Stereochemical course and structure of the products of the enzymic action of endo-1,3-1,4- β -D-glucan 4-glucanohydrolase from *Bacillus licheniformis*

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The stereochemical course of the reaction catalysed by endo-1,3-1,4- β -D-glucan 4-glucanohydrolase (EC 3.2.1.73) has been determined by ^1H n.m.r. The enzyme-catalysed hydrolysis of barley β -glucan proceeds with overall retention of the anomeric configuration, indicating that the enzyme operates through a double-

displacement mechanism. The structures of the final oligosaccharide products, 3- β -O-cellobiosyl D-glucopyranoside and 3- β -O-cellobiosyl D-glucopyranoside, have been completely assigned by ^1H - and ^{13}C -n.m.r. spectroscopy.

INTRODUCTION

Glycosidases catalyse the stereospecific hydrolysis of glycosidic bonds in oligo- and poly-saccharides proceeding either with retention or inversion of configuration at the anomeric centre. It is generally accepted that the mechanism for glycosidases acting upon their natural substrates involves general acid catalysis (Sinnott, 1990), a carboxy group (Asp or Glu) being implicated in most of the known cases [i.e. HEW-lysozyme (Malcolm et al., 1989), T4-lysozyme (Anand et al., 1988), glucoamylase (Sierks et al., 1990)] or a tyrosine residue [β -galactosidase (Ring et al., 1988; Ring and Huber, 1990)], but not a histidine residue. Inverting enzymes are believed to operate through a single-displacement mechanism by a water molecule whose nucleophilicity is enhanced by an enzyme general base, as first suggested by Koshland (1953). For enzymes yielding net retention of the anomeric configuration, a double-displacement mechanism of some kind is fairly well established. Whether it proceeds through a covalent glycosyl-enzyme adduct or the intermediate exists as an oxocarbenium ion-paired to an enzyme carboxylate(s) is subject to current controversy (Sinnott, 1990; Hardy and Poteete, 1991).

Cereal β -glucans containing mixed β -1,3 and β -1,4 glucosidic linkages are hydrolysed by endo-1,3-1,4- β -D-glucan 4-glucanohydrolase (β -1,3-1,4-glucanase; EC 3.2.1.73). Because of its importance in carbohydrate technology (Godfrey, 1983; Dierick, 1989), this enzyme is being studied at the molecular level to establish the details of its enzymic function. Microbial β -1,3-1,4-glucanases are the best characterized, and a number of genes from different *Bacillus* species have been cloned and sequenced (Cantwell and McConnell, 1983; Hofemeister et al., 1986; Borris et al., 1988; Bueno et al., 1990; Gosálbes et al., 1991; Lloberas et al., 1991; Louw et al., 1993). The specificity of the enzyme and deduced minimum binding requirements have been inferred from the structure of the products of complete enzymic hydrolysis of barley and oat β -glucans (Parrish et al., 1960; Moscatelli et al., 1961; Anderson and Stone, 1975), and from the kinetics of irreversible inhibition by active-site-directed inhibitors bearing

an epoxide function (epoxyalkyl oligoglucosides) able to react with enzyme nucleophiles (Høj et al., 1989).

Progress in the identification of active-site residues has been recently reported. Site-directed mutagenesis in our group on the *B. licheniformis* β -1,3-1,4-glucanase (Planas et al., 1992a) has shown that the substitution of Glu-134 with Gln yields an enzyme with nearly abolished activity, suggesting its essential role in catalysis. Consistent with these results, Høj et al. (1992) have reported, for the very sequence-similar *B. amyloliquefaciens* enzyme, that Glu-105 (the equivalent residue to Glu-134 in *B. licheniformis*) is covalently modified by epoxide-based inhibitors, suggesting that this residue is the likely catalytic nucleophile. Further support comes from the recently determined three-dimensional structure of a hybrid β -1,3-1,4-glucanase from *B. amyloliquefaciens* and *B. macerans* by X-ray crystallography at a resolution of 0.2 nm (2.0 Å) for the free enzyme and at 0.28 nm (2.8 Å) for a covalent protein-inhibitor complex with 3,4-epoxybutyl β -D-cellobioside (Keitel et al., 1993). The inhibitor is covalently bound to Glu-105, demonstrating again the central importance of this residue in enzyme catalysis.

The enzymic hydrolysis of barley β -glucan yields 3- β -O-cellobiosyl D-glucopyranose (1) and 3- β -O-cellobiosyl D-glucopyranose (2), indicating that the enzyme cleaves β -1,4 glycosidic bonds adjacent to β -1,3 linkages. The structures of these final products were deduced by chemical methods (Parrish et al., 1960) and by enzymic degradation with cellulases (Lloberas et al., 1988), but no spectral data have been reported so far. Further mechanistic studies require knowledge of the stereospecificity of the catalytic hydrolysis as inverting or retaining to distinguish between possible mechanisms and assign the functional role of specific essential active-site residues.

In this work we have determined the stereochemical course of the hydrolysis of barley β -glucan by monitoring the reaction by ^1H -n.m.r. spectroscopy. We show that recombinant *B. licheniformis* β -1,3-1,4-glucanase acts with retention of the anomeric configuration, and the structures of the tri- and tetra-saccharide products are confirmed by assignment of their ^1H - and ^{13}C -n.m.r. data.

Abbreviations used: β -1,3-1,4-glucanase, endo-1,3-1,4- β -D-glucan 4-glucanohydrolase (EC 3.2.1.73); Dp, degree of polymerization; PNPC, *p*-nitrophenyl β -D-cellobioside; 2D, two-dimensional; TOCSY, total correlation spectroscopy; ROESY, rotating-frame nuclear-Overhauser-enhancement spectroscopy; DQF-COSY, double-quantum-filtered correlated spectroscopy; HMBC, inverse heteronuclear multiple bond correlated spectroscopy; HMQC, inverse heteronuclear multiple quantum correlated spectroscopy.

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MATERIALS AND METHODS

Enzyme

β -1,3-1,4-Glucanase was obtained from recombinant *Escherichia coli* HB101 harbouring a *B. licheniformis* β -glucanase expression plasmid as reported by Planas et al. (1992a,b). Enzyme concentration in all runs was determined by u.v. spectrophotometry [ϵ_{280} 0.0145 cm⁻¹·ml· μ g⁻¹ (Lloberas et al., 1988)].

Stereochemical analysis

¹H-n.m.r. spectra were recorded on a Varian Unity 500 spectrometer. The enzyme and barley β -glucan (Sigma) were freeze-dried three times from 99.8% ²H₂O prior to n.m.r. monitoring of enzymic hydrolysis. Experiments were performed at 28 °C in unbuffered ²H₂O, using a final polysaccharide concentration of 6.4 mg/ml and 1 μ g/ml of enzyme. Spectra were recorded at regular intervals during the first hour, and after 5, 10, 24, 48 and 72 h. The residual ¹H²O signal was minimized using inversion-recovery (time between pulses 4.5 s). Each experiment was run with four scans, using an acquisition time of 4 s per scan and a time delay between every train of pulses of 3 s. Integration of the resonance signals in the anomeric region was performed after baseline correction with a third-order polynomial function.

Reducing-sugar assays

The net release of reducing sugar equivalents during the enzymic hydrolysis of barley β -glucan under the conditions of the ¹H-n.m.r. experiments was monitored by the Somogyi–Nelson method (Somogyi, 1952; Nelson, 1944). Reducing power was expressed as equivalent glucose concentration.

H.p.l.c. monitoring of enzymic hydrolysis

Qualitative oligosaccharide distribution in the degree-of-polymerization (Dp) range 3–10 during the enzymic process and quantitative composition of final tri- and tetra-saccharide was monitored by h.p.l.c. Samples of 200 μ l from a solution of barley β -glucan and enzyme in 99.8% ²H₂O under the same conditions as those described in the ¹H-n.m.r. experiment were taken at regular time intervals, and the reaction was quenched by immersing it in an ice/salt bath and adding 10 μ l of 2% sulphosalicylic acid. H.p.l.c. analysis was performed using an analytical 300 mm $\times$ 7.8 mm Aminex HPX-42A column (Bio-Rad) (water as eluent at 0.6 ml/min; 85 °C; refractive-index detector).

Isolation of oligosaccharides

A solution of 300 mg of barley β -glucan in 37.5 ml of 87.2 mM phosphate/6.5 mM citrate buffer, pH 7.2, was incubated at 55 °C overnight with *B. licheniformis* β -1,3-1,4-glucanase (7.8 μ g). The resulting solution was evaporated to dryness under reduced pressure and extracted ten times with 20 ml of hot methanol. Evaporation of the solvent yielded 370 mg of crude material that was purified by flash chromatography [50 g of silica gel (Macherey–Nagel Kieselgel 60); 0.040–0.063 mm particle size; methanol/water/ethyl acetate, 8:5:20, by vol]. Two main fractions of 131 mg of tri-saccharide **1** and 70 mg of tetrasaccharide **2** were obtained. Both products proved to be chromatographically homogeneous by t.l.c. (Merck precoated silica-gel 60 F₂₅₄ plates; methanol/water/ethyl acetate, 4:3:8, by vol.) and by h.p.l.c.

Structure assignment of tri- and tetra-saccharides

N.m.r. spectra were recorded at 30 and 43 °C on a Varian Unity 500 spectrometer. Proton chemical shifts were referenced to residual ¹H²O at a chemical shift value (δ) of 4.71 or 4.68 p.p.m. Carbon chemical shifts were referenced to external dioxan at 67.4 p.p.m.

The double-quantum-filtered correlation-spectroscopy (DQF-COSY) experiment was performed in the phase-sensitive mode using the method of States et al. (1982). A data matrix of 256 $\times$ 2K points was used to digitize a spectral width of 1500 Hz; 16 scans were used per increment, with a relaxation delay of 2 s. The 90° pulsewidth was 7.5 μ s. Prior to Fourier transformation, zero-filling was used in F₁ to expand the data to 2K $\times$ 2K. The clean two-dimensional (2D) total-correlation-spectroscopy (TOCSY) experiment (Griesinger et al., 1988) was carried out in the phase-sensitive mode using MLEV-17 (Malcolm Levitt sequence) to isotopic mixing. The mixing time was set to 100 ms. A data matrix of 256 $\times$ 1K points was used to digitize a spectra width of 1500 Hz; 16 scans were used per increment with a relaxation delay of 2 s. The 90° pulsewidth during the mixing period was 22.5 μ s. Squared-cosine-bell functions were applied in both dimensions, and zero-filling was used to expand the data to 2K $\times$ 2K. The 2D rotating-frame nuclear-Overhauser-enhancement spectroscopy (Bothner-By et al., 1984) (ROESY) experiment was recorded in the phase-sensitive mode. The spin-lock period consisted of a train of 30° pulses (2.5 μ s), separated by delays of 50 μ s (Kessler et al., 1987). The total mixing time was set to 300 ms. The radio-frequency carrier was set at δ 6.0 p.p.m., downfield from the most deshielded anomeric proton to minimize spurious Hartmann–Hahn effects (Bax and Davis, 1985). A data matrix of 256 $\times$ 2K points was used to resolve a spectral width of 2600 Hz. 16 scans were used per increment with a relaxation delay of 2 s. Squared sine bell functions shifted by $\pi/3$ were applied in both dimensions and zero-filling was used in F₁ to expand the data to 2K $\times$ 2K. The pure absorption one-bond proton–carbon correlation experiment was collected in the ¹H-detection mode using the inverse-heteronuclear-multiple-quantum-correlated-spectroscopy (HMQC) pulse sequence (Bax and Subramanian, 1986) and a reverse probe. A data matrix of 256 $\times$ 2K points was used to resolve a spectral width of 1500 Hz; 16 scans were used per increment with a relaxation delay of 2 s and a delay corresponding to a *J* value of 145 Hz. A binuclear-rotation-decoupling ('BIRD') pulse was used to minimize the proton signals bonded to ¹³C. ¹³C-decoupling was achieved by the WALTZ decoupling scheme. Square-cosine-bell functions were applied in both dimensions, and zero-filling was used in F₁ to expand the data to 2K $\times$ 2K. The multiple-bond proton–carbon correlation experiment was carried out in the ¹H-detection mode using the inverse-heteronuclear-multiple-bond-correlated-spectroscopy (HMBC) pulse sequence (Bax and Summers, 1986) and a reverse probe. A data matrix of 256 $\times$ 2K points was used to resolve a spectral width of 1500 Hz; 64 scans were used per increment with a relaxation delay of 2 s and a delay of 60 ms. The processing was performed in the absolute mode after zero-filling in F₁ to a 2K $\times$ 2K data matrix.

RESULTS

Enzymic hydrolysis

β -1,3-1,4-Glucanase from *B. licheniformis* shows a maximum activity at pH 7.0 and 55 °C (Lloberas et al., 1988; Planas et al., 1992a). Preliminary experiments had shown that the enzyme is active in distilled water, so that the n.m.r. spectra were recorded in unbuffered ²H₂O. Isotopic effects, if present, have not been

Figure 1 ^1H -n.m.r. spectra of the anomeric region for the β -1,3-1,4-glucanase-catalysed hydrolysis of barley β -glucan at different times

The reaction took place in $^2\text{H}_2\text{O}$ at 28°C ; [substrate] = 6.4 mg/ml ; [enzyme] = $1\text{ }\mu\text{g/ml}$. (a) Barley β -glucan before addition of enzyme. (b)–(e) After enzyme addition at the indicated time. (f) Spectrum of the anomerically equilibrated reaction mixture after 72 h. Signals: 5.23 p.p.m. ($J 3\text{ Hz}$), H1_β (αOH); 4.67 p.p.m. ($J 8\text{ Hz}$), H1_α (βOH); 4.54 p.p.m. , H1 in $\beta(1\rightarrow4)$ glycosidic bonds.

taken into account. The temperature of the experiment was set at 28°C (40% of maximum enzyme activity) to avoid overlapping of the residual $^1\text{H}^2\text{HO}$ resonance and the anomeric signals. Unambiguous determination of the anomeric configuration of the reaction products was obtained by selecting an enzyme/substrate ratio such as to obtain a hydrolysis rate higher than that of mutarotation.

Figure 1(a) shows the partial ^1H -n.m.r. spectrum of the initial polymer in the $4.4\text{--}5.3\text{ p.p.m.}$ region. Spectra recorded at 4, 8, 18 and 40 min after the addition of *B. licheniformis* β -1,3-1,4-glucanase are shown in Figures 1(b)–1(e). During the initial minutes of incubation, the broad signal centred at 4.54 p.p.m. , assigned to the anomeric protons in $\beta(1\rightarrow4)$ glycosidic linkages, suffers rapid modifications. Simultaneously, a doublet at 4.67 p.p.m. ($J 8\text{ Hz}$) with increasing intensity is observed. It is characteristic of the anomeric signal of the β -configuration for the newly formed reducing ends. A small doublet at 5.23 p.p.m. ($J 3\text{ Hz}$) starts to be observable only after 18 min of reaction and corresponds to the anomeric α -signal arising from mutarotation of the initially formed β -oligosaccharides. This clearly demonstrates that enzymic hydrolysis occurs with retention of configuration. As a result of the endo activity of the enzyme, the

Figure 2 Time course of the enzyme-catalysed hydrolysis of barley β -glucan

Experimental conditions were as in Figure 1. (a) Changes in the integrals of the ^1H -n.m.r. anomeric signals. β -Anomer and α -anomer correspond to the anomeric protons of the newly formed reducing ends. $\beta(1,4)$ corresponds to anomeric protons in $\beta(1\rightarrow4)$ glycosidic linkages. (b) Total reducing power as determined by the Somogyi–Nelson method ($\circ$) and integral of the ^1H -n.m.r. anomeric signals ($\alpha + \beta$) ($\bullet$); (b) inset: h.p.l.c. profile of the reaction mixture after 18 min in the Dp range of 3–10. (c) H.p.l.c. monitoring of the tri- and tetra-saccharides **1** and **2** expressed as molar fractions calculated from integration of peak areas in h.p.l.c. records.

initial doublets at 4.67 and 5.23 p.p.m. are broad signals arising from a complex oligosaccharide mixture. An estimation of the oligosaccharide distribution in the Dp range 3–10 after 18 min, when mutarotation becomes observable, is shown in the inset of Figure 2(b). The final product composition is a 2.2:1 mixture of the tri- and tetra-saccharides **1** and **2**, respectively. The ^1H -n.m.r. spectrum of the final reaction under mutarotational equilibrium (72 h) is shown in Figure 1(f).

Figure 2 compares the enzymic reaction as monitored by ^1H -n.m.r. integration of the anomeric signals, net reducing-sugar formation and tri- and tetra-saccharide molar formation, measured by h.p.l.c. As expected, the total reducing power and the $\alpha + \beta$ integrals in ^1H -n.m.r. follow the same time course (Figure 2b).

Figure 3 (a) 2D TOCSY and (b) 2D ROESY experiments for compound 1

Relevant cross-peaks have been labelled.

Table 1 ^1H -n.m.r. chemical shifts (δ , p.p.m.) for compounds 1 and 2 in $^2\text{H}_2\text{O}$ solution at 40 °C

Unit		H-1	H-2	H-3	H-4	H-5	H-6A	H-6B
Trisaccharide 1								
β -D-Glcp	C	4.50	3.31	3.50	3.41	3.49	3.91	3.73
4-O- β -D-Glcp	B	4.75	3.40	3.67	3.66	3.63	3.98	3.81
3-O- β -D-Glcp	A_α	5.23	3.72	3.90	3.52	3.87	3.89	3.76
	A_β	4.66	3.43	3.75	3.52	3.50	3.91	3.73
Tetrasaccharide 2								
β -D-Glcp	D	4.51	3.32	3.52	3.43	3.51	3.93	3.75
4-O- β -D-Glcp	C	4.54	3.40	3.70	3.70	3.67	4.02	3.86
4-O- β -D-Glcp	B	4.76	3.41	3.69	3.69	3.65	4.01	3.85
3-O- β -D-Glcp	A_α	5.24	3.74	3.92	3.54	3.89	3.88	3.78
	A_β	4.67	3.46	3.76	3.53	3.52	3.92	3.75

Structure of the tri- and tetra-saccharides 1 and 2

The high-resolution ^1H -n.m.r. spectrum and proton-decoupled ^{13}C -n.m.r. spectra of compound 1 in $^2\text{H}_2\text{O}$ solution showed four anomeric signals, two of them corresponding to the α : β equi-

librium of the reducing end, indicating that 1 is a trisaccharide. Resolution at 40 °C was adequate for DQF-COSY and TOCSY experiments. Coherence transfer to all the protons was achieved by isotropic mixing using 2D-TOCSY with a mixing time of 100 ms (Figure 3a). The ^1H -n.m.r. chemical shifts are listed in Table 1. The residues were labelled A_α , A_β , B and C. The TOCSY subspectra through the anomeric signals showed clear connectivities to the rest of the protons. The assignment of each cross-peak was based also on the information obtained from the DQF-COSY experiment. The chemical-shift and coupling-constant values for the anomeric protons of residues B and C were indicative of β -configurations. Coupling constants were estimated from the DQF-COSY cross peaks and found to be in agreement with the presence of glucopyranose rings in all cases ($\Delta\delta = 0.014$ p.p.m.) due to the α : β equilibrium for residue A, indicating its neighbourhood to the reducing end. Some information on the glycosyl sequence was obtained from the 2D ROESY experiment (Figure 3b). Thus cross-peaks between the anomeric proton of the residue C and the region of H-3, H-4 and H-5 of residue B could be observed, along with other connecting H-1 of residue B with H-3 of unit A. Since nuclear-Overhauser-enhancement cross-peaks are dependent on the conformation around the glycosidic bonds (Neuhaus and Williamson, 1989), the existence of these cross-peaks does not guarantee a knowledge of the exact position of the linkage, although at this stage it can be assumed that β -Glcp-C is (1 $\rightarrow$ 4)- or (1 $\rightarrow$ 3)-linked to Glcp-B, which is connected via a (1 $\rightarrow$ 3) linkage to Glcp-A.

It was possible to discriminate among these alternatives and to ascertain all the configurations of the residues by using an HMQC experiment (Figure 4), which maps the connectivities between carbon atoms and their directly bonded protons. Carbon chemical-shift values are given in Table 2. The chemical shifts of the anomeric carbons indicate unequivocally (Bock et al., 1984, 1982) that monosaccharides B and C are β -Glcp residues. On the other hand, the observed deshielding of C-3 of residue A allows to assign it as a 3-O-substituted β -glucopyranoside unit, while the measured ^{13}C -n.m.r. C-4 chemical shift indicates unequivocally that residue B is a 4-O-substituted β -glucopyranoside unit (Bock et al., 1984, 1982, 1991).

The primary sequence was confirmed by using an HMBC experiment, which shows long-range connectivities between carbon atoms and their coupled protons through two or three bonds. By using this technique, coupling across the glycosidic linkages can be unequivocally detected. The above-discussed connectivities and substitutions were confirmed and permitted one to propose the following structure for trisaccharide 1:

The ^1H -n.m.r. and ^{13}C -n.m.r. spectra of the anomeric region of compound 2 maintained the signals for residues A, B and C with the same pattern and shifts observed for 1 and showed one additional resonance at 4.51 p.p.m., assigned as belonging to residue D. Therefore, compound 2 is a tetrasaccharide. The assignment of the resonances was based on the protocol described above through DQF-COSY, TOCSY, ROESY, HMQC and HMBC experiments. The ^1H -n.m.r. and ^{13}C -n.m.r. chemical-shift values are given in Tables 1 and 2 respectively, and agree with a tetrasaccharide structure that possesses an additional β -glucopyranose moiety 4-O-linked to residue C. Thus tetrasaccharide 2 has the following sequence:

Related structures are formed by enzymic degradation of barley β -glucan with an *Aspergillus niger* β -glucanase (Bock et al., 1991). In that case, one trisaccharide and two tetrasaccharides

Figure 4 HMQC experiment for compound 1 to map the connectivities between carbon atoms and their directly bonded protons

Table 2 ^{13}C -n.m.r. chemical shifts (δ , p.p.m.) for compounds 1 and 2 in $^2\text{H}_2\text{O}$ solution at 40 °C

Unit		C-1	C-2	C-3	C-4	C-5	C-6
Trisaccharide 1							
β -D-Glcp	C	103.4	74.1	76.5	70.4	76.9	61.6
4-O- β -D-Glcp	B	103.5	74.2	75.1	79.6	75.7	62.0
3-O- β -D-Glcp	A _α	92.9	71.9	83.3	69.0	72.2	61.6
	A _β	96.6	74.7	85.6	69.1	76.5	61.7
Tetrasaccharide 2							
β -D-Glcp	D	103.3	74.0	76.3	70.2	76.7	61.4
4-O- β -D-Glcp	C	103.1	73.6	75.5	79.2	76.2	60.7
4-O- β -D-Glcp	B	103.4	74.3	75.5	79.2	76.2	60.7
3-O- β -D-Glcp	A _α	92.7	71.7	83.0	68.8	71.9	61.3
	A _β	96.4	74.8	85.3	68.8	76.7	61.4

were obtained along with a hexasaccharide. However, the isolated trisaccharide was found to have the opposite alternation of β -(1 $\rightarrow$ 3) and β -(1 $\rightarrow$ 4) linkages: β -Glcp-(1 $\rightarrow$ 3)- β -Glcp-(1 $\rightarrow$ 4)-Glcp-, while the tetrasaccharides had also different primary sequences and were identified as:

and

DISCUSSION

The stereochemical course of a number of glycoside-catalysed reactions has been successfully determined by high-resolution ^1H -n.m.r. monitoring. The reported examples on β -glucanases have hitherto involved the use of small substrates and, therefore, the technique is limited to those enzymes for which short

oligosaccharides or derivatives are hydrolysed with rates higher than that of mutarotation. Withers et al. (1986) first described the use of *p*-nitrophenyl β -D-cellobioside (PNPC) as substrate for ^1H -n.m.r. monitoring of a cellobiohydrolase from the cellulase complex of *Cellulomonas fimi*. β -Cellobiosyl fluoride has proved to be a reactive substrate for cellobiohydrolases, and it has been used to establish the stereochemical courses of enzymes from *Trichoderma reesei* (Knowles et al., 1988) and *T. emersonii* (Brooks et al., 1992), showing that cellobiohydrolases I (CBHI) are retaining glycosidases, whereas cellobiohydrolases II (CBHII) are inverting. For endoglucanases with no exo activity, low-molecular-mass oligosaccharide derivatives are often poor substrates, but ^1H n.m.r. has, nonetheless, established the stereochemical course of some of these enzymes. Withers et al. (1986) were able to show inversion of configuration for an endo- β -1,4-glucanase from *C. fimi* with cellotetraose as substrate, while Barras et al. (1992) showed retention for endoglucanase Z from *Erwinia chrysanthemi* on cellotriitol. Also by ^1H -n.m.r. spectroscopy it was determined (Claeyssens et al., 1990) that only β -1,4 bonds were newly synthesized, owing to the transferase activity of endoglucanase I from *T. reesei*, characterizing the enzyme as a retaining glycosidase.

Here we have been able to extend the technique to the enzyme-catalysed hydrolysis of a natural polysaccharide. The relatively high solubility of barley β -glucan in water (up to 10 mg/ml) and the high rate of hydrolysis with *B. licheniformis* endo-1,3-1,4- β -D-glucan 4-glucanohydrolase enable conditions to be found under which the reaction is slow enough to be monitored by ^1H -n.m.r. spectroscopy but faster than mutarotation of the newly formed reducing ends. The enzymic hydrolysis proceeds with overall retention of the anomeric configuration. This is consistent with a double-displacement mechanism and the following conclusions may be put forward. (i) Glu-134 in the *B. licheniformis* enzyme or the equivalent Glu-105 in the *B. amyloliquefaciens* β -1,3-1,4-glucanase, proposed as the likely nucleophile (Høj et al.,

1992), may play the role of the oxocarbenium-stabilizing residue or the true nucleophile (if the reaction on natural substrates proceeds through a covalent intermediate), but not the role of a general base (the latter limited to inverting glycosidases). (ii) As the enzyme is a retaining endoglucanase, transglycosidation might be expected (Sinnott, 1990). This side reaction has not yet been observed, since only studies with large polymeric substrates have so far been performed. The use of low-molecular-mass oligosaccharide substrates will facilitate the detection of transglycosidation products.

The assignment of the ^1H - and ^{13}C -n.m.r. data of the tri- and tetra-saccharide products **1** and **2** confirms the structures previously elucidated by chemical degradation (Parrish et al., 1960). Other β -glucanases of different substrate specificity are also able to depolymerize barley β -glucan. In fact, Bock et al. (1991) have determined the cleavage specificity of the active component of a commercial preparation from *A. niger* (Finizyme; Novo-Nordisk). On the basis of the structures of the final oligosaccharide products assigned by n.m.r. spectroscopy (see the Results section), it was concluded that the enzyme had cellulase (EC 3.2.1.4) specificity, hydrolysing β -(1 $\rightarrow$ 4) linkages, except in structures where the reducing end unit is 3-substituted. Hydrolysis of the latter type of linkage is only observed with the β -1,3-1,4-glucanase as described here, demonstrating that the *A. niger* enzyme is distinct from that of the *Bacillus* species.

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