A NEW SUBSTRATE FOR INVESTIGATING THE SPECIFICITY OF β-GLUCAN HYDROLASES

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1. Introduction

The fine distinctions between the substrate specificities of the β-glucan endo-hydrolases [1] can only be defined precisely by the use of substrates with known, and preferably regular, structures. A new 1,3; 1,4-β-glucan with these properties has been prepared and its use in studies on the specificity of β-glucan endo-hydrolases is described.

The SIII pneumococcal polysaccharide from which the new glucan is denved is composed of a repeating sequence of cellobiuronic acid residues joined through 1,3-β-linkages [2], so that reduction of each uronic acid residue yields a neutral β-glucan with alternating 1,3- and 1,4-linkages. The carboxyl groups on the SIII polysaccharide can be readily reduced via a carbodi-imide derivative using sodium borohydride [3].

The new glucan has been used to confirm and extend the specificity descriptions of enzymes previously described by Parrish, Perlin and Reese [4]. These authors have shown that the *Rhizopus arrhizus* β-glucan endo-hydrolase (E.C. 3.2.1.6) depolymerizes both laminarin, a 1,3-β-glucan and the 1,3; 1,4-β-glucans, lichenin and oat glucan. On the other hand, the Streptomyces β-glucan endo-hydrolase (E.C. 3.2.1.4) was shown to hydrolyse 1,4-β-glucans in addition to lichenin and oat glucan.

Identification of the oligosaccharides produced by these enzymes led to the conclusion [4] that the *Rhizopus* hydrolase cleaves the glycosidic linkage x in the sequence -G3G↓xG-, when x is either a 1,3-linkage or a 1,4-linkage. The *Streptomyces* enzyme was shown to hydrolyse the glycosidic linkage adjacent to a 1,4-linkage as in the sequence -G4G↓xG- which occurs in its substrates cellulose, lichenin and the barley and oat glucans. It appears likely from considerations of the proportions of 1,3- and 1,4-linkages in the mixed-linked substrates and in the hydrolysis products, that for the *Streptomyces* enzyme, x can only be a 1,4-linkage. However, unequivocal proof was not provided by these experiments. An *Aspergillus niger* β-glucan hydrolase [5] and an enzyme from *Trichoderma viride* [6] appear to have the same specificity as the *Streptomyces* enzyme.

In this paper the new β-glucan has been used to provide additional information concerning the substrate specificity of the *Rhizopus* and *Streptomyces* enzymes and also of the β-glucan hydrolase from *Bacillus subtilis* (E.C. 3.2.1.73) [7,8].

2. Materials and methods

The polysaccharide substrates were obtained from the following sources: lichenin ex *Cetraria islandica* and laminarin ex *Laminaria hyperborea,* Koch-Light Laboratories, Colnbrook, Bucks., England. Carboxymethylcellulose, Imperial Chemical Industries, Stevenston, Ayrshire, Scotland. The carboxymethylpachyman was prepared by the method of Stone [9]. The carboxymethylpachyman and carboxymethylcellulose were dispersed in alkali before use [10]. The lichenin was purified before use by dissolving in boiling water, freezing and allowing to thaw. The precipitate was collected, washed with water and dried by solvent exchange.

Pneumococcal SIII polysaccharide was obtained through the courtesy of Dr J. Humphrey, National Institute for Medical Research, Mill Hill, London. The reduced pneumococcal polysaccharide (RSIII) was prepared by the carbodi-imide method of Taylor and Conrad [3] as follows: Pneumococcal SIII polysaccha-

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ride (34 mg; 100 μeq of carboxyl groups) was dissolved in 10 ml of water and solid 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulphonate (0.42 g, 1 mmol) was added after the pH had been adjusted to 4.75 with 0.1 M HCl. During the reaction the pH was maintained at 4.75 by titration with 0.1 M HCl using a pH stat. When hydrogen ion uptake had ceased, a sodium borohydride solution (2 M, 25 ml) was added slowly to the reaction mixture. The pH was allowed to rise to 7 and was maintained at that value by titration with 4 M HCl in a pH stat. The reduction was allowed to proceed for 60 min. The reaction mixture was then dialyzed overnight against 5M NaCl and subsequently against distilled water. The reduced polysaccharide was recovered by freeze drying.

The product showed negligible carboxyl absorption in the infrared spectrum. On methylation [11] followed by hydrolysis, reduction and acethylation, the only products apparent in g.l.c. analysis were 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and these were present in equal amounts. These results indicate that the reduction of the uronic acid residues was virtually complete and that the glucan product contained equal numbers of 1,3- and 1,4-linked residues.

The *Rhizopus arrhizus* QM 6789, and the *Streptomyces* QM B814 enzymes were preparations supplied by Dr E. T. Reese, Pioneering Research Laboratory, U.S. Army Natick Laboratories, Natick, Mass. USA. The *Bacillus subtilis* preparation was obtained from Novo Industri A/S, Copenhagen, Denmark and fractionated by the procedure of Anderson, Dunkley and Stone (unpublished).

Each enzyme preparation was tested for activity against laminarin, CM-pachyman, lichenin, CM-cellulose, SIII and RSIII by incubation of 100 μl of the enzyme solution at 40°C with 1 ml of a substrate–buffer solution (0.1% polysaccharide in 0.05 M acetic acid–sodium acetate (pH 5.0) or 0.05 M sodium maleate (pH 6.5) for *Bacillus subtilis* β-glucanase). The enzyme preparations used were adjusted in concentration so that they had comparable activities towards lichenin, the only common substrate tested, i.e. when incubated with 0.5% lichenin (0.8 ml) in the appropriate buffer, 200 μl of each enzyme produced 3–4 μg of reducing sugar (as glucose)/min. Chromatograms of samples from the reaction mixtures were run on Whatman No. 3 paper in an *n*-propanol:ethyl acetate:water (6:1:3, v/v/v) solvent system and oligosaccharides were located with the alkaline silver nitrate reagent [12].

3. Results and discussion

The action of the three enzyme preparations on laminarin, CM-pachyman, lichenin, SIII polysaccharide, reduced SIII polysaccharide and CM-cellulose is summarised in table 1.

3.1. *Action of Rhizopus arrhizus hydrolase*

The hydrolysis products of this enzyme acting on laminarin, lichenin and oat glucan have been described by Parrish, Perlin and Reese [4] and are shown in table 2.

Table 1
Specificity range of β-glucan hydrolases

Enzyme source	Substrate				
	SIII	RSIII	Laminarin or CM-pachyman	Lichenin or oat glucan	CM-cellulose
Rhizopus arrhizus QM 6789	–	+	+	+	–
Streptomyces QMB814	–	–	–	+	+
Bacillus subtilis	–	+	–	+	–

+, hydrolysis observed; –, no hydrolysis observed.
Tested under the conditions described in Materials and methods.

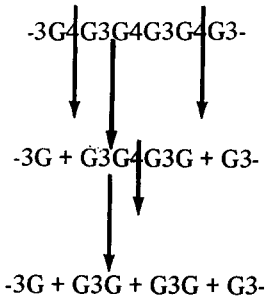
Table 2
Comparison of hydrolysis products from three β-glucan hydrolases acting on their β-glucan substrates

Enzyme source	Cellulose	RSIII	Lichenin or oat glucan	Laminarin
Rhizopus arrhizus	not hydrolysed	[G3G4G3G4G3G4G3G G3G4G3G4G3G G3G4G3G]* G3G	(G4G4G3G (G4G3G ref. 4	(G3G3G G3G ref. 4
Streptomyces QM B814	(G4G4G (G4G ref. 4	not hydrolysed	(G3G4G4G (G4G3G4G (G3G4G (G4G ref. 4	not hydrolysed
Bacillus subtilis	not hydrolysed	[G3G4G3G4G3G4G3G G3G4G3G4G3G G3G4G3G]* G3G	(G4G4G3G (G4G3G refs. 7, 8.	not hydrolysed

[transient products]*

When incubated with RSIII this enzyme ultimately produced a single compound with the R_{Glc} of laminaribiose (0.85), although the transient formation of products with R_{Glc}'s of 0.39, 0.13 and 0.02 was also observed (fig.1). Since the RSIII substrate has a regular sequence it is reasonable to conclude that these compounds represent the series of oligosaccharides with 3-substituted glucose residues at their reducing end as shown in table 2.

This result is entirely consistent with the conclusion of Parrish, Perlin and Reese [4] that the *Rhizopus* enzyme hydrolyses 1,4-glucosidic linkages adjacent to 1,3-linked glucose residues, the RSIII being cleaved as shown:



The enzyme must therefore have a glycosyl binding site which corresponds to a laminaribiosyl residue. It may be further concluded that if additional binding sites exist for the glycosyl part of the substrate, they can accept either a 1,3- or a 1,4-linked glucosyl residue.

3.2. *Action of Streptomyces hydrolase*

The *Streptomyces* preparation depolymerizes cellulosic substrates, lichenin and oat glucan [4] forming the products shown in table 2. However, it failed to hydrolyse the RSIII polysaccharide (table 1). This result is consistent with the conclusion reached by Parrish, Perlin and Reese [4] that the enzyme hydrolyses only 1,4-linkages next to 1,4-linked glucose residues as in the sequence -G4G4G-.

An alternative possibility is that the binding site of the enzyme is complementary to a cellotriosyl residue, so that the sequence -G4G4G4G- would be hydrolysed as shown, whereas RSIII would not be attacked. However, this cannot be the case since the trisaccharide, G3G4G, is a product of hydrolysis of oat glucan and lichenin by this enzyme [4], indicating that the binding site does not have an absolute requirement for -G4G4G-.

It is clear from the failure of the *Streptomyces* enzyme to hydrolyse RSIII that it is not the 1,4-β-hydrolase analogue of the *Rhizopus* 1,3-β-hydrolase.

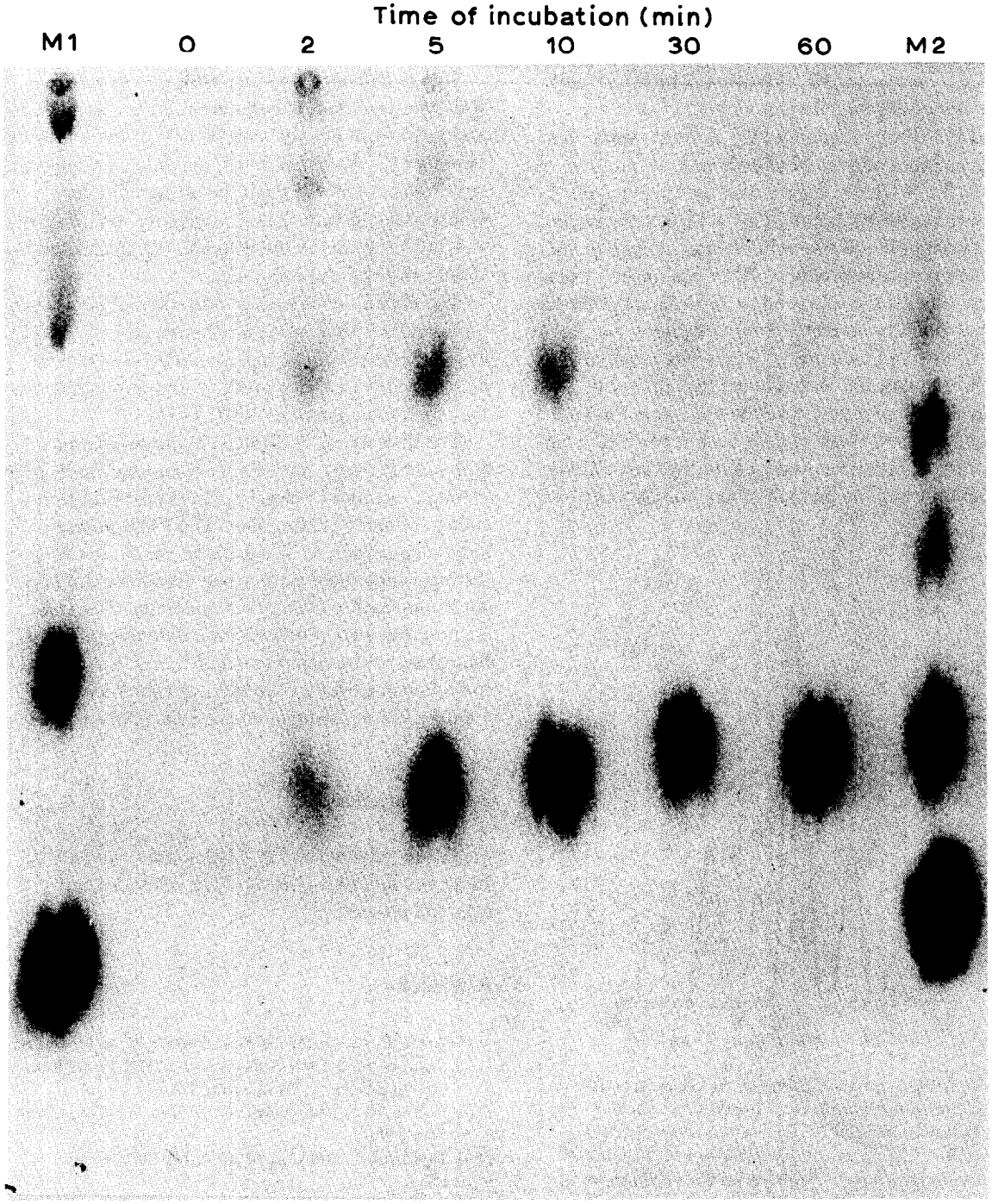


Fig.1. Chromatogram showing hydrolysis products formed during the action of the *Rhizopus arrhizus* hydrolase on RSIII. M_1 : 1,4-oligo-β-glucosides plus glucose; M_2 : 1,3-oligo-β-glucosides plus glucose.

3.3. *Action of the Bacillus subtilis hydrolase*

The *Bacillus subtilis* enzyme hydrolyses neither 1,3-β- nor 1,4-β-glucans, but is restricted in its substrate range to mixed-linked glucans [7,8] (see table 1). It cleaves the RSIII polysaccharide producing a series of oligosaccharides with K_{av} values on Bio-Gel P-2 chromatography [13] corresponding to oligosaccharides of degrees of polymerization, 8, 6, 4 and 2 (fig.2). These oligosaccharides are apparent early in the incubation but later the higher oligosaccharides disappear and the tetramer and dimer become the most prominent products. After prolonged incubation the dimer is the sole product. The disaccharide has the same mobility on paper chromatography as laminaribiose and since the substrate has a regular repeating sequence, it is concluded that the other oligosaccharide products have the sequences shown in table 2. These sequences have been confirmed from the pattern of products produced during their alkaline degradation using the method of Luchsinger and Stone [14].

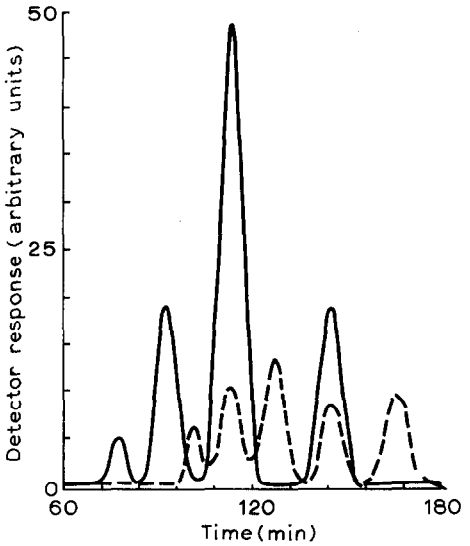


Fig.2. Bio-Gel P-2 chromatography of the oligosaccharides produced during the hydrolysis of RSIII by the *Bacillus subtilis* glucanase. Bio-Gel P-2, minus 400 mesh; 60°C; column, 0.9 × 170 cm; eluant, water; flow rate, 33.6 ml/hr. (————) hydrolysate; (- - - - - - - -) 1,3-oligo-β-glucoside series plus glucose. K_{av} glucose, 0.82; laminaribiose; 0.66; laminaritriose, 0.53; laminaritetraose, 0.43; laminaripentaose, 0.34; K_{av} of products formed from RSIII: 'biose', 0.66; 'tetraose', 0.43; 'hexaose', 0.27; 'octaose', 0.16.

Paper chromatographic evidence shows that the transient products formed from RSIII and from lichenin by the *Bacillus* enzyme are the same as those produced by the *Rhizopus* enzyme (table 2).

It may be concluded that both the *Bacillus* and *Rhizopus* hydrolases have a requirement for a -G3G- arrangement in the glycosyl portion of their substrates. However for the *Bacillus* enzyme this is a minimum requirement, and although the oligosaccharide G3G4G3G is slowly cleaved, a glycosyl sequence of -G4G3G- as found in the lichenin, oat glucan and the RSIII substrates is preferred.

The *Bacillus* enzyme has a formal resemblance to α-glucan hydrolase, mycodextranase (E.C. 3.2.1.61). This enzyme produced nigerose, the 1,3-α-glucose disaccharide as a major product from mycodextran, the α-glucan analogue of RSIII [15].

The three types of β-glucan hydrolases from *Rhizopus arrhizus, Streptomyces* and *Bacillus subtilis* belong to a series of closely related enzymes with subtly different binding sites. The RSIII polysaccharide has proven to be a valuable substrate for distinguishing these types when used in conjunction with information from other substrates. This new β-glucan has a great potential for detecting β-glucan hydrolases with related specificities e.g. the 1,3-β-hydrolase analogue of the *Bacillus* enzyme or the 1,4-β-hydrolase analogue of the *Rhizopus* enzyme.

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