ENZYMIC 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 bio-synthesis. 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→4)-β-linked-D -glucosyl residues by (1→3)-β-linked residues.

Enzymes have found considerable use in the characterization of these polysaccharides[1,2] 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-$\underline{D}$-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-$\underline{D}$-glucans is shown.

Table 1:

Hydrolysis of β-Glucans by Cellulases

Substrate	Structure	Relative Rates of Hydrolysis by Cellulases				
		T.reesei		*P.emersonii*		*A.niger*
		I	II	I	II	I
CM-Cellulose 4M	CM on 13% of -OH groups	100	100	100	100	100
CM-Cellulose 7M	CM on 23% of -OH groups	82	100	32	100	29
Barley β-Glucan	↓ G-G-G ↓ G-G-G ↓	49	53	1	50	250
Annona Xyloglucan	X (low substitution) ↓ -G-G-G-G-G- ↑ ↑	73	76	250	10	8
Tamarind Xyloglucan	X X (high substitution)	100	92	0	0.7	0
Hydrocellulose	insoluble cello-saccharides	43	42	0	8	0.4
Konjac Glucomannan	-G-M-M-M-G-M-G-M- (Glc:Man = 40:60)	10	7	0	2	0
Carob Galactomannan (for β-mannanase contamination)	Gal Gal ↓ ↓ -M-M-M-M-M-M- ↑ Gal	0.3	0.2	0	1	0

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-$\underline{D}$-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-$\underline{D}$-glucanases[3]. The non-specific enzyme also acts on (1→3)-β-$\underline{D}$-glucan. The specific *endo*-(1→3)(1→4)-β-$\underline{D}$-glucanase has also been termed lichenase. Both of these enzymes cleave the (1→4)-β-linkage of a 3-linked $\underline{D}$-

lycosyl residue, whereas cellulase acts on these glucans at the sites shown

n fig. 1. β-$\underline{D}$-Glucomannans, which contain $\underline{D}$-glucose and $\underline{D}$-mannose in the

pproximate ratio of 1:3 to 2:3, are susceptible to hydrolysis by both

ellulase and β-$\underline{D}$-mannanase. Each of these enzymes has specific sub-site

inding requirements in the vicinity of the active site and these requirements

ust be fulfilled for hydrolysis to occur.
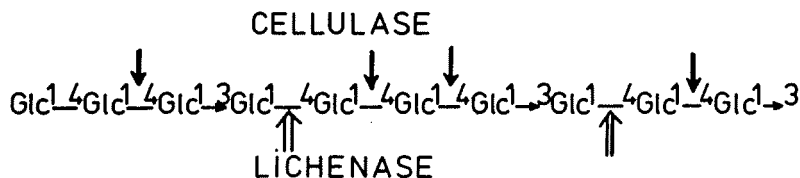

Fig. 1. Sites of action of cellulase and lichenase on (1→3)(1→4)-β-$\underline{D}$-glucans

In this paper I will discuss aspects of my work and the work of others

n the use of enzymes in the quantification, modification and structural

haracterisation of polymers based on a (1→4)-β-$\underline{D}$-glucan backbone with

pecial emphasis on my current work on the (1→3)(1→4)-β-$\underline{D}$-glucans of barley

ndosperm cell-walls.

.   (1→3)(1→4)-β-$\underline{D}$-GLUCANS

.1.   Introduction

(1→3)(1→4)-β-$\underline{D}$-Glucan (barley β-glucan) represents approximately 75% of

he carbohydrate in barley endosperm cell-walls[4]. Although it represents only

.8-5.5% of the total weight of the barley grain it is functionally significant

n the malting and brewing processes. Barley β-glucan is composed of both

soluble" and "insoluble" components[5] and it is considered that it is the

insoluble" component which causes most problems in the brewing industry[6],

venthough the reasons for solubility or insolubility have not been clearly

efined. It has been proposed by some researchers that insolubility may be

ue to binding of the β-$\underline{D}$-glucan through peptide linkages to protein[5], whereas

thers consider it is due to structural variation in the glucan itself i.e.

arley β-glucan subfractions with a high proportion of regions of sequential

1→4)-β-linked $\underline{D}$-glucosyl residues could be expected to have a more

ellulose-like nature and thus be less soluble. Cellosaccharides of degree of

olymerisation greater than 7 are insoluble or sparingly soluble.

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[8]. 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-$\underline{D}$-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 $(1\rightarrow3)(1\rightarrow4)$-β-$\underline{D}$-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 $(1\rightarrow3)(1\rightarrow4)$-β-$\underline{D}$-glucan in barley flour and to define the difference between the soluble and insoluble components.

1.2.   Measurement of $(1\rightarrow3)(1\rightarrow4)$-β-$\underline{D}$-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[8], 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 $\underline{D}$-glucose. However, the analysis can be approached using highly purified enzymes. The theoretical basis of the procedure we have developed using lichenase and β-$\underline{D}$-glucosidase is shown in fig. 2^9. The flour sample is suspended in buffer and "cooked" in a steam bath to hydrate the β-$\underline{D}$-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-$\underline{D}$-glucosidase and the released $\underline{D}$-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-$\underline{D}$-glucosidase. For this assay to be specific and reliable it is vital that the lichenase and β-$\underline{D}$-glucosidase are essentially devoid of contaminating activities which could release $\underline{D}$-glucose from starch, cellulose, sucrose or maltosaccharides. Most importantly, the lichenase must be devoid of β-$\underline{D}$-glucosidase. The enzymes employed in the current studies have been purified to electrophoretic homogeneity and are essentially devoid of interfering activities.
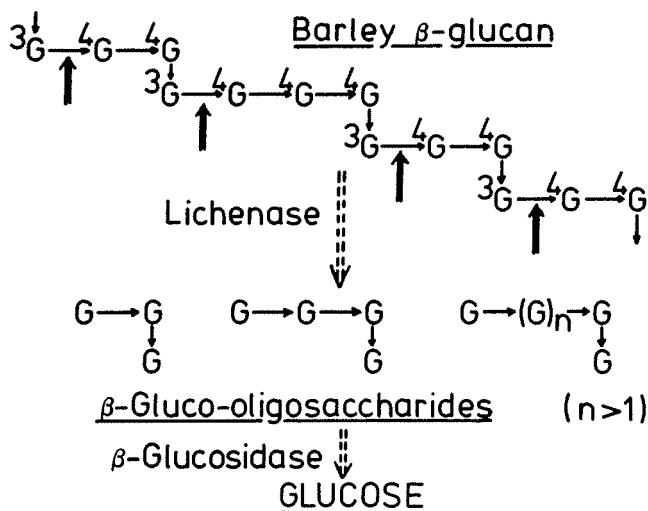
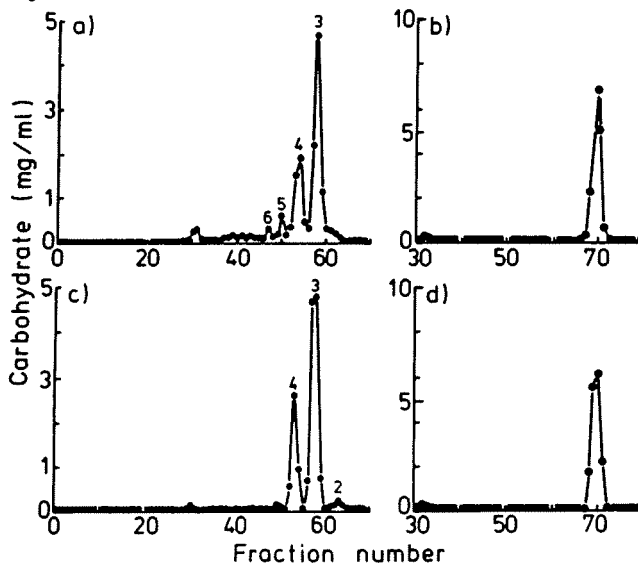Fig. 2. The theoretical basis of the assay procedure for $(1\to3)(1\to4)-\beta-\underline{D}-$ glucan.

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°. 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[6] or to varying patterns of amounts of (1→3)- and (1→4)-β-linked D-glucosyl residues in the glucans[7]. To investigate this further, barley β-glucan has been exhaustively extracted from the flours of several barley varieties with water at 40^0, 65^0 and 90^0, 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 40^0, 65^0 and 90^0 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 40^0, 65^0 and 90^0-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 65^0-soluble fraction with these enzymes are shown in fig. 4. Very similar patterns were obtained on treatment of the 40^0 and 90^0-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, eventhough such differences have previously been reported to occur[10].

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 40^0-soluble fraction of Parwan barley β-glucan has an intrinsic viscosity (limiting viscosity number) of only

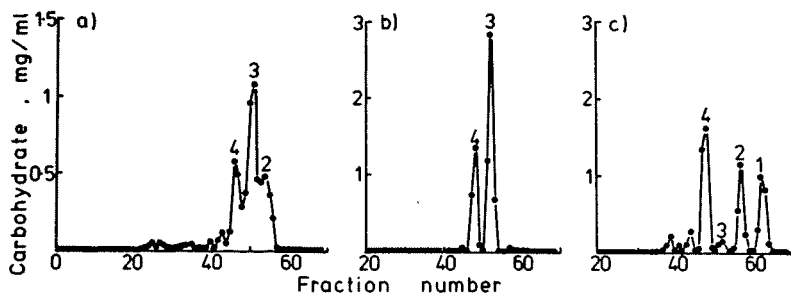Fig. 4. Chromatograms on Bio-Gel P-2 of the oligosaccharides produced on
hydrolysis of the 65°-soluble fraction of Parwan $(1\rightarrow3)(1\rightarrow4)$-β-$\underline{D}$-
glucan by lichenase (a); and by *Trichoderma reesi* (b); and
Aspergillus niger cellulases (c).

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 ηsp/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[11].
However, highly active, non-specific proteases were unable to catalyse this
reaction[11]. 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 40⁰ soluble fraction, the barley β-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 "β-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].
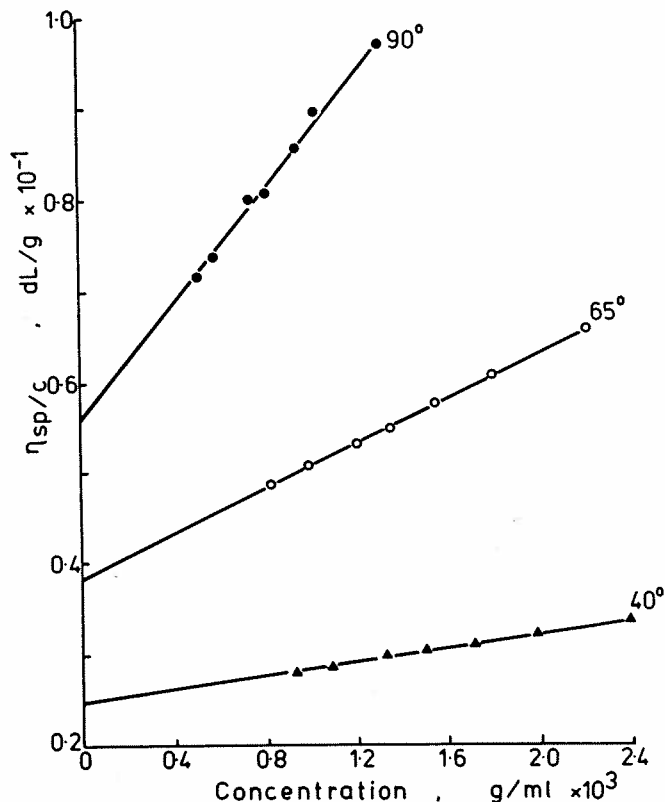

Fig. 5.   Specific viscosity versus concentration curves for the 40⁰, 65⁰ and 90⁰-soluble fractions of barley β-glucan (Parwan variety).

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, β-D-glucosidase, α-L-fucosidase and β-D-galactosidase. Other enzymes such as α-L-arabinofuranosidase and α-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.
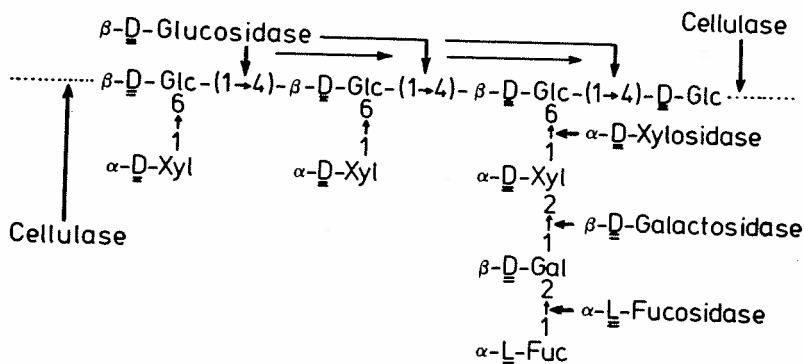

Fig. 6. Sites of action of cellulase and glycosidases on xyloglucan.

3. GLUCOMANNANS

The glucomannans of major commercial significance are those from *morphophallus konjac* (konjac) and *Orchis* spp. (salep). These contain D-glucose and D-mannose in the ratios of 2:3 and 1:3, respectively, and olubility of the polysaccharide is due to partial acetylation of some of the ugar residues. Treatment of the polymers with alkali results in deacetylation nd subsequent precipitation from solution on neutralisation and dialysis. he polymers are rapidly depolymerised by β-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 β-$\underline{D}$-
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[13]. Evidence that the $\underline{D}$-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 β-$\underline{D}$-mannanases.
Information on the patterns of amounts of contiguous $\underline{D}$-mannosyl residues
could potentially be obtained using a highly purified cellulase devoid of
β-$\underline{D}$-mannanase, but such experiments have not as yet been performed.

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

4.   XANTHAN

Xanthan, the extracellular polysaccharide produced by *Xanthomonas
campestris* and related bacterial species, is essentially a substituted (1→4)-
β-$\underline{D}$-glucan to which is attached a trisaccharide side chain on each second $\underline{D}$-
glucosyl residue. $\underline{O}$-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[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[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 *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 D-glucose, cellobiose and D-mannose). The two oligosaccharides were composed of D-glucose, D-mannose and D-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-D-mannosidase, released D-mannose and a tetrasaccharide or an octasaccharide, in which the ratio of D-glucose:D-mannose:D-glucuronic acid was 2:1:1, consistent with the non-reducing terminal location of the β-linked D-mannosyl group.

ACKNOWLEDGEMENTS

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