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Hydrolysis of barley (1→3), (1→4)-β-D-glucan by a cellobiohydrolase II preparation from *Trichoderma reesei*

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The molecular weight of commercial barley β-glucan was 250,000 as determined by dual angle laser light scattering. ^{1}H NMR analysis showed the polymer to contain 29% (1→3)-linkages and 71% (1→4)-linkages. The barley β-glucan was readily hydrolysed by a highly purified cellobiohydrolase II (CBHII) preparation from *Trichoderma reesei*. NMR data demonstrated that the cellulase preparation degraded only (1→4)-linkages in the β-glucan chain. Neither internal G(1→3)G(1→4)G nor reducing end G(1→3)G(1→4)G(1→4)G sequences were hydrolysed. The main hydrolysis products were: cellobiose, β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-D-Glc*p*, β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Glc*p* and β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*. Statistical models of the glucan linkage sequence were fitted to the relative fragment concentrations after CBHII and lichenase degradations. The hydrolysate compositions are well reproduced by a second order Markov chain. All degradation data are consistent with the assumed degradation mechanisms of the two enzymes, including the hypothesis that hydrolysis by CBHII depends on the glycosidic bond orientation.

INTRODUCTION

Mixed-linked (1→3),(1→4)-β-D-glucans are the major cell wall components of barley and oat endosperm, and strongly influence the technical and nutritional value of these cereals. Both barley and oat β-glucans contain about 30% (1→3)-linkages and 70% (1→4)-linkages (Dais & Perlin, 1982; Woodward *et al.*, 1983; Bock *et al.*, 1991) and mainly consist of β-(1→3)-linked cellotriosyl and cellotetraosyl units as well as small amounts of longer (1→4)-linked glucopyranosyl sequences (Woodward *et al.*, 1983; Wood *et al.*, 1991*a*; Edney *et al.*, 1991). Although contiguous (1→3)-linkages have been shown to exist (Goldstein *et al.*, 1965; Bathgate & Palmer, 1974; Fleming & Kawakami, 1977), most recent studies suggest that contiguous (1→3)-linkages are absent or very rare (Dais & Perlin, 1982; Woodward *et al.*, 1983; Vårum & Smidsrød, 1988; Bengtsson *et al.*, 1990; Edney *et al.*, 1991).

The (1→3)-linkages render mixed-linked β-glucans more water soluble and easier to hydrolyse than cellulose, i.e. (1→4)-β-D-glucan (Bamfort, 1982; Woodward *et al.*, 1983). This effect seems to be due to the semi-random linkage sequence, which denies the β-glucan energetically favourable crystal forms such as those of cellulose (L. Kuutti *et al.*, unpublished results). Cereal β-glucans form highly viscous solutions in water, one of their most important physical characteristics. The viscous properties are mainly determined by the amount of β-glucan, its chemical structure and molecular weight. Endogenous β-glucanases may lower viscosity substantially by reducing the molecular weight. This enzymic β-glucan hydrolysis is desired in brewing and improves nutrient accessibility in animal feeds, e.g. for chicken, but is unwanted in the production of nutritionally valuable soluble fibre.

Also enzymes of microbial origin degrade cereal mixed-linked β-glucans in the grain. Lichenase, (1→3),(1→4)-β-D-glucan 4-glucanohydrolase (EC

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Sugar sequences are defined by: G, β-D-Glc*p* if glycosidically linked to another sugar and D-Glc*p* if at the reducing end, G3, β-D-Glc*p*-(1→3)- and G4, β-D-Glc*p*-(1→4)-. Thus, G4G is cellobiose and G3G laminaribiose.

3.2.1.73), which is found both in *Bacillae* and in germinating grain, specifically cleaves (1→4)-β-linkages of a 3-*O*-substituted D-glycopyranose residue. Yin and MacGregor (1988, 1989) found that an endo-type cellulase associated with barley husks attacked longer stretches of (1→4)-β-linkages to produce β-glucan with a molecular weight around 20,000, while Bock *et al.* (1991) reported a fungal cellulase mixture that efficiently hydrolyses β-glucan to principally tri- and tetrasaccharides. For many years fungal cellulase preparations have been used successfully to hydrolyse barley β-glucan in malting and brewing. Brewer's yeasts that secrete *Trichoderma reesei* endo-(1→4)-glucanase represent the most modern technique for β-glucan degradation in brewing (Enari *et al.*, 1987).

The filamentous fungus *Trichoderma reesei* secretes exceptional amounts of cellulolytic enzymes. These belong to the most studied cellulases, but their enzymology has mainly focused on the hydrolysis of cellulose, soluble cello-oligosaccharides and their derivatives (Wood & Garcia-Campayo, 1990). We found earlier that the cellobiohydrolase II (CBHII) of *T. reesei* causes only small changes in the degree of polymerisation of barley β-glucan. This is typical of an exo-acting enzyme (Henriksson *et al.*, 1992). More recently, we obtained considerable variation in extent of β-glucan degradation, despite using similar, highly purified CBHII preparations (Reinikainen *et al.*, 1994). The difference in the mode of action of these preparations was shown to arise from a minor contamination (< 0·4%) with an endo-hydrolase particularly active on β-glucan.

In the present work, the hydrolysis studies of barley β-glucan by a highly purified *T. reesei* CBHII preparation has been continued. The substrate has been further characterised and the principal degradation products have been identified. This information has been combined with published degradation data and knowledge about the enzymic hydrolysis mechanisms to generate statistical sequence models for the β-glucan. These models are a generalization of the sequence models presented by Staudte *et al.* (1983) and by Buliga *et al.* (1986). A number of observations relating to the methods employed are also reported here. For simplicity, we will refer to mixed-linked (1→3),(1→4)-β-D-glucan as β-glucan.

MATERIALS AND METHODS

Hydrolysis experiments

A commercial barley (1→3),(1→4)-β-D-glucan was obtained from Megazyme Pty Ltd, Sydney, Australia. The β-glucan had been extracted from inactivated barley at 65°C with three aliquots of water as described by Bourne and Pierce (1970), precipitated and washed with alcohol and finally lyophilised. The β-glucan content was 96% (dwb), as determined by the enzymic method of McCleary and Codd (1991), the residue being mostly plant cell wall protein. Cellobiohydrolase II from *T. reesei* was purified according to Tomme *et al.* (1988) by means of affinity chromatography. The details of purification and enzyme activity measurements are reported elsewhere (Reinikainen *et al.*, 1995).

Substrate stock solution, 1·1% (w/v), was prepared by solubilizing β-glucan in 50 mM ammonium acetate buffer (pH 5) at 70°C. The hydrolysis reactions were performed in test tubes at 37°C while mixing with a magnetic stirrer. Sodium azide, 100 ppm, was added as preservative. When the time course was studied, 15 mg of enzyme per gram of substrate was added, and barley β-glucan was incubated as 1% (w/v) solution for up to 72 h. During the hydrolysis, samples (5 or 10 ml) were taken and analysed as described below. For characterisation of the hydrolysis end products, 20 ml of substrate solution was hydrolysed at similar conditions for 72 h. The hydrolysis reaction was stopped by heating in a boiling water bath for 10 min. The hydrolysate made for the assignment of end products was lyophilised.

Fractionation of end products

The mono- and oligosaccharides released during 72 h of hydrolysis were fractionated by a semipreparative HPLC method using a reverse phase column. The lyophilised hydrolysate (200 mg) was dissolved in 20 ml of water, relyophilised and redissolved in 2 ml of water. The sample was then fractionated at ambient temperature by reverse phase chromatography with μBondapaK C-18 (19 × 150 mm) with water as eluent at a flow rate of 2 ml/min. A refraction index detector was used. The low molecular weight fractions were analysed by HPLC and selected fractions were pooled for ^{1}H NMR analysis.

Dinitrosalicylic acid (DNS) analysis of reducing sugars

The extent of hydrolysis was estimated from the amount of reducing sugars. The reducing sugars were determined as D-glucose equivalents using the dinitrosalicylic acid (DNS) method as described by Bailey *et al.* (1992). The DNS-reagent was prepared according to Whelan (1964). The amount of reducing sugars was expressed as % of the initial amount of glucose moieties.

Gel permeation chromatography (GPC) and high performance liquid chromatography (HPLC) analysis

The molecular weight of barley β-glucan was characterised by the GPC method of Suortti (1993). For the β-glucan and its hydrolysates, μHydrogel columns 2000, 500 and 250 (Millipore/Waters, Milford, MA, USA) at 70°C were used with 50 mM NaOH as eluent at a flow rate of 0·5 ml/min. The detection was either post-

column staining with Calcofluor or a dual angle laser light scattering detector (DALLS, Precision Detectors, Amherst, MA, USA). The Calcofluor staining method is suitable for detection of β-glucan with a molecular weight over 10,000 (Anderson, 1990). For Calcofluor detection 100 μl injections with 10–100 mg/l of β-glucan in 0·1 M NaOH were used. For DALLS detection the concentration of β-glucan was 1–2 g/l. Standardised high, medium and low molecular weight β-glucan preparations, a generous gift of Barry McCleary, Megazyme Pty Ltd., were used as standards.

For low molecular weight saccharides, a combination of a μHydrogel DP column and two Fast Fruit Juice columns (Millipore/Waters, Milford, MA, USA) at 70°C were employed. The eluent was 0·5% H_3PO_4 applied at a flow rate of 0·5 ml/min. One hundred microlitre injections in eluent with 25–5000 mg/l of oligosaccharide were performed and detected by refractive index. Malto-oligosaccharides and cellobiose were used as standards.

^{1}H NMR Spectroscopy

The saccharides were lyophilised and redissolved in D_2O (99·8 atom-% D, Fluka) to a concentration of 5 mg/ml for the study of hydrolysis time courses, and 6–10 mM for the fractionated end product analysis. ^{1}H NMR spectra were obtained at 599·94 MHz on a Varian UNITY 600 MHz spectrometer using 5 mm NMR tubes containing 0·7 ml of solution. Typical acquisition parameters were a 70° pulse, a spectral width of 3000 Hz, an acquisition time of 1·4 s and a repetition time of 7·4 s. Spectra were obtained at 70°C and 100 transients were collected for each spectrum. Chemical shifts are reported relative to sodium 3-(trimethylsilyl)-3,3,2,2-tetradeuteropropionate. The residual HDO peak was continuously saturated prior to each acquisition.

Polymer sequence models

In order to interpret the degradation results statistically a number of sequence models (Price, 1970) were generated, with the aim of finding the simplest model to explain the experimental data. We will refer to the models by means of their correlation lengths, i.e. the number of preceding linkages that affect the probability of a particular linkage being of the (1→3) kind.

From the NMR data presented below it is clear that, at least in this case, two (1→3)-linkages do not occur in a row in the mixed-linked β-glucan. Thus, the minimal possible model has a correlation length of 1. The conditional probability $p(1\rightarrow3,1\rightarrow3) < 0.01$ and is set to zero for the purpose of sequence modelling. If the relative amount of (1→3)-linkages is denoted p ($p = 0.29$ in our case), the probabilities for (1→3) and (1→4)-linkages (Table 1) are obtained.

Table 1

Preceding linkage	Probability of a linkage of kind 1→3	1→4
1→3	0	1
1→4	$p/(1-p)$	$(1-2p)/(1-p)$

The total probability for (1→3)-linkages is $p*0+(1-p)*p/(1-p)=p$ and that for (1→4)-linkages is $p*1+(1-p)*(1-2p)/(1-p)=1-p$, as desired. We will call this model, which is a first order Markov chain, the C1 model.

The belief that any (1→3)-linkage is normally followed by at least two (1→4)-linkages (Woodward *et al.*, 1983) requires a sequence model with a correlation length of 2. The characteristic property is thus that G3G4G3G sequences do not occur, which, in turn, raises the probability for G4G4G3G sequences in order to keep the total frequency of (1→3)-linkages constant. The conditional probability table is given in Table 2a, where the C2 model has also been included. The C2 model is a second order Markov chain.

Table 2a. Conditional probabilities for the β-glucan linkage sequence models, i.e. the probability that the present linkage is of a certain type, given the preceding two linkages

Preceding linkages	Present linkage	Model C1	Model C2
(1→3)G(1→3)	x[a]	0	0
(1→3)G(1→4)	(1→3)	$\frac{p}{1-p}$	0
(1→3)G(1→4)	(1→4)	$\frac{1-2p}{1-p}$	1
(1→3)G(1→4)	(1→3)	0	0
(1→4)G(1→3)	(1→4)	1	1
(1→4)G(1→4)	(1→3)	$\frac{p}{1-p}$	$\frac{p}{1-2p}$
(1→4)G(1→4)	(1→4)	$\frac{1-2p}{1-p}$	$\frac{1-3p}{1-2p}$

[a]Either (1→3) or (1→4).

Table 2b. Numerical values of coefficients ($p = 0.28$)

Preceding linkages	Present linkage	Model C1	Model C2	Buliga *et al.* (1986)
(1→3)G(1→3)	x[a]	0	0	0
(1→3)G(1→4)	(1→3)	0·389	0	0
(1→3)G(1→4)	(1→4)	0·611	1	1
(1→4)G(1→3)	(1→3)	0	0	0
(1→4)G(1→3)	(1→4)	1	1	1
(1→4)G(1→4)	(1→3)	0·389	0·636	0·65
(1→4)G(1→4)	(1→4)	0·611	0·364	0·35

[a]Either (1→3) or (1→4).

Table 3

Preceding sequence	Probability of a (1→3)-linkage
G3G	0
G3G4G	0
$G3(G4)_2G$	0·70
$G3(G4)_3G$	0·80
$G3(G4)_4G$	0·50
$G3(G4)_5G$	0·50
$G3(G4)_{n>5}G$	0·30

The total probability of (1→3)-linkages is verified from Table 2a as follows. For the C1 model it is:

$$0 + p * 1 * p/(1-p) + 0+ \\ + (1-p) * (1-2p)/(1-p) * p/(1-p) = \\ = (p^2 + p - 2p^2)/(1-p) = p$$

as desired. For the C2 model we have, similarly:

$$0 + p * 1 * 0 + 0+ \\ + (1-p) * (1-2p)/(1-p) * p/(1-2p) = p$$

again as desired.

The hydrolysate composition obtained by Woodward *et al.* (1983) for a commercial β-glucan with 30% (1→3)-linkages can be transformed exactly into a sequence model. This model is the C5 model, formally a fifth order Markov chain, and it is defined in Table 3.

This model gives a total probability for (1→3)-linkages of

$$1/1[1 + 0*0 + 1*1*0 + 2*1*1*0{\cdot}70 + 3*1*1*0{\cdot}30*0{\cdot}80 \\ +4*1*1*0{\cdot}30*0{\cdot}2*0{\cdot}5 + \ldots] = 0{\cdot}293,$$

which verifies the last of the sequence models. The hydrolysate composition given by Woodward *et al.* (1983, Table 2) thus corresponds to $p = 0{\cdot}293$ rather than $p = 0{\cdot}30$. This difference is probably within the experimental uncertainty, but may reflect a small portion of non-glucose moieties or of consecutive (1→3)-linkages.

Simulation of sequences and hydrolysis

The C1, C2 and C5 models were implemented in a Fortran progam that generated random sequences according to the models. About 10^6 residues were generated for each model, in order to attain a numerical accuracy of close to 3 digits.

The model polymers were then cut into oligosaccharides according to enzyme mechanism. For CBHII the cleaving procedure considered of the following criteria: (i) only (1→4)-linkages are cut; (ii) a (1→4)-linkage that follows a (1→3)-linkage is not cut; (iii) a reducing end G3G4G4G sequence is not cut; (iv) only linkages in the 'up' orientation are cut. The basis of this mechanism is given below, including definitions of the 'up' and 'down' orientations.

The average DP after cleavage can be obtained analytically for the C1 model. The probability of a stretch of two (1→4)-linkages is $(1-p)^*(1-2p)/(1-p) = 1-2p$. The requirement of 'up' for cleavage reduces probability by a factor of two $(1-2p)/2 = 0{\cdot}21$ for $p = 0{\cdot}29$. The inverse of this is the average oligosaccharide length immediately after cleavage of the polymer, i.e. 4·76.

Because of criterion (iv) also some of the resulting oligosaccharides are substrates for CBHII, i.e. when they, at a later stage, re-enter the enzyme in the other orientation. To account for this, the oligosaccharide distribution was re-degraded several times until no oligosaccharides were substrates for CBHII.

For lichenase, the cleaving criterion was simpler: a (1→4)-linkage that follows a (1→3)-linkage is cut. This is the reverse of criterion (ii) for CBHII.

The accuracy of the C1–C5 models was assessed by comparison with experimental data. These consisted of: (i) relative oligosaccharide concentrations after CBHII hydrolysis (this work); (ii) average degree of polymerisation (DP) after end of hydrolysis (this work); (iii) relative oligosaccharide concentrations after lichenase hydrolysis (Woodward *et al.*, 1983). For this comparison, the C1 and C2 models were used with $p = 0{\cdot}30$. For the C2 model, a run with $p = 0{\cdot}29$ was also performed. We have further assumed that the same sequence model applies to our β-glucan and to that used by Woodward *et al.* (1983), since they are of similar origin.

RESULTS AND DISCUSSION

Characterisation of barley β-glucan

The molecular weight of the β-glucan used here was found to be 250,000 as determined by DALLS (Fig. 1). Our substrate had a lower molecular weight than the high and medium molecular weight β-glucan preparations obtained from Megazyme Pty Ltd and also than some previously analysed commercial barley β-glucan preparations (Fig. 2 and unpublished results).

According to recent knowledge, GPC is the most suitable method for determination of molecular weights of β-glucans. The development of new analysis methods, such as post column Calcofluor detection (Wood *et al.*, 1991*b*; Suortti, 1993), viscosimetric detection (Vårum *et al.*, 1991) and laser light scattering (LALLS; Wood *et al.*, 1991*b*; Suortti, 1993) has enabled the determination of absolute molecular weights, with better selectivity and sensitivity. Wood *et al.* (1991*b*) reported β-glucan molecular weights as high as 2,660,000 (barley) and 3,140,000 (oat) from GPC experiments detected by Calcofluor staining. Reduction of the molecular weight during isolation and/or purification, as reported by several groups (Morgan, 1971; Wood *et al.*, 1989, 1991*b*; Vårum & Smidsrød, 1988;

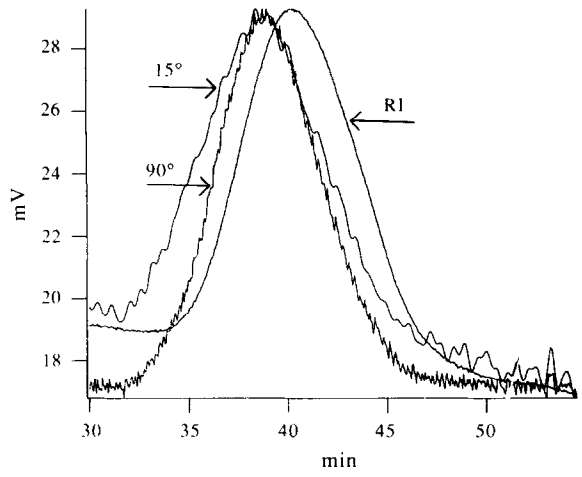

Fig. 1. Gel permeation chromatogram of the commercial barley β-glucan (Megazyme Pty Ltd, Australia) with refractive index (RI) and DALLS detection at angles of 15° and 90°.

Autio *et al.*, 1992), is the most likely explanation of the relatively low molecular weight obtained here for the commercial barley β-glucan.

In the proton NMR spectra of β-glucan and its hydrolysis products the resonances outside the crowded region between 3·2 and 4·1 ppm can be used as structural reporter signals. These include the resonances for all anomeric protons, as they have chemical shifts between 4·3 and 5·3 ppm. Quantitative data can also be obtained from this region by integration and their assignment is known previously (Usui *et al.*, 1975; Bock *et al.*, 1991). The anomeric proton resonances can be divided into three categories: (i) reducing end protons in equilibrium between the α- and β-anomers; (ii) β-(1→3)-linkage protons with two sets of resonances depending on whether the linkage is terminal (non-reducing end) or internal; and (iii) β-(1→4)-linkage protons with the same subdivision into terminal and internal (Fig. 3).

Our ^{1}H NMR data showed barley β-glucan to contain three different types of glucose units: 4G3, 3G4 and 4G4, while the amount of 3G3 was below the detection limit. The amount of (1→3) linkages was 29% and the amount of (1→4)-linkages 71% of the total (Fig. 3), in agreement with previous data (Dais & Perlin, 1982; Bock *et al.*, 1991).

Hydrolysis of barley β-glucan

Barley β-glucan was readily degraded by the cellulose preparation. No high molecular weight hydrolysis products could be detected after 2·5 h, i.e. the average molecular weight had decreased to below 10,000. The formation of low molecular weight products already after 5 h (Fig. 4) and the rapid increase in the amount of reducing sugars in the samples during the first 24 h of hydrolysis (Fig. 5) also indicate rapid depolymerisation of the barley β-glucan. After the first 24 h, the hydrolysis slowed down. As seen in Figs 4–6, no significant changes occurred between 48 and 72 h. The dominant hydrolysis end products were di-, tri- and tetrasaccharides. The average oligosaccharide length was 3·1 after 72 h of hydrolysis, calculated from the amount of reducing ends as determined by proton NMR. From resonance intensities in the NMR spectrum of the total hydrolysate (Fig. 3B), the amount of free glucose was found to be about 0·7% by weight.

The NMR analysis also showed that the CBHII preparation hydrolysed only (1→4)-linkages of barley β-glucan. The number of (1→4)-linkages decreased

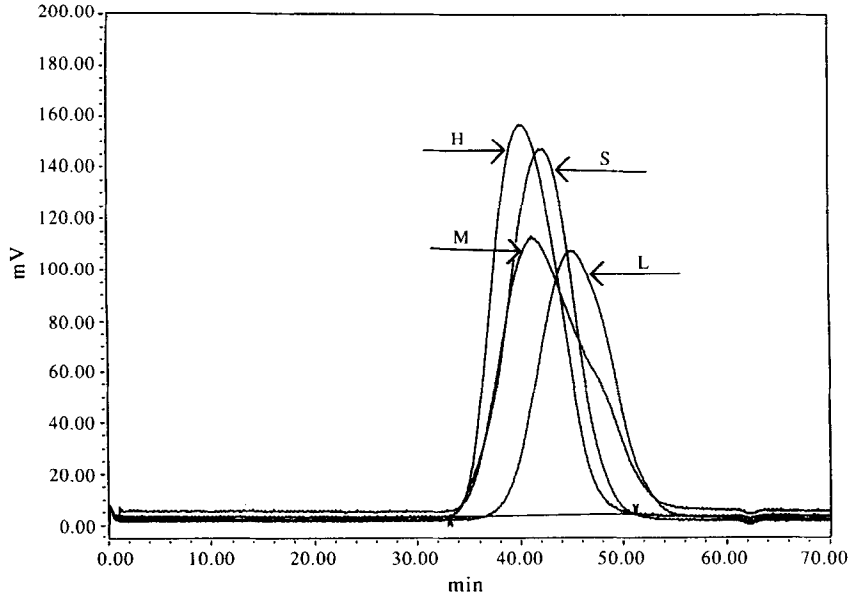

Fig. 2. Gel permeation chromatogram of the commercial barley β-glucan (S) and the high (H), medium (M) and low (L) molecular weight β-glucan preparations (Megazyme Pty Ltd, Australia) with fluorescence detection after post-column Calcofluor staining.

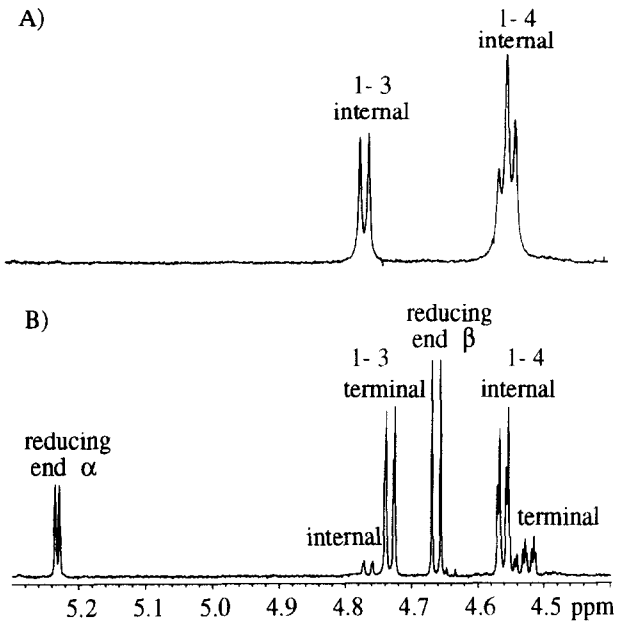

Fig. 3. The anomeric region of ^{1}H NMR spectra of 0·5% (w/v) solution of (A) barley β-glucan; (B) barley β-glucan hydrolysate. The spectra were obtained at 600 MHz and 70°C.

from the initial 71% (mol-% of total glucose moieties) to 40% in 72 h of hydrolysis, whereas the number of (1→3)-linkages was unchanged, 29% (Fig. 6A). Figure 6 also presents the relative amounts of internal and terminal (1→3)-linkages as functions of time.

The DNS method yielded higher reducing sugar concentrations in the hydrolysates than ^{1}H NMR analysis (Fig. 5). Partial hydrolysis of oligosaccharides due to alkali has earlier been reported to increase the number of free reducing groups during the incubation with colouring reagents (Miller, 1959; Bailey *et al.*, 1992). In consequence, DNS-based values cannot be used for the absolute degree of hydrolysis. Nevertheless, the DNS method displayed the course of hydrolysis rather well in a qualitative way, despite the alkaline hydrolysis possibly causing different oligosaccharides to yield different colour intensities. In what follows, no conclusion or discussion is based on DNS data alone.

Identification of hydrolysis end products

Fractionation of the barley β-glucan hydrolysis end products by reverse phase chromatography gave three major fractions (Fig. 7) containing di, tri- and tetrasaccharides. The disaccharide fraction also contains glucose. The presence of G_2, G_3 and G_4 was confirmed by analytical HPLC (data not shown). The structures of the end product oligosaccharides were assigned from chemical shifts (Bock *et al.*, 1991) and integrals in the one-dimensional ^{1}H NMR spectra. Oligosaccharide end products larger than G_4, although detectable by HPLC, could not be isolated in amounts sufficient for NMR analysis. The main hydrolysis products were: cellobiose, β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-D-Glc*p* (or G3G4G), β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Glc*p* (or G3G4G4G) and β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-D-Glc*p* (or G4G3G4). The ^{1}H NMR spectra of the three fractions are shown in Fig. 8, together with the structures of the identified saccharides. Bock *et al.* (1991) obtained the same hydrolysis products, except cellobiose, in the degradation of barley β-glucan with a fungal cellulolytic enzyme mixture, Finizyme.

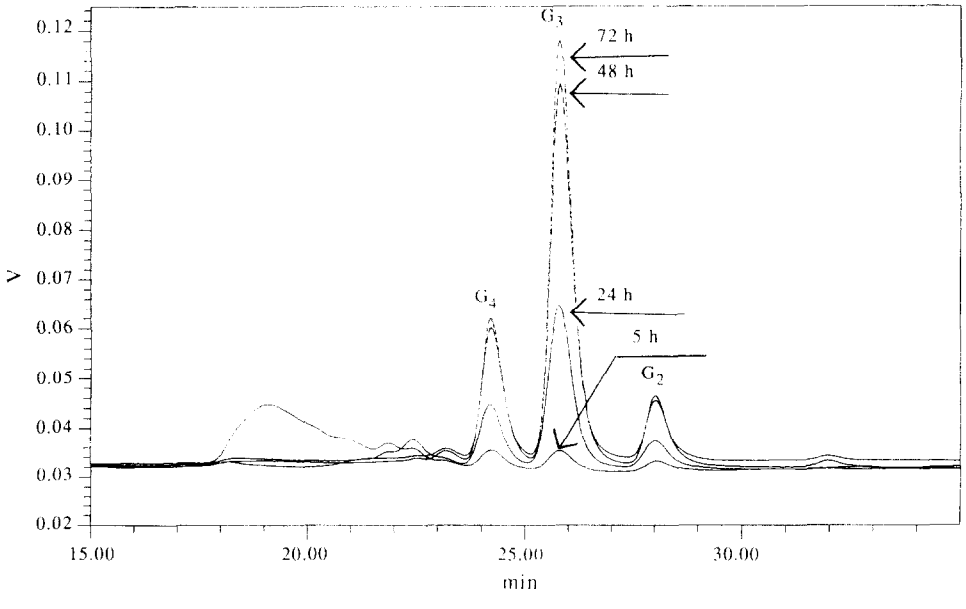

Fig. 4. HPLC chromatogram showing the formation of low molecular weight hydrolysis products from barley β-glucan by the cellobiohydrolase II preparation from *T. reesei* (15 mg CBHII/g, pH 5, 37°C). Refractive index detection.

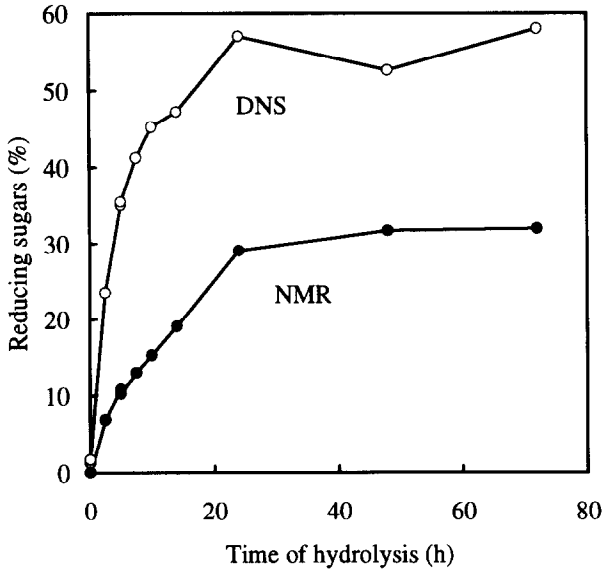

Fig. 5. Comparison of the DNS method and ^{1}H NMR analysis for the detection of reducing sugars in the hydrolysis of barley β-glucan using the cellobiohydrolase II preparation from *T. reesei* (15 mg CBHII/g, pH 5, 37°C).

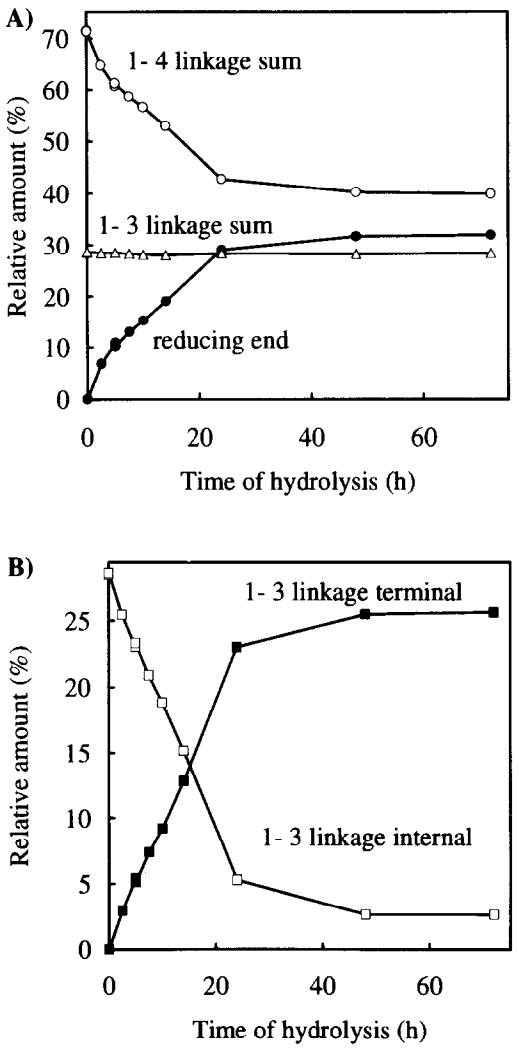

Fig. 6. Hydrolysis of barley β-glucan by the cellobiohydrolase II preparation of *T. reesei* (15 mg CBHII/g, pH 5, 37°C). (A) Amount of linkages and reducing ends. (B) Amounts of different (1→3)-linkages.

The total relative concentration of glucose was 0·7% by weight or about 2 mol-%. Also by weight, the disaccharide fraction contained 87% cellobiose, 6% glucose and 7% other small sugars (Fig. 8A). Comparison of the resonances for the reducing end β-anomers gives a molar cellobiose concentration that is 6·5 times that of glucose. As all glucose is eluted in the pooled cellobiose fraction, this gives a total relative concentration of cellobiose of 13%. These figures are quite uncertain as they are based on the glucose concentration.

Expressed with respect to the number of anomeric protons, the relative concentration of terminal (1→3)-linkages was 26% at the end, and thus 3% are internal. Only the G4G3G4G contributes to these 3%, as the concentration of longer oligosaccharides is very small. From Fig. 8C, [G3G4G4G] ≈ 1·5[G4G3G4G] ≈ 4%, with the consequence that 26% − 4% = 22% is the relative G3G4G concentration. Thus [G3G4G]:[G3G4G4g]:[G4G3G4G] is about 22:4:3. These amounts are consistent with the HPLC data (Figs 4 and 7). The relative concentrations are collected in Table 4 together with estimated uncertainties.

Already in 1965, Goldstein *et al.* demonstrated the existence of contiguous (1→3)-linkages. Bathgate and Palmer (1974) gave a quantitative assessment of such sequences at about 100 instances per 10^6 Da, i.e. once per 62 glucose units, while Fleming and Kawakami (1977) found them to be more common in β-D-glucans extracted at high temperature. We could not detect any β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-D-Glc*p* (G3G3G4G). The detection limit in the NMR experiment is about 1% of the G3G4G4G intensities, which gives a conditional probability for a G3G3G sequence of <0·01. The occurrence probability of such a sequence is in consequence 0·29 * 0·01 = 0·0029, i.e., at most once per 350 glucose units. Several other studies report no or rare contiguous (1→3)-linkages (Dais & Perlin, 1982; Woodward *et al.*, 1983; Vårum & Smidsrød, 1988; Bengtsson and Palmer, 1990; Edney *et al.*, 1991) and the discrepancy with the results of Bathgate and Palmer may be due to a differing β-glucan preparation.

Mechanism of degradation

The *Trichoderma* cellobiohydrolases are exo-acting enzymes, which degrade cellulose by releasing cellobiose from the polyglucose chain ends (Wood & Garcia-Campayo, 1990). Electron microscopy data show that *T. reesei* CBHII attacks cellulose microcrystals from the non-reducing end, which supports the claim that it is a true exo-enzyme (Chanzy & Henrissat, 1985) and,

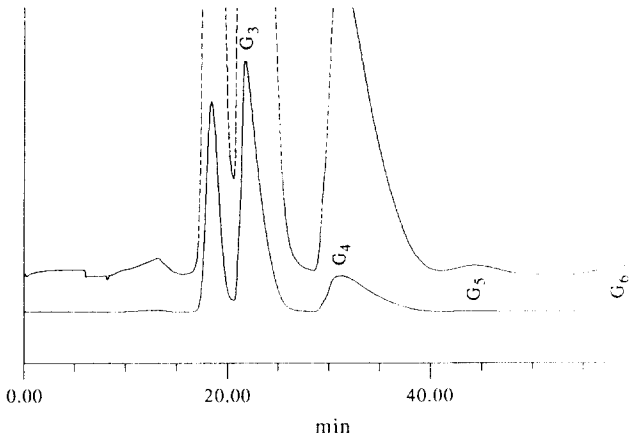

Fig. 7. A semipreparative fractionation of the barley β-glucan hydrolysis end products (15 mg CBHII/g, pH 5, 37 C, 72 h). The top curve is an expansion of the lower curve by a factor of 10. The peak preceding the cellotriose peak (G3) contains cellobiose and other low molecular weight compounds. Due to the semipreparative nature of the experiment, peak integrals are not totally linear in concentration.

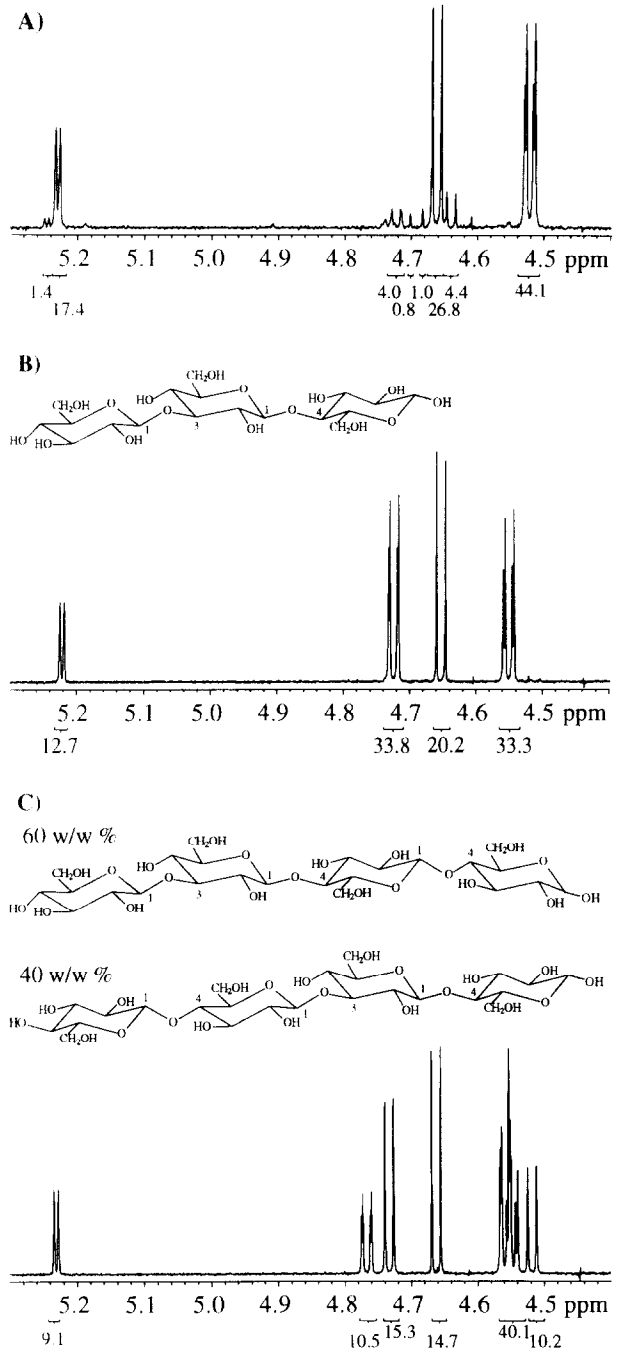

Fig. 8. The ^{1}H NMR spectra and the structures of the main end products in the hydrolysis of barley β-glucan by the cellobiohydrolase II preparation of *T. reesei* (15 mg CBHII/g, pH 5, 37 C, 72 h). The spectra of 6–10 mM solutions of (A) mono- and disaccharides; (B) trisaccharides; and (C) tetrasaccharides in D_2O were obtained at 600 MHz and 70 C.

therefore, causes only a slow change in the degree of polymerisation. Penttilä *et al.* (1988) used CBHII expressed in a heterologous host, *Saccharomyces cerevisiae*, that lacks endoglucanases to demonstrate that CBHII hydrolyses β-glucan.

The three-dimensional structures of CBHII (Rouvinen *et al.*, 1990) and CBHI (Divne *et al.*, 1994) have enclosed tunnel-like active sites. In CBHII the substrate is bound to at least four subsites (A, B, C and D), mostly by interactions with tryptophan sidechains. Cellulose threads into the active site tunnel from the D end, while cellobiose is released from the other end. The glycosidic linkage between subsites B and C is cleaved when the cellulose occupies all four subsites (Rouvinen *et al.*, 1990), which is also the reason why CBHII releases no glucose. In the active site tunnel, the saccharide is confined to a ribbon-like shape and each glycosidic linkage is situated on one of the two faces of the ribbon. We will refer to these orientations as 'up' and 'down'. Cleavage requires a certain geometry with respect to the catalytically important residues Tyr 169, Asp 175 and Asp 221, i.e. that the glycosidic linkage between subsites B and C is in the 'up' orientation. If not, the polymer eventually shifts one subsite further and is then cleaved, releasing an oligosaccharide that is one glucose residue longer. Ruohonen *et al.* (1993) demonstrated this for the cello-hexasaccharide, which CBHII degrades to a mixture of cellobiose and cellotriose.

If a (1→4)-linkage is 'up' then the following linkage is always 'down' and vice versa, so that in purely (1→4)-linked stretches, like cellulose, the 'up' and 'down' orientations alternate. This also explains why CBHII degradation of cellulose only produces cellobiose. A (1→3)-linkage in the 'up' orientation is, on the other hand, followed by an 'up' linkage and likewise for 'down' linkages. This is easily seen from the oligosaccharide structural formulae in Fig. 8.

No hydrolysis end product with a (1→3)-linkage adjacent to the reducing end was obtained and we conclude that two sequential (1→4)-linkages are necessary for hydrolysis. This can be formulated such that the minimum epitope for binding and hydrolysis is three

Table 4. Relative concentrations of oligosaccharides after completed β-glucan hydrolysis by CBHII

Compound	Conc. (mol-%)
Glucose	2 ± 1
Cellobiose	13 ± 10
G3G4G	64 ± 10
G4G3G4G	9 ± 3
G3G4G4G	12 ± 3
G_5	< 1
$G_{n>5}$	< 1
Non-glucose sugars	1

(1→4)-linked glucose residues. In this respect CBHII differs from lichenase, which cleaves a (1→4)-linkage that is preceded by a (1→3)-linkage.

The tetrasaccharide G3G4G4G was obtained in about equal amounts as G4G3G4G, so that it does not appear to be a substrate of CBHII. As 344 sequences are hydrolysed when internal in the polymer, it seems likely that the substrate is insufficiently anchored when subsite D is unoccupied. This tallies with the observation that subsite C is fairly spacious (Rouvinen *et al.*, 1990) and that cellotriose, G4G4G, is a poor substrate for CBHII (Ruohonen *et al.*, 1993). We obtained only small amounts of free glucose in the hydrolysate and, therefore, *a fortiori*, no glucose is cleaved off from the reducing end. We thus conclude that CBHII does not cleave reducing end sequences -G3G4G4G at the last (1→4)-linkage.

Polymer models

In 1983, Staudte *et al.* reported an attempt to model the sequence of β-glucan. They treated the polymer as a chain of (1→4)-linked tri- and tetraosyl moieties and relied on composition analysis at the 'penultimate' stage of hydrolysis. A more complete study was performed by Buliga *et al.* (1986), who treated each linkage individually and modelled the linkage sequence by a second order Markov chain. The coefficients were derived by fitting to the total amount of (1→3)-linkages and using the fact that consecutive (1→3)-linkages were very rare. With this knowledge, the sequence model is without parameters.

As described above, we have generated three sequence models in a similar way. Their Markov chain orders range from 1 to 5. The second order model was made parameterless by assuming that (1→3)-linkages are always separated by at least two (1→4)-linkages, as demonstrated by our NMR data. The numerical values of the coefficients are compared in Table 2b.

By means of a computer program, sequence stretches of ≈1 million glucose units were generated, corresponding to about 600 β-glucan molecules of the present DP. Sample stretches of the three models are given in Table 5.

Table 5. Sample polymer stretches for the C1, C2 and C5 models

Model
C1
34343443434343434444444344434443443443443443443443443434434434444434443
44444444343444434444434343443443443434344344344344344344344344344344
34343434443434344444434344443434443443444444434344434344444344443444
44444344434434443434344444443443443434344344344344344344344444444444
C2
34434434434434444434443443443443443443443443443443443443443444344443
44344344344444344344344344344344344344344344344344344344443444344344
34434434434444344344344344344344344344344344344344344344344344344434
44344434434434434434443443443443443443444434434434434434434434434434
C5
44344344343443444344443443443443443443443444344344344344344344434443
44344434434434434434443443443443443443443443443443443443443443443344
34443443444344344344344344344344344344344344344344344344344344344344
43444344434434434443443443443443443443443443443443443443443443443434

Here, we have used a very compact notation, where 3 means G3- and 4 G4-, and where the reducing end G is suppressed. Thus, 343 means G3G4G3G, 443 means G4G4G3G and 4 means cellobiose, G4G.

The polymer models were then degraded according to the mechanism of CBHII and lichenase. Because of the 'up' requirement in the CBHII mechanism, some of the initially produced oligosaccharides are substrates of CBHII. Repeated degradation of the obtained oligosaccharides resulted in a converged average DP in about eight cycles. The oligosaccharide distribution will contain an uncertainty of 1–2 percentage units that arise from non-glucose residues in the polymer.

The final oligosaccharide distributions are compared to experimental data in Table 6. The C1 model fails to reproduce the CBHII degradation data, see e.g. the DP values. It also fails for lichenase hydrolysis and we conclude that a first order Markov chain cannot describe the linkage sequence. The C1 model predicts a high portion of G3G, a compound which is not experimentally observed. Instead, Woodward *et al.* (1983) observed traces of cellobiose. The occurrence of cellobiose cannot be explained by existing knowledge of the lichenase mechanism and will be disregarded here.

The C2 model reproduces the oligosaccharide composition quite well for both enzymic processes. In the case of lichenase, the agreement is better with $p = 0{\cdot}29$, rather than with the nominal value of $p = 0{\cdot}30$. The C5 model accurately reproduces the lichenase hydrolysis data, since it was derived from these. It however also produces degradation data for the CBHII case in reasonable agreement with experimental data.

We now address the question of whether the C5 model can be reduced in order. The second order probabilities were evaluated from the computer run of the C5 model and compared with those of the C2 model in Table 7. The autocorrelation along the polymer was calculated (see Staudte *et al.*, 1983, p. 309 for a defini-

Table 6. Comparison of results from the degradation of model polymers with experimental data

	Degradation by CBHII			
	C1	C2	C5	Experiment
DP_l	4·76	4·76	4·80	
DP_x	4·08	3·23	3·24	3·1
[glucose]	0	0	0	2 ± 1
[G4G]	30	7	6	13 ± 10
[G3G4G]	24	69	69	64 ± 10
[G4G3G4G]	5	10	10	9 ± 3
[G3G4G4G]	5	10	10	12 ± 3
[G_5]	14	5	5	< 1

	Degradation by lichenase				
	C1	C2 (0·29)	C2 (0·30)	C5	Experiment
DP	3·45	3·45	3·33	3·44	3·47
[glucose]	0	0	0	0	0
[G4G]	0	0	0	0	trace
[G3G]	41	0	0	0	0
[G4G3G]	24	69	75	70	70
[G4G4G3G]	14	21	19	24	24
[G4G4G4G3G]	9	7	5	3	3
[G4G4G4G4G3G]	5	2	1	2	1

DP_l is the average degree of polymerisation when the polymer has threaded through the enzyme once, DP_x that when CBHII hydrolysis has reached completion. All concentrations are given in mol-%. The experimental data are our CBHII data, and taken from Woodward *et al.* (1983) in the case of lichenase.

Table 7. Comparison of the coefficients for C2 ($p = 0·29$) and C5 models

Preceding linkages	Present linkage	Model C2 ($p = 0·29$)	Model C5
(1→3)G(1→3)	x[a]	0	0
(1→3)G(1→4)	(1→3)	0	0
(1→3)G(1→4)	(1→4)	1	1
(1→4)G(1→3)	(1→3)	0	0
(1→4)G(1→3)	(1→4)	1	1
(1→4)G(1→4)	(1→3)	0·690	0·693
(1→4)G(1→4)	(1→4)	0·310	0·307

[a]Either (1→3)G(1→4).

tion), and shows that sequence 'memory' falls off over some 10–20 glucose units (Fig. 9). The behaviour of the C2 and C5 models is identical. This and the coefficients in Table 7 show that the C5 model is indeed equivalent to the C2 model, i.e. all composition data of Woodward *et al.* (1983, Table 2) are reproduced by a second order Markov chain. As this model also reproduces our data, it is probably a general property of β-glucan that the linkage sequence does not depend on preceding linkages further away than two glucose moieties. That there is significant autocorrelation even after seven glucose

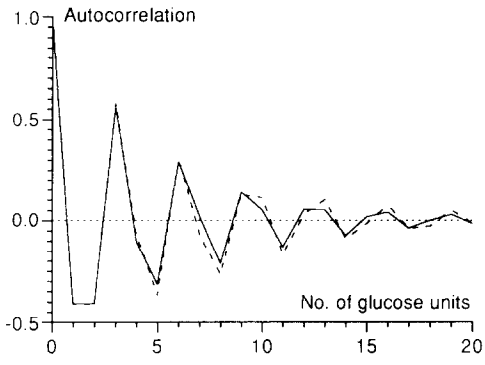

Fig. 9. The correlation between glycosidic linkages as a function of separation along the polymer for the C2 ($p = 0·29$) model (solid line) and the C5 model (dashed line).

units indicates that the second order Markov chain is a more accurate sequence model than a Bernoullian (random) polymer consisting of cellotriosyl and cellotetraosyl moieties (Buliga *et al.*, 1986).

The C2 model produces an accurate hydrolysate composition for both enzymic processes, and we can conclude that the hydrolysis mechanisms arrived at for the two enzymes are good models for the actual processes. The work presented here combines well characterised enzymes as hydrolysis tools with statistical methods for the interpretation of results. This approach enabled more far-reaching conclusions on the structure of β-glucan, but also added to the knowledge on cellobiohydrolase II. We expect that similar approaches can be also applied to other biopolymers.

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