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Simulation of endo-PG digest patterns and implications for the determination of pectin fine structure

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Abstract

Novel stepwise approaches to the calculation of enzyme digest patterns are described and used in the validation of a computer simulation. Results obtained using the simulation show that, while a previously proposed model of endo-PG action captures some of the salient features of this enzymes behaviour, it is not sufficient to successfully predict experimental digest patterns from pectic substrates. Subsequently, it has been shown that a modified model incorporating existing information regarding subsite architecture and speculative site tolerances for esterified residues, goes someway towards improving the situation.

Keywords: Pectin; Polygalacturonase; Degree of methylesterification; Intramolecular DE distribution; Endo-PG digest pattern

1. Introduction

While the industrial exploitation of pectin-modifying enzymes has been commonplace for many decades, only recently has there been an increasing renewed interest in the fundamental biochemical aspects of the enzyme–substrate interaction. Lately, literature in this field has become more prevalent and studies are unearthing a wealth of information, particularly regarding the active sites and the mechanism of action of polygalacturonases.[1–5] In the last year alone the crystal structure of endo-PG II has been determined,[4] attempts have been made to map its subsites by measuring bond cleavage frequencies,[5] and progress has been made in the investigation of its substrate specificity.[6–8]

In parallel with this biochemical renaissance, endo-PG has been promoted in contemporary carbohydrate literature as a useful tool for the elucidation of pectin fine structures and in particular the intramolecular distribution of methylesterification.[9–13] Broadly speaking this problem has been addressed by two approaches. The first of these uses direct measurements on the polymer using NMR methods and discriminates intramolecular distributions based on observed chemical shift differences.[14,15] Alternatively, enzymatic[9–13,16,17] and chemical methods[17,18] utilise a fragmentation approach in which the polymer chains are disassembled and attempts are made, based on derived digests, to infer properties of the pre-digested substrate.

However, despite a large amount of experimental work in this latter area, calculations of digest patterns as an avenue of enquiry has, to date, been largely neglected. While the biopolymer literature abounds with comments regarding the results of preliminary computer calculations,[9,11,16,19,20] details of the simulations are, at best, sketchy. As outlined above,

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recent work has begun to advance the understanding of pectin-degrading enzymes and it is timely, therefore, to investigate how the derived rules of engagement impact on the digests that would be obtained from pectin substrates. This paper seeks to address this issue using a validated computer simulation.

2. Experimental

The experiments carried out in the work reported here take the form of computer simulations. The details given below are intended in the same spirit as more conventional experimental sections, in order to provide sufficient information regarding methodology such that an interested reader could repeat the reported work.

Basic simulation philosophy.—A single chain of pectic substrate is simulated by two one-dimensional arrays, with each array element representing one residue. One of the arrays keeps track of the residue type (GalA or methylesterified GalA), while the other monitors the residue position with regard to the fragment of which it is a part (non-reducing end, middle, reducing end). (While it is the goal of further work to incorporate rhamnose into the simulated substrate in a realistic fashion, the present work is restricted to homogalactan.) All substrates are initialised as polygalacturonic acid (PGA) and subsequently attain their required degree of methylesterification (DE) via a methylation algorithm. For random distributions, Bernoulian statistics are used. Distributions in the degree of polymerisation (DP) of the chains can be accounted for by generating a set of chains, with the relevant number modelled by arrays of different length. Intermolecular DE distributions are simulated in a similar way with relevant numbers of chains being assigned slightly different DE values in order to build up a selected DE distribution for the simulation set. Typically 1000–10,000 chains were used in any one simulation.

The actual endo-PG digestion is simulated according to two models (i) a previously claimed situation (designated forthwith as the 4, 3 model) in which the enzyme requires four unesterified residues in order to dock and cuts the chain exclusively between the 4th and 3rd residues from the non-reducing end,[21–26] and (ii) a modified version taking into account experimentally measured bond cutting frequencies when the substrate is smaller than the total subsite size, and postulated subsite tolerances for methylesterified residues obtained from the consideration of the known ester distribution of surviving fragments. It should be noted that this latter model is based on the consideration of experimental results obtained from digests of a particular polygalacturonase (endo-PG II), and this should be borne in mind when making comparisons of the predicted digest patterns with experiment.

More specifically, in this second proposed model, the subsite size is assumed to be seven,[5] with subsites running, in conventional notation, from -5 through to $+2$.[27] Within the context of the 4, 3 model, this is equivalent of a 7, 5 variant. Owing to the nature of the subsites and the preferential binding of some of these for the substrate, oligimers that are smaller than the subsite extent will have a preferred binding position within the cleft. Therefore, not all cleavage sites within such substrates will be cut with the same frequency. These bond cleavage frequencies have been measured for endo-PG II digests of GalA oligomers[5] and these values have been used in this simulation for the digestion of oligomers ($n < 7$). The detailed effect of methylesterification of the substrate here is not known, i.e., the way in which the free energies of substrate binding, and hence bond-cleavage frequencies, are modified by the presence of esterifed residues at different positions is unknown. However, work on synthesised monoesterified substrates has unequivocally demonstrated that subsites -1 and $+1$ must be unesterified for enzymatic action to occur.[6] Furthermore, recent work has recovered actual intramolecular ester distributions for the digest fragments of pectic substrates using endo-PG II.[8,13] Studying these gives clues to the rules of enzyme engagement with respect to methylesterification. Firstly, runs present within the body of the fragments must be enzyme resistant, while the ends of the surviving fragments

show tolerance for the sections either side of the active site, $-5\ldots-1$ for the reducing end, and $+1\ldots+2$ at the non-reducing end (neglecting the small amount of fragments that originate from cleavage that occurs at the ends of chains). We propose that the form of the fragments observed in the work described[8,13] can largely be accounted for by assuming (i) that subsites -1 and $+1$ must be unesterified; (ii) that three sites out of the remaining -5, -4, -3, -2, and $+2$ positions must also be GalA and (iii) that the tetramer is an exception to (ii) and can successfully bind to the enzyme despite only being bound at four subsites, spanning -3 to $+1$. (It should be noted that it has been shown experimentally that trigalacturonic acid, can also, in fact, be digested by endo-PG II, but over extended periods, with a rate some 20 times less than the tetramer.[23] Indeed, over long enough periods even 3- and 1-methyl trigalacturonic acid can be digested,[6] with rates yet further orders of magnitude down on the unesterified trimer. These rates yield time-scales that are long enough to essentially decouple the further digestion of trimers from the main digestion activity and make the vast majority of experimental data unconcerned with such a secondary process. Hence, in this work, the GalA trimer is considered as an end product.)

In the absence of further information on the modification of bond-cutting frequencies by different methylesterification arrangements, the rules described here are used in the first case to determine the success or otherwise of a chain scission. The order of tasks carried out by the newly proposed digest algorithm is as follows. Firstly, the set of chains is attacked by randomly selecting a residue (the selected residue is arbitrarily assigned to subsite $+2$). Secondly, if the fragment selected has $n>6$, then the substrate structure in the proximity is assessed to determine if it fulfils the methylesterification tolerance rules and if it does, the chain is split in the relevant position according to the subsite architecture. The characters of the residues surrounding the sight of enzyme action are modified in the relevant descriptor array in order to mark them as non-reducing and reducing ends, respectively. Alternatively, if the fragment has $n<7$ then, if the ester tolerance criteria are satisfied, the bond to be cut is selected by comparison with the cumulative percentage bond-cleavage frequencies, and the substrate array modified as described. At each stage of the simulation (both the 4, 3 model and newly proposed variant) the substrate arrays are interrogated and plots of the intermolecular DE distribution, the DP distribution and the GalA block length distribution are displayed.

Program validation.—In order to be assured that the written code represented a realistic simulation, and did not contain programming artefacts, several criteria were selected and deemed as vital checks on the program performance. Firstly, the generation of random intramolecular DE distributions of selected average DE was essential. It is straightforward to show that for a random intramolecular distribution of methylesterified residues, the DE value can be extracted from the non-esterified GalA blocklength distribution. In fact, plotting log(frequency) versus blocklength should result in a linear plot with a gradient of $\log((100-\mathrm{DE})/\mathrm{DE})$. This was checked and was indeed found to be the case.

Secondly, the previously described 4, 3 model of enzyme action was programmed and this algorithm used to digest a straightforward PGA substrate. As expected the ultimate products were solely mono-, di- and trigalacturonic acid. It was required that the ratio of these simulated digest products be comparable with that obtained from an independent calculation that was performed in the following fashion. Assuming all possible allowed first scission points have an equal probability of being cut, it is possible to calculate the relative ratios of mono-, di- and trigalacturonic acid, resulting from the digestion of a GalA oligomer of length n, in a recursive fashion, using the ratios obtained from the calculation for smaller oligomers. When normalised to the monomer, the predicted ratios soon converge as n increases. The convergent values (1:0.420:1.243) were found to be in excellent agreement with the results obtained from the simulation algorithm for a simulation of 1000 chains of DP 500 (1:0.422:1.243).

Lastly, in order to further confirm the correct functioning of the 4, 3 model code, the

digest fragment distribution for randomly methylesterified substrates was calculated in steps, in a more analytical fashion, and compared with the results of the straightforward simulation. The starting point for the stepwise calculation is the identification of all possible scenarios in the intramolecular methylesterification distribution of a particular product *n*-mer. This is illustrated in Fig. 1(a), which shows all possible intramolecular motifs for a surviving decamer digest fragment within the framework of the 4, 3 model. It is immediately apparent that the problem is substantially more complex than calculation of the probability of a run of 10 residues that cannot be cut by the enzyme (i.e., contains no runs of

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Surviving Decamer Fragments from the Body of the Chain

GE - - 4_U - - EGGG (i)
GGE - - 3_U - - EGGG (ii)
GGGE - - 2_U - - EGGG (iii)

Surviving Decamer Fragments from the Reducing End of the Chain

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GE - - 8_U (iv)
GGE - - 7_U (v)
GGGE - - 6_U (vi)

Surviving Decamer Fragments from the Non-Reducing End of the Chain

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7_U - -GGG (vii)

Probability of *n*-mer being formed =

(For fragments cut from the body of the chain)

Probability cut leaves monomer (- - G) × Probability of $(n\text{-}4)_U$ with ends both E (E - - $(n\text{-}6)_U$ - - E) × Probability cuttable at back (GGG-G)

+Probability cut leaves dimer (- - GG) × Probability of $(n\text{-}5)_U$ with ends both E (E - - $(n\text{-}7)_U$ - - E) × Probability cuttable at back (GGG-G)

+Probability cut leaves trimer (- - GGG) × Probability of $(n\text{-}6)_U$ with ends both E (E - - $(n\text{-}8)_U$ - - E) × Probability cuttable at back (GGG-G)

(For fragments cut from the reducing end)

+Probability cut leaves monomer (- - G) × Probability of $(n\text{-}1)_U$ starting in E (E - - $(n\text{-}2)_U$)

+Probability cut leaves dimer (- - GG) × Probability of $(n\text{-}2)_U$ starting in E (E - - $(n\text{-}3)_U$)

+Probability cut leaves trimer (- - GGG) × Probability of $(n\text{-}3)_U$ starting in E (E - - $(n\text{-}4)_U$)

(For fragments cut from the non-reducing end)

+ Probability of $(n\text{-}5)_U$ ending in E ($(n\text{-}4)_U$ - - E) × Probability cuttable at back (GGG-G)

Fig. 1. (a) The form of surviving decamer fragments from the digestion of a pectic substrate, within the framework of the 4, 3 model. GalA groups are denoted by G, methylesterified GalA groups are denoted by E. The subscript U is used to denote a sequence of residues that is resistant to degradation by the enzyme, e.g., 7_U denotes all sequences of 7 residues in length that do not contain a run of 4 or more consecutive GalA. (b) An extension of (a) to the general *n*-mer case and outline of the calculation strategy.

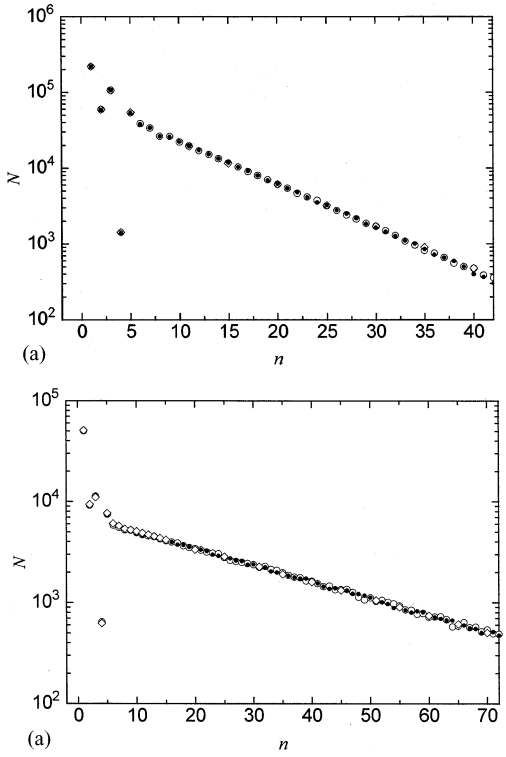

Fig. 2. (a) Endo-PG digest patterns expected from randomly methylesterified substrates calculated within the framework of the 4, 3 model in a stepwise fashion (◇), and by computer simulation (○, repeat ●), for 30% DE. (b) Endo-PG digest patterns expected from randomly methylesterified substrates calculated within the framework of the 4, 3 model in a stepwise fashion (◇), and by computer simulation (○, repeat ●), for 50% DE.

GalA equal to or greater than four residues). Indeed, owing to the nature of the 4, 3 model it is possible that a decamer can be generated simply by the existence of a run of four residues with an methylesterified residue at either end (route (iii)). The scheme is shown for the general n-mer case in Fig. 1(b), which also serves as a schematic of the calculation strategy. It can be seen that the calculation of the probability of the occurrence of each fragment with a DP larger than four involves (a) the calculation of the probability of the occurrence of enzyme resistant runs of lengths $n-6$, $n-5, \ldots, n-1$, that are terminated at either end by an methylesterified residue; (b) the calculation of the probability that a cuttable run follows such a sequence and (c) the calculation of the relative probability that the enzyme attack leaves one, two or three unesterified GalA residues to the right (non-reducing end) of the cut.

Using the approach detailed above it has been possible to calculate endo-PG digest patterns expected from randomly methylesterified substrates within the framework of the described 4, 3 model. The results are shown in Fig. 2(a and b) for two different degrees of methylesterification (30 and 50%). An excellent agreement can be seen between these data and the computer simulation, and this serves to deflect concerns regarding the rigour of the computer simulation code itself when comparing theoretical digest patterns with those obtained experimentally.

3. Results and discussion

The results presented take the form of a comparison of simulations with reported experimental results from existing literature. There have been a number of studies that have generated data on the digestion of PGA and/or pectic substrates by polygalacturonase. Unfortunately, most of these are not appropriate for comparison with the simulations carried out here either because the digestion has not gone to completion, or the enzyme used originated from a different source. Both of these comments are worthy of some expansion.

Firstly, it should be clarified what is meant by completion in the context of this comment. It is not intended here to refer to the very slow degradation of trigalacturonic acid oligomers, as mentioned previously, but to indicate that digests of PGA have been reported in which there is still a significant amount of tetramer present at the conclusion of the experiment.[5]

Secondly, it must be noted that enzymes from different sources, and indeed different isozymes encoded by the same organism, are likely to possess differing subsite architectures, bond-cleavage frequencies and methylesterification tolerances. It is inappropriate then to compare previous work involving endo-PG from *Erwinia carotovora*[17,26] and *Kluyveromyces fragilis*[9–11,16] with the simulation described here, that has been wholly based on results obtained with endo-PG II from *Aspergillus niger*. However, it is still noteworthy that comparison of results ob-

Table 1
A comparison of the calculated and experimental molar ratios of mono- (M), di- (D) and trigalacturonic acid (T) liberated by a complete digestion of PGA

M	D	T	Source
1	0.62	1.2	Ref. 25
1	0.6	1.1	Ref. 28
1	0.42	1.24	4, 3 model
1	0.62	0.97	this work
1	2	1.76	Ref. 10

While the majority of work is focussed on *A. niger* (Refs. 25,28, this work) an experimental result obtained using an endopolygalacturonase from *K. fragilis* (Ref. 10) is included for comparison. The calculations are described in detail in the text.

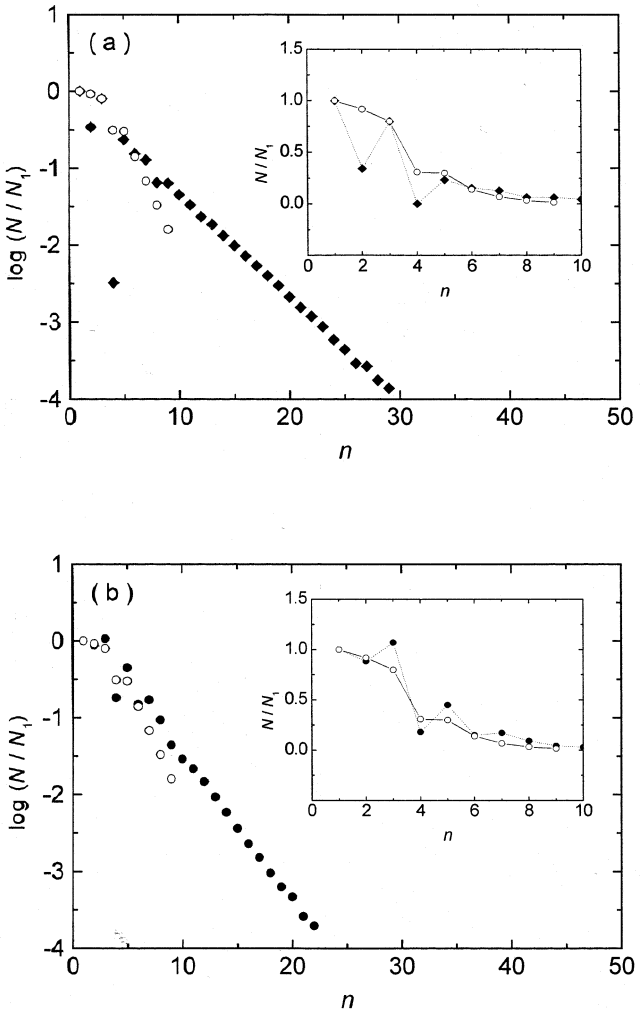

Fig. 3. Endo-PG digest patterns calculated for a randomly methylesterified substrate of 15% DE using (a) the 4, 3 model (◆) and (b) the extended model described herein (●) compared with reported experimental data (○). The number of fragments of length n, (N), have been normalised to the monomer (N_1).

tained from the digestion of PGA with an endo-PG from *K. fragilis*[10] with those obtained by the 4, 3 model demonstrate the inappropriateness of this model for the description of this enzymes action (Table 1).

Polygalacturonic acid.—Two studies that are amenable to comparison with the theoretical work described here have been carried out using endo-PG from *A. niger*[25,28] and although these involve commercial samples as the enzyme source, it is believed that these consist predominantly of endo-PG II.[29] The results are collected in Table 1 and it can be seen that while the results of the 4, 3 model are not too far removed from observation, the more specific model proposed here gives a slightly better agreement. The sum of the squared deviations of the predicted ratios from the average of the reported experimental work for the more involved model is found to be 3/4 of that for the 4, 3 model. Progressing to pectic substrates allows a more rigorous assessment of the applicability of the proposed models by involving the methylesterification toleration schemes.

It is worth noting that an analytical model for the calculation of endo-PG digest patterns from randomly methylesterified pectic substrates has previously been proposed in existing literature.[17] This is based on considering the probability that a run of n residues does not contain an enzyme degradable site but unfortunately has been based on a flawed statistical assumption that the probability of producing an oligomer of a particular length decreases with each extra residue by a factor of $(1-P)$, where P is the probability of the existence of the minimum degradable site.

Pectic substrates.—A recent study has provided data on digest patterns obtained from pectic substrates using endo-PG II from *A. niger* and thus forms the ideal comparison for the models described here. Figs. 3–5 show the comparison of the predictions from the 4, 3 methodology and the more specific model expounded herein, with experimental digest patterns measured for substrates of 15, 34 and 43% DE with random ester distributions. More specifically, all three experimental samples have been alkali de-esterified from a mother pectin of 81% DE, and their classifica-

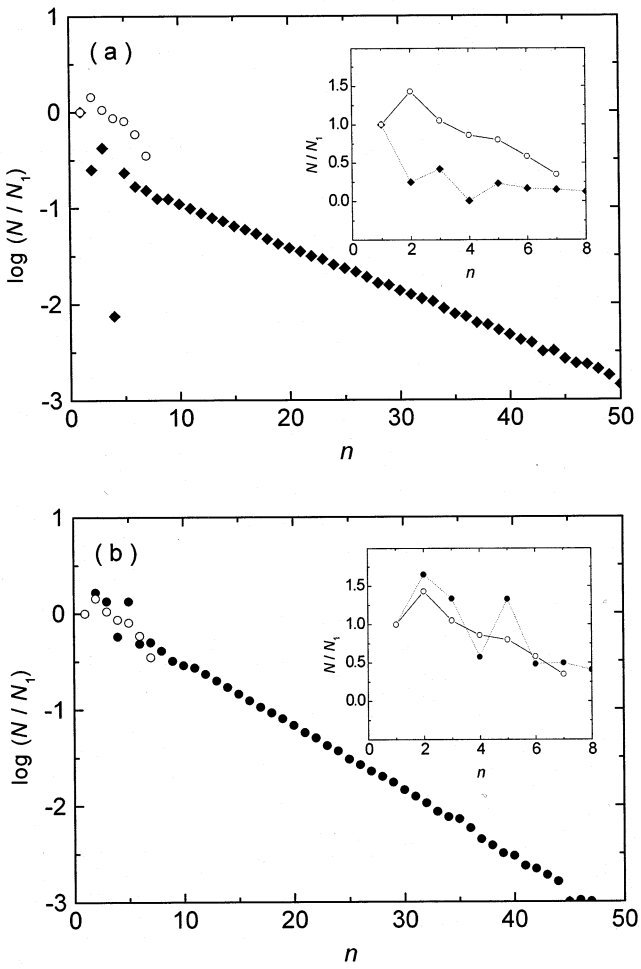

Fig. 4. Endo-PG digest patterns calculated for a randomly methylesterified substrate of 34% DE using (a) the 4, 3 model (◆) and (b) the extended model described herein (●) compared with reported experimental data (○). The number of fragments of length n, (N), have been normalised to the monomer (N_1).

tion as random implies that this starting material also possesses a random DE distribution. Logarithmic scales and extended n axes are used in the figures in order to view the power law behaviour of the predicted digests at larger degrees of polymerisation, while inserts show the plots in more detail over the experimental range. (Although data in the referenced work was obtained up to DP 12, that displayed here represents points that could be confidently read from graphs displayed therein.) It can be seen that, overall, the more specific model proposed here is more successful in matching the experimental digest patterns than the 4, 3 model. It is worth noting that, above about the decamer, the digest pattern calculated using this alternative model still clearly exhibits a power law dependence for the frequency of fragments with their DP, although the exponent of such a relationship is substantially modified compared to the 4, 3 model.

It should be noted that the experimental literature data were obtained using chromatographic analysis at high pH and, as such, any partially methylesterified fragments present in digests would have been completely de-esterified in the separation process. This means that there is another rich vein of data that could be tapped into, by, for example, running experiments with a post-column reactor, as has been described for other polygalacturonases.[9,10] This would allow data to be obtained not just on the total amount of oligomers of a particular DP present in the

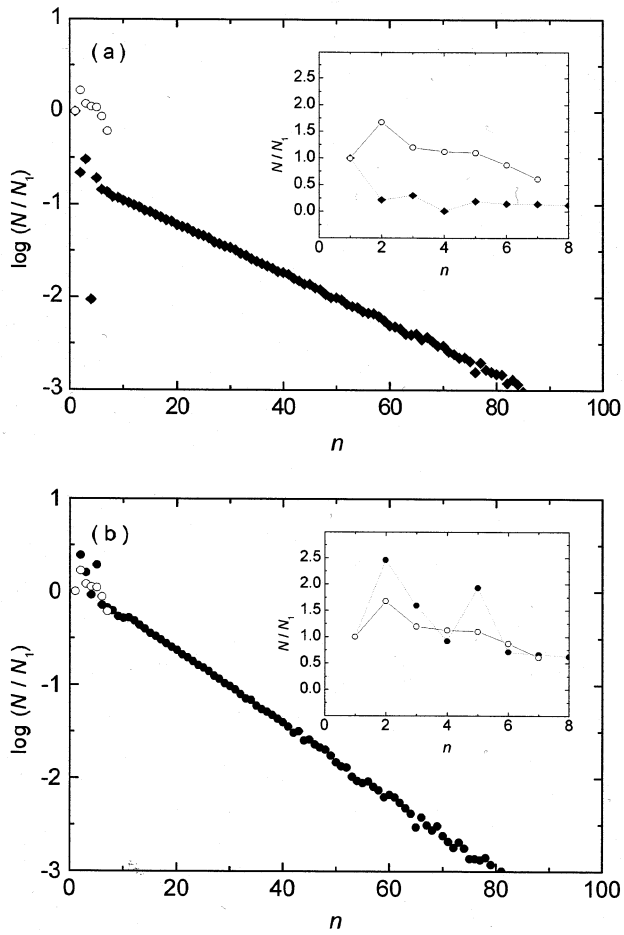

Fig. 5. Endo-PG digest patterns calculated for a randomly methylesterified substrate of 43% DE using (a) the 4, 3 model (◆) and (b) the extended model described herein (●) compared with reported experimental data (○). The number of fragments of length n, (N), have been normalised to the monomer (N_1).

digests, but on the amount of different methylesterified species within that size-class. Such work would provide a more rigorous experimental digest characterisation for comparison with the enzyme models that routinely predict this information.

While much progress has been made recently in the investigation of pectin fine structure, particularly using enzymes, previous work has concentrated on constructing comparative scales and establishing a degree of discrimination between substrates engineered to have different characteristics. This endeavour is, of course, valuable in its own right. The work described here attempts to extend the area by offering another avenue of enquiry in which digest patterns can be compared with simulated data, as well as with experimental digest patterns obtained from standard substrates. The hope is that as experiments in the biochemical arena progress and more data becomes available that the model of enzyme action will become more sophisticated and provide a usable connection between experimental digest patterns and tangible descriptors of substrate fine structure.

4. Conclusions

Novel stepwise approaches to the calculation of enzyme digest patterns have been described and have been used in the validation of a computer simulation. Simulation results show that while the previously proposed 4, 3 model of endo-PG action captures some of the salient features of the enzymes behaviour, it is not sufficient to successfully simulate experimental digest patterns obtained from pectic substrates. Subsequently, it has been shown that a modified model incorporating existing information regarding subsite architecture, in addition to speculative methylesterified residue tolerances, improves the situation somewhat.

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