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ENDO-(1→4)-β-D-GLUCANASES FROM *Sclerotium rolfsii*. PURIFICATION, SUBSTRATE SPECIFICITY, AND MODE OF ACTION*

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ABSTRACT

Three purified endo-(1→4)-β-D-glucanases (EC 3.2.1.4), A, B, and C, from *Sclerotium rolfsii* culture filtrates showed homogeneity in disc-gel electrophoresis and in analytical isoelectric-focusing in polyacrylamide gel. The three endo-D-glucanases are glycoproteins, endo B and endo C being composed of a single polypeptide chain, and endo A of two dissimilar polypeptide chains that are covalently bound by a disulfide bridge. Endo B and endo C do not contain half-cystine residues. With carboxymethylcellulose as substrate, the liquifying activity of the three enzymes was inhibited by cellobiose but not by D-glucose. The specificity of the enzymes is restricted to β-(1→4) linkages, but they showed some differences in the mode of attack on cellodextrins, phosphoric acid-swollen cellulose, and lichenan to give cellobiose, cellotriose, and small proportions of D-glucose. Endo B in addition showed endo-D-xylanase activity.

INTRODUCTION

Sclerotium rolfsii produces a highly active cellulase which hydrolyzes highly ordered celluloses[1–6]. In order to study the stepwise enzymic hydrolysis of crystalline cellulose to D-glucose, isolation of the various components of the cellulase complex in a homogeneous state was attempted. The purification, characterization, and properties of four β-D-glucosidase enzymes from *S. rolfsii* and their mode of action have been reported previously[1,7]. The β-D-glucosidases behaved rather as exo-(1→4)-β-D-glucan glucohydrolases[7]. Each of the cellulase component of the cellulase complex displayed some multiplicity[8–10]. The present study shows that endo-(1→4)-β-D-glucanase (EC 3.2.1.4) activity in the culture filtrates of *S. rolfsii* can be separated into three fractions.

EXPERIMENTAL

Materials and methods. — The sources of the materials used have been de-

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scribed previously[1–7]. CM-cellulose [*O*-(carboxymethyl)cellulose] sodium salt having a degree of substitution (d.s.) of 0.7 and average degree of polymerization (d.p.) of 400 (C-8758) was obtained from Sigma (U.S.A.). A cello-oligosaccharide having a degree of polymerization of 37 (G_{37}) was prepared according to the method of Kaustinen *et al.*[11], and phosphoric acid-swollen Avicel P.H. 101 (H_3PO_4-swollen cellulose) according to the method of Wood[12]. Carboxymethylpachyman was kindly supplied by Prof. B. A. Stone (La Trobe University, Australia).

Analytical methods. — The reducing sugars, expressed as glucose, were determined by the *p*-hydroxybenzoic acid hydrazide method (*p*-HBAH method)[13] or by the Nelson–Somogyi method[14,15], D-glucose was determined by the D-glucose oxidase–peroxidase method[16] using "Glox" reagent, total carbohydrate by the anthrone–sulfuric acid (anthrone) method[17], and protein by the method of Lowry *et al.*[18]. In column effluents, protein was estimated by measuring the absorbancy at 280 nm. The sugars produced by the endo-D-glucanases from soluble and insoluble substrates were qualitatively examined by filter-paper-chromatography[5]. For paper-chromatography analysis, the enzyme (3 μg) was incubated with the cellodextrin (2 mg) or an insoluble substrate (3 mg) at 50° in 50mM citrate buffer, at pH 4.0 for endo A and C, and at pH 2.8 for endo B.

Polyacrylamide gel electrophoresis and isoelectric focusing. — Analytical disc-gel electrophoresis using a 7.5% polyacrylamide gel was performed at pH 8.9 according to Davis[19], and pH 2.9 according to Maurer[20], as described previously[1].

The procedure described by O'Farrell[21] was used for isoelectric focusing in 7.5% polyacrylamide gel. The pH gradient was obtained with 0.1 mL of pH 3.5–10, and 0.4 mL of pH 5–7 Ampholine carrier ampholytes (40%) per 10 mL of gel. Gels were stained with 0.01% Coomassie Brilliant Blue in 10% trichloroacetic acid for 1 h, and destained in 5:2:13 (v/v) ethanol–acetic acid–water[20]. Preparative isoelectric focusing was carried out in a 110-mL LKB electrofocusing column at 5–7° according to the procedure described in the LKB Instruction Manual.

The molecular weight of the purified enzymes was determined by molecular-sieve chromatography[22], and by gel electrophoresis as described by Hedrick and Smith[23]. The use of spacer gel was found unneccessary. $NaDodSO_4$-gel electrophoresis containing 7.5% polyacrylamide and 0.1% $NaDodSO_4$ was carried out as described by Weber and Osborn[24], and Shapiro *et al.*[25], except that samples, after treatment with 1% $NaDodSO_4$, 1% 2-mercaptoethanol, and 0.5% iodoacetamide at pH 7.0, were incubated for 4 h at 37° and not dialyzed prior to electrophoresis.

Amino acid composition. — Samples of the enzymes (0.5–1.0 mg) were hydrolyzed as described previously[26], and analyzed in a Beckman model 120-B amino acid analyzer by the method of Spackman *et al.*[27]. The tryptophan and tyrosine contents were determined by the spectrophotometric method of Goodwin and Morton[28].

Enzyme assays. — (*a*) *Reducing sugars method.* The endo-D-glucanase activity (carboxymethylcellulase) was determined by mixing an aliquot of diluted enzyme (0.5 mL) with 1% CM-cellulose sodium salt (0.5 mL) in 50mM citrate buffer

(pH 4.0) for endo A and C, and 50mM potassium acid phthalate–hydrochloric acid buffer (pH 2.8) for endo B, and incubating for 30 min at 74° for endo A, and 50° for endo B and C. The reducing sugars produced were determined as glucose equivalents by one of the methods described earlier and the plotted results were linear up to 0.25 mg under the conditions of assay. The rate of CM-cellulose hydrolysis against the amount of enzyme protein was linear between 0.1 to 0.5 μg of enzyme. The products of endo-D-glucanases reaction with CM-cellulose are heterogeneous polymers. It is not, therefore, valid and technically correct to express the endo-D-glucanase activity as μmol of glucose equivalents when D-glucose is produced in the least amount, if at all. In order to establish a relation with the data previously reported in the literature, one unit of endo-D-glucanase activity was defined as the amount of enzyme that releases 180 μg of reducing sugars (expressed as glucose equivalents) per min from CM-cellulose, at optimum pH and temperature of the enzyme, under the defined assay conditions.

(*b*) *Viscosity method.* The CM-cellulose-liquifying activity of endo-D-glucanase was determined in arbitrary units by the decrease in viscosity of a CM-cellulose solution according to Thomas[29]. The reaction mixture contained 0.5% of CM-cellulose sodium salt, d.s. 0.7 (10 mL), in 50mM acetate buffer, pH 4.8, in an Ubbelöhde viscometer at 40°. Diluted enzyme (3 mL, 0.1–0.5 μg) was then added and the decrease in the flow time recorded at 3-min intervals. A plot of fluidity against time was constructed with various concentrations of endo-D-glucanase. The value of slope of these curves were plotted against enzyme concentration. Under the conditions of assay, the rate of increase of fluidity against enzyme concentration was linear up to a value of 0.02 min^{-1}.

β-D-Glucosidase and D-xylanase activity. β-D-Glucosidase activity was determined with *p*-nitrophenyl β-D-glucopyranoside or cellobiose as substrate as described previously[7]. For determination of D-xylanase activity, the reaction mixture contained 1% of D-xylan (larch wood) (0.5 mL) in 50mM citrate buffer, pH 4.2, and a suitably diluted solution of enzyme (0.5 mL); the mixture was incubated at optimum temperature for 30 min, and the reducing sugars produced determined as xylose equivalents. The formation of reducing sugars was linear up to 0.6 mg.

Preparation of crude extract. — Sclerotium rolfsii CPC 142 used as the enzyme source[2,5] was grown for 14 days on the modified Reese and Mandel's medium[30] with Cellulose-123 as the sole carbon source as described previously[5].

RESULTS

The following operations were carried out at 0–4° unless otherwise indicated. The purification steps, ammonium sulfate precipitation (Step 1) and fractionation by Sephadex G-75 gel chromatography (Step 2), have been described previously for the purification of the β-D-glucosidase enzymes[1]. Fraction A (110–170 mL) and Fraction B (175–254 mL) contained 169 700 and 77 210 endo-D-glucanase units (a total of 4 runs which were pooled), respectively. Fraction B was processed further

for purification of endo-D-glucanase A (endo A) and endo-D-glucanase B (endo B), and Fraction A was processed for purification of endo-D-glucanase C (endo C).

Purification of endo A and endo B. — Fraction B from Step 2 was dialyzed in a collodion bag for 3–4 h against 50mM phosphate buffer, pH 7.3, and chromatographed on a DEAE-Sephadex A-50 column (1.8 × 100 cm) (Step 3). Endo-D-glucanase and β-D-glucosidase activities were not adsorbed on the column. β-D-Glucosidase emerged just after the void volume and ahead of endo-D-glucanases which were practically free from β-D-glucosidase activity. Peak 2 (170–240 mL) containing endo-D-glucanase of 103 specific activity was dialyzed against 2.5mM Tris·HCl buffer, pH 8.1, lyophilized to a small volume (4–6 mL), and dialyzed against 25mM Tris·HCl, pH 8.1, overnight. The dialyzed fraction (25 mL) was chromatographed on a QAE-Sephadex A-50 column (1.5 × 30 cm) (Step 4). The endo-D-glucanase activity was eluted in a stepwise manner by increasing the molarity and lowering the pH of the buffer. Peak A (95–140 mL, endo A) was eluted with 50mM phosphate buffer, pH 7.0, and contained 27% of endo-D-glucanase (of the total endo-D-glucanase loaded), and peak B (220–265 mL, endo B), which was eluted with 100mM citrate buffer, pH 4.8, contained 13% of endo-D-glucanase activity. These two fractions were concentrated by lyophilization and dialyzed overnight against 1% glycine, pH 7.4. At this stage of purification, the endo A fraction gave two major and three minor protein bands in analytical disc-gel electrophoresis, and the endo B fraction two major and four minor protein bands. The two fractions were purified further by isoelectric focusing using pH 3.5–10 Ampholine carrier ampholytes (40%, 3 mL) (Step 5). Fractions (1 mL) were immediately processed for pH, activity, and protein determination. The fractions having the higher specific activity were freed from sucrose by dialysis against 50mM citrate buffer, pH 4.5.

Purification of endo C. — Fraction A from Step 2 was concentrated with a Diaflo membrane XM-50 ultrafiltration system (Amicon Corporation, U.S.A.), which also removed the low-molecular-weight proteins (Step 6). The concentrated top enzyme solution was dialyzed against 50mM phosphate buffer pH 7.3, for 3–4 h and chromatographed on a DEAE-Sephadex A-50 column (1.8 × 100 cm) (Step 7) in a manner similar to that described for Step 3. Peak 2 (175–245 mL) contained the major endo-D-glucanase activity and was almost free of β-D-glucosidase. The pooled, Peak-2 fractions were concentrated by lyophilization and dialyzed against 5mM Tris–glycine buffer, pH 8.5. Total volume after dialysis was 25 mL containing 700 mg of protein. Further purification of endo C was obtained by preparative polyacrylamide gel-electrophoresis (Step 8) according to the procedure described by Husain and Sadana[31]. The protein (~50 mg, 2 mL) was loaded on the polyacrylamide gel column (1.5 × 6 cm) in each run. Fractions (1.5–2 mL) were collected every 60 min and analyzed for activity and protein concentration. The enzyme was collected in a dialysis bag between 6 and 18 h. Fractions 16–18 were pooled on the basis of their specific activity and dialyzed against 25mM citrate buffer, pH 4.5. Table I summarizes the purification procedure, recoveries, and specific activities of endo A, B, and C.

TABLE I

PURIFICATION OF ENDO-(1→4)-β-D-GLUCANASES (ENDO A, ENDO B, AND ENDO C) FROM *S. rolfsii*

Fraction	*Total protein (mg)*	*Endo-(1→4)-β-D-glucanase*			*β-D-Glucosidase*		
		Total units	*Specific activity*	*Recovery (%)*	*Total units*	*Specific activity*	*Recovery (%)*
Culture filtrate	17 232	520 550	30	100	48 824	2.8	100
Ammonium sulfate 0–90% saturation	13 500	442 370	33	85	43 865	3.0	90
Sephadex G-75 chromatography							
(a) Fraction A	10 450	169 700	16	32	31 370	3.0	64
(b) Fraction B	1380	77 210	56	15	180	0.1	0.4
Purification of endo A and B:							
DEAE-Sephadex A-50 chromatography of Fraction B from Step 2	495	50 958	103	10	0	0	0
QAE-Sephadex A-50 chroamtography							
(a) Peak A (Endo A)	130	13 758	106	3	0	0	0
(b) Peak B (Endo B)	60	6624	110	1	0	0	0
Isoelectric focusing							
Endo A	16	1664	104	0.3	0	0	0
Endo B	7.2	576	80	0.1	0	0	0
Purification of endo C:							
Ultrafiltration of Fraction A (Amicon XM-50), Top	5390	88 900	16.5	17	25 340	5.0	52
DEAE-Sephadex A-50 chromatography							
(a) Peak 1	76	108	1.5	0.02	3300	43.4	7
(b) Peak 2	700	46 200	66	9	224	0.3	0.5
Preparative electrophoresis of Peak 2	14.5	1116	77	0.2	0	0	0

TABLE II

AMINO ACID COMPOSITION OF HOMOGENEOUS ENDO-(1→4)-β-D-GLUCANASES FROM *Sclerotium rolfsii*[a]

Amino acid	*Endo A*		*Endo B*		*Endo C*	
	Residues	*Residues per 100 residues*	*Residues*	*Residues per 100 residues*	*Residues*	*Residues per 100 residues*
Aspartic acid	58	11.0	20	6.7	71	9.3
Threonine[b]	49	9.3	20	6.7	67	8.8
Serine[b]	42	8.0	20	6.7	51	6.7
Glutamic acid	32	6.1	24	8.0	85	11.2
Proline	30	5.7	28	9.4	48	6.3
Glycine	58	11.0	38	12.7	115	15.1
Alanine	54	10.3	26	8.7	81	10.7
Half-cystine[c]	6	1.1	0	0	0	0
Valine[d]	40	7.6	28	9.4	18	2.4
Methionine[e]	11	2.1	14	4.7	5	0.7
Isoleucine[d]	36	6.9	13	4.4	33	4.3
Leucine[d]	28	5.3	6	2.0	66	8.7
Tyrosine[f]	12 (14)	2.3	13 (15)	4.4	26 (24)	3.4
Phenylalanine	22	4.2	18	6.0	32	4.2
Lysine	14	2.7	15	5.0	23	3.0
Histidine	10	1.9	4	1.3	4	0.5
Arginine	5	0.95	0	0	6	0.8
Tryptophan[f]	(18)	3.4	(12)	4.0	(29)	3.8
Total residues	525		299		760	
Molecular weight	52 000		27 000		78 000	

[a]The calculated number of residues per mol of *S. rolfsii* endo-(1→4)-β-D-glucanases are based upon molecular weights of 52 000, 27 000, and 78 000 for endo A, B, C, respectively. [b]Extrapolated to zero time. [c]Determined as cysteic acid after perfomic acid oxidation[26]. [d]72-h value used. [e]Determined as methionine sulfone after performic acid oxidation[26]. [f]Values given in parentheses determined by the method of Goodwin and Morton[28].

Physical properties. — Purified endo A, B, and C were free from β-D-glucosidase activity when determined with *p*-nitrophenyl β-D-glucopyranoside or cellobiose as substrate. Electrophoresis of each of the three enzymes at pH 2.9 and 8.9 in polyacrylamide gel, with and without $NaDodSO_4$, and isoelectric focusing[21] in 7.5% polyacrylamide gel over the pH range 3.5–10 revealed only one protein band. The molecular weights determined[22] with Bio-Gel P-100 for endo A and B, and Bio-Gel P-150 for endo C, were 50 000, 27 500, and 78 000, respectively; and by the slope method[23] 52 000, 28 000, and 78 000, respectively. The isoelectric points of endo A, B, and C, determined with 1% of 40% Ampholine carrier ampholytes, were 4.55, 4.2, and 4.51, respectively.

Chemical properties. — On $NaDodSO_4$-gel electrophoresis[24,25], the carboxyamidomethylated derivatives of the reduced form of endo B and C gave one protein band (M_r 27 000 and 77 600) indicating single polypeptide chains. On similar treatment, endo A gave two protein bands indicating two subunits, M_r 32 000 and 22 000, that are linked by a disulfide bond. On $NaDodSO_4$-gel electrophoresis,

Clostridium thermocellum endo-D-glucanase, pretreated with 1% $NaDocSO_4$ for 4 min at 90°, showed two additional bands, but since the enzyme did not contain cysteine, subunits were ruled out[32].

Polyacrylamide gels, stained with Coomassie Brilliant Blue and with periodate–fuchsin[33], showed single, coinciding bands for endo A, B, and C, respectively, which suggests that the three enzymes are glycoproteins. Endo-D-glucanase from *Sporotrichum pulverulentum*[34], *Irpex lacteus*[35], and *Trichoderma viride*[36] have been reported to be glycoproteins, whereas that from *Aspergillus niger* is devoid of carbohydrate[13].

The three enzymes contain high proportions of glycine, alanine, aspartic acid, and glutamic acid (Table II). The proportions of basic amino acids are low in all the endo-D-glucanases examined[34,37–39]. Endo B is particularly low in leucine and histidine; and arginine and half-cystine were not detected. Endo A and C are low in methionine, histidine, and arginine, and half-cystine was absent in endo C.

The three enzymes were adsorbed on a concanavalin A-Sepharose column, and eluted with 1% methyl α-D-glucopyranoside or -D-mannopyranoside, indicating the enzymes to be glycoproteins[40].

Enzymic properties. — The purified endo-D-glucanases were stable when stored at −15° at pH 4.5, and to repeated freezing and thawing, but were completely inactivated at 100° for 2 min. Endo A and C were most stable at pH 4.5, endo B was most stable at pH 2.8–3.0. At pH and temperature optima (4.0, 74°; 2.8–3.0, 50°; 4.0, 50°, respectively), and with CM-cellulose as substrate, 0.5 μg of endo A, B, and C produced 8.8, 7.4 and 7.0 μg, respectively, of reducing sugars per min, which are higher activities as compared to those of endo-D-glucanases from other sources[38,41]. Endo B exhibited a bimodel pH-activity profile, at 2.8–3.0

TABLE III

KINETIC CONSTANTS FOR *Sclerotium rolfsii* ENDO-(1→4)-β-D-GLUCANASES[a]

Substrate	*Endo A*			*Endo B*			*Endo C*			*Endo A*[b]		
	$[S]_{0.5v}$ (mM)	V_{max}	K_f	$[S]_{0.5v}$ (mM)	V_{max}	K_f	$[S]_{0.5v}$ (mM)	V_{max}	K_f	$[S]_{0.5v}$ (mM)	V_{max}	K_f
G_{37}	1.8	34.1	18.8	1.2	19.8	16.9	1.1	17.4	15.6	1.5	25.2	17.0
Cellohexaose	2.2	23.1	10.5	1.4	17.4	12.1	1.2	15.3	15.3	1.7	14.6	8.8
Cellopentaose	2.5	19.8	7.9	1.7	13.9	8.4	1.8	12.6	6.9	2.1	11.6	5.5
Cellotetraose	4.4	9.9	2.3	4.0	11.6	2.9	3.3	9.9	3.0	5.0	7.9	1.5
Cellotriose	13.3	6.1	0.5	10.0	4.6	0.5	8.0	5.5	0.7	13.3	4.0	0.3
CM-cellulose[c]	2.5	28.3		4.8	29.1		2.2	24.1				

[a]Kinetic studies were done by incubating substrates at various concentrations from 0.1 to 0.5mM (except for CM-cellulose which was 0.1 to 0.5%) with 0.3 μg of enzyme for 30 min at 74°, pH 4.0 for endo A; at 50°, pH 2.8 for endo B; and at 50°, pH 4.0 for endo C. The apparent $[S]_{0.5v}$ and V_{max} values were calculated from Lineweaver–Burk plots, which were linear in all cases. The reducing sugars were determined by the *p*-HBAH method; V_{max} = mg RS · min^{-1} · mg^{-1} of protein; $K_f = V_{max}/[S]_{0.5v}$. [b]Assays carried out at 50°, pH 4.0. [c]$[S]_{0.5v}$ values expressed as mg/mL.

(100% of activity), and at 3.6 (85% of activity). From Arrhenius plots, the energy of activation was calculated to be 33, 20, and 11.5 kJ for endo A, B, and C, respectively. The three endo-D-glucanases did not show any transglycosylation or transferase activity when incubated with cellobiose and D-glucose, together or separately.

The $[S]_{0.5v}$, V_{max}, and K_f ($V_{max}/[S]_{0.5v}$) values for CM-cellulose, cellodextrins, and G_{37} of the three endo-D-glucanases are shown in Table III. Cellobiose was not hydrolyzed by any of the three enzymes, whereas cellotriose was hydrolyzed extremely slowly to give cellobiose and D-glucose, indicating that the purified endo-D-glucanases were free of cellobiase activity. The turnover rate of the enzymes, as represented by V_{max}, for cello-tetraose, -pentaose, and -hexaose, and G_{37} increased while the empirical $[S]_{0.5v}$ decreased with the chain length of the substrate (Table III). (The numerical values of the constants might be of limited significance as only the end-reducing sugars, and not the intermediate products produced, were determined). The lower $[S]_{0.5v}$ values for substrates with higher degree of polymerization support the preferred order of attack and suggest that higher cello-oligosaccharides, at least up to G_{37}. are the preferred substrates for endo A, B, and C.

Endo-D-glucanases (0.5 μg) rapidly decreased the viscosity of a 0.5% CM-cellulose solution with simultaneous, slow release of reducing sugars in the initial stages of reaction up to 10 min. Reducing sugars continued to be generated at a

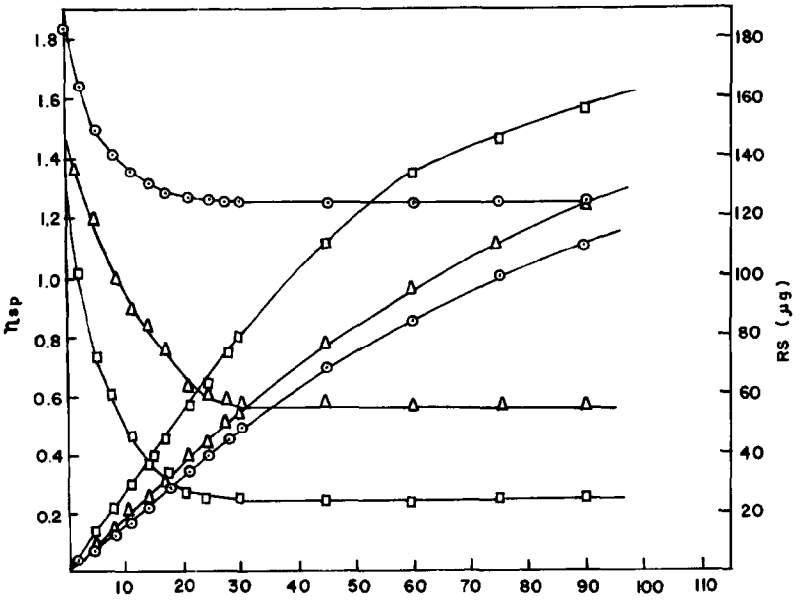

Fig. 1. Fall in specific viscosity (η sp) of a CM-cellulose solution having d.s. 0.7 (0.5%) in citrate buffer, pH 4.8, caused by the action of endo A, B, and C (each 0.5 μg), and corresponding reducing sugars production. The decrease in flow time was recorded at 3-min intervals for 90 min, and simultaneously aliquots were removed and boiled for 3 min, and reducing sugars estimated: □, Endo A; △, endo B; and ⊙, endo C.

TABLE IV

HYDROLYSIS OF PHOSPHORIC ACID-SWOLLEN CELLULOSE, CELLULOSE-123, AND AVICEL BY *Sclerotium rolfsii* ENDO-(1→4)-β-D-GLUCANASES[a]

Substrate	*Endo A*			*Endo B*			*Endo C*		
	RS (glucose equiv.)[b] *(μg)*	D-*Glucose*[c] *(μg)*	*Total sugar (glucose equiv.)*[d] *(μg)*	*RS (glucose equiv.)*[b] *(μg)*	D-*Glucose*[c] *(μg)*	*Total sugar (glucose equiv.)*[d] *(μg)*	*RS (glucose equiv.)*[b] *(μg)*	D-*Glucose*[c] *(μg)*	*Total sugar (glucose equiv.)*[d] *(μg)*
H_3PO_4-swollen cellulose	302	34	678	204	28	488	190	24	466
Avicel P.H. 101	12	2	29	12	2	25	16	3	24
Cellulose-123	68	12	142	48	8	104	46	8	99

[a]The reaction mixture (2 mL) contained 20 mg of substrate, 3 μg of enzyme for H_3PO_4-swollen cellulose and Cellulose-123, and 25 μg of enzyme for Avicel for 4 h at 74°, pH 4.0 for endo A; at 50°, pH 2.8 for endo B; and at 50°, pH 4.0 for endo C. [b]The glucose equivalent was measured by the *p*-HBAH method. [c]The D-glucose content was measured by the D-glucose oxidase–peroxidase method. [d]The total sugar content was measured as glucose equivalents by the anthrone method.

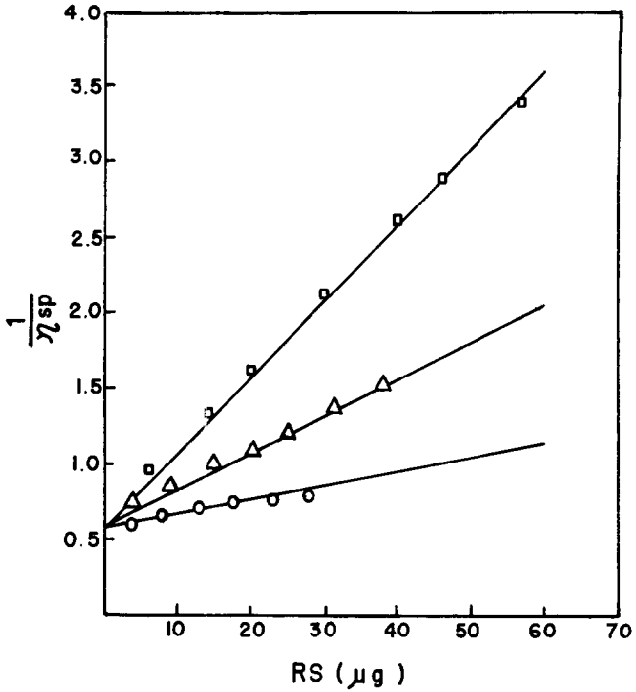

Fig. 2. Degree of randomness activity of endo A, B, and C. Relationship between increase in fluidity and production of reducing sugars during hydrolysis of CM-cellulose (d.s. 0.7) by endo A, B, and C. The enzymes (0.5 μg) wee incubated with 0.5% CM-cellulose (13 mL) at 40°, and the decrease in flow time was recorded at 3-min intervals: □, Endo A; △, endo B; and ⊙, endo C.

near-linear rate up to 90 min, though viscosity losses were essentially complete after 20 min (Fig. 1), indicating the endo action of the enzymes.

The reducing sugar values obtained on hydrolysis of H_3PO_4-swollen cellulose, Cellulose-123, and Avicel, as determined by the anthrone method, were 2.3–2.5 and 18–20 times higher than the end-reducing group values (determined by the *p*-HBAH method) and D-glucose values (determined by the D-glucose oxidase–peroxidase method), respectively (Table IV), which further supports the endo action of the enzymes. The results presented in Table IV also suggest that endo A formed higher-chain-degradation products than did endo B and C.

The decrease in viscosity of CM-cellulose-incubation mixture *vs.* reducing-sugar formation has been used as a measure of the degree of randomness of the endo-D-glucanase activity[42,43]. The slopes obtained for endo A, B, and C were 0.05, 0.025, 0.009, respectively (see Fig. 2), indicating an increase in randomness of attack of CM-cellulose from endo A, to B, to C.

The uptake of alkali (18% NaOH) (S-factor activity[44,45]) by 100 mg of cotton treated, at 37° for 2 h, with endo A, B, and C (15 μg each) was 20, 22, and 10 mg of NaOH, respectively. No marked increase in alkali uptake was observed with endo A plus B, A plus C, and B plus C, as compared to the sum of that obtained

by the two endo-D-glucanases separately. (1→4)-β-D-Glucan cellobiohydrolase (purified from *S. rolfsii*) did not show any S-factor activity. However, addition of (1→4)-β-D-glucan cellobiohydrolase (15 μg) to either endo A, B, or C caused an increase of 105–112% in uptake. (1→4)-β-D-Glucan cellobiohydrolases were reported to act synergistically with endo-D-glucanases in this respect[45,46].

The cations Mg^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+}, Ca^{2+}, and Zn^{2+} at a 5mM concentration did not stimulate the activity of the enzymes, whereas Mn^{2+} at the same concentration caused a 28–40% stimulation.

Inhibition of endo-D-glucanases. — Inhibition by cellobiose and D-glucose of endo A, B, and C of the degradation of a 0.5% CM-cellulose solution was examined with the viscosimetric method[29]. Cellobiose at 1% and 10% inhibited endo A, B, and C activity by 25–35% and 80–85%, respectively. Cellobiose inhibits endo-D-glucanases of most organisms[47]. No inhibition of endo A, B, and C was observed with D-glucose at 1% or 10% final concentration. *p*-Hydroxymercuribenzoate at 0.5mM concentration caused significant inhibition (70–80%) of endo A, B,

TABLE V

HYDROLYSIS OF VARIOUS D-GLUCANS BY ENDO-(1→4)-β-D-GLUCANASES OF *Sclerotium rolfsii*[a]

Substrate	*Components*	*Linkage of D-glycosyl group*	*Reducing sugars · h^{-1} · μg^{-1} of enzyme*		
			Endo A	*Endo B*	*Endo C*
CM-cellulose[b]	D-Glc→D-Glc	β-(1→4)	1040	820	790
Phosphoric acid-treated cellulose	D-Glc→D-Glc	β-(1→4)	36	28	25
Alkali-swollen cellulose	D-Glc→D-Glc	β-(1→4)	13	11	10
Avicel P.H. 101	D-Glc→D-Glc	β-(1→4)	0.5	0.5	0.8
Cotton sliver	D-Glc→D-Glc	β-(1→4)	0.5	0.5	0.8
Filter paper	D-Glc→D-Glc	β-(1→4)	1.0	1.0	1.5
α-Cellulose	D-Glc→D-Glc	β-(1→4)	0.5	0.5	0.8
Solka-Floc SW 40	D-Glc→D-Glc	β-(1→4)	0.5	0.5	0.7
Sólka-Floc BW 200	D-Glc→D-Glc	β-(1→4)	3.0	3.0	4.0
Cellulose-123	D-Glc→D-Glc	β-(1→4)	8.0	5.0	4.5
G_{37} Cellooligosaccharide	D-Glc→D-Glc	β-(1→4)	84	60	60
Lichenan	D-Glc→D-Glc	β-(1→3) and β-(1→4)	63	49	45
Laminaran	D-Glc→D-Glc	β-(1→3) and β-(1→6)	0	0	0
Sophorose	D-Glc→D-Glc	β-(1→2)	0	0	[c]
Carboxymethylpachyman	D-Glc→D-Glc	β-(1→3)	0	0	0
Dextrin	D-Glc→D-Glc	α-(1→4)	0	0	0
Dextran	D-Glc→D-Glc	α-(1→6)	0	0	0
Starch	D-Glc→D-Glc	α-(1→4)	0	0	0
Xylan	D-Xyl→D-Xyl	β-(1→4)	0	30	0
Chitin	GlcNAc→GlcNAc	β-(1→4)	0	0	0

[a]The reaction mixture (2 mL) contained substrate (5 mg) and enzyme (3 μg) in 50mM citrate buffer, pH 4.0, for 4 h at 50°. The reducing sugars (μg) produced were measured in the supernatant solution by the *p*-HBAH method. [b]With CM-cellulose (d.s. 0.7, DP 400) as substrate and 0.3 μg of enzyme for 30 min. [c]Not determined.

and C, which is not due to its effect on thiol groups as endo B and C do not contain half-cystine residues. Nojirimycin at a 5mM concentration inhibited the three enzymes by 25–40%; nojirimycin is reported to have only weak inhibitory effect on enzymes that degrade (1→4)-β-D-glucans[48,49].

Substrate specificity. — The hydrolysis patterns of several substrates are reported in Table V. The specificity of the *S. rolfsii* endo-D-glucanases was restricted to β-D-(1→4)-linkages.

It has been reported that some endo-D-cellulases show D-xylanase activity[50]. Endo B was active with CM-cellulose and D-xylan as substrates, whereas endo A and C were devoid of D-xylanase activity. The following data show that CM-cellulose and D-xylan are hydrolyzed by endo B at two different sites: (a) The enzyme showed two pH optima for CM-cellulose hydrolysis, whereas the pH optimum for D-xylan hydrolysis was 4.2 and the optimum temperature for D-xylan hydrolysis was 65°. (b) On heating at 65° for 10 min in 50mM citrate buffer, at pH 3.0 and 4.0, the enzyme retained 59 and 29% of D-xylanase, and 23 and 59% of CM-cellulase activity, respectively. On heating for 10 min at 80° in 50mM citrate buffer, pH 4.0, CM-cellulase activity was lost, but 20% of D-xylanase activity was retained. (c) The $[S]_{0.5v}$ values at pH 2.8, 50° for CM-cellulose and D-xylan were 4.8 and 8.3 mg/mL, respectively. The $[S]_{0.5v}$ values at pH 4.2, 65°, for CM-cellulose and D-xylan were 6.4 and 5 mg/mL, respectively. (d) The CM-cellulose- and D-xylan-hydrolyzing activities of endo B showed different sensitivity to inhibition by *p*-hydroxymercuribenzoate. At a 0.5mM concentration, *p*-hydroxymercuribenzoate inhibited the CM-cellulase and D-xylanase activities by 77 and 28%, respectively. (e) On treatment of endo B with urea, a denaturing agent, the CM-cellulose- and D-xylan-hydrolyzing activities of endo B were inactivated to different extents. However, on dialysis, the return of CM-cellulose-hydrolyzing activity was approximately paralleled with reappearance of D-xylan-hydrolyzing activity. (f) One might except that if CM-cellulose and D-xylan were hydrolyzed at different catalytic sites, the rate of formation of reducing sugars from CM-cellulose (5 mg/mL) + D-xylan (8.3 mg/mL), *i.e.*, at their $[S]_{0.5v}$ values, should be equal to the sum of the rates obtained with CM-cellulose and D-xylan, respectively. The production of reducing sugars, at pH 3 and 50°, from CM-cellulose, D-xylan, and CM-cellulose + D-xylan, was 0.2, 0.075, and 0.255 mg, respectively, which suggests different catalytic sites.

Mode of action. — The rates of specific glycosidic-bond hydrolysis by the endo-D-glucanases were examined from the time-course formation of specific products from different cellodextrins, H_3PO_4-swollen cellulose, and lichenan. Small differences were observed in the mode of action of the three endo-D-glucanases. After 4 h of incubation with cellotriose, barely visible spots corresponding to D-glucose and cellobiose were seen on the paper chromatograms with the three endo-D-glucanases. With cellotetraose and endo A, cellobiose was the only product detected. With endo B and C, on the other hand, hydrolysis of cellotetraose gave small quantities of D-glucose and cellotriose, in addition to cellobiose, as the major

product. This indicated that with endo A the C-1″–O-4′ glycosidic bond was selectively cleaved, whereas endo B and C cleaved, in addition, the C-1′–O-4 or C-1‴–O-4″ (or both) bond at a low rate.

Hydrolysis of cellopentaose with endo A, B, and C yielded cellobiose and cellotriose, indicating that the C-1″–O-4′ or C-1‴–O-4″ (or both) glycosidic bond was cleaved. On prolonged incubation, D-glucose was also detected, but no cellotetraose, indicating the slow cleavage of cellotriose to cellobiose and D-glucose. With endo A, B, and C, the products of hydrolysis of cellopentaitol (in which the terminal reducing D-glucose residue of cellopentaose was converted into a D-glucitol residue by $NaBH_4$) were again cellobiose and cellotriose; these compounds were produced apparently in equal amounts, either at 15 min or 4 h. No spot was detected earlier to 15 min. This indicated cleavage of the C-1″–O-4′ or C-1‴–O-4″ bond. Alternatively, the three endo-D-glucanases produced cellobiose and cellotriose from the reducing and nonreducing end with equal ease. Similar data have been reported for the degradation of cellopentaose and cellopentaitol by enzymes of *Trichoderma koningii*[51] and *C. thermocellum*[32]. At 4 h, a faint spot corresponding to D-glucose was detected, indicating cleavage of cellotriose to D-glucose and cellobiose. Hydrolysis of cellohexaose with endo C yielded initially cellotriose (15 min), and subsequently cellotetraose, cellotriose, and cellobiose (30 min to 4 h). With endo A and B, however, cellobiose, cellotriose, and cellotetraose were detected even at 2 min. This indicated preferential cleavage of the C-1‴–O-4″ bond over the C-1″–O-4′ or C-1⁗–O-4‴ (or both) bond of cellohexoase by endo C, whereas endo A and B cleaved the C-1‴–O-4″ and C-1″–O-4′ or C-1⁗–O-4‴ (or both) bonds with equal ease. With endo A, cellopentaose and D-glucose were also detected at 1, 4, and 24 h, suggesting slower cleavage of the C-1′–O-4 or C-1⁗′–O-4⁗ (or both) bond of cellohexaose.

Hydrolysis of H_3PO_4-swollen cellulose and lichenan. — With endo A, B, and C, the major soluble products from H_3PO_4-swollen-cellulose hydrolysis were cellobiose and cellotriose, though cellotriose was the first product detected at 3 min. The three enzymes produced D-glucose in small proportions, but the amount of cellobiose was comparable to that of cellotriose. The amount of D-glucose, cellobiose, and cellotriose increased with time. Other cellodextrins, cellotetraose, cellopentaose, and cellohexaose were not detected in the hydrolysis products of any of the three enzymes.

With lichenan and endo A or B, D-glucose, cellobiose and cellotriose, were detected at 15 min (and not earlier); with endo C, cellotriose alone was detected at 3 min; cellobiose and cellotriose at 5 min; and D-glucose, cellobiose, and cellotriose at 4 h. Cellotetraose was not detected at any time by the action of any of the *S. rolfsii* endo-D-glucanases on lichenan.

DISCUSSION

The three endo-(1→4)-β-D-glucanases from *S. rolfsii,* which have been ob-

tained in an homogeneous state, differ in their molecular weights, pH and temperature optima, subunit structure, S-factor activity, degree of randomness of their activity, apparent $[S]_{0.5v}$ and V_{max} values, and their ability to hydrolyze various substrates. Some functional differences and preferred mode of attack on cellodextrins, phosphoric acid- and alkali-swollen cellulose, and lichenan have been found. Unlike endo A, endo B and C do not contain half-cystine residues. The apparent molecular weights of native endo-(1→4)-β-D-glucanases and those obtained under dissociating conditions indicated that endo B and C are composed of single polypeptide chains, and endo A consists of two dissimilar subunits. To our knowledge, this is the first report of an endo-D-glucanase having two subunits. The pH optimum of 2.8–3.0 for endo B with CM-cellulose as substrate is also, to our knowledge, the lowest reported in the literature. In addition, endo B showed D-xylanase activity whereas endo A and C were devoid of it. In contrast to the findings of Kanda *et al.*[50] with F_1 endo-D-cellulase from *I. lacteus,* which hydrolyzes CM-cellulose and D-xylan at the same site, endo B hydrolyzes CM-cellulose and D-xylan at different sites.

The cellulase components of *S. rolfsii* cellulase-complex display multiplicity. Comparison of the amino acid contents of the three endo-D-glucanases and their subunit structure precludes derivation of one from the other by addition or deletion of polypeptides or polysaccharides.

Cellobiose was not hydrolyzed by any of the three endo-D-glucanases from *S. rolfsii,* whereas cellotriose was hydrolyzed extremely slowly. An endo-D-glucanase from *Trichoderma reesei* has been reported to hydrolyze cellobiose to D-glucose[52]. The *C. thermocellum* endo-D-glucanase, on the other hand, was inactive on cellobiose and cellotriose[32].

Endo A, B, and C produced cellobiose, cellotriose, and D-glucose from H_3PO_4-swollen cellulose and lichenan. Shoemaker and Brown[38,53] also could not detect cellotetraose, cellopentaose, or cellohexaose in the hydrolysis products of H_3PO_4-swollen cellulose by *T. viride* endo-D-glucanases, though Kanda *et al.*[43] reported the presence of small proportions of cellotetraose by *I. lacteus* endo-D-cellulase. D-Glucose was the principal product of hydrolysis by *T. koningii* E_4 endo-D-glucanase from H_3PO_4-swollen cellulose[54]. The major products of lichenan hydrolysis by Penicillium endo-D-glucanase[55] were reported to be cellopentaose and cellohexaose, whereas Clarke and Stone[56] found cellotriose to be the major product from the exhaustive hydrolysis of lichenan by *A. niger* endo-D-cellulase.

On the basis of the equal degradation of the C-1‴–O-4″ and C-1⁗–O-4‴ bonds of cellopentaosylglucitol by the endo-D-glucanases from *A. niger,* and as observed by us for cellohexaose, Hurst *et al.*[57] suggested that the hexamer is one residue too large to be completely accomodated in the active centre and that the specificity region of the endo-D-cellulase is five D-glucose units long. The kinetics of splitting of β-(1→4)-cello-oligosaccharides by endo A, B, and C are dependent on chain length of the substrate as are those of other endo-D-glucanases[55,57–59]. According to Hanson[60], the trend in the drastic increase in the K_f values, which tapers off from

cellopentaose to higher cello-oligosaccharides, indicates that the specificity region of the enzyme is five D-glucose units in length.

Although *S. rolfsii* endo-D-glucanases hydrolyze H_3PO_4-swollen cellulose (which is a model substrate of the amorphous components of the native cellulose fibres), cello-oligosacharides, and higher cellodextrins to cellobiose, cellotriose, and small proportions of D-glucose, comparison of the apparent $[S]_{0.5v}$ and V_{max} values of β-D-glucosidase[7] and endo-D-glucanase components from *S. rolfsii* suggest that the major route of D-glucose formation from cellulose is by hydrolysis (through β-D-glucosidase components of the cellulase complex) of cellodextrins produced by endo-D-glucanase.

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