Enzymic Hydrolysis of Barley and other β -Glucans by a β -(1 $\rightarrow$ 4)-Glucan Hydrolase

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1. A barley glucan with 68% of β -(1 $\rightarrow$ 4)-linkages and 32% of β -(1 $\rightarrow$ 3)-linkages was exhaustively hydrolysed with an *Aspergillus niger* β -(1 $\rightarrow$ 4)-glucan 4-glucanohydrolase (EC 3.2.1.4) (Clarke & Stone, 1965b). The hydrolysis products were separated and estimated. 2. The lower-molecular-weight products were identified as: glucose, 1.4%; cellobiose, 11.9%; 3²-O- β -glucosylcellobiose, 45.0%; a tetrasaccharide(s), which was a substituted cellobiose, 16.4%. A series of unidentified higher-molecular-weight products (26.5%) were also found. 3. The identity of the products suggests that the *A. niger* β -(1 $\rightarrow$ 4)-glucan hydrolase hydrolyses β -glucosidic linkages joining 4-O-substituted glucose residues. 4. When an enzyme fraction containing the β -(1 $\rightarrow$ 4)-glucan hydrolase and an exo- β -(1 $\rightarrow$ 3)-glucan hydrolase was used, the same products were found, but the higher-molecular-weight products were observed to have only a transient existence in the hydrolysate and were virtually absent after prolonged incubation. It is suggested that these oligosaccharides are resistant to attack by β -(1 $\rightarrow$ 4)-glucan hydrolase but are partially hydrolysed by the exo- β -(1 $\rightarrow$ 3)-glucan hydrolase and therefore possess one or more (1 $\rightarrow$ 3)-linked glucose residues at their non-reducing end.

Three polysaccharides have been described that are composed solely of β -D-glucopyranose units joined by both β -(1 $\rightarrow$ 3)- and -(1 $\rightarrow$ 4)-glucosidic linkages to form essentially linear molecules. These are lichenin from Iceland moss (*Cetraria islandica*) and the glucans from oat and barley endosperm. A number of workers (Aitken, Eddy, Ingram & Weurman, 1956; Parrish, Perlin & Reese, 1960; Preece, Garg & Hoggan, 1960; Cunningham & Manners, 1961, 1964; Preece & Garg, 1961; Perlin & Suzuki, 1962; Reese & Perlin, 1963) have examined the hydrolysis of these mixed linked glucans with various β -glucan-hydrolase preparations. The results have been used both to deduce the structural arrangement of linkages in the substrates and to give information about the specificity of the enzymes (for a discussion see Clarke & Stone, 1963).

This paper reports observations on the action of an endo- β -(1 $\rightarrow$ 4)-glucan hydrolase (EC 3.2.1.4) from *Aspergillus niger* (Clarke & Stone, 1965b) on these mixed linked β -glucans.

MATERIALS AND METHODS

Substrates. Lichenin was extracted from the dried fronds of *Cetraria islandica* (see Krishna Murti & Stone, 1961). This sample contained 96.2% of polyglucose. On periodate oxidation the reduction of periodate [moles/mole (hr.)] was:

0.46 (48); 0.65 (115); 0.70 (168); 0.71 (216); 0.70 (288).

Oat glucan was prepared by a modification of the method of Preece & Hobkirk (1953). Freshly harvested oats (5 kg.) were ground to pass the 1 mm. sieve of a hammer mill; the meal was refluxed twice with 12.5 l. of 80% (v/v) ethanol for 30 min. periods, filtered and dried in air. A water extract of the meal was obtained by using three 30 min. extractions at 40° (2500 ml./kg. of dried meal). The extract was centrifuged and filtered to a clear pale-yellow fluid, the volume of which was reduced to one-fifteenth of the original in a climbing film evaporator, and frozen. On thawing a gelatinous precipitate appeared; the extract was heated at 85° for 1 hr. and the insoluble material discarded.

The water extract was brought to 20% saturation with solid (NH₄)₂SO₄. The precipitate obtained was further purified by alternate precipitations (four each) with 20% saturated (NH₄)₂SO₄ and 50% (v/v) acetone; the final precipitate was dialysed exhaustively against water and freeze-dried. The final preparation gave a negative iodine test for starch. On periodate oxidation the reduction of periodate [moles/mole (hr.)] was: 0.52 (48); 0.65 (97); 0.70 (168); 0.72 (260); 0.73 (312); 0.72 (384).

Barley glucan was prepared from barley by the same procedure used for oat glucan. The 20% saturated (NH₄)₂SO₄ fraction (F20) gave a positive iodine test for starch and was treated with crude salivary amylase as follows. The 35–70% (v/v) acetone precipitate from 150 ml. of saliva was dissolved in 10 ml. of water and 1 ml. was incubated for 4 hr. at pH 7.0 and 40° with a 1% solution of fraction F20. After this treatment had been repeated, the solution was heated at 85° for 1 hr. and filtered. The

Table 1. *Results of analyses of oat and barley glucans*

	Oat glucan	Barley glucan
Sugars present after complete hydrolysis	Glucose only	Glucose only
Glucose recovery on hydrolysis	96%	96%
Nitrogen content	0.1%	0.1%
Infrared spectrum	Strong peak at 891 cm. ⁻¹ ; no peak at 844 cm. ⁻¹	Strong peak at 891 cm. ⁻¹ ; no peak at 844 cm. ⁻¹
Acetyl content	0%	< 1%
Relative viscosity at 25° (0.5% solutions)	262	295
[α] _{589 mμ} (c 0.5 in water)	-12.4	-11.2
[α] _{436 mμ} (c 0.5 in water)	+32.4	+30.2
Periodate consumption (moles/mole of anhydroglucose)	0.72*	0.68*
(1→4)-linkages from optical rotation in cuprammonium soln.	67 ± 2%	Solution too turbid for accurate measurement

* The values quoted by Clarke & Stone (1963) for these glucans were based on a spectrophotometric periodate estimation and have been revised on the basis of results from a titrimetric periodate estimation.

glucan was alternately precipitated with 50% (v/v) acetone and 20% saturated (NH₄)₂SO₄, dialysed and freeze-dried. The resulting F20 fraction gave a negative iodine test for starch. On periodate oxidation the reduction of periodate [moles/mole (hr.)] was: 0.39 (48); 0.66 (97); 0.68 (260); 0.68 (312); 0.68 (384).

The analyses of the cereal glucans are summarized in Table 1.

Analytical methods

Hydrolysis. The glucan was refluxed with N-H₂SO₄ (1mg./ml.). Samples were withdrawn at intervals up to 8 hr. and neutralized to phenolphthalein with N-NaOH, and the reducing sugar was estimated as glucose by the Nelson (1944) colorimetric method with the Somogyi (1952) alkaline copper reagent. A correction for loss of glucose under these conditions was applied. A portion of the hydrolysate was neutralized with solid BaCO₃, and the BaSO₄ was removed by centrifugation. The reducing sugar present in the supernatant ran as glucose in solvent systems A, B and D (see below).

Viscosity. Viscosities were measured in a B.S.S. no. 2 glass-bulb viscometer.

Periodate oxidation. Samples of the glucan were weighed into separate tubes and dissolved in 1 ml. of water by heating to 70°; on cooling 1 ml. of 0.1M-sodium metaperiodate was added. The tubes were closed with glass stoppers and shaken in the dark at 4°. At intervals duplicate tubes were removed and the contents added to 15 ml. of 0.5M-phosphate buffer, pH 7.0, containing KI (1.67%, w/v) (Neumuller & Vasseur, 1953), and the liberated I₂ was titrated with a freshly prepared solution of 0.01M-Na₂S₂O₃. Blank digests containing water in place of glucan solution were also titrated.

Spectroscopy. The infrared spectra were recorded by Dr H. Higgins, C.S.I.R.O. Division of Forest Products.

Optical rotations. Optical rotations were measured with a Zeiss Oberkochen Sugar Polarimeter 0.05°S. Optical rotations of 5% solutions of the glucans in cuprammonium solution were measured at 436 m μ . The cuprammonium solution was added to the glucan under N₂ and the solution

shaken in a dark bottle for 20 hr. Samples (2 ml.) of the solution were neutralized with 5N-H₂SO₄ and diluted to 100 ml., and the glucan content was determined by the anthrone method. The cuprammonium solution used contained 11.50 g. of Cu/l. and 206.5 g. of NH₃/l., and was supplied through the courtesy of Dr A. Meller of Australian Paper Manufacturers Ltd., Fairfield, Victoria, Australia.

The use of measurements of optical rotation in cuprammonium solution for estimation of the proportion of (1→4)-linkages in the molecule depends on the assumption that the change in optical rotation due to the complex-forming reaction is proportional to the number of (1→4)-linkages in the molecule (Peat, Whelan & Roberts, 1957). The errors introduced are due to the difficulty of reading the polarimeter with mercury blue light (436 m μ). The oat-glucan samples gave optically clear solutions, but the barley glucan was more difficult to dissolve and the final solution used was slightly turbid, lowering the accuracy of the measurements.

Acetyl determinations. Acetyl content was determined, by the CrO₃-H₂SO₄ method, by Dr K. W. Zimmerman, Australian Microanalytical Service, C.S.I.R.O., University of Melbourne.

Paper chromatography. Chromatograms were run on Whatman no. 3 paper by downward development. The solvent systems used were: A, pentan-1-ol-pyridine-water (1:1:1, by vol.; organic phase); B, propan-1-ol-ethyl acetate-water (6:1:3, by vol.); C, butan-1-ol-acetic acid-water (4:1:5, by vol.); D, butan-2-one-acetic acid-aq. saturated boric acid (9:1:1, by vol.). Reducing sugars were detected with alkaline AgNO₃ by the method of Trevelyan, Procter & Harrison (1950), as modified by E. A. Moscatelli (personal communication cited by Mayer & Lerner, 1959). Solutions were deionized with Bio-Deminrolit (HCO₃⁻ form) (Woolf, 1953).

For quantitative chromatography Whatman no. 3 papers were washed extensively with 50% (v/v) ethanol and then with water and dried before use. Known volumes of the barley-glucan hydrolysates (10 mg./ml.) were applied to the washed paper with an Agla micrometer syringe (Burroughs Wellcome and Co., London). For estimation of the total amount of sugar in the hydrolysate, washed papers

that had been run in solvent *B* for 18 hr. were used. Six 30 μ l. lots (containing 300 μ g.) were applied to the irrigated papers (5.6 cm. $\times$ 5.6 cm.), eluted and taken up in 3 ml. of water, and the total sugars present were estimated.

For estimation of the individual components of the hydrolysate, six 30 μ l. samples and six 60 μ l. samples were applied to washed papers between guide strips, and the papers run in solvent *B*. The guide strips were developed and the individual components of the test strips cut out and eluted. Blanks were obtained by eluting, with water, equivalent areas of papers that had been run in solvent *B* and dried.

Elution procedure. This procedure was based on an unpublished method due to Dr R. Markham. The test strip was cut out and a point cut at one end. The sugar on the paper was run to the tip by allowing 50% (v/v) ethanol to ascend the paper from the non-pointed end. When the solvent had reached the tip, the paper was removed and dried. This procedure was repeated twice. To elute the sugar the paper was rewetted as before, the non-pointed end wrapped in aluminium foil and attached to the outside of a 15 ml. centrifuge tube with a rubber band and the exposed tip bent into the tube. The tube was then centrifuged for 10 min. at 500g. The elution step was repeated and the solvent collected in the tube evaporated to dryness in a desiccator.

Sugar estimation. The sugars were dissolved in water to give solutions containing approx. 100 μ g./ml. The amount of sugar eluted from each paper was estimated by a modification of the method of Whittaker (1954). Samples (1 ml.) of the sugar solutions were added to Folin-Wu tubes containing 0.05 ml. of 4N-HCl, and the tubes were shaken, covered with aluminium foil and autoclaved at 121° (15 lb./in.²) for 75 min. After cooling, 0.05 ml. of 4N-NaOH was added to each tube followed by 1 ml. of Somogyi-Nelson copper reagent and the reducing sugar estimated. Blanks and standard glucose solutions were treated with acid, autoclaved and neutralized in the same way. In a series of six replicate determinations of glucose the recovery was $100 \pm 2.4\%$.

Paper ionophoresis. Paper ionophoresis was performed on Whatman no. 1 paper in an apparatus similar to that described by Frahn & Mills (1959). The electrolyte was either 0.05M-sodium borate solution, pH 9.2 (Frahn & Mills, 1956), or 0.1M-sodium molybdate solution adjusted to pH 5.0 with H₂SO₄ (Bourne, Hutson & Weigel, 1959, 1961). Borate ionophoresis was run at 15–20 ma at 10 v/cm. for 4 hr. Molybdate ionophoresis was run at 15 ma, at 10 v/cm. for 5 hr. To enable corrections for electro-osmosis the non-migrating marker 2,3,4,6-tetra-O-methyl-D-glucose was applied in borate ionophoresis, and glycerol was used in molybdate ionophoresis.

RESULTS

Hydrolysis of β -(1 $\rightarrow$ 3)- β -(1 $\rightarrow$ 4)-linked glucans by β -(1 $\rightarrow$ 4)-glucan-hydrolase fractions from *A. niger*. Each glucan was incubated separately with two enzyme fractions obtained by chromatography of an *A. niger* preparation. One fraction, E, contained a β -(1 $\rightarrow$ 4)-glucan 4-glucanohydrolase (EC 3.2.1.4) and an exo- β -(1 $\rightarrow$ 3)-glucan hydrolase. The second fraction, LE, was prepared from fraction

Table 2. *Percentage hydrolysis of β -(1 $\rightarrow$ 3)- β -(1 $\rightarrow$ 4)-glucans by fractions E and LE*

Glucan (aq. 2% solution, 8 ml.), fraction E (253 μ g. of protein/ml.) or fraction LE (212 μ g. of protein/ml.) (8 ml.) and phosphate-citrate buffer, pH 5.0 (0.2 ml.), were incubated at 40°. Samples (1 ml.) were removed at intervals and deproteinized, and the reducing sugar was determined as glucose as described in the text. After incubation for 48 hr. there was no further increase in reducing sugar and the final value was not altered by two further incubations of 24 hr. with fresh enzyme (2 ml.).

Substrate	Percentage hydrolysis	
	By fraction E	By fraction LE
Barley glucan	29.6	13.8
Oat glucan	32.6	15.1
Lichenin	19.9	10.0

E by treatment with insoluble laminarin to remove the exo- β -(1 $\rightarrow$ 3)-glucan hydrolase (Clarke & Stone, 1965a). The details of the hydrolysis procedure and the percentage hydrolysis (as glucose) of each glucan by the two enzyme preparations are shown in Table 2.

Products of hydrolysis of β -(1 $\rightarrow$ 3)- β -(1 $\rightarrow$ 4)-glucans by fraction E and fraction LE

Hydrolysis by fraction E. Fig. 1 shows diagrammatically the chromatographic separation in solvent *A* of the products of hydrolysis of barley glucan by fraction E.

The first products were slow-moving reducing oligosaccharides (A and B). After incubation for 5 hr. there had appeared two sugars (C and D) moving between cellotetraose and cellotriose, one (E) moving just ahead of cellotriose, one (F) moving just behind laminaritetraose, and one (G) corresponding to cellobiose. As the reaction proceeded, the slower-moving oligosaccharides (A and B) disappeared and a small amount of a sugar (H) moving with glucose was produced. After incubation for 47 hr., sugars C and D had disappeared and sugars E, F, G and H were the only products detected. There was no apparent change in their relative amounts after incubation for a further 25 hr.

The hydrolysis of oat glucan by fraction E gave the same products in the same order; hydrolysis of lichenin gave the same final products of hydrolysis, but no transient production of oligosaccharides A and B was detected. In contrast with the final hydrolysates of oat and barley glucan there was some material in the lichenin hydrolysate that could be precipitated by 4 vol. of acetone.

Fig. 1. Diagram of a chromatogram of the products appearing during the hydrolysis of barley glucan by fraction E. The chromatogram was run in solvent A and the reducing sugars were detected with alkaline AgNO_3 .

Hydrolysis by fraction LE. Hydrolysis of barley glucan, oat glucan and lichenin by fraction LE followed a pattern somewhat different to that by fraction E. The first detectable product of hydrolysis was sugar G at 1 hr. At 5 hr. a series of partly resolved slow-moving oligosaccharides and a reducing substance moving in the same position as sugar E had also appeared. At 12 hr. a reducing substance corresponding to sugar F had appeared. At 24 hr. the hydrolysis products detected corresponded in mobility to sugars C, D, E, F, G and H in the fraction E hydrolysate. A number of very slow-moving sugars was also apparent. After incubation for 72 hr., and after a further addition of fraction LE and incubation, there was little apparent change in the nature of the hydrolysis products.

The possibility that the oligosaccharides arising during the hydrolysis were transglycosylation products was tested by incubating cellobiose (final concn. 1%) under the same conditions used for the glucan hydrolysis. Chromatographic examination of the incubation mixture in solvent B showed no reducing substances other than cellobiose (see also Clarke & Stone, 1965b).

Estimation of the products of hydrolysis of barley glucan

Hydrolysis of samples of barley glucan by fraction E and fraction LE was taken to completion

Table 3. Amounts of oligosaccharides produced by exhaustive enzymic hydrolysis of barley glucan

Barley glucan was exhaustively hydrolysed as described in the text. Samples were applied to Whatman no. 3 paper and chromatographed in solvent B, and the components were located, cut out, eluted and estimated as described in the text. The results are expressed as percentages of the total amount of sugar present in the hydrolysate estimated as described, and are calculated from the mean of six individual estimations.

	Oligosaccharides produced (% of total)	
	By untreated enzyme (fraction E)	By laminarin- treated enzyme (fraction LE)
Glucose (sugar H)	4.9	1.4
Cellobiose (sugar G)	17.2	11.9
'Trisaccharide' (sugar F)	58.0	45.0
'Tetrasaccharide' (sugar E)	16.9	16.4
'Higher oligosaccharides' (including sugars A, B, C and D)	2.0	26.5
Total sugar recovered	99.0	101.2

under the conditions described. The reaction mixtures were deionized with Bio-Deminrolit and freeze-dried. Aqueous solutions (10 mg./ml.) of the freeze-dried reaction mixtures were prepared and applied to washed Whatman no. 3 papers. After chromatography in solvent B sugars E, F, G and H were eluted separately and estimated as described. The slower-moving oligosaccharides, including sugars C and D, were eluted together; these are referred to as 'higher oligosaccharides'. The results are shown in Table 3.

Structural examination of the products of hydrolysis of barley glucan by fraction LE

The products of the hydrolysis of barley glucan by fraction LE were separated in small amounts by the method described in the preceding section and examined as follows.

Sugar H. Sugar H was a reducing sugar with the same chromatographic mobility as glucose in solvents A and C. It had an M_{Glc} (mobility relative to glucose) 1 on borate ionophoresis.

Sugar G. Sugar G was a reducing sugar with the same chromatographic mobility as cellobiose in solvents A and C. During ionophoresis in borate it migrated with the same M_{Glc} as cellobiose (0.22). Sugar G was reduced with sodium borohydride and the solution deionized with Bio-Deminrolit and freeze-dried. On ionophoresis in molybdate the products had the same M_s (mobility relative to sorbitol) as cellobi-itol (0.4).

Sugar F. Sugar F was a reducing sugar with degree of polymerization 2.9 as estimated by the method of Peat, Whelan & Roberts (1956). On paper chromatography in solvents *A* and *C* it migrated as a single spot and the chromatographic mobility relative to the β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-oligoglucosides in solvent *A* may be seen from Fig. 1. In solvent *C* it had R_{glc} 0.32 (cellobiose, 0.52; cellotriose, 0.24; cellotetraose, 0.06; laminaribiose, 0.68; laminaritriose, 0.43; laminaritetraose, 0.27). On borate ionophoresis it had M_{glc} 0.22 (cellobiose, 0.22; cellotriose, 0.19).

Sugar F (100 μ g. dissolved in 50 μ l. of water) was heated in a sealed capillary tube on a boiling-water bath with 10 μ l. of 2*N*-hydrochloric acid for 1 hr. The solution was neutralized on a waxed microscope slide with Bio-Deminrolit beads and removed to another slide with a syringe. The resin beads were washed with water on the slide and the washings combined with the original solution. The combined solutions were evaporated to dryness in a desiccator and the residue was dissolved in 10 μ l. of water. On paper chromatography in solvent *A* the hydrolysate was shown to contain glucose, cellobiose, laminaribiose and sugar F. After reduction with sodium borohydride, sugar F had M_s close to that of cellobi-itol (0.32) on ionophoresis in molybdate. On partial acid hydrolysis (0.33*N*-hydrochloric acid for 25 min. at 100°) and chromatography (solvent *A*) glucose, sorbitol, cellobi-itol and laminaribiose were detected.

Sugar E. Sugar E was a reducing sugar that migrated as a single spot on chromatography in solvents *A* and *C*. Its chromatographic mobility relative to the β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-oligoglucosides in solvent *A* may be seen from Fig. 1. In solvent *C* it had R_{glc} 0.16 (laminaritetraose, 0.27; laminaripentaose, 0.17; cellotetraose, 0.06; cello-pentaose, 0.0). In borate ionophoresis it had M_{glc} 0.23–0.24. After partial acid hydrolysis (0.33*N*-hydrochloric acid for 1 hr. at 100°) and chromatography in solvent *A* glucose, cellobiose, laminaribiose and a sugar with the mobility of a trisaccharide were detected. After reduction with sodium borohydride sugar E had M_s 0.27 on ionophoresis in molybdate (cellobi-itol, 0.40; cellobi-itol, 0.32; cellotetraitol, 0.25).

Hydrolysis by fraction E of higher oligosaccharides produced by the action of fraction LE on barley glucan

The higher oligosaccharides, including sugars C and D, produced by exhaustive hydrolysis of barley glucan by fraction LE were recovered from chromatograms of the hydrolysate run in solvent *B* by elution with 50% (v/v) ethanol. The eluates were evaporated to dryness in a desiccator, redissolved in water and freeze-dried.

The product (1 mg.) was dissolved in water (50 μ l.) and incubated at 40° with fraction E (50 μ l.) and phosphate-citrate buffer, pH 5.0 (12.5 μ l.). After 0, 3, 8, 24 and 48 hr., 20 μ l. samples of the incubation mixture were removed and applied to Whatman no. 3 paper and run in solvent *A*. After incubation for 3 hr., sugars corresponding to compounds H, F and E in hydrolysates of barley glucan by fraction E and fraction LE had appeared. These increased in amount during the incubation and a small amount of a product corresponding to sugar G appeared after incubation for 24 hr. The higher oligosaccharides decreased in amount during the incubation but were still present in detectable amounts after 48 hr. The amount of glucose present in the final hydrolysate was relatively much greater than observed in the final products of hydrolysis of the barley glucan by fraction E or fraction LE.

DISCUSSION

The barley glucan used in this investigation has a similar content of (1 $\rightarrow$ 4)-linkages (70%) to that used by Parrish *et al.* (1960) and resembles in this respect the oat and lichenin samples also examined (Perlin & Suzuki, 1962) and those used in the present study. The enzymic hydrolysis of the barley glucan was investigated in detail.

Of the several products of exhaustive hydrolysis of barley glucan by fraction LE, sugars H, G, F and E were examined structurally. The slower-moving sugars A, B, C and D were not identified. The evidence presented indicates that sugar H and sugar G are glucose and cellobiose respectively. Sugar F appears to be a single glucose trisaccharide and the partial-hydrolysis products indicate the presence of a (1 $\rightarrow$ 3)- and a (1 $\rightarrow$ 4)-linkage. The mobility of sugar F on borate ionophoresis (Foster, 1957) and of the reduced product on molybdate ionophoresis (Bourne *et al.* 1959, 1961) together with the appearance of cellobi-itol among the partial hydrolysis products of reduced sugar F indicate that the (1 $\rightarrow$ 4)-glucosidic linkage is to the terminal reducing glucose residue. Together these results indicate that sugar F is 3²-*O*- β -glucosyl-cellobiose. Sugar E has a chromatographic mobility compatible with a tetrasaccharide linked by (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 4)-linkages, which were shown to be present by partial acid hydrolysis. Its mobility in borate ionophoresis and of the reduced sugar in molybdate ionophoresis indicate that the linkage to the terminal reducing glucose is of the (1 $\rightarrow$ 4)-type. The positions of the remaining glucosidic linkages were not elucidated.

The products of exhaustive hydrolysis of barley glucan by fraction E were chromatographically identical with compounds H, G, F and E. Comparison of the chromatographic mobility of the

final products of exhaustive hydrolysis of oat glucan and lichenin by both enzyme fractions suggested that they were identical with the products from barley glucan. These products of hydrolysis of β -(1 $\rightarrow$ 3)- β -(1 $\rightarrow$ 4)-linked glucans are similar to those found by Perlin and co-workers (Parrish *et al.* 1960; Perlin & Suzuki, 1962) after the hydrolysis of the glucans by a *Streptomyces* cellulase preparation. The final products of hydrolysis by this enzyme preparation were glucose, cellobiose, 3 β -glucosylcellobiose and two tetrasaccharides identified as 4 β -laminaribiosylcellobiose and 3 β -cellobiosylcellobiose. Since all the oligosaccharide products were terminated at the reducing end by (1 $\rightarrow$ 4)-linked glucose residues Parrish *et al.* (1960) proposed that their *Streptomyces* enzyme specifically hydrolyses β -glucosidic linkages attached to 4-O-substituted glucose residues, i.e. in the sequence -Glc- α -Glc- γ -Glc-, for linkage γ to be hydrolysed linkage α must be of the (1 $\rightarrow$ 4)-type. The identities of the oligosaccharides produced by the *A. niger* glucan hydrolase may be adduced as evidence for a like specificity for this enzyme. However, the present data do not allow a direct conclusion as to whether γ must always be a (1 $\rightarrow$ 4)-linkage or whether it may also be a (1 $\rightarrow$ 3)-linkage.

Since the *A. niger* enzyme hydrolyses both β -(1 $\rightarrow$ 4)-glucans and β -(1 $\rightarrow$ 3)- β -(1 $\rightarrow$ 4)-glucans, its specificity is different from those enzymes that hydrolyse only β -(1 $\rightarrow$ 3)- β -(1 $\rightarrow$ 4)-glucans. Such enzymes have been reported from micro-organisms (Moscatelli, Ham & Rickes, 1961; Reese & Perlin, 1963) and from germinating barley (Chen & Luchsinger, 1964).

Comparison of the actions of fraction E and fraction LE on barley glucan. The differences in the extent of hydrolysis of barley glucan by the two enzyme fractions (Table 2) can be accounted for by the difference in amounts of higher oligosaccharides present in the final hydrolysates (see Table 3).

In the experiments described, two enzyme preparations were used: fraction E, which showed both endo- β -(1 $\rightarrow$ 4)-glucan-hydrolase [β -(1 $\rightarrow$ 4)-glucan 4-glucanohydrolase] and exo- β -(1 $\rightarrow$ 3)-glucan hydrolase [β -(1 $\rightarrow$ 3)-glucan 3-glucosyl-

hydrolase) activity, and fraction LE, which showed only endo- β -(1 $\rightarrow$ 4)-glucan-hydrolase activity (Clarke & Stone, 1965*a,b*). Specificity studies have shown that the endo- β -(1 $\rightarrow$ 4)-glucan hydrolase hydrolysed β -(1 $\rightarrow$ 4)-linked oligoglucoside substrates of degree of polymerization 4 or more; the exo- β -(1 $\rightarrow$ 3)-glucan hydrolase apparently acted by endwise removal of glucose units from β -(1 $\rightarrow$ 3)-linked oligoglucosides of degree of polymerization 3 or more; the observation that the higher oligosaccharides A, B, C and D, which were resistant to hydrolysis by the endo- β -(1 $\rightarrow$ 4)-glucan hydrolase (fraction LE), were hydrolysed by fraction E, which contained both endo- β -(1 $\rightarrow$ 4)-glucan hydrolase and exo- β -(1 $\rightarrow$ 3)-glucan hydrolase, suggests that these higher oligosaccharides are susceptible to hydrolysis by the exo- β -(1 $\rightarrow$ 3)-glucan hydrolase. This is supported by the observation that oligosaccharides A, B, C and D appear transiently during the hydrolysis of barley glucan by fraction E. These oligosaccharides are presumably produced by the action of the endo- β -(1 $\rightarrow$ 4)-glucan hydrolase and have at least two terminal (1 $\rightarrow$ 3)-linkages at the non-reducing end, one or more of which is susceptible to exo- β -(1 $\rightarrow$ 3)-glucan-hydrolase action, e.g. in the sequence shown in Scheme 1.

A similar explanation for the low degree of hydrolysis of oat glucan and lichenin by the purified β -(1 $\rightarrow$ 4)-glucan hydrolase (fraction LE) may also be given. Large amounts of slow-moving oligosaccharides were observed to be present on chromatography of the hydrolysates after exhaustive enzymolysis of these glucans. As with barley glucan, treatment of oat glucan and lichenin with the mixed enzyme (fraction E) produced a hydrolysate from which the slow-moving oligosaccharides were absent. A similar synergistic action has been described by Preece & Garg (1961) for barley-glucan hydrolases acting on barley glucan.

These findings may be compared with the results of Perlin & Suzuki (1962), who reported values for the percentage hydrolysis by a *Streptomyces* enzyme of the three glucans that are comparable with that found for the mixed enzyme acting on oat glucan in the present study. The data (Parrish,

Scheme 1.

Perlin & Reese, 1960) for the composition of the hydrolysates of exhaustively treated oat-glucan and barley-glucan samples suggest that only 6.7% was present as high-molecular-weight oligosaccharides. It is possible that these results may also be explained by the presence of other glucoside hydrolases in the *Streptomyces* enzyme preparation used.

The extent of hydrolysis of lichenin by both fractions E and LE was lower than for barley and oat glucans and is consistent with the findings of acetone-precipitable material in the hydrolysate of lichenin by fraction E. An acetone-precipitable residue was also observed by Preece & Garg (1961) in hydrolysates of their barley glucan by a barley β -glucan-hydrolase preparation. The reason for the resistance of the lichenin sample to hydrolysis remains to be explored but may be due to differences in the arrangement of the linkages in the lichenin molecule as compared with the cereal glucans (for a summary see Clarke & Stone, 1963).

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