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A β-1,3-GLUCAN HYDROLASE FROM *NICOTIANA GLUTINOSA* II. SPECIFICITY, ACTION PATTERN AND INHIBITOR STUDIES

A. E. MOORE AND B. A. STONE

Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria 3052 (Australia)

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SUMMARY

1. It has been shown that the β-1,3-glucan hydrolase (β-1,3-glucan glucanohydrolase, EC 3.2.1.39) from *Nicotiana glutinosa* has an endo-action pattern and is highly specific for linear β-1,3-glucan substrates. These glucans are depolymerized to yield a mixture of β-1,3-oligoglucosides of degree of polymerization 2–7 and a trace of glucose.

2. Laminaribiose and -triose are not hydrolysed by the enzyme and laminaritetraose and -pentaose are only slowly attacked. The enzyme does not hydrolyse Claviceps β-glucan and the rate of hydrolysis of *O*-carboxymethylpachyman (CM-pachyman) decreases as the degree of substitution increases. These findings suggest a requirement for at least three unsubstituted β-1,3-glucosyl residues in substrates of this enzyme. The very limited hydrolysis of the β-1,3;1,4-glucans from cereals is probably due to action at the few consecutive β-1,3-linked glucose residues which are present in these substrates.

3. No transglycosylase activity was detected.

4. A method for purifying the *Rhizopus arrhizus* β-1,3-glucan endohydrolase is described and its action on a series of β-glucan substrates is compared with that of the Nicotiana enzyme.

5. The Nicotiana enzyme was inhibited by Hg^{2+} (0.2 mM), Cu^{2+} (20 mM), phenylmercurinitrate (2.0 mM), I_2 in KI (2.0 mM), *N*-bromosuccinimide (5 mM), 2-hydroxy-5-nitrobenzylbromide (10 mM), *N*-acetylimidazole (23 mM), carbodiimide (100 mM), and the *Lespedeza cuneata* cellulase inhibitor (0.2 mg/ml).

INTRODUCTION

This paper describes the specificity and action pattern of a β-1,3-glucan hydrolase (β-1,3-glucan glucanohydrolase, EC 3.2.1.39) from *Nicotiana glutinosa* leaves and the effect of a number of inhibitors on its activity. The extraction and purification of the enzyme are described in the accompanying paper[1].

Abbreviation: CM-pachyman, *O*-carboxymethylpachyman.

EXPERIMENTAL PROCEDURE

Substrates

Insoluble pachydextrins. These were obtained by centrifuging the undissolved material remaining after phosphoric acid hydrolysis of pachyman by the procedure described for the preparation of soluble pachydextrins[2].

β-1,3-Xylan. The sample, prepared by Dr. A. E. Clarke, was the residue remaining after exhaustive extraction of *Caulerpa brownii* cell walls and consisted almost entirely of β-1,3-xylan.

Lichenin. Sample A was prepared as previously described[3]. Sample B was a gift of Professor D. J. Manners, Department of Brewing and Biochemistry, Heriot-Watt University, Edinburgh, Scotland[4].

Eisenia bicyclis laminarin[5,6]. The sample was a gift of Professor K. Nisizawa, Botanical Institute, Tokyo University of Education and was dialysed before use to remove oligosaccharides.

Lutean. The sample was prepared from *Penicillium luteum* culture filtrates[7].

Yeast glucan. Baker's yeast was extracted as described by BELL AND NORTHCOTE[8] and the residue was treated with salivary amylase and deproteinized[9].

Claviceps fusiformis glucan[10]. This glucan was the gift of Dr. K. W. Buck, Imperial College, London.

Dispersed pachyman, Caulerpa xylan and lutean were prepared essentially as described for dispersed paramylon[11].

Other glucans and glucosides. The following compounds were obtained as described in the references quoted: *O*-carboxymethylpachyman[12,13], insoluble laminarin[11], pachyman[11], β-1,3-oligoglucosides[11], paramylon granules[14], Mycoplasma β-1,2-glucan[2], carboxymethylcellulose[3], oat and barley glucans[15], β-1,4-oligoglucosides[3], sophorose[2], methyl β-glucoside[16], *p*-nitrophenyl β-glucoside[17].

Soluble starch, salicin, cellobiose and gentiobiose were commercial preparations.

Enzymes

N. glutinosa β-1,3-glucan hydrolase. Purified enzyme samples (Preparations A and C) were obtained as described in the accompanying paper[1]. Preparation A was used except where otherwise specified.

Rhizopus arrhizus β-1,3-glucan hydrolase. A culture of *R. arrhizus* donated by Dr. E. T. Reese, U.S. Quartermasters Laboratory, Natick, Mass., U.S.A. was grown as described by REESE AND MANDELS[18]. The β-1,3-glucan hydrolase from the culture filtrate was partially purified as follows: The culture filtrate was concentrated by rotary evaporation at pH 4.8 and 40° and was then added to a column of CM-cellulose in 0.05 M acetate buffer (pH 5.0). The enzyme was eluted in a gradient of Na_2SO_4 in the same buffer, dialysed against distilled water and freeze-dried. Further purification was achieved by chromatography on Bio-Gel P-200 in 0.05 M acetate buffer (pH 5.0) and the recovered enzyme was stored frozen.

Euglena gracilis β-1,3-glucan hydrolase. The enzyme was the purified preparation described by BARRAS AND STONE[19].

Inhibitors

Reagents used in inhibition studies were commercial analytical grade reagents

or were obtained as follows: (1→5) gluconolactone (British Drug Houses, Poole, England); tosyl lysyl chloroketone (Calbiochem, Los Angeles, U.S.A.); *N*-ethylmaleimide, *N*-acetylimidazole, oxidized glutathione, *N*-bromosuccinimide (Sigma Chemical Co., St. Louis, Mo., U.S.A.); mercaptoethanol (Eastman Kodak Co., Rochester, N.Y.); 2-hydroxy-5-nitrobenzylbromide (Pierce Chemicals, Ill.); 1-cyclohexyl-3-(2-morpholino-ethyl)-carbodiimide metho-*p*-toluene sulfonate (Aldrich Chem. Co. Inc., Milwaukee, Wisc.); conduritol B epoxide (gift of Dr. M. A. Jermyn, C.S.I.R.O., Division of Protein Chemistry, Parkville, Victoria); cellulase inhibitor from *Lespedeza cuneata* (gift of Dr. T. A. Bell, Nth. Carolina Agricultural Experiment Station, Raleigh.)

Radiochemicals

^{14}C-labelled D-glucose (specific activity 15.6 μC/mg) was purchased from the Radiochemical Centre, Amersham, Bucks., U.K.

Enzyme assays

Reductometric assays were performed as described in the accompanying paper[1] and viscometric assays by the method described previously[12] using 1% substrate in 0.05 M acetate buffer (pH 5.0), 5 mM EDTA, 0.2 M NaCl, 6.25 mM NaN_3 and 20 μg/ml bovine serum albumin.

Chromatography

The nature of the products of enzymic hydrolysis was examined by the following procedure. 5 vols. of substrate (1% (w/v) polysaccharide or 0.25% (w/v) oligosaccharide) were mixed with 5 vol. of enzyme and 1 vol. of 0.05 M acetate buffer (pH 5.0). The mixtures, which also contained bovine serum albumin and NaN_3 at final concentrations of 20 μg per ml and 5 mM, respectively, were incubated at 40° and were shaken during the incubation when the substrate was insoluble. At zero time and after suitable incubation periods, samples (0.1 or 0.2 ml) were removed, heated in a boiling water bath for 3 min to inactivate the enzyme, cooled and deionised, either with Bio-Deminrolit (The Permutit Co. of Australia, Sydney, N.S.W.) or with Dowex mixed-bed resin (AG 501-X8) in the HCO_3^- form[20]. The supernatant was combined with two 0.1 ml water washings of the resin and was dried in a vacuum desiccator. The residue was redissolved in 50 μl water and applied to a sheet of Whatman No. 3 paper for chromatography.

Chromatograms were developed by descending chromatography in a propan-1-ol–ethyl acetate–water (6:1:3, by vol.) solvent. For separation of β-1,4-oligoglucosides pentan-1-ol–pyridine–water (1:1:1, by vol., organic phase) was used. Reducing sugars on the chromatograms were detected with the alkaline $AgNO_3$ reagent[21].

Inhibitor assays

Procedure A. Two volumes of a 1:100 (v/v) dilution of the enzyme in 0.1 M acetate buffer (pH 5.0) containing 100 μg bovine serum albumin per ml was allowed to react for 30 min at room temperature with 3 vol. of a solution of the inhibitor in water adjusted to pH 5.0. The enzyme was then assayed by the reductometric procedure, following addition of 5 vol. of 1.6% (w/v) laminarin in 0.2 M acetate buffer (pH 5.0). Activity was compared with that of a control in which the inhibitor solu-

tion was replaced by water and in all cases appropriate enzyme, substrate and inhibitor blanks were estimated.

Procedure B. 2 vols. of a 1:40 (v/v) dilution of the enzyme in 0.05 M acetate buffer (pH 5.0) containing 250 μg bovine serum albumin per ml was allowed to react with 3 vol. of inhibitor solution as described for Procedure A. Enzyme activity was then estimated viscometrically, following the addition of 20 vol. of *O*-carboxymethylpachyman (CM-pachyman) substrate solution. Initial viscosities were measured using substrate–inhibitor mixtures without enzyme. The time (*T*) required for 10% reduction in specific viscosity was determined and enzyme activity was compared with the control value[12].

Procedure C for assay of inhibition by 2-hydroxy-5-nitrobenzyl bromide. 1 vol. of 0.2 M 2-hydroxy-5-nitrobenzyl bromide in acetone was added to 20 vol. of a 1:10 (v/v) dilution of the enzyme in 0.5 M acetate buffer (pH 5.0) containing 20 μg bovine serum albumin per ml. After 30, 60 and 120 min in the dark at room temperature, samples of the mixture were diluted 10-fold with aqueous bovine serum albumin (100 μg/ml) and enzyme activities were assayed reductometrically with laminarin as substrate. The 2-hydroxy-5-nitrobenzyl bromide solution was replaced by acetone in control assays and enzyme, substrate and inhibitor blanks were also estimated.

In a second experiment an enzyme–inhibitor mixture was prepared as described above and after 30 min, an additional volume of the reagent was added. After a further 30 min the sample was diluted and assayed as before.

Procedure D for assay of inhibition by N-acetylimidazole. The inhibitor was stored in dry benzene and evaporated to dryness immediately before use. For the inhibition experiment solid *N*-acetylimidazole was added to a sample of the enzyme to give a 23 mM solution. To a second enzyme sample four separate equal additions of solid *N*-acetylimidazole were made at 15 min intervals to give a final concentration of 92 mM (equivalent to 100 mg reagent per mg enzyme protein). A third enzyme sample served as a control. The solutions were kept at 25° (pH 7.5) and samples were withdrawn at intervals, cooled on ice and dialysed overnight against 0.01 M Tris–HCl buffer (pH 8.0) at 4°. The enzyme was assayed against laminarin by the reductometric procedure.

Procedure E for assay of inhibition by carbodiimide. The reaction mixture contained enzyme, 1 M glycine methylester, 0.1 M carbodiimide reagent and 1 M pyridine–pyridinium chloride buffer (pH 4.75). In control mixtures the ester, carbodiimide or both were omitted. The reaction temperature was 25° and at intervals samples were diluted with 5 vol. 1 M acetate buffer (pH 4.75) containing 100 μg bovine serum albumin per ml. After 15 min at room temperature the samples were dialysed against 0.01 M Tris–HCl buffer (pH 8.0) at 4° for 18 h and activity was measured with laminarin as substrate.

Sepharose 4B chromatography

Sepharose 4B (Pharmacia, Uppsala, Sweden) suspended in a solution of 0.3% NaCl containing 5 mM NaN_3 was packed in a dimethyldichlorosilane treated glass column (39 cm × 1 cm). Oat glucan samples (3 mg) were dissolved in 0.5 ml of 20 mM phosphate buffer (pH 7.5) containing 4 mg of *Escherichia coli* cells and were applied to the columns. Columns were run at 20° with a constant flow rate (3.5 ml/20 min) maintained by a Mariotte bottle. The eluates were collected in 3.5 ml samples

which were assayed for protein by the Folin–Lowry method[22], total carbohydrate by the phenol–H_2SO_4 method[23], reducing end groups by the ferricyanide procedure[24] and phosphate by the method of ALLEN[25].

RESULTS

Action of the purified Nicotiana enzyme on various glycosidic substrates

The results shown in Table I indicate that the most readily hydrolysed sub-

TABLE I

SUBSTRATE SPECIFICITY OF NICOTIANA ENZYME

The incubations for reductometric assay and for chromatography were performed as described under EXPERIMENTAL PROCEDURE. Samples were removed after 48 h. Key: +++, high concentration of products; ++, moderate concentration of products; +, low concentration of products; —, not examined or estimated. Abbreviations: Glc, glucose; G_2, laminaribiose; G_3, laminaritriose, *etc.* Negative results were obtained with soluble starch, CM-cellulose, cellopentaose, cellotetraose, cellotriose, cellobiose, lutean, dispersed lutean, gentiobiose, Mycoplasma glucan, sophorose, Cualerpa xylan, salicin, methyl β-glucoside and *p*-nitrophenyl β-glucoside.

Compound	*Linkage type(s)*	*μg reducing sugar (as glucose) per ml incubation mixture per 48 h*	*Products of hydrolysis and relative amounts*
Pachyman (dispersed)	β-1,3-	182	G_2–G_7 (+++) Glc (trace)
Pachydextrins (insoluble)	β-1,3-	67	G_2–G_7 (+)
CM-pachyman	β-1,3-	375	—
Paramylon (granules)	β-1,3-	0	—
Paramylon (dispersed)	β-1,3-	216	G_2–G_7 (+++) Glc (trace)
Laminarin (insoluble)	β-1,3-	648	G_2–G_7 (+++) Glc (trace)
Laminaripentaose	β-1,3-	—	G_2 and G_3 (++) Glc and G_4 (trace)
Laminaritetraose	β-1,3-	—	G_2 (++), Glc and G_3 (trace)
Laminaritriose	β-1,3-	—	Nil
Laminaribiose	β-1,3-	—	Nil
Eisenia bicyclis laminarin	β-1,3; β-1,6-	—	Nil
Claviceps fusiformis glucan	β-1,3; β-1,6-	0	Nil
Yeast glucan	β-1,3; β-1,6	23	Oligosaccharides close to G_2 (++), G_3 (+) and G_4 (trace)
Barley glucan	β-1,3; β-1,4-	0	Nil
Oat glucan	β-1,3; β-1,4-	0	Nil
Lichenin (Sample A)	β-1,3; β-1,4-	79	Glc (++), traces of four oligosaccharides and higher oligosaccharides of low R_{Glc} (+++)
Lichenin (Sample B)	β-1,3; β-1,4-	—	Oligosaccharide (trace) moving more slowly than G_5

strates were the linear β-1,3-glucans, although some other glucans containing β-1,3-glucosidic linkages were also hydrolysed.

Action pattern of the Nicotiana enzyme on β-1,3-glucan substrates

A diagram of a chromatogram of the products formed during the progressive hydrolysis of dispersed paramylon is shown in Fig. 1 and this pattern is typical of the hydrolysis products of all the β-1,3-glucan substrates listed in Table I. The chromatographic mobilities suggest that the products of hydrolysis of β-1,3-glucans are the members of the homologous series of β-1,3-linked oligoglucosides, together with a trace of glucose. In the case of paramylon (Fig. 1), the chromatographic

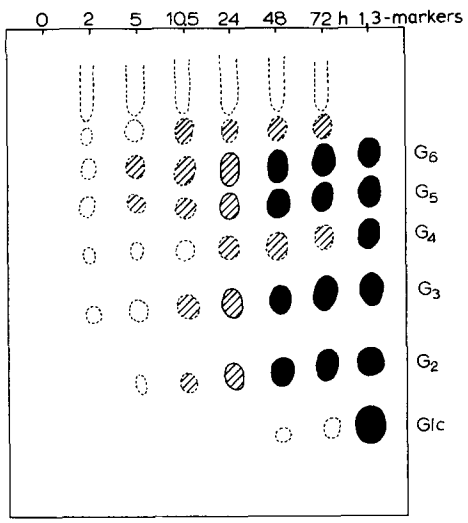

Fig. 1. Diagram of a chromatogram of the products of hydrolysis of dispersed paramylon. The conditions of incubation and the treatment of samples are described in EXPERIMENTAL PROCEDURE, with the modification that the substrate concentration was increased to 2% (w/v). From left to right the chromatogram shows samples of the reaction mixture after 0, 2, 5, 10.5, 24, 48 and 72 h incubation. On the right is a marker containing glucose and the series of β-1,3-oligoglucosides.

identification of an homologous series has been further substantiated by the demonstration of a linear relationship between $-\log R_{Glc}$ for the products shown in Fig. 1 and degree of polymerization. Fig. 1 also shows that traces of all products except glucose were detectable after 2 h incubation and that the concentration of each increased with time. After 72 h incubation, laminaribiose and oligosaccharides up to laminariheptaose are still present, together with traces of glucose and of higher oligosaccharides. It is also apparent that the level of laminaritetraose is lower than that of laminaribiose, -triose, -pentaose or -hexaose.

An additional experiment was performed to determine if the pattern of oligosaccharide products could be altered by prolonged hydrolysis. Dispersed paramylon (1%, w/v) was incubated with the enzyme for 48 h as described above. Further enzyme was then added and the incubation continued, with addition of another

aliquot of enzyme at 72 h. At 96 h the incubation mixture was centrifuged to remove the remaining paramylon and the clear supernatant solution was again incubated, with the addition of fresh aliquots of enzyme at 96 h and 120 h. Samples were removed and chromatographed at 48, 72, 96, 120 and 144 h after the start of the experiment. All showed the typical pattern of sugars illustrated in Fig. 1, except that after 96 h there was a decrease in the amount of the higher oligosaccharides with degree of polymerization greater than seven.

Since the pattern of oligosaccharides observed could have been produced by the combined action of the endo-hydrolase and any contaminating hydrolases present in Preparation A the action of the electrophoretically homogeneous Preparation C was examined. When dispersed paramylon was incubated with Preparation C the same final pattern of oligosaccharides was obtained indicating that they were products of the Nicotiana endo-hydrolase acting alone.

Hydrolysis of carboxymethylpachyman

As shown in Fig. 2 the viscosity of a solution of CM-pachyman was very rapidly

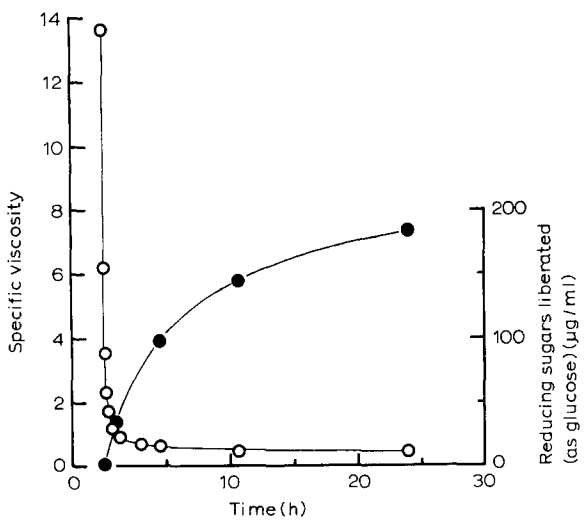

Fig. 2. Reduction of viscosity and release of reducing sugars from a solution of CM-pachyman. A reaction mixture of total volume 10 ml was prepared as described for viscometric assay. 2 ml was taken for viscosity measurement and the remainder used for measurement of reducing sugars released as described under EXPERIMENTAL PROCEDURE. ○—○, specific viscosity; ●—●, reducing sugars, as glucose (µg/ml).

reduced on incubation with the Nicotiana enzyme, whilst reducing sugars were only slowly released.

Hydrolysis of β-1,3; 1,4-glucans

Although no hydrolysis products were detected reductometrically or chromatographically following incubation of oat and barley glucans with the enzyme (Table I),

TABLE II

EFFECT OF NICOTIANA ENZYME ON THE VISCOSITY OF SOLUTIONS OF CM-CELLULOSE, CEREAL GLUCANS AND CM-PACHYMAN

The viscometric assays were performed as described in EXPERIMENTAL PROCEDURE except that the substrate solution for CM-cellulose contained 0.75% (w/v) CM-cellulose in phosphate–citrate buffer (pH 5.0), 0.25 M NaCl, 5 mM EDTA, 6.25 mM NaN_3 and 25 μg bovine serum albumin per ml. Unit activity for each substrate refers to the amount of enzyme in 2.0 ml incubation mixture required to cause a 10% reduction in η_{sp} in 100 min.

Substrate	*Enzyme dilution*	*Incubation time (h)*	η_{sp}	*% Reduction in initial* η_{sp}	*Activity of undiluted enzyme (units)*
CM-cellulose	Not diluted	0	5.33		
		0.18	5.30		
		1.0	5.28	< 1	< 0.015
		17.5	5.17		
		25	5.14		
		45	5.07		
		111	4.88		
CM-pachyman	1:200	0	13.6		
		0.23	2.34	83	48 000
		1.03	1.03	93	
		2.87	0.70		
		10.67	0.52		
		24	0.48	97	
Barley glucan	1:10	0	3.50		
		0.16	3.44		
		0.52	3.30		
		1.0	3.16	10	17
		2.0	2.88		
		20.0	1.62		
Oat glucan	1:10	0	3.87		
		0.18	3.63		
		0.55	3.39		
		1.0	3.16	18	41
		2.0	2.82		
		18.0	1.67		

there is evidence that limited hydrolysis does occur. Table II shows the results obtained using the more sensitive viscometric method of detection of enzymic hydrolysis of oat and barley β-glucans, CM-pachyman and CM-cellulose. The units of activity with the different substrates cannot be directly compared, but it can be seen that there is neglible attack on CM-cellulose, slow but significant attack on the cereal β-glucans and very rapid hydrolysis of CM-pachyman.

The molecular size distribution of oat glucan before and after prolonged incubation with the enzyme was examined by molecular exclusion chromatography on Sepharose 4B. Fig. 3 shows that a limited alteration in the relative molecular size distribution had occurred during enzyme treatment.

Transglycosylation reaction

The occurrence of enzymic transglycosylation from substrate to glucose or of

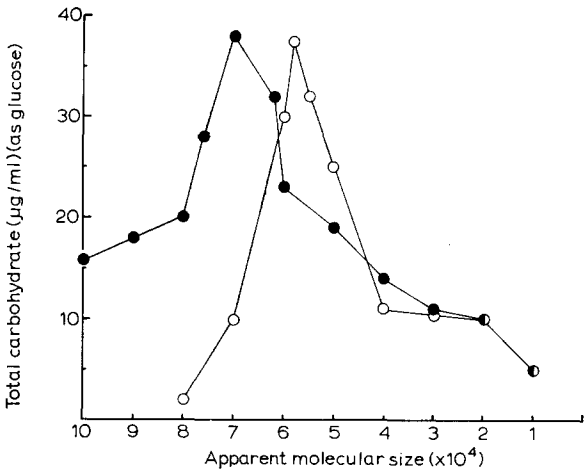

Fig. 3. Molecular size distribution of oat glucan before and after enzymic hydrolysis. Samples of oat glucan before (●—●) and after (○—○) prolonged incubation with Nicotiana enzyme, were fractionated on a column of Sepharose 4B as described in EXPERIMENTAL PROCEDURE. The apparent molecular size of the glucan in each fraction was calculated from the ratio of total carbohydrate, measured by the phenol–H_2SO_4 method, to the reducing value, measured by the ferricyanide method. This data was used to construct the curves which show the concentration of glucan as a function of apparent molecular size.

reversion reaction with glucose alone was examined. The enzyme was incubated with glucose at concentrations of 2.5, 25 and 50% (w/v) containing uniformly labelled D-[^{14}C]glucose (spec. act. 0.036 μC/mg glucose), with or without the addition of 5 mg/ml laminarin. After 24 and 48 h incubation, samples were removed and aliquots containing 500 μg glucose were chromatographed (see EXPERIMENTAL PROCEDURE). The chromatograms were auto-radiographed on Ilford X-ray film with a 12-day exposure, after which reducing sugars were detected with the alkaline $AgNO_3$ reagent. No radioactive areas other than those corresponding to glucose were seen. In the incubations containing laminarin the usual pattern of hydrolysis products was

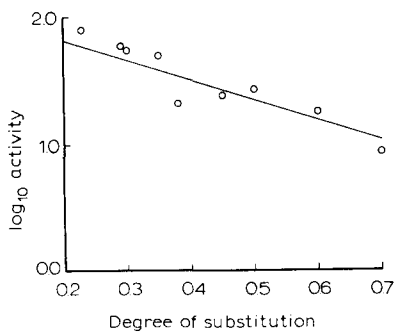

Fig. 4. Relationship between rate of hydrolysis of CM-pachyman and degree of substitution of the substrate. The enzyme was assayed reductometrically with CM-pachyman samples of various degrees of substitution as substrates. Log of rate of hydrolysis is plotted against degree of substitution and the regression equation has been calculated to be $\log_{10}$ rate of hydrolysis $= 2.13 - 1.55 \times$ degree of substitution. The standard errors of slope and intercept, respectively, are 0.572 and 0.256, with $P < 0.05$ and $P < 0.001$.

observed, though there was a marked reduction in their concentration in the presence of 25 and 50% glucose.

Effect of degree of substitution of CM-pachyman on rate of hydrolysis

The effect of degree of substitution of CM-pachyman on the rate of its hydrolysis by the Nicotiana enzyme was investigated using a number of samples of CM-pachyman of varying degree of substitution and the results are shown in Fig. 4. The regression and the P values (<0.05 and <0.001) indicate a negative correlation between the degree of substitution of CM-pachyman and the logarithm of the rate of hydrolysis by the Nicotiana enzyme. The scatter of points on the graph is likely to be due to the effect of uneven distribution of substituent groups in some of the CM-pachyman samples. In view of the dependence of rate of hydrolysis on degree of substitution it should be noted that the same sample of CM-pachyman was used for all experiments in which comparisons of enzyme activity were made.

Comparison of the action patterns of the Nicotiana and Rhizopus endo-hydrolases

The action patterns and specificities of the purified *N. glutinosa* and *R. arrhizus* enzymes were compared by chromatographic examination of the products of their

TABLE III

COMPARISON OF THE SPECIFICITY OF NICOTIANA AND RHIZOPUS β-GLUCAN HYDROLASES

The enzymic hydrolyses and paper chromatographic analyses of products were performed as described under EXPERIMENTAL PROCEDURES. The R_{Glc} values of unidentified products are recorded in the table. Abbreviations and symbols: Glc, glucose; G_2, laminaribiose; G_3, laminaritriose; *etc.* Intensity of spots, tr, trace; +, very low; ++, low; +++, moderate; ++++, strong. R_{Glc} values of standard oligosaccharides: G_2 (0.84), G_3 (0.67), G_4 (0.53), G_5 (0.41); cellobiose (0.72), cellotriose (0.37), cellotetraose (0.09), cellopentaose (0.01); gentiobiose (0.67), gentiotriose (0.42), gentiotetraose (0.25).

Substrate	*N. glutinosa enzyme products*	*R. arrhizus enzyme products*
CM-cellulose	Nil	Nil
Paramylon	Glc (+), G_2 (++++), G_3 (++++) G_4 (+++), G_5 (++), G_6 (+), slower sugar, (tr)	Glc (+), G_2 (+++), G_3 (+++), G_4 (tr)
Yeast glucan	Glc (+), G_2 (++), 0.65 (+)	Glc (++), G_2 (++++), 0.63 (+++), 0.50 (tr), 0.33 (+), 0.22 (tr)
E. bicyclis laminarin	Nil	Glc (++), G_2 (+), 0.67 (+), 0.55 (+), 0.36 (++), 0.21 (+), 0.14 (+), slower moving sugars (++)
L. hyperborea laminarin	Glc (+), G_2 (++++), 0.70 (+), G_3 (++++), G_4 (+), G_5 (++), G_6 (+++), series of slower sugars (+).	Glc (++), G_2 (+++), 0.73 (tr,) G_3 (++++), 0.57 (+), G_4 (++), 0.45 (+), G_5 (+), series of slower moving sugars (+)
Lutean	Nil	Glc (+), 0.84 (+), 0.65 (tr).
Lichenin (Sample A)	Glc (++), 0.69 (+), 0.55 (tr), 0.26 (tr), sugars at and near origin (++)	Glc (+), G_2 (+), 0.69 (+), 0.49 (+++), 0.27 (tr), 0.12 (+), 0.08 (++), sugars at and near origin (++).
Lichenin (Sample B)	0.28 (tr)	Glc (++), G_2 (+++), 0.68 (+), 0.55 (++++), 0.41 (++), 0.20 (+), 0.13 (+), 0.04 (+).

action on a range of β-glucan substrates. The results are summarised in Table III.

The relationship between change in fluidity (φ) and reducing sugar (RS) production for the *N. glutinosa* and *R. arrhizus* enzymes acting on CM-pachyman was compared. Fig. 5 shows that the $d\varphi/dRS$ relationships were similar for the two en-

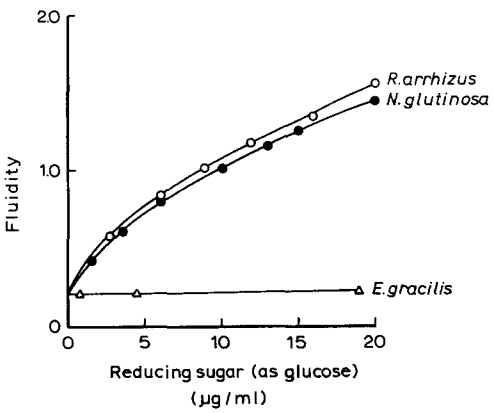

Fig. 5. Fluidity ($\varphi = 1/\eta_{sp}$) as a function of reducing sugar production for the hydrolysis of CM-pachyman by three β-1,3-glucan hydrolases. 4 vol. of CM-pachyman solution and one volume of enzyme solution were incubated at 40°. The *N. glutinosa* enzyme sample was Preparation C and the *R. arrhizus* and *E. gracilis* enzymes were those described in EXPERIMENTAL PROCEDURE. Specific viscosities and reducing sugar concentrations were measured at intervals during the incubation. ○—○, *R. arrhizus*; ●—●, *N. glutinosa*; △—△, *E. gracilis*.

zymes and had values characteristic of endo-hydrolases. By comparison the value of $d\varphi/dRS$ for the *E. gracilis* exo-hydrolase was near zero.

Inhibitor studies

The effect of potential inhibitors on the Nicotiana enzyme was investigated by incubating the enzyme with each compound and assaying the residual activity. Where possible the reductometric procedure (A) was used (see EXPERIMENTAL PROCEDURE—*Inhibitor assays*) but for compounds which interfered with the Nelson–Somogyi reducing sugar method the activity was assayed viscometrically (Procedure B). For 2-hydroxy-5-nitrobenzyl bromide, *N*-acetylimidazole and carbodiimide, Procedures C, D and E, respectively, were used. The results of the inhibitor tests are shown in Table IV.

DISCUSSION

Specificity and action pattern

The *N. glutinosa* enzyme has been shown to have a high specificity for β-1,3-glucans. The homomorphous β-1,3-xylan is not a substrate and there is no action on a number of β-glucosides or oligo- and polysaccharides containing α-1,4-, β-1,2-, β-1,4- or β-1,6-linked D-glucopyranose residues.

The products of hydrolysis of linear β-1,3-glucans such as dispersed paramylon, pachyman and insoluble laminarin are a mixture of β-1,3-oligoglucosides with degree of polymerization from 2 to 7, together with traces of glucose. These appear to be the

TABLE IV

INHIBITION DATA

Methods for reaction of the enzyme with potential inhibitors and for assay of residual enzyme activity are described under EXPERIMENTAL PROCEDURE. Except where stated the enzyme and inhibitor were reacted for 30 min at pH 5.0. Using assay Procedure A negative results were obtained with EDTA (10 mM), fluoride (2 mM), azide (20 mM), cyanide (20 mM), mercaptoethanol (20 mM), Mn^{2+} (2 mM), (1 → 5) gluconolactone (5 mM), tosyl lysyl chloroketone (2 mM, reaction time 30 and 120 min), diisopropyl phosphofluoridate (0.2 mM, reaction temperature 30°), conduritol B epoxide (0.2 mM, reaction at pH 4.0 in 0.05 M acetate buffer for 30 min or 24 h), cyanate (200 mM, reaction in 0.01 M Tris–HCl buffer, pH 8.0, followed by dialysis against the same buffer for 24 h before assay), *p*-chloromercuribenzoate (0.2 mM), *N*-ethylmaleimide (1.0 mM, reaction in 0.01 M phosphate buffer, pH 7.0 for 30 and 60 min).

Compound	*Inhibitor concn. in enzyme–substrate mixture (mM)*	*% inhibition*	*Assay method*	*Comment*
Cysteine	5	10	B	
	50	10		
Cu^{2+}	0.2	0	A	
	2	15		
	20	90		
Hg^{2+}	0.2	97	A	
Cellulase inhibitor from *L. cuneata*	0.002 mg/ml	0	A	
	0.02 mg/ml	80		
	0.2 mg/ml	100		
Iodoacetate	10	7	A	Reaction in 0.01 M Tris-HCl buffer (pH 8.0) followed by dialysis *vs.* same buffer for 24 h before assay
Iodoacetamide	10	8	A	
Phenyl mercurinitrate	0.2	39	A	
	2.0	91		
Methylmercuric iodide	1	3	A	
Oxidized glutathione	50	0	B	
Iodine in KI	2.0	99	B	
N-Bromosuccinimide	5	99.6	B	
2-Hydroxy-5-nitrobenzyl bromide	10	79	C	Reaction time 30 min
	10 or 20	87		Reaction time 60 min
	10	100		Reaction time 120 min
N. Acetylimidazole	23	94	D	Reaction time 30 or 60 min
	92	99		Reaction time 30 min
Carbodiimide: 1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulphonate				*In precence of glycine methyl ester*
	100	100	E	Reaction time 15 min
				In absence of glycine methyl ester.
	100	40	E	Reaction time 15 min
	100	83	E	Reaction time 120 min

end products of the reaction since the pattern was unaltered even after prolonged hydrolysis or after incubation of the soluble reaction products with fresh enzyme. Furthermore these oligosaccharides apparently result from the action of the β-1,3-glucan hydrolase itself, and not from the combined action of the enzyme and of

others present as contaminants, since both Preparation A and the more purified Preparation C formed the same products.

Laminaribiose and laminaritriose are not hydrolysed by the enzyme, whilst laminaritetraose and laminaripentaose are slowly cleaved, yielding a disaccharide or a disaccharide *plus* a trisaccharide, respectively (Table I). These results indicate that hydrolysis must occur at the non-terminal linkages of these oligosaccharides and, together with the fact that a series of oligosaccharides are formed from β-1,3-glucans, suggest that the hydrolase has an endo-action pattern. The endo-action of the enzyme has been confirmed by the demonstration that it rapidly reduces the viscosity of CM-pachyman solutions with the simultaneous very slow release of reducing sugars (Fig. 2).

Of all the linear β-1,3-glucans tested as substrates, only the native, granular form of paramylon resisted hydrolysis. This is presumably due to the organization of the β-1,3-glucan molecules within the granule, since disruption of the structure by swelling in NaOH[26] converted the glucan to a form which was susceptible to attack by the enzyme.

It has been shown however[27] that the enzymic attack on alkali swollen paramylon is incomplete and ceases after only a fraction of the substrate has been solubilized. The residual paramylon, after washing and re-suspension in fresh enzyme–buffer solution, was only slightly attacked, but could be rendered susceptible to further hydrolysis by re-dispersion in alkali. Apparently the hydrolysis of the alkali swollen paramylon is limited by the number of sites accessible to the enzyme and the further alkali treatment makes new sites available. Similar conclusions were reported by WALSETH[28] in an investigation of the attack of cellulase on swollen cellulose.

Since laminaritetraose and -pentaose were hydrolysed only slowly and since the products of hydrolysis of β-1,3-glucans have a relatively high average degree of polymerization, it seems probable that the enzyme requires at least three adjacent β-1,3-linked glucose residues in its substrates.

The β-glucans from cereal endosperm in which 1,3- and 1,4-linked glucose residues are distributed either singly or as short runs in the linear molecule were hydrolysed to a limited extent. It is thus apparent that there are some sites in these molecules capable of binding productively with the enzyme and from its specificity requirements it may be suggested that these consist of runs of three or more 1,3-linked glucose residues. This conclusion is supported by the demonstration[29–31] that small proportions of four and five consecutive 1,3-linked glucosyl units are present in these substrates.

The action of the Nicotiana enzyme on lichenin samples further supports the conclusion that runs of several 1,3-linkages are required by the enzyme in its substrates. Lichenin (Sample B) does not possess long runs of 1,3-linkages[4] and no hydrolysis of this sample was detected chromatographically. On the other hand, Sample A was hydrolysed to a much greater extent than the cereal glucans and there is evidence that this sample may contain a proportion of consecutive 1,3-linkages. This possibility is suggested by the finding of an acetone-precipitable residue after exhaustive hydrolysis of the lichenin by a purified β-1,4-glucan hydrolase from *Aspergillus niger*[15].

Claviceps glucan has a high proportion of glucosyl side chains and these apparently prevent productive enzyme–substrate interaction with the Nicotiana en-

zyme. The carboxymethyl substituents in CM-pachyman also apparently interfere with such interactions, since the rate of hydrolysis decreases as the degree of substitution increases.

The exact structure of *Eisenia bicyclis* laminarin is at present uncertain so that its resistance to hydrolysis by the Nicotiana enzyme cannot be fully explained. However, if the conclusions already drawn as to the substrate requirements of the enzyme are correct, it is apparent that few, if any, runs of three or more 1,3-linkages can be present.

The Nicotiana enzyme resembles the *R. arrhizus* β-1,3-glucan hydrolase in that both are endo-enzymes, but they differ in the pattern of end-products formed from the hydrolysis of linear β-1,3-glucans and also in their action on mixed-linked glucans. The differences in the action patterns of these β-glucan hydrolases have been discussed in detail elsewhere[32]. It appears likely that the active centre of the Nicotiana enzyme is of the same general type as those proposed for the α-amylases[33] and for lysozyme[34], with binding sub-sites for a number of β-1,3-linked glucose residues, each with a particular binding affinity. An active centre with nine binding sub-sites has been reported for the α-glucan hydrolase from *Bacillus subtilis*[35] which forms oligosaccharide end products with a high average degree of polymerization. The nature of the end products of Nicotiana hydrolase action suggest that this enzyme may also have a relatively large number of binding sub-sites. The Rhizopus hydrolase on the other hand, probably contains fewer binding sub-sites since the end-products of its action have a lower average degree of polymerization. Since this enzyme can cause extensive hydrolysis of mixed-linked glucans with few, if any, adjacent 1,3-linkages, it may be necessary for only one of the glucose residues bound at the active centre to be 3-substituted. Although the Rhizopus and Nicotiana enzymes are both β-1,3-glucan glucanohydrolases they may be distinguished by their action on β-1,3;1,4-glucans and belong to the classes EC 3.2.1.6 and EC 3.2.1.39, respectively.

Glucan hydrolases with action patterns similar to that of the Nicotiana enzyme have been described from pepper callus[36], wheat and barley root callus[37] and germinating barley[38].

Transfer action

No transglycosylase activity was detected. However, catalysis of transglycosylation and reversion reactions by certain glycan endo-hydrolases has been demonstrated[32,39–42] and it is possible that such transfer reactions could occur with the Nicotiana enzyme under different conditions. Transglycosylase activity of certain amylases has been detected using short incubations with donors of appropriate anomeric configuration[42].

Inhibition

The results of inhibitor tests (Table IV) provide some information concerning the effect of metal ions on the activity of the Nicotiana enzyme and suggest the identity of some of the amino acid residues which may be essential for activity.

Since the activity was retained following treatment of the enzyme with EDTA and after prolonged dialysis against buffer, it is apparent that metal ions or other co-factors are not essential, unless they are so firmly bound that they cannot be re-

moved by these methods. On the other hand fairly high concentrations of heavy metal ions, Cu^{2+} and Hg^{2+}, strongly inhibited the enzyme.

The importance of particular functional groups for the activity of the Nicotiana enzyme has been investigated by the use of reagents which react more or less specifically with these residues. Thiol and disulphide groups are known to be important in a number of ways for the activity of certain enzymes. They do not however seem to be necessary for the activity of the Nicotiana enzyme since no significant inhibition was observed with *p*-chloromercuribenzoate, *N*-ethylmaleimide, methylmercuric iodide, oxidised glutathione, mercaptoethanol or cysteine. The enzyme was however inhibited by phenylmercurinitrate, but it is unlikely that this is due to its reaction with thiol groups since, as has been noted, other thiol reagents did not inhibit. In particular it would be expected that the small and reactive methylmercuric iodide molecule would react more readily with an available thiol than would the bulky phenylmercurinitrate molecule[43]. The reaction of phenylmercurinitrate with groups other than thiols has been shown to occur with α-amylases[44] and catalase[45] which are inhibited by organomercurials, even though thiol groups are either absent or not essential for activity. The slight inhibition due to iodoacetamide and iodoacetate may also be attributable to reactions with other groups, such as lysyl, tyrosyl, methionyl residues or possibly carboxyl groups. However, since cyanate did not inhibit it is unlikely that lysysl (or N-terminal) residues are essential for activity.

Tryptophan residues which react with 2-hydroxy-5-nitrobenzyl bromide, *N*-bromosuccinimide and I_2 are probably essential for activity. Similarly, tyrosyl residues which react with *N*-acetylimidazole, *N*-bromosuccinimide, I_2 and carbodiimide are probably also required. It is also probable that a carboxyl group, reacting in the carbodiimide-nucleophile procedure, could be essential, indicating the involvement of a glutamyl, aspartyl or a C-terminal amino acid residue. The participation of histidyl residues which react with *N*-bromosuccinimide, I_2 and possibly with heavy metals, has not been excluded.

The cellulase and pectinase inhibitor from the legume *Lespedeza cuneata*[46] which has a structure related to the leucoanthocyanidins[46,47] is a relatively strong inhibitor of the Nicotiana enzyme.

Glucono-(1→5)-lactone, which is a competitive inhibitor of β-glucoside hydrolases but not of β-glucan endo-hydrolases[48], does not inhibit this enzyme. Some β-glucoside hydrolases[49,50] have been shown to be inhibited by their substrate analogue, conduritol B epoxide, which reacts with a carboxyl group at the active site. However the Nicotiana enzyme is not inhibited by this reagent, although a carboxyl group may form part of its active site. This may be because the conduritol B epoxide does not satisfy the binding requirements of this enzyme, suggesting that it could be of interest to test the effect of substrate analogues such as the 2',3'-epoxypropyl glycosides of β-1,3-linked glucose oligosaccharides. Analagous derivatives of *N*-acetyl glucosamine have recently been shown to be effective inhibitors of lysozyme in which carboxyl groups participate in catalysis[51].

The limited data available on inhibitors of β-glucan hydrolases has been reviewed[52]. More recently the cellulase from *Penicillium notatum* has been shown to require tryptophan for binding and possibly histidine for catalysis[53,54], whilst tryptophan and aspartic acid participate in binding by lysozyme and glutamic and aspartic acids in catalysis[34]. From a comparative viewpoint it is of interest that in

α-amylases from a wide variety of sources, carboxyl and imidazole groups are also probably involved in catalysis[55].

Even from this restricted information, some similarities are apparent between the amino acids essential for the activity of glucan endo-hydrolases and the residues suggested for the Nicotiana enzyme by the present inhibition data. The occurrence of active site homologies in these glucan hydrolases would be analagous to those well established in the serine protease and esterase families of enzymes[56,57].

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