for such a model, such as K_M values, inhibition constants and denaturation constants, must be obtained by other methods. The advantage of the fingerprinting method is that it removes the effects of these phenomena from the analysis [18], but this means that it cannot quantify these phenomena.

Additionally, the fingerprinting method can only be used to analyze reaction schemes in which all reactions are catalyzed by the same enzyme [18]. It cannot be applied to saccharification processes of complex polysaccharides that involve several different enzymes. Even if a single enzyme were to be used with a com-

plex polysaccharide, the method requires that all intermediates be quantified, and this is a significant challenge when a highly complex mixture of intermediates is produced. It is usually possible to obtain adequate resolution of smaller oligosaccharides, and appropriate standards are often available or easy to produce. However, for larger oligosaccharides, quantification can be more difficult, if not impossible, since they may be present in low concentrations and peaks may not be adequately resolved [9]. Also, appropriate standards for the analytical method are unlikely to be available and will be difficult to prepare in-house.

5. Conclusions

Mathematical models are important tools for guiding the development of processes for the hydrolysis of polysaccharides. In order to use these mathematical models, it is necessary not only to have correctly formulated equations, but also to have good estimates of the parameters of the model. Models of the saccharification of polysaccharides will need to include descriptions of the later stages of the reaction, when the enzymes are attacking oligosaccharides, in complex, branched reaction schemes. The preferences of an enzyme for the various reactions that it can catalyze within such reaction schemes depend on the length of the oligomer and on the position of the bond within the oligomer. In the current work, we have demonstrated that the fingerprinting method of determining relative specificity constants can be applied to branched reaction schemes. This method is simpler to apply than methods that have been used to date, such as those based on the determination of initial rates and the bond cleavage frequency method: Estimates of all relative specificity constants can be obtained from one complete time course experiment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2016.05.012.

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