ENZYMATIC MODIFICATION AND QUANTIFICATION OF POLYMERS BASED ON A (1→4)-β-D-GLUCAN BACKBONE

B.V. McCLEARY

Biological and Chemical Research Institute, N.S.W. Department of Agriculture, Rydalmere, Australia

SYNOPSIS

In this paper, examples of the use of enzymes in the modification, quantification and investigation of fine-structural details of mixed-linkage (1→3)(1→4)-β-D-glucans, xyloglucans, glucomannans and xanthan are presented and discussed.

INTRODUCTION

There occur in Nature a wide range of polysaccharides based on a (1→4)-β-D-glucan backbone. The simplest of these is the homoglycan, cellulose. In xyloglucans (amyloids) the β-D-glucan main chain is irregularly substituted at C-6 by α-D-xylosyl residues or by short-chain oligosaccharides containing α-D-xylosyl, β-D-galactosyl, α-L-fucosyl and α-L-arabinosyl residues. Xanthan, the extracellular polysaccharide produced by Xanthomonas sp., also consists of a β-D-glucan main chain substituted by short-chain branch units. However, in this polysaccharide the pattern of distribution of branch units is essentially regular, which in turn is a consequence of the specific mechanism of biosynthesis. In glucomannan, which is a (1→4)-linked β-D-glycan, a majority of the D-glucosyl residues are replaced by D-mannose; and in cereal cell-wall β-D-glucans and in lichenan there is irregular replacement of some of the (1→3)-3-linked-D-glucosyl residues by (1→3)-β-linked residues.

Enzymes have found considerable use in the characterization of these polysaccharides and the general approach has been to depolymerise the polymer with one or more specific endo-glycanases followed by characterisation of fragments by chemical procedures, by nuclear magnetic resonance or by the use of specific glycosidases. All of the (1→4)-β-D-glucan type poly-
Saccharides are susceptible to hydrolysis by *endo-(1→4)*-β-D-glucanase (cellulase), but to vastly differing degrees as shown in table 1. In this table, the relative action of highly purified cellulases on a range of β-D-glucans is shown.

**Table 1:**

Hydrolysis of β-Glucans by Cellulases

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>Relative Rates of Hydrolysis by Cellulases</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM-Cellulose 4M</td>
<td>CM on 13% of -OH groups</td>
<td>T. reesei I 100  P. emersonii I 100</td>
</tr>
<tr>
<td>CM-Cellulose 7M</td>
<td>CM on 23% of -OH groups</td>
<td>P. emersonii II 100</td>
</tr>
<tr>
<td>Barley β-Glucan</td>
<td>G-G-G</td>
<td>A. niger I 100</td>
</tr>
<tr>
<td>Annona Xyloglucan</td>
<td>X (low substitution)</td>
<td></td>
</tr>
<tr>
<td>Tamarind Xyloglucan</td>
<td>-G-G-G-G-G-G</td>
<td></td>
</tr>
<tr>
<td>Hydrocellulose</td>
<td>Insoluble cello-saccharides</td>
<td></td>
</tr>
<tr>
<td>Carob Galactomannan</td>
<td>Gal Gal</td>
<td></td>
</tr>
<tr>
<td>(for β-mannanase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>contamination)</td>
<td>G=M-M-M-M-M-M-M-M</td>
<td></td>
</tr>
</tbody>
</table>

*Endo*-depolymerases other than cellulases have been of use in the characterisation of some of the polymers mentioned. Thus the (1→3)(1→4)*-β-D-glucan present in cereal cell-walls and in Icelandic moss (namely lichenan) is also susceptible to depolymerisation by specific and non-specific *endo-(1→3)* (1→4)*-β-D-glucanases. The non-specific enzyme also acts on (1→3)*-β-D-glucan. The specific *endo-(1→3)(1→4)*-β-D-glucanase has also been termed lichenase. Both of these enzymes cleave the (1→4)*-β-linkage of a 3-linked Q-
lycosyl residue, whereas cellulase acts on these glucans at the sites shown in Fig. 1. β-Ω-Glucomannans, which contain Ω-glucose and Ω-mannose in the approximate ratio of 1:3 to 2:3, are susceptible to hydrolysis by both cellulase and β-Ω-mannanase. Each of these enzymes has specific sub-site binding requirements in the vicinity of the active site and these requirements must be fulfilled for hydrolysis to occur.

\[
\text{CELLULASE} \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad
Barley β-glucans form highly viscous solutions and/or gelatinous precipitates and in the brewing industry they can cause problems in wort and beer filtration and can also lead to haze or gel formation in the packaged beer. This problem can be avoided by using well-modified malts or by the addition of commercial enzymes to the mash-tun or the beer. The problems associated with β-D-glucans occur mainly in high-gravity beers and in beers where unmalted barley is the major adjunct. One of the criteria applied for the selection of barley for malting and for use as an adjunct is the level of (1→3)(1→4)-β-D-glucan, thus one of the aims of most barley breeding programmes is the selection of varieties low in this component. Such work has been hindered by the unavailability of a reliable and rapid method for the assay of this glucan. Consequently, the specific aims of my current research programme are to develop a rapid method for the determination of total (1→3)(1→4)-β-D-glucan in barley flour and to define the difference between the soluble and insoluble components.

1.2. Measurement of (1→3)(1→4)-β-D-glucan levels

Measurement of barley β-glucan can involve the extraction and purification of this component followed by quantification using a specific or non-specific procedure, or alternatively, assay in situ. The latter format is complicated by the fact that barley flour contains a range of glucans, glucose containing saccharides and some D-glucose. However, the analysis can be approached using highly purified enzymes. The theoretical basis of the procedure we have developed using lichenase and β-D-glucosidase is shown in Fig. 2. The flour sample is suspended in buffer and "cooked" in a steam bath to hydrate the β-D-glucan and to inactivate endogenous enzymes. The slurry is then treated with lichenase which depolymerises the barley β-glucan and after adjustment to a set volume, aliquots are treated with β-D-glucosidase and the released D-glucose measured with glucose oxidase/peroxidase reagent. The patterns of amounts of oligosaccharides produced on hydrolysis of pure barley β-glucan by lichenase (Bacillus subtilis) or cellulase (Aspergillus niger) are shown in Fig. 3. Also shown is the effect of treatment of these mixtures with Aspergillus niger β-D-glucosidase. For this assay to be specific and reliable it is vital that the lichenase and β-D-glucosidase are essentially devoid of contaminating activities which could release D-glucose from starch, cellulose, sucrose or maltosaccharides. Most importantly, the lichenase must be devoid of β-D-glucosidase. The enzymes employed in the current studies have been purified to electrophoretic homogeneity and are essentially devoid of interfering activities.
Enzymic Modification and Quantification of Polymers

\[ 3\text{G} \xrightarrow{\text{Lichenase}} \text{Barley } \beta\text{-glucan} \]
\[ \text{G} \xrightarrow{\text{Lichenase}} \text{β-Gluco-oligosaccharides } (n>1) \]
\[ \text{β-Glucosidase} \xrightarrow{\text{GLUCOSE}} \]

Fig. 2. The theoretical basis of the assay procedure for \((1\rightarrow3)(1\rightarrow4)\beta\)-D-glucan.

Fig. 3. Chromatograms on Bio-Gel P-2 of the oligosaccharides produced on hydrolysis of barley β-glucan with either lichenase (a) or cellulase (c) for 24 h at 40°C. The pattern obtained on treatment of (a) with β-glucosidase is shown in (b), and on treatment of (c) with β-glucosidase, is shown in (d).
The procedure described allows the analysis of up to 50 samples per day by a single operator, and it is currently used in the barley breeding programme of the N.S.W. Department of Agriculture. The method has also been adapted for the measurement of barley β-glucan in malt, wort and beer and is currently being evaluated in several breweries and malt houses.

1.3. Soluble and insoluble (1→3)(1→4)-β-D-glucan

As previously mentioned, the difference between "soluble" and "insoluble" barley β-D-glucan is considered to be due either to the binding of the latter to protein or to varying patterns of amounts of (1→3)- and (1→4)-β-linked D-glucosyl residues in the glucans. To investigate this further, barley β-glucan has been exhaustively extracted from the flours of several barley varieties with water at 40°, 65° and 90°, and then with sodium hydroxide (10% w/v) plus sodium borohydride at room temperature. These barley β-glucan subfractions were then purified by a series of washing, filtration and centrifugation steps and using a highly purified temperature-stable α-amylase to remove starch. For the variety, Parwan, the percentage of total barley β-glucan extracted at 40°, 65° and 90° was 17, 63 and 18% respectively. The amount of this glucan extracted with sodium hydroxide plus sodium borohydride, conditions under which peptide linkages to protein are likely to be split, represented less than 2% of the total extracted (this material was highly contaminated with arabinoxylan), and the amount left in the pellet was insignificant, being less than 1% of the total. The protein content of the 40°, 65° and 90°-soluble fractions were similar, being 0.62, 0.52 and 0.58%, respectively.

Comparative structural features of the barley β-glucan fractions were studied employing highly purified lichenase and cellulases from Trichoderma reesei and Aspergillus niger. The patterns of amounts of oligosaccharides produced on treatment of the 65°-soluble fraction with these enzymes are shown in fig. 4. Very similar patterns were obtained on treatment of the 40° and 90°-soluble fractions with the respective enzyme, indicating that the differentiation between "soluble" and "insoluble" fractions is not due to fine-structural differences in these polymer fractions, even though such differences have previously been reported to occur.

Why then is there a clear differentiation between the barley β-glucan fractions which extract at the three temperatures employed? The results shown in fig. 5 demonstrate that, in fact, it would appear to be due simply to differences in molecular size. The 40°-soluble fraction of Parwan barley β-glucan has an intrinsic viscosity (limiting viscosity number) of only
Fig. 4. Chromatograms on Bio-Gel P-2 of the oligosaccharides produced on hydrolysis of the 65°-soluble fraction of Parwan (1+3)(1+4)-β-Ω-glucan by lichenase (a); and by Trichoderma reesi (b); and Aspergillus niger cellulases (c).

2.5 dL/g, whereas that soluble at 65° has a value of 3.8 dL/g, and that soluble at 90° has an even higher value (5.6 dL/g). Furthermore, the much steeper gradient of the nsp/c versus concentration curve for the 90° soluble material indicates that polymer molecules in this fraction display a high degree of molecular interaction and that this is highly concentration dependent. This contrasts with the viscosity curve for the 45° soluble fraction. The difference in the self association/interaction of these fractions is clearly evident when the purified polymers are dissolved. After wetting with ethanol, the 40°-soluble fraction can be readily dissolved in hot water (70°), whereas dissolution of the 65°- and the 90°-soluble fractions requires hydration over extended periods, followed by vigorous homogenization in hot water. Once dissolved, the polymers tend to remain in solution.

It has been suggested that the solubilisation of "insoluble" barley β-glucan is effected by a specific peptidase termed "β-glucan solubilase" which cleaves specific peptide linkages between barley β-glucan and protein. However, highly active, non-specific proteases were unable to catalyse this reaction. We have confirmed this latter finding by demonstrating that treatment with papain, protease or proteinase K did not induce solubilisation of the 65°- and 90°-soluble fractions, at 40°. However, after extraction of
the $40^\circ$ soluble fraction, the barley $\beta$-glucan remaining in the residue can be solubilised by treatment of the residue with trace amounts of cellulase. Cellulase enzymes, such as those from Trichoderma and Aspergillus spp. are very heat stable. These results lead us to conclude that the reported "$\beta$-glucan solubilise", rather than being a peptidase could also be a cellulase. Cellulase activity has been found in barley flour extracts and most, if not all, of this is derived from fungi, such as Aspergillus fumigatus, which grow in the outer seed coat and husk.$^{12}$

![Graph showing specific viscosity versus concentration for the $40^\circ$, $65^\circ$, and $90^\circ$-soluble fractions of barley $\beta$-glucan (Farwan variety).]
2. XYLOGLUCANS (AMYLOIDS)

Enzymes have been extensively employed in the characterisation of xyloglucans from such diverse sources as the seed of *Tamarindus indica*, *Annona muricata*, soybean and jojoba, the midribs of leaves of *Nicotiana tabacum*, the cell-walls of oat-coleoptile tissue, rice endosperm and immature barley and from suspension-cultured sycamore and soybean cells\(^1,2\). Enzymes employed have included cellulase, \(\beta-D\)-glucosidase, \(\alpha-L\)-fucosidase and \(\beta-D\)-galactosidase. Other enzymes such as \(\alpha-L\)-arabinofuranosidase and \(\alpha-D\)-xylosidase could also be potentially useful. The action of these enzymes on xyloglucan are summarized in fig. 6. Generally, on depolymerisation by cellulase, the fragments are separated by gel-permeation chromatography and then characterised using n.m.r., chemical and enzymic procedures.

![Reaction diagram](image)

Fig. 6. Sites of action of cellulase and glycosidases on xyloglucan.

3. GLUCOMANNANS

The glucomannans of major commercial significance are those from *Amorphophallus konjac* (konjac) and *Orchis* spp. (salep). These contain \(D\)-glucose and \(D\)-mannose in the ratios of 2:3 and 1:3, respectively, and solubility of the polysaccharide is due to partial acetylation of some of the sugar residues. Treatment of the polymers with alkali results in deacetylation and subsequent precipitation from solution on neutralisation and dialysis. The polymers are rapidly depolymerised by \(\beta-D\)-mannanase, but cleavage by
cellulase proceeds at a much reduced rate. Some cellulases have no action on glucomannan (table 1) and this is undoubtedly due to the fact that the specific sub-site binding requirements around the active site are not being fulfilled. Some information on the fine-structures of glucomannans can be obtained by characterising the fragments produced on cleavage by $\beta$-$D$-mannanase or cellulase, but the significance of the results is limited by the high degree of transglycosylation catalyzed by these enzymes with the reaction products\textsuperscript{13}. Evidence that the $D$-glucosyl units in salep glucomannan occur essentially as isolated residues, has been obtained by characterising the fragments produced on cleavage by $A. niger$ and guar-seed $\beta$-$D$-mannanases. Information on the patterns of amounts of contiguous $D$-mannosyl residues could potentially be obtained using a highly purified cellulase devoid of $\beta$-$D$-mannanase, but such experiments have not as yet been performed.

Knowing the ratio of $D$-glucose to $D$-mannose in a particular glucomannan, the amount of this polymer in plant tissues could potentially be assayed by depolymerising the polymer with $\beta$-$D$-mannanase followed by hydrolysis of oligosaccharides to $D$-glucose and $D$-mannose using a mixture of $\beta$-$D$-glucosidase and $D$-$D$-mannosidase and subsequent measurement of $D$-glucose using glucose oxidase/peroxidase reagent.

4. XANTHAN

Xanthan, the extracellular polysaccharide produced by \textit{Xanthomonas campestris} and related bacterial species, is essentially a substituted (1→4)-$\beta$-$D$-glucan to which is attached a trisaccharide side chain on each second $D$-glucosyl residue. $D$-acetyl and pyruvic acetals are also present. Xanthan in the native form exists in a highly ordered conformation and is resistant to enzymic hydrolysis. However, after removal of excess ions it can be dissolved in water to give the unordered conformation, and in this form it is susceptible to random degradation by fungal cellulases at 40-50°C\textsuperscript{14,15}. The action of various cellulase preparations on solutions of commercial xanthans and those from laboratory strains, containing both pyruvate and acetate, pyruvate only or acetate only, in the unordered state, has been studied\textsuperscript{15}. The pattern of amounts of fragments obtained on gel-permeation chromatography depended on the substrate and the enzyme preparation used, indicating that such an approach may prove useful in comparing fine-structural details of various xanthan preparations. Treatment of xanthan with various cellulase preparations such as that from \textit{Trichoderma viride} (Novo Industri), followed
by gel-permeation chromatography, gave as reaction products, large fragments, two major oligosaccharides and products of lower molecular weight (identified as β-glucose, cellobiose and β-mannose). The two oligosaccharides were composed of β-glucose, β-mannose and β-glucuronic acid in the expected molar ratios of 2:2:1. Treatment of the non-pyruvylated penta- and deca-saccharide fractions from the hydrolyzate of strain 1128 xanthan with β-β-mannosidase, released β-mannose and a tetrasaccharide or an octasaccharide, in which the ratio of β-glucose:β-mannose:β-glucuronic acid was 2:1:1, consistent with the non-reducing terminal location of the β-linked β-mannosyl group.

ACKNOWLEDGEMENTS

The author thanks Mrs E. Nurthen and Mrs H. Taylor for competent technical assistance and Mrs C. Edge and Mr. M. Hill for assistance in the preparation of this manuscript. This research was funded by a grant from the Australian Barley Industry Research Council.

REFERENCES


