β -Glucan Hydrolases from *Aspergillus niger*

ISOLATION OF A β -(1 $\rightarrow$ 4)-GLUCAN HYDROLASE AND SOME PROPERTIES OF THE β -(1 $\rightarrow$ 3)-GLUCAN-HYDROLASE COMPONENTS

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1. The components of an enzyme preparation from *Aspergillus niger*, which hydrolysed substrates containing β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-glucosidic linkages, were separated by calcium phosphate and Dowex 1 column chromatography. 2. The hydrolytic activity of each fraction from both types of column towards laminaribiose, laminarin, carboxymethylpachyman, pachydestrins, salicin, cellobiose, cellopentaose and swollen cellulose was tested. 3. The activity towards the β -(1 $\rightarrow$ 3)-glucosidic substrates was found in three well-separated groups of fractions. The differences in action pattern of these groups is discussed. 4. Preparative-scale chromatography that enabled the separation of a β -(1 $\rightarrow$ 4)-glucan-glucanohydrolase component substantially free of activity towards β -(1 $\rightarrow$ 3)-glucosidic substrates is described. Residual β -(1 $\rightarrow$ 3)-glucan-hydrolase activity was removed by adsorption on to insoluble laminarin at pH 3.5.

In a previous paper, Krishna Murti & Stone (1961) described column chromatography of *Aspergillus niger* culture filtrates that allowed the resolution of several β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-glucan-hydrolase components. The present paper reports further experiments on the chromatography of these culture filtrates that allow the description of some properties of the β -(1 $\rightarrow$ 3)-glucan-hydrolase components and the isolation of one of the β -(1 $\rightarrow$ 4)-glucan-hydrolase components. Further details of the properties of this β -(1 $\rightarrow$ 4)-glucan-hydrolase and of its action on some β -glucans are reported by Clarke & Stone (1965a,b).

MATERIALS AND METHODS

Substrates. The β -(1 $\rightarrow$ 4)-glucosidic substrates were as used by Krishna Murti & Stone (1961). The β -(1 $\rightarrow$ 3)-oligo-glucosides were prepared by partial acid hydrolysis of soluble laminarin followed by charcoal-column chromatography and preparative paper chromatography. Pachyman and CM-pachyman† were prepared as described by Clarke & Stone (1962). Soluble oligosaccharides from pachyman (pachydestrins) were prepared as follows. A 1 g. portion of pachyman was swollen by continuous stirring at 4° in 100 ml. of 70% (v/v) phosphoric acid for 48 hr. The suspension was then brought to pH 7.0 by the addition of 40% (w/v) NaOH and dialysed exhaustively against distilled water. The

volume was reduced to 100 ml. and the insoluble material sedimented by centrifuging at 1000g for 15 min. The supernatant was freeze-dried to a white hygroscopic powder and is referred to as soluble pachydestrins (P content 4.2%). Insoluble and soluble laminarin were obtained from the Institute of Seaweed Research, Inveresk, Scotland. Sophorose was prepared by the method of Coxon & Fletcher (1961). A β -(1 $\rightarrow$ 2)-glucan from a mycoplasma, isolated from a case of bovine arthritis, was a gift from Dr P. Plackett, C.S.I.R.O. Division of Animal Health, Parkville, Victoria, Australia. Gentibiose was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Pustulan, from the fronds of *Umbilicaria pustulata*, was a gift from Dr B. G. Lindberg, Swedish Forest Products Research Laboratories, Stockholm, Sweden. Glucono-(1 $\rightarrow$ 5)-lactone and phenylmercuric nitrate were from British Drug Houses Ltd., Poole, Dorset. Salicin was from L. Light and Co. Ltd., Colnbrook, Bucks. Bio-Deminrolit was obtained from The Permutit Co. o Australia, Sydney, New South Wales, Australia.

Enzyme preparation. The commercial preparation E19 (Rohm and Haas Co., Philadelphia 5, Pa., U.S.A.) was treated as described by Krishna Murti & Stone (1961) to give the starting material (crude enzyme).

Enzyme assays. Enzyme activity on the various substrates was measured by determining the reducing sugar produced in a standard assay system. Conditions were chosen so that the kinetics of the system were zero order for soluble substrates and so that for insoluble substrates there was less than 5% hydrolysis. The substrate (0.8 ml.) in McIlvaine (1921) phosphate-citrate buffer, pH 5.0, and the enzyme solution (0.2 ml.) were incubated at 40° for a suitable period. The substrate concentrations and incubation times used were as follows: cellobiose, 0.5%, 0.5 hr.; cellopentaose, 0.1%, 0.5 hr.; swollen cellulose, 0.25%, 2 hr.; sodium CM-cellulose, 0.75%, 0.5 hr.; laminaribiose, 0.5%, 6 hr.; soluble

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† Abbreviation: CM-pachyman, carboxymethylpachyman.

pachydestrins, 0.6%, 6 hr.; soluble laminarin, 0.75%, 6 hr.; sodium CM-pachyman, 0.75%, 6 hr.; salicin, 0.5%, 6 hr.

With the exception of reactions with disaccharide substrates all reactions were stopped by the addition of 1 ml. of the Somogyi (1952) alkaline copper reagent and the amount of reducing sugar was determined as glucose by the Nelson (1944) colorimetric method. Under the conditions used in these assay systems there was no demonstrable effect of citrate on the Somogyi-Nelson determination (cf. Paleg, 1959). Where disaccharides were substrates the reactions were stopped by the addition of 1 ml. of 0.3 N-Ba(OH)₂ and 1 ml. of 5% (w/v) ZnSO₄ (Somogyi, 1945). After centrifugation the glucose present in the supernatant solution was determined by the glucose oxidase-peroxidase method of Huggett & Nixon (1957). High blanks were encountered when laminaribiose was the substrate and were shown to be due to the hydrolytic activity of the peroxidase preparations. Three different peroxidase samples [a crude horse-radish preparation, Sigma type 1 (Sigma Chemical Co.) and a sample from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany] were tested and all were found to have hydrolytic activity towards laminaribiose (determined chromatographically). Sigma glucose oxidase showed no action on laminaribiose. The effect of the peroxidase could be minimized by using the lowest possible non-limiting concentration of peroxidase, rather than the excess recommended by Huggett & Nixon (1957). At this concentration (5 μ g. of crude horse-radish peroxidase/ml. of enzyme-dye reagent), the blank corresponded to a 1% hydrolysis in assays of about 15% total hydrolysis of laminaribiose.

Activity units. The I.U.B. definition of 1 unit of β -glucanhydrolase activity as the amount that will catalyse the hydrolysis of 1 μ equiv. of the glucosidic linkage/min. has not been applied as no stoichiometric method for the estimation of reducing groups suitable for routine analysis is available. In the present work the reducing sugar produced has been determined as glucose and the number of μ moles of reducing sugar (expressed as glucose) used as an approximation to the number of glucosidic bonds hydrolysed.

The activity of the fractions from the column chromatograms is expressed in terms of μ g. of reducing sugar (as glucose) produced under the standard conditions rather than as units. The activity, in units, of the eluates is about 12 times as great on the (1 $\rightarrow$ 4)-glucosidic substrates as on the (1 $\rightarrow$ 3)-glucosidic substrates, as the incubation times used to obtain comparable amounts of reducing sugar were 0.5 and 6 hr. respectively.

Calcium phosphate columns. Calcium phosphate was prepared and packed into columns as described by Krishna Murti & Stone (1961).

Dowex 1 columns. In preliminary experiments Dowex 1 (X4; 200-400 mesh), a quaternary ammonium anion-exchange resin obtained from V. A. Howe and Co., London, and from the same batch used by Krishna Murti & Stone (1961), was employed. Further batches of this resin from the same source were markedly different in appearance and flow characteristics and failed to show separation of the components. In subsequent experiments a washed Dowex 1 from Bio-Rad Laboratories, Richmond, Calif., U.S.A., was used. This resin had the following specifications: effective pore size, large; total capacity 5m-equiv./g. dry wt., 1.2m-equiv./ml. of resin bed. This resin gave similar elution patterns to those obtained by Krishna Murti & Stone (1961). The resin was cycled through the OH⁻ and the Cl⁻ form

before packing and equilibration as described by Krishna Murti & Stone (1961).

Elution procedure. Chromatograms were run as described by Krishna Murti & Stone (1961), except that the temperature was maintained at 4°.

Concentration of pooled fractions. Pooled fractions from large-scale columns were dialysed against frequently changed distilled water and concentrated by a modification of the partial-freezing methods of Shapiro (1961) and Gibor (1961). Batches of dialysed fractions (10l.) were placed in a stainless-steel bucket in a vertical freezing unit (-15°); the solution was stirred by a paddle, driven by an externally mounted motor. Ice crystals appeared in the solution after about 2 hr. and the side and the bottom of the bucket were gradually lined by a layer of ice. As freezing continued the ice lining thickened and the concentrated enzyme solution remained in the cone-shaped enclosure formed by the ice lining. As the solution became more concentrated losses to the solid phase increased. This loss could be reduced by allowing the freezing to proceed more slowly as the solution became more concentrated. Recoveries of 90-97% of the enzyme activity were obtained for 50-fold concentrations of CM-cellulose- and CM-pachyman-hydrolysing activity.

Paper chromatography. Chromatograms were run on Whatman no. 3 paper by downward development. The solvent systems used were: solvent A, pentan-1-ol-pyridine-water (1:1:1, by vol.; organic phase); solvent B, propan-1-ol-ethyl acetate-water (6:1:3, by vol.). Reducing sugars were detected by the alkaline AgNO₃ method of Trevelyan, Procter & Harrison (1950), as modified by E. A. Moscatelli (personal communication cited by Mayer & Lerner, 1959).

RESULTS

The following experiments were performed to establish the concentrations of eluant required at 4° to reproduce the type of elution pattern obtained at room temperature by Krishna Murti & Stone (1961) and also to establish the elution patterns of activity towards some additional β -(1 $\rightarrow$ 3)-glucosidic substrates.

Elution analysis of the A. niger enzyme preparation on calcium phosphate columns. Crude enzyme (15mg.) in 1 ml. of 5mm-phosphate buffer, pH 5.8, was applied to a column (70 cm. $\times$ 0.7 cm.) of calcium phosphate that had been equilibrated with the same buffer. Elution was effected with 100ml. of this buffer followed by a phosphate concentration gradient (mixing vessel: 600ml. of 5mm-phosphate buffer, pH 5.8; reservoir: 600ml. of 50mm-phosphate buffer, pH 5.8). The column was run under a pressure of 6lb./in.² of air, giving a flow rate of 0.4 ml./min., and 10ml. fractions were collected.

Fig. 1 shows the elution diagrams of hydrolytic activity towards cellobiose, CM-cellulose, laminarin, pachydestrins, CM-pachyman and salicin. The elution of the hydrolytic activity towards the β -(1 $\rightarrow$ 4)-glucosidic substrates follows a pattern similar to that described by Krishna Murti & Stone (1961). Two differences were noted. First, the concentration of phosphate required to complete the elution at 4°

Fig. 1. Chromatography of crude enzyme (15 mg.) on a calcium phosphate column (70 cm. $\times$ 0.7 cm.) by gradient elution with 5–50 mm-phosphate buffer, pH 5.8. Patterns of hydrolytic activity towards CM-pachyman (○), laminarin (■), pachydextrins (Δ), CM-cellulose (●), cellobiose (□) and salicin (▽), and the phosphate concentration gradient (continuous line), were determined as described in the text.

is 45 mm, whereas at room temperature it is 140 mm. Secondly, an additional peak of activity towards CM-cellulose eluted at 10 mm-phosphate was a constant feature of the columns run at 4°.

Two distinct groups of components hydrolysing β -(1 $\rightarrow$ 3)-glucosidic substrates were demonstrated: one group was eluted at 12–25 mm-phosphate and overlapped the group of β -(1 $\rightarrow$ 4)-glucan-hydrolysing components eluted at 15–25 mm-phosphate; the second group of components hydrolysing the β -(1 $\rightarrow$ 3)-glucosidic substrates was eluted at 30–50 mm-phosphate together with the cellobiose-hydrolysing activity and one of the two groups of salicin-hydrolysing components, as shown in Fig. 1.

Elution analysis of the A. niger enzyme preparation on Dowex 1 columns. Crude enzyme (15 mg.) in 1 ml. of 5 mm-tris-hydrochloric acid buffer, pH 7.0, was applied to a column (70 cm. $\times$ 0.7 cm.) of Dowex 1 that had been equilibrated with the same buffer. Elution was effected with 100 ml. of this buffer followed by a chloride concentration gradient (mixing vessel: 600 ml. of 5 mm-tris-hydrochloric acid buffer, pH 7.0; reservoir: 600 ml. of 0.4 M-sodium chloride in 5 mm-tris-hydrochloric acid buffer, pH 7.0). The flow rate was restricted to 0.4 ml./min., and 10 ml. fractions were collected.

Fig. 2 shows the elution patterns of hydrolytic activity towards cellobiose, CM-cellulose, laminari-

Fig. 2. Chromatography of crude enzyme (15 mg.) on a Dowex 1 column (70 cm. $\times$ 0.7 cm.) by gradient elution with 5–400 mM-NaCl in 5 mM-tris-HCl buffer, pH 7.0. Patterns of hydrolytic activity towards CM-pachyman ($\circ$), laminaribiose ($\blacktriangle$), laminarin ($\blacksquare$), pachydextrins ($\triangle$), CM-cellulose ($\bullet$), cellobiose ($\square$) and salicin (∇), and the chloride concentration gradient (continuous line), were determined as described in the text.

biose, laminarin, pachydextrins, CM-pachyman, and salicin. The elution pattern of hydrolytic activity towards the β -(1 $\rightarrow$ 4)-glucosidic substrates closely follows the pattern described by Krishna Murti & Stone (1961). As with the calcium phosphate column the components were eluted at lower chloride concentrations at 4° than at room temperature.

At least three groups of components hydrolysing the β -(1 $\rightarrow$ 3)-glucosidic substrates are apparent, as judged by their position of elution and substrate ranges. Two of these are eluted at 0.09–0.18 M-chloride and the third at 0.34–0.40 M-chloride. The last group of components was not observed in the experiments of Krishna Murti & Stone (1961), when

laminarin alone was used as a β -(1 $\rightarrow$ 3)-glucosidic substrate.

Preparative chromatography on calcium phosphate. It is apparent from Figs. 1 and 2 that a combination of Dowex 1 and calcium phosphate chromatography would allow separation of CM-cellulose-, laminarin- and cellobiose-hydrolysing activity. For large-scale preparation of these groups of components calcium phosphate chromatography was used for the primary fractionation and groups of components from this chromatogram were then rechromatographed on Dowex 1.

Crude enzyme (2 g.) in 20 ml. of 5 mM-phosphate buffer, pH 5.8, was applied to a column (70 cm. $\times$ 4 cm.) of calcium phosphate and the enzyme allowed

Fig. 3. (a) Preparative chromatography of crude enzyme (2g.) on a calcium phosphate column (70 cm. $\times$ 4 cm.) by gradient elution with 5–50 mM-phosphate buffer, pH 5.8. Patterns of hydrolytic activity towards CM-pachyman ($\circ$), CM-cellulose ($\bullet$) and cellobiose ($\square$), and the phosphate concentration gradient (continuous line), were determined as described in the text. Fractions were pooled as indicated to give fractions A, B and C. After dialysis and concentration, fractions were run separately on analytical-scale calcium phosphate columns under the conditions described in the text. The elution patterns are shown in (b), (c) and (d).

Fig. 4. Preparative-scale chromatography of fraction B on a Dowex 1 column (70 cm. $\times$ 4 cm.) by gradient elution with 5–400 mM-NaCl in 5 mM-tris-HCl buffer, pH 7.0. Patterns of hydrolytic activity towards CM-pachyman ($\circ$), CM-cellulose ($\bullet$), cellopentaose ($\blacktriangledown$), swollen cellulose ($\square$), laminarin ($\blacksquare$), laminaribiose ($\blacktriangle$) and pachydestrins ($\triangle$), and the chloride concentration gradient (continuous line), were determined as described in the text.

to adsorb during 3 hr. Elution was effected with 10l. of the same buffer followed by a phosphate concentration gradient (mixing vessel: 45l. of 5 mM-phosphate buffer, pH 5.8; reservoir: 45l. of 50 mM-phosphate buffer, pH 5.8). The flow rate was 2 ml./min., and fractions (approx. 1l.) were collected manually. The elution patterns (Fig. 3a) obtained for three substrates, CM-cellulose, CM-pachyman and cellobiose, taken as representative of the components detected in previous experiments, were similar to the elution patterns obtained from the analytical-scale columns (Fig. 1), but the different components were not as well separated. The fractions were pooled as shown in Fig. 3(a) to give fraction A, which showed hydrolytic activity towards CM-cellulose only, fraction B, which showed activity towards CM-pachyman and CM-cellulose, and fraction C, which was active towards cellobiose and CM-pachyman.

The pooled fractions were dialysed until free of buffer salts and concentrated by partial slow-freezing. Suitable volumes of the concentrated fractions (A, 10 ml., 1.0 mg. of protein; B, 3 ml., 1.8 mg. of protein; C, 4 ml., 1.4 mg. of protein) were dialysed in 10/32 Visking tubing against frequently changed 5 mM-phosphate buffer, pH 5.8, for 6 hr. before analytical-scale chromatography on calcium phosphate under the same conditions as those used for the crude enzyme. The elution patterns of the chromatograms are shown in Figs. 3(b), 3(c) and 3(d).

Fraction B, which contained the main component hydrolysing CM-cellulose, was chosen for further preparative-scale fractionation. Analytical-scale fractionation on Dowex 1 indicated that the CM-cellulose-hydrolysing components could be well separated from the residual CM-pachyman-hydrolysing components.

Table 1. *Substrate specificity of fractions E and LE*

Reaction mixtures containing substrate (disaccharides, 0.5%; polymeric substrates, 0.7%) in Mollvaine buffer, pH 5.0 (5.6 ml.), and enzyme (1.4 ml.) were incubated at 40°. The amount of reducing sugar produced was measured at intervals. Results are expressed as μ g. of reducing sugar (as glucose) for the polymeric substrates and as μ g. of glucose for the disaccharides. N.D., Not determined.

Substrate	Fraction E					Fraction LE				
	2	5	8	27	53	2	5	8	27	53
Time of incubation (hr.)...										
Laminarin	0	0	0	4.7	14.6	0	0	0	0	0
CM-pachyman	0	0	0	3.4	10.3	0	0	0	0	0
Laminaribiose	0	0	0	2.1	1.4	0	0	0	0	0
Swollen cellulose	0	0	1.7	11.7	20.7	0	0	0	0	0
CM-cellulose	40	75	123	186	266	18	40	62	161	275
Cellobiose	0	0	0	0	0	0	0	0	0	0
β -(1 $\rightarrow$ 2)-Glucan	0	0	0	0	0	N.D.	N.D.	N.D.	N.D.	N.D.
Sophorose	0	1.4	1.0	1.4	0	0	0	0	0	0
Pustulan	0	0	0	0	0	N.D.	N.D.	N.D.	N.D.	N.D.
Gentiobiose	0	0	0	0	0	N.D.	N.D.	N.D.	N.D.	N.D.

Preparative-scale chromatography of fraction B on Dowex 1. Concentrated fraction B (240mg. of protein) was dialysed against 5mm-tris-hydrochloric acid buffer, pH 7.0, at 4° for 24hr. and applied to a column of Dowex 1 (70cm. $\times$ 4cm.). Elution was effected with 3l. of 5mm-tris-hydrochloric acid buffer, pH 7.0, followed by a chloride concentration gradient (mixing vessel: 18l. of 5mm-tris-hydrochloric acid buffer, pH 7.0; reservoir: 18l. of 0.4M-sodium chloride in 5mm-tris-hydrochloric acid buffer, pH 7.0). The flow rate was 1.5ml./min. and 500ml. fractions were collected manually.

The elution pattern of activity towards a number of β -(1 $\rightarrow$ 4)- and β -(1 $\rightarrow$ 3)-glucosidic substrates is shown in Fig. 4. A good measure of separation of the components hydrolysing the β -(1 $\rightarrow$ 4)- and the β -(1 $\rightarrow$ 3)-glucosidic substrates was attained. The fractions eluted between 0.22M- and 0.25M-chloride contained the greater part of the activity towards the β -(1 $\rightarrow$ 4)-glucosidic substrates. A subfraction from this group of fractions was obtained by pooling the eluate from 0.23M- to 0.26M-chloride, as shown in Fig. 4(a). This was designated fraction E and contained the components hydrolysing the polymeric β -(1 $\rightarrow$ 4)-glucosidic substrates, CM-cellulose, swollen cellulose and cellopentaose, but not cellobiose or salicin. This fraction was expected to be substantially free of activity towards the β -(1 $\rightarrow$ 3)-glucosidic substrates.

Substrate specificity range of fraction E. Table 1 shows the results of incubation of fraction E with disaccharides and polysaccharides containing β -(1 $\rightarrow$ 6)-, β -(1 $\rightarrow$ 4)-, β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 2)-glucosidic linkages. The reaction conditions were as follows: substrate (disaccharides, 0.5%; polymeric substrates, 0.7%) in Mollvaine buffer, pH 5.0 (5.6ml.),

was incubated with enzyme solution (1.4ml.) at 40°. Samples were removed after suitable incubation times, the reaction was stopped with barium hydroxide and zinc sulphate, and the amount of reducing sugar was estimated.

As expected, there was considerable hydrolysis of the polymeric β -(1 $\rightarrow$ 4)-glucan substrates, but there was also a low but detectable activity towards the β -(1 $\rightarrow$ 3)-glucosidic substrates. The remaining substrates tested were not hydrolysed.

Further information on the specificity range of fraction E was provided by investigating the identities of the products of hydrolysis of the series of β -(1 $\rightarrow$ 4)- and β -(1 $\rightarrow$ 3)-oligoglucosides by chromatography. Substrate (0.25% in water) (0.25ml.), enzyme (0.25ml.) and Mollvaine buffer, pH 5.0 (0.05ml.), were incubated together at 40°. At suitable intervals 0.1ml. samples were removed and applied to Whatman no. 3 paper. The chromatograms of the hydrolysis products of the β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-series of oligoglucosides were run in solvents B and A respectively. The results are summarized in Table 2.

Of the β -(1 $\rightarrow$ 4)-series of oligoglucosides tested, cellobiose was not hydrolysed, cellotriose was hydrolysed very slowly, cellotetraose gave cellobiose as the major product, and cellopentaose was hydrolysed to give predominantly cellobiose and cellotriose. These hydrolysis products are consistent with a random (endo-) action of the enzyme, and this conclusion is supported by evidence presented by Clarke & Stone (1965a).

On the other hand, during the hydrolysis of the β -(1 $\rightarrow$ 3)-series of oligoglucosides, glucose and oligosaccharide having one less glucose unit than the substrate were the first hydrolysis products to appear. This action is consistent with an endwise

Table 2. Action of fraction E on β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-oligoglucosides

Reaction mixtures containing oligoglucoside (0.25% in water) (0.25 ml.), enzyme (0.25 ml.) and McIlvaine buffer, pH 5.0 (0.05 ml.), were incubated at 40°. At the times indicated 0.1 ml. samples were removed and chromatographed, as described in the text. Abbreviations: G, glucose; tr., trace; —, substrate unchanged.

Substrate	Time of incubation (hr.) ...	1	2	5	8	24	48
β -(1 $\rightarrow$ 4)-Oligosaccharides							
Cellobiose (Cb)	—	—	—	—	—	—	—
Cellotriose (Ct)	—	—	—	—	—	—	Ct, G, (tr.) Cb
Cellotetraose (Ctet)	—	Ctet, Cb	—	—	Cb, G (tr.), Ct	Cb, G (tr.), Ct	Cb, G (tr.), Ct
					(tr.)	(tr.)	(tr.)
Cellopentaose (Cp)	—	Cp, Cb, Ct	Cb, Ct, G (tr.)	Cb, Ct, G (tr.)	Cb, Ct, G (tr.)	Cb, Ct, G (tr.)	Cb, Ct, G (tr.)
β -(1 $\rightarrow$ 3)-Oligosaccharides							
Laminaribiose (Lb)	—	—	—	—	—	—	G (tr.)
Laminaritriose (Lt)	—	—	—	—	Lt, Lb, G	Lt, Lb, G	Lb, G
Laminaritetraose (Ltet)	—	—	Ltet, Lt, G	Ltet, Lt, G	Ltet, Lt, G	Lt, Lb, G	Lb, G
Laminaripentaose (Lp)	—	—	Lp, Ltet, G	Ltet, Lt, G	Ltet, Lt, G	Lt, Lb, G	Lb, G

(exo-) attack by the enzyme involving the removal of single glucose units.

Attempts to inactivate or remove the residual β -(1 $\rightarrow$ 3)-glucan-hydrolase activity from fraction E. A number of attempts were made to remove or differentially inactivate the β -(1 $\rightarrow$ 3)-glucan-hydrolase activity and these were followed chromatographically.

Fractionation with ammonium sulphate did not separate the components and cation-exchange resins failed to adsorb either enzyme. Glucono-(1 $\rightarrow$ 5)-lactone (10mm) (Conchie & Levvy, 1957) and phenyl mercuric nitrate (Preece & Hoggan, 1956) were not effective inhibitors of the β -(1 $\rightarrow$ 3)-glucan-hydrolase activity.

Heating for 4 hr. at 75° inactivated the β -(1 $\rightarrow$ 4)-glucan-hydrolase activity completely, but the preparation retained its activity towards the β -(1 $\rightarrow$ 3)-glucosidic substrates, suggesting that the two types of activity in the preparation are due to different enzyme components.

The ability of Whatman powdered cellulose, pachyman and insoluble laminarin to adsorb the enzymes in fraction E was tested at several pH values: 3.5 and 5.0 (McIlvaine phosphate-citrate buffer), and 7.0 and 8.9 (tris-hydrochloric acid buffer). At the low pH cellulose adsorbs both components, whereas soluble laminarin at pH 3.5 preferentially removes the β -(1 $\rightarrow$ 3)-glucan-hydrolase component. Pachyman was not an adsorbent for either enzyme under the conditions tested.

Adsorption of β -(1 $\rightarrow$ 3)-glucan-hydrolase activity of fraction E on to insoluble laminarin. Insoluble laminarin was prepared for use as an adsorbent by exhaustive extraction with McIlvaine buffer diluted 1:4 (pH 3.5). When no further reducing substances were detected in the extracts the laminarin was dialysed against water and freeze-dried. Laminarin

treated in this way was not suitable for column chromatography, but batchwise adsorption under the following conditions was successful. Laminarin (1.7 g.) was made into a slurry with 5 ml. of McIlvaine buffer, pH 3.5, and 10 ml. of fraction E. After standing at 4° with periodic stirring for 2 hr. the laminarin was sedimented at 2000g and the supernatant treated in the same way with a further batch of laminarin. The final supernatant was dialysed against distilled water.

The substrate-specificity ranges of fraction E before and after laminarin adsorption are compared in Table 1. When the action of laminarin-adsorbed fraction E (designated fraction LE) was tested on the β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-oligoglucosides under the conditions used for fraction E, no hydrolysis of the β -(1 $\rightarrow$ 3)-oligoglucosides was observed but the β -(1 $\rightarrow$ 4)-oligoglucosides were hydrolysed to give the same products and in the same order as shown in Table 2.

DISCUSSION

The elution patterns of the hydrolases obtained on analytical-scale columns of calcium phosphate and Dowex 1 at 4° were similar to those found by Krishna Murti & Stone (1961) at room temperature. It was noted, however, that for both types of columns elution was effected at lower concentrations of eluent at the lower temperature.

Components hydrolysing β -(1 $\rightarrow$ 3)-glucosidic linkages. Among β -(1 $\rightarrow$ 3)-glucan hydrolases so far described from micro-organisms there occur two types: endo- β -(1 $\rightarrow$ 3)-glucan hydrolases [β -(1 $\rightarrow$ 3)-glucan 3-glucanohydrolases, EC 3.2.1.6] (Reese & Mandels, 1959; Mandels, Miller & Slater, 1961; Chesters & Bull, 1963) and exo- β -(1 $\rightarrow$ 3)-glucan hydrolases [β -(1 $\rightarrow$ 3)-glucan 3-glucosylhydrolases]

(Reese & Mandels, 1959; Mandels *et al.* 1961; Chesters & Bull, 1963; Nelson, Scalletti, Smith & Kirkwood, 1963). Occasionally only one type is produced by the organism but more often both types occur together in the culture filtrates (Reese & Mandels, 1959; Chesters & Bull, 1963), and this appears to be the case with *A. niger*.

Krishna Murti & Stone (1961) noted two groups of components hydrolysing laminarin, one of which was associated with cellobiose-hydrolysing activity in eluates from both types of column. The present experiments with additional β -(1 $\rightarrow$ 3)-glucosidic substrates suggest the presence of at least three different groups of components hydrolysing β -(1 $\rightarrow$ 3)-glucosidic substrates. They are most clearly shown in eluates from Dowex 1 columns (Fig. 2b).

No definite conclusions can be drawn on the action patterns of these three groups of β -(1 $\rightarrow$ 3)-glucan-hydrolase components from *A. niger*. However, the nature of the substrates hydrolysed by each group clearly shows a difference in the specificity of the enzymes involved. Thus the substrate specificity of the group eluted at 0.09–0.13M-chloride is consistent with the presence of glucanohydrolases or conceivably with the presence of a glucohydrolase whose lower substrate range does not include laminaribiose and whose action was not blocked by the presence of carboxymethyl or phosphate substituents as found in the CM-pachyman and pachy-dextrin substrates.

The second group of components eluted at 0.13–0.18M-chloride hydrolyses laminaribiose and laminarin but not CM-pachyman or pachydextrins and is associated with fractions hydrolysing cellobiose and salicin. The possibility that this situation is due to a glucohydrolase component with a wide specificity range with respect to the position of the glycosidic linkage, i.e. to a β -glucoside glucohydrolase or to the presence of physically similar enzymes having different linkage specificities, has been discussed by Krishna Murti & Stone (1961). The failure to attack CM-pachyman and soluble pachydextrins might be attributed to the blocking of hydrolysis by the presence of substituted glucose residues near the non-reducing ends of these substrates or to the lowered affinity of the enzyme for the substrate with a higher degree of polymerization. The action pattern of the third group of components eluted at 0.34–0.39M-chloride and hydrolysing laminaribiose and β -(1 $\rightarrow$ 3)-glucan substrates with a higher degree of polymerization but not cellobiose or salicin is not apparent but it is clearly different from the first and second components.

The existence of a fourth component hydrolysing β -(1 $\rightarrow$ 3)-glucosidic linkages was made evident when fraction E was tested against the β -(1 $\rightarrow$ 3)-oligoglucoside series. Its pattern of hydrolysis of these substrates is consistent with the presence of a

glucohydrolase having a minimum requirement of three glucose units in the substrate.

Compared with the activities of the β -(1 $\rightarrow$ 4)-glucan hydrolases the activities of the *A. niger* β -(1 $\rightarrow$ 3)-glucan hydrolases are low and the number and complexity of their components make their further separation a formidable task.

Components hydrolysing β -(1 $\rightarrow$ 4)-glucosidic linkages. Krishna Murti & Stone (1961) distinguished two groups of components hydrolysing β -(1 $\rightarrow$ 4)-glucosidic linkages in the crude *A. niger* preparation. These were tentatively identified as β -(1 $\rightarrow$ 4)-glucan 4-glucanohydrolases (EC 3.2.1.4) and β -(1 $\rightarrow$ 4)-glucan glucohydrolases. By successive calcium phosphate and Dowex 1 chromatography it has now been possible to obtain a fraction (E) that hydrolyses the β -(1 $\rightarrow$ 4)-oligoglucosides in a pattern typical of a glucanohydrolase (Clarke & Stone, 1965a) and with a requirement for a minimum chain length of four glucose units. The small amount of β -(1 $\rightarrow$ 3)-glucan-hydrolase activity present in this fraction was selectively removed by adsorption on to laminarin at pH 3.5. Further characteristics of this enzyme and a discussion of its properties appear in the next paper (Clarke & Stone, 1965a).

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