

Oligomer distribution of depolymerase digests: comparison of theory and experiment

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Enzyme hydrolysis of linear β -1,3-glucan was carried out, with *p*-nitrophenylglucoside (in a high concentration) involved in simultaneous transglucosylation reaction. The *p*-nitrophenyloligosides were next separated by gel chromatography and the product distribution shape was studied. In good agreement with earlier theoretical predictions the distribution shape was close to an exponential one for oligomer chain length exceeding the size of the enzyme-binding region.

Enzyme degradation Product distribution Action pattern β -1,3-Glucanase Transglycosylation

1. INTRODUCTION

The composition of oligomers formed during enzyme hydrolysis of a linear polysaccharide has long been used to study the specificity and mode of action of endoglycanases (*O*-glycoside hydrolases which split the internal bonds of linear substrates, EC 3.2.1) [1]. In recent years, experimental data in the field were successfully interpreted based on the extended active centre model [2–4]. The model postulates that multiple-site interactions of the enzyme with the polysaccharide molecule, when several substrate monomer units are bound in the active centre, result in specific distribution of the bond cleavage frequencies along the polysaccharide chain. A computer simulation of polysaccharide degradation by some endoglycanases showed that the oligomer compositions predicted with this model are sufficiently close to those experimentally observed [5–7]. In these works, the oligosaccharide concentrations (chain length n 1–7) were measured and calculated.

In our recent paper [8], the theoretical problem was studied with reference to the character of the influence of interactions within the enzyme binding region on the chain length distribution of

products having higher M_r . As shown [8], the size of the active centre and its individual features should not affect the shape of the polymer part of product distribution. That is, if l is the maximum number of monosaccharide units bound in the active centre, then for chain length $n \geq l - 1$ the exponential form of the distribution function should persist during enzyme degradation. These results give new possibilities in studying depolymerizing enzymes and have already been successfully applied for critical analysis of experimental data [9,10]. However, the shape of the chain length distribution for $n \geq l - 1$ has not yet been directly tested in experiments because the common size of the binding region of endoglycanases ($l \approx 10$) is approximately equivalent to the limit chain length of oligosaccharides, which can be separated by gel filtration. In this work, we studied the composition of polysaccharide degradation products with degrees of polymerization in the region where comparison with theoretical results [8] was possible. To avoid the above-mentioned complication, we used a low- M_r endoglycanase and a special reaction scheme (transglycosylation) leading to accumulation of reducing end-‘labelled’ aryloligosides which can be separated by gel filtration with much higher resolution [11].

2. MATERIALS AND METHODS

The reaction was carried out by disc electrophoresis homogeneous endo- β -1,3-glycanase L IV prepared from the marine mollusc *Spisula sacchalinesis* [12], using laminarin (β -1,3-glucan) from the seaweed *Laminaria cycharioides* [13] as a substrate and *p*-nitrophenyl β -D-glucopyranoside (commercial preparation, 'Chemapol', Czechoslovakia) as acceptor in the transglycosylation reaction. Normally, substrate and acceptor concentrations were 0.5 and 33 mM, respectively, except for the results presented in fig. 1a, where a 7.7 mM substrate concentration was chosen to give a clearer view.

Quantitative analysis of products of reaction mixtures consisting of *p*-nitrophenyl- β -laminaribioside G_2Ar , triside G_3Ar , etc. was performed with a Jeol LCH-6AH carbohydrate auto-analyser (Japan) on a Bio-Gel P-2 (-400 mesh) column (0.9×98 cm); temperature of the jacket, 50°C ; elution rate, 7–9 ml/h; 0.05 M Na-acetate in 0.2 M NaCl buffer (pH 5.2–5.5) as eluent. The orcinol-sulfuric acid procedure was used for determining polysaccharide concentrations [11]. Simultaneously, *p*-nitrophenylglucosides G_1Ar – G_nAr were tested with a UV detector at 280 nm.

The composition of *p*-nitrophenol-labelled products was also analyzed on Bio-Gel P-4 (-400 mesh), using 2 consecutive columns (1.6×100 and 1.6×80 cm; elution rate, 15 ml/h) with a Uvicord (LKB) UV detector.

3. RESULTS AND DISCUSSION

The endo- β -1,3-glycanase L IV from *S. sacchalinesis* has a relatively small molecular mass (22 kDa) [12]. Its binding region can supposedly hold 4–5 monosaccharide units [7,9,14]. Earlier, we reported that this enzyme has a high transferase activity with a wide range of acceptors including *p*-nitrophenyl β -D-glucoside [11]. The oligomer products of the transglycosylation reaction, *p*-nitrophenyllaminarioligosides, can be readily detected with light absorption techniques and, moreover, gel filtration of these products gives a resolution which is sufficiently higher than that for unmodified oligosaccharides. Hence, it would be convenient to use *p*-nitrophenol as a 'label' for

studying the molecular mass distribution of polysaccharide degradation products.

The scheme of enzyme splitting of a polysaccharide in the presence of the acceptor is shown below (scheme 1).

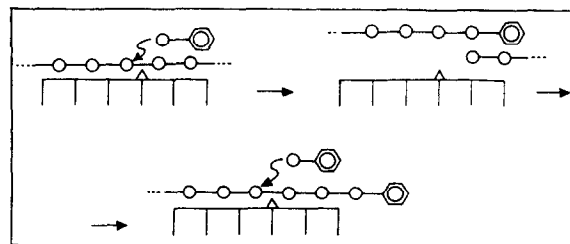
As the transglycosylation kinetic constant for L IV is more than 10^4 -times that for hydrolysis [15], under the conditions used practically all enzyme attacks on the initial homopolymer should be accompanied by transglycosylation. As a result of these initial attacks a set of polysaccharide fragments labelled by *p*-nitrophenol at the reducing end appear. The composition of this mixture is determined by the relative bond cleavage frequencies. With further degradation, the reaction mixture would contain both labelled and unmodified products, with gradually changing molecular mass distributions (see fig. 1a).

In [7,8], we presented a theoretical study of polymer distributions of enzyme degradation products. In particular, the situation was considered when the substrate initially has a nearly fixed chain length. It is easy to see that the same mathematical treatment can be applied to the distribution of *p*-nitrophenol-labelled oligosaccharides in our experiments. According to [8], for a not very high degree of polymerization n , the distribution shape would be qualitatively the same as when the initial polysaccharide has the most probable distribution. More accurately, for $n \geq l - 1$ it should be described by the relation:

$$C_n(t) = C_l(t)[q(t)]^{n-l} \quad (1)$$

where C_n is the concentration of n -mer, and $q(t)$ is a gradually decreasing function, while at $n < l - 1$ the oligomer distribution would depend on the individual features of the enzyme, i.e. on the structure of its active centre.

Although the theoretical treatment of [8] does not consider explicitly the degradation of the



Scheme 1.

reducing end-labelled substrate, the main theoretical result should also be valid in this case, the reasons being as follows. Two conditions are necessary for eqn 1 to appear: (i) the rate of n -mer formation from $(n + i)$ -mer should depend only on i ; (ii) the probability of polymer chain rupture in the solution should increase with chain length in a linear fashion. One can readily see that both conditions are satisfied when only the reducing end-labelled polysaccharides are measured in the solution. The lower limit of oligomers fitting eqn 1 is the same as in [8] because it is determined by the second condition.

Typical oligomer compositions of the reaction mixture at some intermediate stages of the reaction are shown in fig.1. The 2 elution profiles in fig.1a obtained by different detection techniques reflect

both the integral molecular mass distribution of products and the chain length distribution of p -nitrophenol-labelled oligosaccharides. Fig.1a and b, shows that in the second case it is possible to measure the concentrations of products with a chain length n up to 12.

As is apparent from fig.1b, the product distribution shape resembles an exponential one. For more accurate comparison, it would be convenient to plot the molecular mass distribution on semilogarithmic coordinates. Some of the typical plots thus obtained are shown in fig.2, from which it is apparent that the form of the high-molecular-mass part of the distribution is practically indistinguishable from the exponential one. However, with oligosaccharide chain lengths less than 4, deviation of the experimental points from the least-squares straight lines is observed. According to theory [8], the deviation is possible only when the oligosaccharide chain length is 2 units less than that of the enzyme binding region. The end-labelled oligosaccharides used in our experiment had a label (p -nitrophenol) size approximately equal to that of glucose. Thus, the labelled oligosaccharide of 3 glucose units corresponds to the tetramer homooligosaccharide. Consequently, the endoglycanase L IV can have an active centre of 6 monosaccharide binding subsites. This value is consistent with earlier estimates [7,9,14].

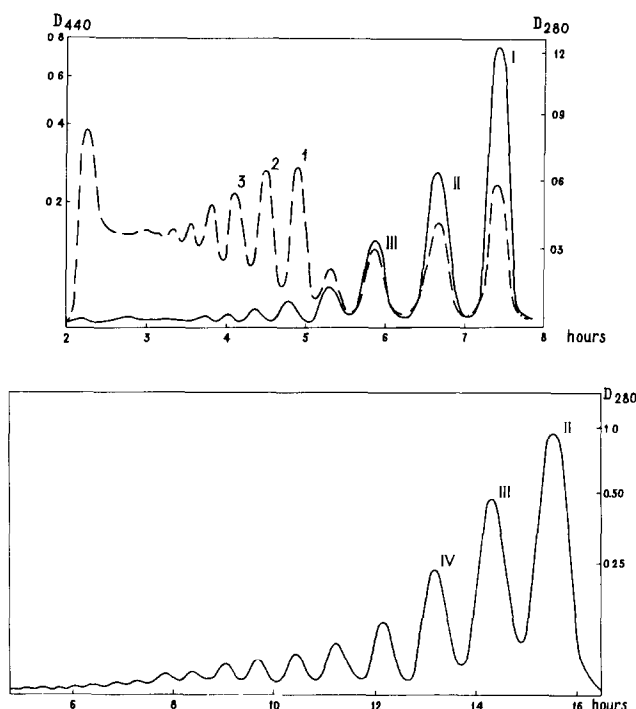


Fig.1. Gel filtration of products of enzyme digestion of laminarin and p -nitrophenyl- β -D-glucoside on Bio-Gel P-2 (-400 mesh) column (a) and Bio-Gel P-4 (-400 mesh) columns (b). (—) Elution profile of p -nitrophenyloligosides measured by absorbance at 280 nm. (----) Elution profile detected by the orcinol-sulfuric acid procedure. Peaks I–III... and 1–3... correspond to G_1Ar , G_2Ar , G_3Ar , etc. and glucose, biose, triose, etc., respectively.

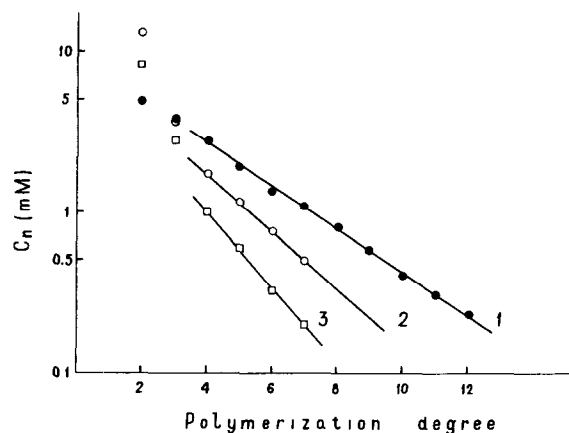


Fig.2. Chain length distribution of aryloligosides at 3 different stages of degradation. Degrees of polymerization were measured by the number of glucose units in the substance. Lines through points are the least-squares straight lines.

However, as indicated in [8], the number obtained is an estimate of the length of the substrate chain fragment interacting with the enzyme when the latter is bound to the polysaccharide. It may be somewhat longer than the true size of the enzyme active centre.

Thus, the experimental results obtained are in excellent agreement with theory [8] and serve to confirm and illustrate it directly.

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