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Studies on Carbohydrate-Metabolizing Enzymes

11. THE HYDROLYSIS OF LICHENIN BY ENZYME PREPARATIONS FROM MALTED BARLEY AND *RHIZOPUS ARRHIZUS**

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Lichenin, the reserve carbohydrate of Iceland moss (*Cetraria islandica*), is an essentially linear polymer of β -D-glucopyranose containing 30 % of (1 $\rightarrow$ 3)- and 70 % of (1 $\rightarrow$ 4)-linkages (Chanda, Hirst & Manners, 1957). The sequence of the linkages has not been rigidly established. A partial acid hydrolysate contained the disaccharides cellobiose and laminaribiose, and the trisaccharides cellotriose, 4²- β -glucosyl-laminaribiose and 3²- β -glucosylcellobiose (Peat, Whelan & Roberts, 1957; for nomenclature of oligosaccharides see Whelan, 1960). Since laminaritriose was absent, it was suggested that the molecule consisted of cellotriose units joined to-

gether by (1 $\rightarrow$ 3)-linkages. A structure of lichenin proposed by Peat *et al.* (1957) is shown in Fig. 1.

During the period 1920–40, many workers reported 'lichenase' activity in enzyme preparations from a wide variety of biological sources (for a review, see Pigman, 1951). In view of the similarity in structure of lichenin and cellulose (although the presence of (1 $\rightarrow$ 3)-linkages in lichenin was not then known), and in their behaviour with various enzyme preparations, it was suggested that 'lichenase' and 'cellulase' were identical or closely related enzymes. For example, Otto (1929) could not separate the two activities in a malted-barley preparation. However, later workers (e.g. Freudenberg & Ploetz, 1939) obtained some separation of

* Part 10: Anderson, Cunningham & Manners (1964).

Fig. 1. Structure of lichenin proposed by Peat, Whelan & Roberts (1957), on the basis of partial acid hydrolysis studies. G, β -Glucopyranose residue. The oligosaccharide fragments are also indicated.

activities in preparations from *Aspergillus oryzae* and *Helix pomatia*.

Anderson, Cunningham & Manners (1964) found that extracts of ungerminated barley which showed lichenase activity contained separate cellobiase, laminariase, β -(1 $\rightarrow$ 4)-glucosanase and β -(1 $\rightarrow$ 3)-glucosanase activities. In the presence of 8 mM-glucono-(1 $\rightarrow$ 4)-lactone all the activities except that of β -(1 $\rightarrow$ 3)-glucosanase were inhibited. Since malted-barley preparations show a generally higher level of β -glucosidase activity, the action of such a preparation on lichenin, in the presence of the lactone, has been examined. Several fungi produce β -(1 $\rightarrow$ 3)-glucosanases (Reese & Mandels, 1959), and the action of one of these preparations, from *Rhizopus arrhizus*, on lichenin has also been studied. Since a preliminary account of this work was published (Cunningham & Manners, 1961), Perlin & Suzuki (1962) have described the degradation of lichenin by preparations from *R. arrhizus* and a *Streptomyces* sp. and their general results are in agreement with ours.

METHODS AND MATERIALS

Analytical methods. The methods used were those described by Anderson *et al.* (1964). The degree of polymerization of oligosaccharides was measured: (a) from the reducing power, before and after acid hydrolysis; (b) from the weight of borohydride-reduced oligosaccharide which produced 2 mol.prop. of formaldehyde on periodate oxidation. Laminarinase assays of the *Rhizopus arrhizus* preparations were carried out by the method of Reese & Mandels (1959). The apparatus for continuous electrophoresis was that used by Cunningham, Manners & Wright (1962). Paper electrophoresis of sugars was carried out as described by Anderson & Manners (1959). The solvents used for paper chromatography were: A, ethyl acetate-pyridine-water (10:4:3, by vol.); B, ethyl acetate-acetic acid-formic acid-water (18:3:1:4, by vol.); C, propan-2-ol-acetic acid-water (3:1:1, by vol.).

Materials. (a) Malted-barley preparations. Malted Ymer barley flour (200 g.) was extracted with 1% (w/v) sodium chloride solution (800 ml.) for 29 hr. at 0°. After centrifuging, the extract was saturated with ammonium sulphate. The precipitate which formed overnight was collected, dissolved in water and dialysed against running tap-water at 0° for 48 hr. The solution was then freeze-dried to give preparation M.1; the yield was 4 g. In a second experiment, flour (1 kg.) was extracted with 0.2M-sodium acetate buffer, pH 5.0 (2.3 l.), at 20° for 2 hr. The grain residue was removed and the solution dialysed for 16 hr. against running tap-water. Ammonium sulphate was then added to the cooled (0°) solution, and the protein precipitated by 0.28, 28–55 and 55–100% saturation collected; the precipitates were dissolved in water and dialysed against running distilled water at 0° for 48 hr. The solutions were then freeze-dried to give respectively 3.7, 2.5 and 0.05 g. of enzyme preparation. Qualitative tests showed that the fraction precipitated at 28–55% saturation with ammonium sulphate was the most active; this is referred to as preparation M.2.

(b) *Rhizopus arrhizus* preparations. A culture of *Rhizopus arrhizus* IMI83711 from the Commonwealth Mycological Institute, Kew Gardens, Surrey, was grown in a medium containing 0.6% of cellobiose as carbon source together with (per l.): potassium dihydrogen phosphate, 2.0 g.; ammonium sulphate, 1.4 g.; urea, 0.3 g.; magnesium sulphate heptahydrate, 0.3 g.; calcium chloride, 0.3 g.; yeast extract, 0.1 g.; proteose peptone, 1.0 g. The cultures (100 ml. of medium in 500 ml. conical flasks) were grown for 7–19 days at 27° with shaking. All media were sterilized at a pressure of 10 lb./in.² for 10 min. The cultures were filtered through sintered glass (G3 or G4) and cooled, and the enzyme protein was precipitated by the addition of acetone at –15°. Preparation R.1 was obtained from 500 ml. of medium grown for 7 days, and R.2 from 850 ml. of medium combined with 20 ml. of 4% cellobiose solution, after 19 days of growth. The respective yields and laminarinase activities were: R.1, 1400 mg., 0.4 unit/mg.; R.2, 970 mg., 0.7 unit/mg.

RESULTS

β -Glucosidase activity of malted-barley preparation. Preliminary experiments showed that preparation M.1 slowly hydrolysed cellobiose, laminariobiose, laminarin and cellodextrin. The fact that these activities are due to different enzymes was shown by the distribution of activities after chromatography on alumina or continuous paper electrophoresis.

Preparation M.1 (300 mg.) was adsorbed on a column (20 cm. $\times$ 2 cm.) of Spence (activated) alumina (type H; Peter Spence and Sons Ltd., Widnes), and protein eluted with a sodium phosphate-citric acid buffer increasing in pH from 4, as described by MacWilliam & Harris (1957). Forty-five fractions (5 ml.) were collected, the extinctions at 280 m μ measured, and samples (0.25 ml.) mixed with cellobiose or laminarin (1.0 ml. of 2% solutions in sodium phosphate-citrate buffer, pH 5.9) and examined by paper chromatography after incubation at 37° for 24 hr. Column fractions (2 ml.) were also incubated with 0.25% Cellofas B (sodium carboxymethylcellulose) (10 ml.) and pH 5.9 buffer (3 ml.), and the specific viscosity measured at intervals. β -(1 $\rightarrow$ 4)-Glucosanase activities were calculated as described by Anderson *et al.* (1964).

The distribution of protein in the fractions, and the various enzymic activities, are recorded in Table 1. Although complete separation of activities was not obtained, they show that enzymes catalysing the random hydrolysis of Cellofas B [β -(1 $\rightarrow$ 4)-glucosanase], and laminarin [β -(1 $\rightarrow$ 3)-glucosanase], and the hydrolysis of cellobiose are different (fractions 18–28). The presence of an oligosaccharide with R_G , i.e. R_F relative to that of glucose, 0.41 (gentiobiose) in the laminarin and cellobiose digests is attributed to trans- β -glucosylation at the disaccharide level (cf. Anderson & Manners, 1959).

In the electrophoresis studies, fraction M.1 (100 mg.) was dissolved in 0.05 M-sodium phosphate buffer, pH 7.8, and applied to the filter-paper curtain. After electrophoresis for 40 hr. at 600 v and 24 ma at 0°, the distribution of protein on the filter-paper curtain was determined by the Nigrosine method. In this time fractions of about 3 ml. had been collected. Two main bands of protein were present, covering fractions 12–14 and 15–18. The relative distribution of protein was obtained from the extinction at 280 m μ . For enzymic assay, column fractions (0.3 ml.) were incubated with substrate (4–5 mg. in 1 ml. of water) and 0.2 M-sodium acetate buffer, pH 5.6 (8.7 ml.), at 37° for 2 hr. The reducing power of samples (4 ml.) measured by the Somogyi procedure, after 0 and 2 hr., and expressed in ml. of 0.01 N-sodium thiosulphate solution, was taken as an estimate of the relative activity. The results (Table 2) show considerable separation of laminaribiase activity (fraction 14) from the cellobiase, laminarinase and cellodextrinase activities, and the highest lichenase activity is present in fraction 17.

The results in Tables 1 and 2 illustrate the complexity of the mixture of enzymes in malted barley, and suggest that simple protein-fractionation methods will not readily yield enzymically homogeneous β -glucosidase preparations. Accordingly, the technique of selective enzyme inhibition was applied to the malted-barley preparation.

Selective inhibition of certain β -glucosidases in malted-barley preparations. Anderson *et al.* (1964) showed that with barley preparations, glucono-(1 $\rightarrow$ 4)-lactone selectively inhibited the β -glucosidases acting on β -(1 $\rightarrow$ 4)-linkages. With 8 mm-lactone, 22 % of the original laminarinase activity was retained.

The effect of this inhibitor on the β -glucosidase activity of malted-barley preparation M.2 was investigated. By using the assay conditions described by Anderson *et al.* (1964), and lactone concentrations in the range 2–12 mm, the results shown in Fig. 2 were obtained. In 8 mm-lactone the cellobiase and cellobiase activities were completely inhibited, but one-third of the original laminarinase and lichenase activities remained.

The lichenase activity involved random degradation. Viscometric measurements showed that the activity of preparation M.2 in the presence and absence of 4 mm-lactone were 4.7 and 5.8 units respectively; i.e. the lactone had only a limited effect (19 % inhibition) on the viscosity-decreasing action of the malted-barley preparation.

Enzymic hydrolysis of lichenin by a malted-barley preparation. In a preliminary experiment, lichenin (20 mg. in 6 ml. of water), malted-barley preparation M.2 (20 mg. in 4 ml. of 0.2 M-sodium acetate buffer, pH 5.6) and 0.02 M-glucono-(1 $\rightarrow$ 4)-lactone or water (10 ml.) were incubated at 37° for 1 hr. The digests were then heated (5 min. at 98°), filtered

Table 1. *Distribution of enzymic activities in malted-barley protein fractions eluted from an alumina column*

The relative amounts of the products on paper chromatograms are indicated by: 0, nil; $\pm$, trace; +, light; ++, medium; +++, heavy.

Fraction no. ...		18	19	21	23	25	28	30	31	35	39	43	45
Substrate		Distribution of enzymic activities											
Assay													
Cellofas B	Viscometry	0.34	.	0.51	.	0.52	0.56	.	.	0.14	.	0.11	.
Cellobiose	Paper chromatography												
	(a) Glucose	$\pm$	+	+	++	+++	+++	+++	+++	+++	+	+	+
	(b) R_G 0.41	0	0	0	0	+	+	+	+	$\pm$	0	0	0
Laminarin	Paper chromatography												
	(a) Glucose	$\pm$	$\pm$	+	++	+++	+++	++	++	+	+	$\pm$	.
	(b) Laminaribiase	0	$\pm$	+	+	$\pm$	0	0	0	0	0	0	.
	(c) Laminarisaccharides	0	$\pm$	+	+	0	0	0	0	0	0	0	.
	(d) R_G 0.41	0	0	0	0	+	+	+	+	+	0	0	.
Relative protein concentration (E_{280} m μ)		0.58	0.56	0.53	0.68	0.96	∞	1.30	0.70	0.25	0.13	0.09	.

Table 2. *Distribution of enzymic activities in malted-barley protein fractions from continuous electrophoresis*

Fraction no. ...		11	12	13	14	15	16	17	18	19
Enzyme		Distribution of enzymic activities								
Relative protein concentration (E_{280} m μ)		0.6	1.6	1.6	1.8	1.9	2.0	2.0	0.8	0.2
Cellobiase		0.1	0.5	0.4	0.8	1.4	3.2	3.8	0.6	0.0
Laminaribiase		0.2	0.4	1.1	5.8	4.8	5.8	3.1	1.7	0.6
Cellodextrinase		0.0	0.3	0.4	1.0	1.4	2.6	2.4	0.3	0.0
Laminarinase		0.0	0.2	0.5	2.2	3.4	2.8	3.6	1.1	0.3
Lichenase		0.2	0.2	0.4	0.8	0.9	1.4	2.5	0.4	0.0

through sintered glass (G2), deionized with Bio-Deminolit (CO_3^{2-} form) ion-exchange resin and concentrated *in vacuo*. The results of paper-chromatographic analysis are shown in Table 3, tentative identification being based on a comparison of R_G values with authentic samples. Sugar X_M was isolated by preparative paper chromatography from the lactone-containing digest. Paper chromatographic analysis showed that: (a) partial acid hydrolysis gave glucose, laminaribiose and cellobiose; (b) further incubation with preparation M.2 gave only glucose. The degree of polymerization, from measurements of reducing power, was 3.2, and the electrophoretic mobility was similar to that of laminaribiose. Since this property is related to the type of linkage at the reducing group (Foster, 1953), these results suggest that sugar X_M is 4²- β -glucosyl-laminaribiose.

In a larger-scale hydrolysis, lichenin (1.9 g. in 190 ml. of water) was incubated at 37° with enzyme preparation M.2 (0.5 g. in 50 ml. of pH 5.6 buffer) and 0.25 M-lactone (10 ml.) for 1 hr. The increase in reducing power corresponded to 14% conversion into glucose. The digest was then deproteinized (with zinc sulphate-barium hydroxide) and centrifuged, and an equal volume of ethanol was added to the supernatant solution to remove residual polysaccharide. After deionization and concentration to 50 ml., a portion was chromatographed on paper with solvent A. The results are shown in Table 4. The identity of glucose was confirmed by treatment with glucose oxidase; the laminaribiose and sugar X_M had similar R_G values in two solvents to the products from the preliminary experiment. Sugar Y_M is probably a mixture of two tetrasaccharides.

The degree of polymerization of the whole mixture was 3.7, indicating the presence of a considerable proportion of higher oligosaccharides. Electrophoresis of the mixture showed the presence of glucose, and only one other zone, which had the same mobility as laminaribiose. This suggests that all the higher oligosaccharides possessed a terminal β -(1 $\rightarrow$ 3)-glucosidic linkage at the reducing end of the molecule (cf. Foster, 1953).

β -Glucosidase activity of *Rhizopus arrhizus* preparation. Paper-chromatographic analysis of digests of preparation R.1 and various β -glucosides showed the presence of considerable cellobiase, laminaribiase and laminarinase activities, and weak gentiobiase and celloedextrinase activities. Provided that incubation periods were restricted to 3 hr., only the first three activities could be detected. The general pattern of carbohydrates produced by this strain of *R. arrhizus* is therefore similar to that of the QM1032 strain examined by Reese & Mandels (1959).

Enzymic hydrolysis of lichenin by a *Rhizopus arrhizus* preparation. Lichenin (20 mg. in 2 ml. of

water) was incubated with preparation R.2 (4 mg. in 1 ml. of 0.05 M-sodium citrate buffer, pH 4.8) at 40°. Samples (0.5 ml.) were removed at intervals for the measurement of reducing power. The apparent percentage conversions into glucose after 50, 125, 160 and 360 min. were 22, 26, 28 and 30 respectively. Paper-chromatographic analysis showed that random hydrolysis had occurred, with the formation of oligosaccharides with R_G 0.80 and R_G 0.41 together with glucose (with solvent A).

Fig. 2. Effect of various concentrations of glucono-(1 $\rightarrow$ 4)-lactone on the enzymic activity of a malted-barley preparation. The activities were: $\square$, cellobiase; $\circ$, celloedextrinase; $\blacksquare$, lichenase; $\bullet$, laminarinase.

Table 3. *Products of hydrolysis of lichenin by malted-barley preparation M.2 in the presence and absence of glucono-(1 $\rightarrow$ 4)-lactone*

The composition of the digest is given in the text. Ethyl acetate-acetic acid-formic acid-water (18:3:1:4, by vol.) was used as the paper-chromatographic solvent.

Products	R_G	Approximate yield	
		Control digest	Lactone present
Gluconic acid	1.25	—	+++
Glucose	1.00	+++	+++
Laminaribiose	0.41	$\pm$	+
Cellobiose	0.32	+	—
Sugar X_M	0.14	$\pm$	++

Table 4. *Products from malted-barley lichenase in the presence of 10 mM-glucono-(1 $\rightarrow$ 4)-lactone*

The composition of the digest is given in the text.

Product	R_G (solvent A)	Yield	
		mg.	mM
Glucose	1.00	34	0.21
Laminaribiose	0.80	20	0.06
Sugar X_M	0.41	236	0.47
Sugar Y_M	0.22, 0.15	29	0.04
Higher oligosaccharides	—	878	—

On a preparative scale, lichenin (2.7 g.) and preparation R.2 (500 mg. in 50 ml. of pH 4.8 buffer) in a total volume of 350 ml. were incubated at 40° for 3.25 hr. The digest was then heated (10 min. at 98°), cooled, filtered (G3 sintered glass) and de-ionized [on Bio-Deminrolit (CO₃²⁻ form) resin]. The concentrate was then separated by chromatography on Whatman 3MM paper with solvent A, portions of about 100 mg. being applied to each sheet of paper. Appropriate areas of the sheets were eluted with water, filtered, concentrated and further purified by passage through Bio-Deminrolit resin. The yields of the products are given in Table 5.

The monosaccharide was identified as D-glucose by treatment with glucose oxidase. The disaccharide was characterized as laminaribiose by comparison of the R_G values in two solvents with an authentic sample, by electrophoretic mobility, by hydrolysis to glucose by almond emulsin and by the specific rotation +20.2° (c 1.1 in water) [cf. $[\alpha]_D + 20.4^\circ$ reported by Peat, Whelan & Lawley (1958)].

Sugar X_R had a degree of polymerization of 3.1 from measurements of reducing power, and 3.3 by the periodate-oxidation method. Partial acid hydrolysis gave products with the R_G values of laminaribiose, cellobiose and glucose. Incubation with emulsin gave glucose and laminaribiose; since this β -glucosidase degrades oligosaccharides from the non-reducing end, this suggests that there was a β -(1→3)-linkage at the reducing end of trisaccharide X_R. This conclusion was substantiated by electrophoresis studies which showed a mobility similar to that of laminaribiose, and much greater than that of cellobiose (cf. Foster, 1953). In all these properties, sugar X_R was identical with the trisaccharide X_M from the malted-barley experiment, to which the structure 4²- β -glucosyl-laminaribiose had been tentatively assigned.

Confirmation of this structure was obtained in the following manner: (a) Sugar X_R had $[\alpha]_D + 12.3^\circ$ (c 2.1 in water), in good agreement with the values of +12.7° and +12.1° reported by Peat *et al.* (1957) and Parrish, Perlin & Reese (1960) for the same trisaccharide. (b) Oxidation with alkaline hypoiodite followed by partial acid hydrolysis gave cello-

biose and gluconic acid. (c) Periodate oxidation at 0° for 100 hr. gave 1.0 mol.prop. of formic acid, which was increased to 1.8 mol.prop. on raising the temperature to 20° to allow intermediary formyl esters to be hydrolysed. The theoretical values for 4²- β -glucosyl-laminaribiose are: initial release, 1.0 mol.prop.; total release, 2.0 mol.prop.; for the isomeric 3²- β -glucosylcellobiose: initial release, 2.0 mol.prop.; total release, 3.0 mol.prop.; and for the 'branched' trisaccharide 3¹,4¹- β -diglucosylglucose: initial release, 2 mol.prop.; total release, 3.0 mol.prop. (d) Acetylation of 65 mg. of trisaccharide gave 80 mg. of an acetate, recrystallized from ethanol, with m.p. 103–104° (Found: C, 49.3; H, 5.8. C₄₀H₆₄O₂₇ requires C, 49.7; H, 5.6%) [cf. m.p. 104–105° reported by Parrish *et al.* (1960) and m.p. 108–110° reported by Peat *et al.* (1957)].

Sugar Y_R had the chromatographic properties of a tetrasaccharide, and an electrophoretic mobility similar to that of laminaribiose. Since partial acid hydrolysis gave products with the R_G values of glucose, laminaribiose, cellobiose, cellotriose and 4²- β -glucosyl-laminaribiose, sugar Y_R is tentatively identified as 4²- β -cellobiosyl-laminaribiose:

The hydrolysis of lichenin by both *Rhizopus arrhizus* and malted-barley preparations therefore yields a mixture of oligosaccharides, all of which possess a terminal β -(1→3)-linkage at the reducing end of the molecule. With both enzyme preparations, the major product on both a weight and molar basis is the trisaccharide 4²- β -glucosyl-laminaribiose.

Enzymic hydrolysis of complex β -glucosides by a Rhizopus arrhizus preparation. The substrates listed in Table 6 (5–10 mg. in 0.5 ml. of water) and preparation R.2 (2.5 mg. in 0.5 ml. of 0.05M-sodium citrate buffer, pH 4.8) were incubated at 40° for 3 hr. The results of paper-chromatographic analyses with solvents A and C (Table 6) show that the preparation rapidly hydrolyses a β -(1→3)-linkage which is already attached to a 3-O-substituted glucose residue, but cannot attack a β -glucosyl-mannitol linkage.

DISCUSSION

β -Glucosidases of malted barley. The presence of a complex mixture of β -glucosidases in extracts of malted barley has been shown by several workers, e.g. Preece & Hoggan (1956), Bass & Meredith (1960), Luchsinger (1962), and by the results reported in Tables 1 and 2. Preece and Meredith, and their respective co-workers, have studied the degradation of barley β -glucosan by malted-barley preparations, and consider that at least three types of activity are involved: (a) endo- β -glucanase;

Table 5. *Products from Rhizopus arrhizus lichenase*

The composition of the digest is given in the text.

Product	R_G (solvent A)	Yield	
		mg.	mM
Glucose	1.00	201	1.24
Laminaribiose	0.80	194	0.57
Sugar X _R	0.41	924	1.83
Sugar Y _R	0.12	137	0.20
Higher oligo-saccharides	—	505	—

Table 6. *Products of the action of a Rhizopus arrhizus preparation on some oligosaccharides*

The composition of the digests is given in the text.

Substrate	Products			
	Glucose	Laminaribiose	Glucosyl-mannitol	Laminaribiosyl-mannitol
Laminaribiose	++	-	-	-
Laminaribiosylmannitol	++	-	+	-
Laminaritetraosylmannitol	++	++	±	+
4 ² -β-Glucosyl-laminaribiose*	+	+	-	-
Sugar Y _R *	+	±	-	-

* Cellobiose was not a product.

(b) exo-β-glucosanase in which cellobiose is the product of a stepwise hydrolysis of alternate linkages; (c) cellobiase. With the two polysaccharase activities, the nature of the β-glucosidic linkage hydrolysed was not specified.

Bass & Meredith (1960) attempted a chromatographic separation of these activities on an alumina column by using increasing concentrations of ammonium sulphate solution as eluent. They obtained one fraction showing only endo-β-glucosanase activity, and a second fraction with exo-β-glucosanase and cellobiase activities. Luchsinger, Cochrane & Kneen (1960) obtained evidence, by ammonium sulphate fractionation, for two endo-β-glucosanase enzymes which differed markedly in heat stability, and slightly in pH optima. These studies were continued by Luchsinger (1962), who concluded that a third endo-β-glucosanase was also present. One enzyme rapidly decreased the viscosity of barley β-glucosan but not that of carboxymethylcellulose. The obvious conclusion that this enzyme was a β-(1→3)-glucosanase was not accepted by this author, although no evidence against this view was cited. The presence of such an enzyme in malted barley is clearly established by the present work (Tables 1 and 2 and Fig. 2). The other two enzymes examined by Luchsinger (1962) hydrolysed both barley β-glucosan and carboxymethylcellulose but differed in stability, particularly on dilution.

The fractions (Table 1) obtained by chromatography of an extract of Ymer malted barley on alumina showed both β-(1→4)-glucosanase and β-(1→3)-glucosanase activities, together with cellobiase and an associated carbohydrase that could hydrolyse laminarisaccharides to glucose. There was some separation of β-(1→4)-glucosanase and cellobiase activities, but no appreciable resolution of cellobiase and laminarinase as was reported by MacWilliam & Harris (1957) during the chromatography of a malt extract on Brockmann alumina. The complexity of the β-glucosidases of malted barley was confirmed by the electrophoresis experiments (Table 2) which provided definite evidence for the presence of separate cellobiase and lamina-

ribose enzymes, and separate cellodextrinase and laminarinase activities. Maximum hydrolysis of lichenin was observed in fraction 17 which showed high cellodextrinase, laminarinase and cellobiase activities. Attempts to separate these activities completely by chromatography on diethylaminoethylcellulose have not yet been successful, although in unreported experiments a separation of cellobiase from laminarinase was obtained.

Since the degradation of lichenin by such a mixed enzyme preparation would be of limited value, the action of relatively homogeneous β-(1→3)-glucosanase preparations has been examined: (a) by the selective inhibition of the β-(1→4)-glucosidases present in malted barley by glucono-(1→4)-lactone; (b) by the selective production of a laminarinase by *Rhizopus arrhizus*.

Enzymic degradation of lichenin. The degradation of lichenin by the malted-barley preparation in the presence of 10 mM-glucono-(1→4)-lactone, and by the *Rhizopus arrhizus* 'laminarinase', gave similar results (Tables 4 and 5). The major products were the trisaccharide 4²-β-glucosyl-laminaribiose [3-O-β-cellobiosyl-D-glucose; also known as cellamiose (Ono & Dazai, 1959)] and glucose, with laminaribiose and a tetrasaccharide based on the above trisaccharide as minor products. Cellobiose and the isomeric trisaccharide 3²-β-glucosylcellobiose were absent. In all cases, the oligosaccharide products contained a (1→3)-linkage adjacent to the reducing group, and contained a higher proportion of (1→3)-linkages than the original substrate.

In terms of the basic structure of lichenin, it is clear that 'laminarinase' action had hydrolysed β-(1→4)-glucosidic linkages, and not, as might be expected, β-(1→3)-linkages. The enzyme may therefore be regarded as being specific for a β-glucosidic linkage attached to a 3-O-substituted glucose residue, i.e. in the diagram:

the ability of the enzyme to hydrolyse linkage (b) is controlled by the nature of linkage (a). These results are in agreement with the studies of Parrish,

et al. (1960). With the fungal enzyme: (a) must be a β -(1 $\rightarrow$ 3)-glucosidic linkage, but (b) can be a β -(1 $\rightarrow$ 3)- or β -(1 $\rightarrow$ 4)-glucosidic linkage, but not a β -(1 $\rightarrow$ 1)-mannitol linkage.

It is therefore clear that deductions on the structures of polysaccharides containing mixed linkages, made from enzymic degradation studies, require especial caution, and that the nature of the linkages hydrolysed cannot be unambiguously identified from specificity studies carried out with substrates containing only one type of linkage.

The formation of the trisaccharide 4²- β -glucosyl-laminaribiose as a major product of enzymic degradation confirms that a large portion of the lichenin molecule must consist of the sequence shown in Fig. 1. However, the isolation of a tetrasaccharide, and the absence of positive evidence for sequences of adjacent β -(1 $\rightarrow$ 3)-linked glucose residues, indicates that there must be some sequences of three β -(1 $\rightarrow$ 4)-linkages joined by a β -(1 $\rightarrow$ 3)-glucosidic linkage. To account for the ratio of linkages obtained by chemical analysis, there must be minor sequences of alternating β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-linkages.

Laminaribiose was present in both digests, the molar ratio to trisaccharide being 1:8 with the malted-barley 'laminarinase' and 1:3 with the fungal enzyme. In the presence of the lactone, the malted-barley preparation does not show β -glucosidase activity, so that the laminaribiose may represent a primary product of polysaccharase action. This would be in accordance with the postulated minor sequences of alternating linkages. With the *Rhizopus arrhizus* preparation, part of the laminaribiose may arise directly from lichenin, and part from 4²- β -glucosyl-laminaribiose (see Table 6) and the corresponding tetrasaccharide, as a result of the weak activity of a contaminating β -glucosidase.

From the present work, it is concluded that lichenin consists of a major proportion of cellobiosyl units joined together by β -(1 $\rightarrow$ 3)-glucosidic linkages, with a minor proportion of randomly situated cellobiosyl and cellobiosyl units joined by the same type of linkage. The general results are in agreement with those reported by Perlin & Suzuki (1962). These workers also examined the action of a β -(1 $\rightarrow$ 4)-glucosidase preparation from a *Streptomyces* sp. on lichenin, and isolated 3²- β -glucosylcellobiose from the hydrolysis products. We also planned a similar study but were unable to complete the work since the strain of *Streptomyces* available to us (IMI 45560 from the Commonwealth Mycological Institute) showed appreciable β -(1 $\rightarrow$ 3)-glucosidase activity, in addition to β -(1 $\rightarrow$ 4)-glucosidase, when grown on a medium containing 1% cellulose powder as carbon source.

SUMMARY

1. The hydrolysis of lichenin by a malted-barley preparation yields a complex mixture of oligosaccharides.

2. In the presence of 10 mM-glucono-(1 $\rightarrow$ 4)-lactone, which inhibits cellobiase and β -(1 $\rightarrow$ 4)-glucosidase activities, the products are a trisaccharide, laminaribiose, and glucose.

3. The hydrolysis of lichenin by a *Rhizopus arrhizus* preparation also gives laminaribiose and the same trisaccharide.

4. The trisaccharide has been characterized as O- β -D-glucopyranosyl-(1 $\rightarrow$ 4)-O- β -D-glucopyranosyl-(1 $\rightarrow$ 3)-D-glucose, and related to the sequence of linkages in lichenin.

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