Properties of a β -(1 $\rightarrow$ 4)-Glucan Hydrolase from *Aspergillus niger*

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(Received 23 November 1964)

1. A β -(1 $\rightarrow$ 4)-glucan hydrolase prepared from *Aspergillus niger*, as described by Clarke & Stone (1965a), showed a pH optimum in the range 4.5–6 and K_m 0.25% when acting on a cellulose dextrin sulphate substrate. 2. The hydrolase rapidly decreased the specific viscosity of carboxymethylcellulose with a small increase in the production of reducing sugars. The identity of the products of hydrolysis of cellotetraose, cellopentaose and their reduced analogues indicate a preferential cleavage of non-terminal glucosidic linkages. The enzyme may be described as β -(1 $\rightarrow$ 4)-glucan 4-glucanohydrolase (EC 3.2.1.4). 3. In addition to carboxymethylcellulose, cellulose dextrans, cellopentaose and cellotetraose the enzyme fraction hydrolysed lichenin, oat and barley glucans, ivory-nut mannan and a glucomannan from Konjak flour. No hydrolysis of wheat-straw β -(1 $\rightarrow$ 4)-xylan, *Lupinus albus* β -(1 $\rightarrow$ 4)-galactan, pneumococcal type III polysaccharide, chitin, hyaluronic acid, laminarin, pachydextrins, carboxymethylpachyman or β -(1 $\rightarrow$ 3)-oligoglucosides was detected. 4. The hydrolase showed no transglycosylase activity from cello-dextrin or cellopentaose substrates to glucose or methanol acceptors. 5. The hydrolysis of cellodextrins was inhibited completely by 1.0 mM-Hg²⁺, 0.7 mM-phenylmercuric nitrate and 1.0 mM-iodine.

The isolation of a fraction containing an active β -(1 $\rightarrow$ 4)-glucan hydrolase from a commercial preparation of the extracellular enzymes of *Aspergillus niger* has been described in the preceding paper (Clarke & Stone, 1965a). Some properties of this fraction are described below.

MATERIALS AND METHODS

Substrates. The β -(1 $\rightarrow$ 4)-glucosidic substrates used were those described by Clarke & Stone (1965a). The two cellodextrin samples used had D.P.† 58 and 125 and sulphate contents 6.04 and 6.11% respectively. Reduced β -(1 $\rightarrow$ 4)-oligoglucosides were prepared by treating oligoglucoside (100 mg.) with 200 mg. of NaBH₄ in 10 ml. of water at 30° for 24 hr., deionizing with Bio-Deminrolit (HCO₃-form) (The Permutit Co. of Australia, Sydney, New South Wales, Australia) and freeze-drying. The reduced oligoglucosides migrated as single compounds in solvent A and failed to give the aniline-diphenylamine reaction (Buchan & Savage, 1952). Ivory-nut mannan was kindly provided by Dr M. A. Jermyn, C.S.I.R.O., Division of Protein Chemistry, Melbourne; chitin by Mr M. V. Tracey, C.S.I.R.O., Wheat Research Unit, Sydney; pneumococcal type III polysaccharide (E. R. Squibb, lot 186) by Dr M.

Heidelberger, Rutgers University, N.J., U.S.A.; hyaluronic acid by Dr D. Lowther, Monash University, Melbourne, and the mannobiose and mannotriose markers by Dr C. T. Bishop, National Research Council, Ottawa, Canada. Wheat-straw xylan was prepared according to the method of Aspinall & Mahomed (1954). A glucomannan was prepared from Konjak flour kindly provided by Professor F. Smith by the method of Smith & Srivastava (1959). A β -(1 $\rightarrow$ 4)-galactan from *Lupinus albus* seeds containing residual pectin and araban was prepared by the method of Hirst, Jones & Walder (1947). The pectin was removed by passing a solution of the crude galactan, which had been dialysed successively against EDTA (sodium salt), water and 25 mM-phosphate buffer, pH 5.5, through a column of DEAE-cellulose that had been equilibrated with the same phosphate buffer. The initial carbohydrate-positive fractions were pooled, dialysed and freeze-dried. Chromatography in solvent D (see below), after complete hydrolysis in nitric acid (Jermyn & Isherwood, 1956), showed galactose, a trace of arabinose but no uronic acid. [¹⁴C]Glucose of high specific activity was obtained from The Radiochemical Centre, Amersham, Bucks.

Enzyme preparations. Fraction E, and fraction E after treatment with laminarin (referred to as fraction LE), were those described by Clarke & Stone (1965a).

Enzyme assays. The β -(1 $\rightarrow$ 4)-glucan-hydrolase activity was measured by the amount of reducing sugar produced under the reaction conditions described in the text. The reducing sugar was determined as glucose by the Somogyi-Nelson colorimetric method (Somogyi, 1952; Nelson, 1945). Under these conditions zero-order kinetics were

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† Abbreviation: D.P., degree of polymerization.

obeyed. Viscometric assays were made by using the method described by Clarke & Stone (1962).

In constructing the pH-activity curves the activity was measured by incubating 0.8 ml. of phosphate-citrate buffer, 0.8 ml. of substrate [sodium CM-cellulose (1.37%) or celloextrins (D.P. 58) (0.5%)] and 0.4 ml. of enzyme at 40°. After incubation for 7 hr. the action on CM-cellulose was stopped by heating the incubation mixture in a boiling-water bath for 10 min. After cooling, a 1 ml. sample was removed and made just alkaline to phenolphthalein by the dropwise addition of *N*-NaOH; the phenolphthalein colour was then just discharged by the addition of 0.5 *N*-HCl and the final volume adjusted to 2.0 ml. The reducing sugar was measured in 1 ml. samples by the Somogyi-Nelson method. Incubations with celloextrins were stopped after 11 hr. by the addition of 1 ml. of 0.3 *N*-Ba(OH)₂ and 1 ml. of 5% (w/v) ZnSO₄ (Somogyi, 1945) and the amounts of soluble sugar in the supernatant solutions determined as glucose by the anthrone method (Trevelyan & Harrison, 1952).

Chromatography. Chromatograms were run on Whatman no. 3 paper by downward development. The solvents 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.); solvent *C*, butan-1-ol-pyridine-water (6:4:3, by vol.); solvent *D*, ethyl acetate-pyridine-water (2:2:1, by vol.); solvent *E*, butan-1-ol-ethanol-water-aq. ammonia (sp.gr. 0.88) (40:10:49:1, by vol.); solvent *F*, 0.6% formic acid in 72% (v/v) ethanol; solvent *G*, butan-1-ol-acetic acid-water (50:12:25, by vol.); solvent *H*, butan-1-ol-pyridine-water (10:1:2, by vol.).

Sugars and sugar alcohols 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). Reducing sugars in the presence of sugar alcohols were detected by the aniline-diphenylamine reagent (Buchan & Savage, 1952).

RESULTS

pH-activity relationships. The effect of pH on the activity of fraction LE with CM-cellulose and celloextrin (D.P. 58) substrates and phosphate-citrate buffers in the range pH 3.0–8.0 is shown in Fig. 1. The enzyme showed a broad optimum in the acid range, pH 4.5–6.0, with celloextrins and a narrower optimum, pH 4.0–4.5, with CM-cellulose.

Substrate concentration-activity relationships. The activity of fraction E at various concentrations of celloextrins (D.P. 125) is shown in Fig. 2(a). The

Fig. 1. Effect of pH on the activity of fraction LE. Activity was measured by the amount of reducing sugar (as glucose) produced during incubation of enzyme (0.4 ml.), phosphate-citrate buffer (0.8 ml.) and substrate (0.8 ml.). Substrates used were CM-cellulose (1.37%) (○) and celloextrins (D.P. 58) (0.5%) (●).

Fig. 2. (a) Effect of substrate concentration on the activity of fraction LE. Activity was measured by the amount of reducing sugar (as glucose) produced during incubation of enzyme (0.2 ml.) and celloextrins (D.P. 125) in phosphate-citrate buffer, pH 5.0 (0.8 ml.), to give the final concentrations shown. After incubation for 2.5 hr. at 40° the reaction was stopped by the addition of 0.3 *N*-Ba(OH)₂ (1 ml.) and 5% ZnSO₄ (1 ml.). The reducing sugar in the supernatant was measured and corrected for the reducing value of the substrate at each concentration. (b) Plot of 1/*v* against 1/*s* for the same data. Several points are omitted at high substrate concentrations for clarity.

Lineweaver-Burk reciprocal plot obtained from these data is shown in Fig. 2(b).

Effect of inhibitors. The effect of various inhibitors on the activity of fraction E towards cellodextrins (D.P. 125) is shown in Table 1.

Action pattern. The time-course of hydrolysis of CM-cellulose by fraction LE was followed viscometrically and reductometrically. The results are compared in Fig. 3. When fraction LE was tested for hydrolytic activity against the β -(1 $\rightarrow$ 4)-series of oligoglucosides, 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 (Table 2 in Clarke & Stone, 1965a). A similar experiment was conducted with the series of alcohols derived from the β -(1 $\rightarrow$ 4)-oligoglucosides. The results are shown in Table 2.

Substrate specificity. The ability of fraction LE to hydrolyse a number of β -glucosides and β -glycans was tested by chromatographic examination of the

enzyme-substrate mixture for the products of hydrolysis after incubation for 48 hr., and in certain cases by measuring the change in viscosity of the enzyme-substrate mixture.

Chromatographically there was no detectable hydrolysis of wheat-straw xylan (solvent C), a β -(1 $\rightarrow$ 4)-galactan (solvent D), pneumococcal type III polysaccharide (solvent F), chitin (solvent E), hyaluronic acid (solvent G), laminarin (solvent B), pachydestrins (solvent B), carboxymethylpachyman (solvent B), or the β -(1 $\rightarrow$ 3)-oligoglucosides (solvent B). The β -(1 $\rightarrow$ 4)-mannan from ivory nuts was hydrolysed to give two main products, which correspond to the mannobiose and mannotriose markers, and a trace of a reducing substance running as mannose (solvent C). Konjak glucomannan was hydrolysed to give five distinct reducing substances, one of which corresponds to mannobiose (R_{mannose} 0.59) and the others had R_{mannose} 0.45, 0.35, 0.22 and 0.04 (solvent C). Lichenin, oat glucan and barley glucan were also hydrolysed (Clarke & Stone, 1965b).

Viscosity measurements on the pneumococcal type III polysaccharide and hyaluronic acid enzyme-substrate mixtures showed a 3% decrease in η_{sp} for pneumococcal type III polysaccharide and a 5% decrease for the hyaluronic acid during a 24 hr. incubation. These changes are not considered significant. Under comparable conditions

Table 1. *Effect of various inhibitors on fraction E*

The incubation mixture contained: 0.2% cellodextrins (D.P. 125), 0.4 ml.; 0.5 M-phosphate-citrate buffer, 0.1 ml.; inhibitor, 0.4 ml.; enzyme, 0.1 ml. The enzyme was added 5 min. after the addition of inhibitor to substrate and buffer. After incubation for 2.5 hr. at 40° the reaction was stopped by the addition of 0.3 N-Ba(OH)₂ (1 ml.) and 5% ZnSO₄ (1 ml.) and the reducing sugar estimated by the Somogyi-Nelson method.

Inhibitor	Final concn. in reaction mixture (mm)	Inhibition (%)
Mn ²⁺	1.0	0
Hg ²⁺	0.1	87
	1.0	97
Cu ²⁺	0.1	0
	1.0	0
	10.0	23
<i>p</i> -Chloromercuribenzoate	0.1	0
Iodoacetate	0.1	0
	1.0	10
Phenylmercuric nitrate	0.1	31
	0.7	100
Di-isopropyl phosphorofluoridate	0.001	0
	0.1	0
Oxidized glutathione	2.5	0
	10.0	0
Glucono-(1 $\rightarrow$ 5)-lactone	2.5	0
EDTA	5.0	0
Iodine	1.0	93
Serum albumin	0.01%	0

Fig. 3. Time-course of hydrolysis of CM-cellulose followed viscometrically and reductometrically. Fraction LE (2 ml.) and CM-cellulose [8 ml. of a 0.7% solution in phosphate-citrate buffer, pH 5.0, containing EDTA (5.0 mm) and NaCl (0.2 M)] were separately brought to 40° and mixed. One portion (1.95 ml.) was immediately transferred to a viscometer and the decrease in viscosity measured during incubation at 40°. The remainder of the enzyme-substrate mixture was incubated at 40°; 1 ml. samples were withdrawn at suitable intervals and the amounts of reducing sugar present measured as glucose.

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

Reaction mixtures containing 0.25% reduced oligoglucoside (1.8 ml.), enzyme (1.8 ml.) and phosphate-citrate buffer, pH 5.0 (0.36 ml.), were incubated at 40°. At the times indicated 0.6 ml. samples were removed, heated on a boiling-water bath for 10 min., cooled and evaporated to dryness in a desiccator over P₂O₅. The dry residue was dissolved in 100 μ l. of water and chromatographed in solvent A, as described in the text. Abbreviations: G, glucose; G-G, cellobiose; G-G-G, cellotriose; G-S, cellobi-itol; G-G-S, cellotri-itol; tr., trace; —, substrate unchanged.

Substrate	Time of incubation (hr.)	...	2	5	8	24	48
Cellobi-itol		—	—	—	—	—	—
Cellotri-itol		—	—	—	G (tr.)	G (tr.), G-S (tr.)	G (tr.), G-S (tr.)
Cellotetraitol		—	—	—	G (tr.), G-S (tr.)	G (tr.), G-S (tr.), G-G-S (tr.)	G (tr.), G-S (tr.), G-G-S (tr.)
Cellopentaitol		*G-G-G, G-G, G-S, G-G-S	G-G-G, G-G, G-S, G-G-S	G (tr.), G-G-G, G-G, G-S, G-G-S	G (tr.), G-G-G, G-G, G-S, G-G-S	G (tr.), G-G-G, G-G, G-S, G-G-S	G (tr.), G-G-G, G-G, G-S, G-G-S

* Cellopentaitol present.

there was a 95% decrease in the η_{sp} for CM-cellulose in 24 hr.

Transglycosylase activity. The possibility of enzymic transglycosylation from oligosaccharides to glucose was tested by incubating glucose (4 mg.) and [¹⁴C]glucose (1 μ C; specific activity 484 μ C/mg.) alone, and with either cellopentaose (0.6 mg.) or celloextrins (D.P. 125) (0.6 mg.) in 45 μ l. of phosphate-citrate buffer, pH 5.0, with 10 μ l. of fraction LE for 19 hr. at 40°. The reaction mixtures were applied to Whatman no. 3 paper and chromatographed in solvent A. Strips were cut from the paper and scanned in a Nuclear-Chicago Actigraph II (model C100B) and also radioautographed on Ilford X-ray film with a 7-day exposure. No radioactive areas other than a spot corresponding to a glucose marker were detected by either method on any of the strips. Reaction mixtures with cellopentaose or celloextrins and enzyme, but without added glucose, were incubated and chromatographed as controls. The hydrolysis products detected by alkaline silver nitrate from cellopentaose were cellobiose and cellotriose with small amounts of glucose and cellotetraose; from celloextrins glucose, cellobiose and higher oligosaccharides were detected.

The possibility of transglycosylation to methanol was also tested by incubating cellopentaose (1.25 mg./ml.) in the presence of methanol (1M) and enzyme (0.7 ml.) in 20 mm-phosphate-citrate buffer, pH 5.0. Samples were removed at intervals up to 42 hr., applied to Whatman no. 3 paper and chromatographed in solvent H. With the alkaline silver nitrate reagent cellobiose, cellotriose and traces of glucose were detected but no spots corresponding to a methyl β -glucoside marker or with the $R_{glucose}$ of methyl β -cellobioside (Nisizawa & Hashimoto, 1959).

DISCUSSION

The range of soluble polymeric β -(1 $\rightarrow$ 4)-glucoside substrates suitable for kinetic studies on β -(1 $\rightarrow$ 4)-glucan hydrolases is limited to partially substituted, more or less degraded celluloses. The substrates used in the present study were prepared by ethanol fractionation of a sulphuric acid hydrolysate of cellulose. The sulphur contents of the two fractions used (6.04 and 6.11%) corresponded to degrees of substitution by sulphate of 0.36 and 0.37 respectively. The use of these sulphated celloextrins is probably subject to similar limitations as is the use of CM-cellulose: the linkages may not all be equally susceptible to enzymic hydrolysis (Holden & Tracey, 1950) and the substrate may act as a complex-forming agent (Datta, Hanson & Whitaker, 1961) in inhibition studies.

The plot of $1/v$ against $1/s$ (Fig. 2b) obtained by using data for the lower substrate concentrations gives a value for K_m , the substrate concentration at half-maximum rate of hydrolysis, 0.25%. These plots, however, show a decrease in rate of hydrolysis in the higher substrate concentration range. A similar but more marked decrease in reaction velocity with increase in substrate concentration is shown by the *Trichoderma viride* β -(1 $\rightarrow$ 4)-glucan hydrolase acting on CM-cellulose, but not on cellulose sulphate (Mandels & Reese, 1963). This type of kinetic behaviour has been generally interpreted in terms of inhibition of the enzyme by high concentrations of the substrate.

Action pattern. The rapid decrease in specific viscosity of CM-cellulose accompanied by a small increase in the production of reducing sugar (Fig. 3) is compatible with a random hydrolysis of this substituted cellulose. The products formed during the hydrolysis of the β -(1 $\rightarrow$ 4)-oligoglucosides

(Clarke & Stone, 1965a) and their reduced analogues are also consistent with an endo-action pattern for the *A. niger* enzyme. Both cellopentaose and cellopentaol are hydrolysed to give products that would be expected from the cleavage of linkages 2 and 3 as shown:

No conclusions about the frequency of cleavage of these two linkages can be drawn from the chromatographic data. Cellotetraose was hydrolysed more slowly than cellopentaose. The hydrolysis occurred preferentially at linkage 2, although other cleavages appear to occur to a slight extent. Cellotriose and cellotri-itol were extremely slowly hydrolysed, cellobiose and cellobi-itol not at all. These findings indicate that the enzyme has a preference for the hydrolysis of non-terminal linkages in those oligosaccharides that are substrates. Cellotetraose is the smallest oligosaccharide substrate having a non-terminal linkage, but reduction of the terminal glucose residue to a sorbitol residue effectively prevents its hydrolysis. In Jermyn's (1962) nomenclature both the 'head' and 'tail' specificities of the enzyme are fulfilled by two glucose units. The enzyme may be classified as a β -(1 $\rightarrow$ 4)-glucan 4-glucanohydrolase (EC 3.2.1.4).

The production of small amounts of glucose from cello-pentaose, -tetraose and -triose, as well as from cello-pentaol, -tetraol and -tri-itol, indicates that linkages to terminal non-reducing glucose residues are slowly hydrolysed. Whether this represents the activity of the endo-glucan hydrolase or is due to the presence of traces of an enzyme with a different action pattern has not been resolved. No sorbitol was detected in any of the reaction products, indicating that attack at the glucosyl-sorbitol linkage in the reduced oligosaccharides does not occur.

Glycosyl group specificity. The β -(1 $\rightarrow$ 4)-xylan and the β -(1 $\rightarrow$ 4)-galactan were not hydrolysed by the purified enzyme, indicating that the enzyme requires both the primary alcoholic group at C-5 and the glucose configuration at C-4 for activity. Further, as chitin is not hydrolysed, the substitution of an acetamido group for the hydroxyl at C-2 is not tolerated by the enzyme, although it is possible that a simpler substitution may be acceptable. Both the pneumococcal type III polysaccharide and hyaluronic acid contain potentially hydrolysable β -glycosidic linkages, but these are not split by the *A. niger* enzyme. Both the nature of the glycosyl residues and the position of the glycosidic linkages in these polysaccharides could contribute to their resistance to hydrolysis.

The hydrolysis of the β -(1 $\rightarrow$ 4)-mannan indicates

that the enzyme may be able to tolerate the inversion of the configuration of the hydroxyl at C-2; alternatively, a specific β -(1 $\rightarrow$ 4)-mannan hydrolase may be present. This last possibility cannot be excluded on the present evidence, for although the enzyme fraction used was highly purified it is not claimed to contain a single enzyme. The hydrolysis of the glucomannan to give mannobiose and a series of higher oligosaccharides probably containing both glucose and mannose could again be due to the action of the β -(1 $\rightarrow$ 4)-glucan hydrolase or to the combined action of specific β -(1 $\rightarrow$ 4)-glucan and β -(1 $\rightarrow$ 4)-mannan hydrolases. The hydrolysis of the glucans containing β -(1 $\rightarrow$ 4)- and β -(1 $\rightarrow$ 3)-linkages is discussed by Clarke & Stone (1965b).

The ability of fungal β -(1 $\rightarrow$ 4)-glucan hydrolases to catalyse transglycosylation has been shown to vary with the source of the enzyme (Whitaker, 1956; Nisizawa, Hashimoto & Shibata, 1963). Under conditions comparable with those used by Nisizawa *et al.* (1963) to demonstrate transglycosylation by an *Irpex lacteus* enzyme, no evidence of transglycosylation from cello-dextrins to glucose or methanol by the *A. niger* enzyme was obtained.

The inhibition studies indicate that, on the whole, the *A. niger* β -(1 $\rightarrow$ 4)-glucan hydrolase is relatively unaffected by a wide range of enzyme inhibitors. Thiol reagents such as *p*-chloromercuribenzoate, iodoacetate and oxidized glutathione were ineffective, although phenylmercuric nitrate (0.7mM) completely inhibited the enzyme. Hg^{2+} (0.1mM) and iodine (1mM) were the other effective inhibitors. Di-isopropyl phosphorofluoridate was without activity, suggesting that serine hydroxyl groups are not involved at the active site. The inhibition behaviour of other fungal β -(1 $\rightarrow$ 4)-glucan hydrolases has been summarized by Mandels & Reese (1963) and may be compared with the present findings.

The authors thank Professor V. M. Trikojus for his interest during the course of this work. A. E. C. was in receipt of a Commonwealth Post-Graduate Scholarship and the investigation was financially supported by Barrett's Food Co. (Pty) Ltd.

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