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CHARACTERIZATION OF ENDO-1,4-β-D-GLUCANASES PURIFIED FROM *TRICHODERMA VIRIDE*

S.P. SHOEMAKER and R.D. BROWN, Jr. *

Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Va. 24061 (U.S.A.)

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Summary

Four electrophoretically distinct endo-1,4-β-D-glucanases (EC 3.2.1.4) from *Trichoderma viride* have been identified and named as isozymes, Endoglucanases I, II, III and IV, according to their electrophoretic mobilities on polyacrylamide gels. Endoglucanases II, III and IV, the homogeneity of each of which was established by discontinuous gel electrophoresis and ultracentrifugation, had specific activities on CM-cellulose of 1010, 60 and 250 specific fluidity units/mg protein, respectively. These enzymes have similar pH optima (pH 4.0—4.5) and are labile at pH values greater than 8.0.

The endoglucanases are high in acidic and hydroxylated amino acids and glycine, but low in basic amino acids. Values of 12.0, 10.3 and 13.1 have been determined for the $\epsilon_{280}^{1\%}$ of purified Endoglucanases II, III and IV, respectively. Sedimentation equilibrium analysis has established the molecular weights of Endoglucanases II, III and IV to be 37 200, 52 000 and 49 500, respectively.

The three endoglucanases contain mannose, galactose, glucose and glucosamine. Mannose is the principal neutral sugar in each enzyme. Endoglucanase II is distinguished by its low carbohydrate content, 4.5% (w/w), compared to Endoglucanases III and IV which contain 15.0% and 15.2% carbohydrate, respectively.

Introduction

Originally thought to be one enzyme [1], cellulases of true cellulolytic organisms are now well established to be multicomponent enzyme systems [2—6]. These enzyme components include the 1,4-β-D-glucan 4-glucanohydrolases (EC 3.2.1.4), the 1,4-β-D-glucan cellobiohydrolases (EC 3.2.1.91) and the β-glucosi-

* To whom correspondence should be addressed.

dases (EC 3.2.1.21). Investigators from this laboratory have purified to homogeneity an exo-cellobiohydrolase C [7] and a β-glucosidase [2] from *Trichoderma viride*. This paper reports the puridication of endo-1,4-β-D-glucanases, or endoglucanases, from *T. viride*.

Several investigators have purified endo-1,4-β-D-glucanases to varying degrees of homogeneity from cellulolytic fungi including *T. viride* [4,8-10], *Trichoderma koningii* [11], *Fusarium solani* [12], *Sporotrichum pulverulentum* [13] and *Irpex lacteus* [14,15]. Endoglucanases usually have been identified according to their liquefying (viscosimetric) and saccharifying activities using CM-cellulose as substrate. In the case of each organism studied, multiple endo-1,4-β-D-glucanases were demonstrated in culture filtrates. However, adequate evidence of purity has been established only for the endoglucanases from *T. viride* [8—10]. *S. pulverulentum* [13] and *I. lacteus* [14,15]. Since studies have provided only limited information regarding the structure and properties of the endoglucanases, this paper reports the purification and characterization of each of the enzymes from *T. viride* exhibiting endoglucanase activity as measured by the viscosimetric assay.

Materials and Methods

Materials

Pancellase SS, a commercial cellulase preparation derived from *T. viride*, was purchased from Yakult Biochemicals Co., Ltd., Shingikancho, Japan. High viscosity CM-cellulose 7 HP (DS = 0.83) was obtained from Hercules Powder Company, Hopewell, Va. Phosphoric acid swollen cellulose (Walseth cellulose) was prepared by swelling Avicel PH 101 in phosphoric acid [12].

General procedures

The protein concentration of crude cellulase solutions was determined using an ultraviolet absorption method [16]. The concentration of solutions of purified Endoglucanases II, III or IV was calculated using experimentally determined extinction coefficients ($\epsilon_{280}^{1\%}$ = 12.0, 10.3 and 13.1, respectively).

A viscosimetric assay was used to measure endoglucanase activity. A prefiltered 0.22% solution of CM-cellulose in 50 mM sodium acetate/3 mM sodium azide buffer, pH 4.5, was used as substrate. The decrease in viscosity upon incubation of the enzyme at 40°C for 30 min with CM-cellulose was measured by the procedure of Hash and King [17] as modified by Liu [18]. Units of activity are expressed in terms of the change in specific fluidity ($\Delta\phi_{sp}$) per min. In order to obtain a linear relationship between units and amount of enzyme only drain times ranging from 45 to 70 s ($1.04 \geqslant \phi \leqslant 0.49$) were used in activity calculations [19].

A conductivity meter, calibrated with NaCl solutions, was used to adjust buffers to ionic strengths which are expressed as the NaCl concentrations yielding equivalent conductivity values.

The method of Nelson [20] and Somogyi [21] was used to estimate reducing sugars. Discontinuous gel electrophoresis using buffer system No. 1 described by Maurer [22] was employed to assess homogeneity of enzyme solutions during purification. Protein was stained with Coomassie blue and carbohydrate was stained with the periodic acid-Schiff reagent.

Purification of "water fraction" endoglucanases

An Avicel column as described by Gum and Brown [7] was used in the initial separation of cellulase components from Pancellase. A dialyzed enzyme solution containing 4 g protein in 500 ml of 50 mM sodium acetate buffer, pH 5.0, was applied to a column (15 cm diameter × 1 cm height) containing 70 g of Avicel equilibrated in the same buffer. The non-adsorbed proteins which were eluted by washing with 3 l of this buffer were termed the "buffer fraction" enzymes. The adsorbed enzymes were subsequently eluted with 2 l distilled water and the resulting turbid "water fraction" eluate was clarified by the incubation procedure of Gum and Brown [7].

The "water fraction" enzymes from the Avicel column, after concentration on an Amicon PM-30 membrane (Amicon Corporation, Lexington, Mass.) were separated further by the batch process of Gum and Brown [7]. Fractions I, II and III were obtained by stepwise elution with 0.05 M sodium succinate buffer at pH 5.35, 5.0 and 3.6, respectively. All buffers contained 3.0 mM sodium azide and the pH 3.6 buffer had been adjusted with NaCl to an ionic strength equivalent to a 0.5 M NaCl solution. These fractions were concentrated by ultrafiltration using a PM-10 membrane. Most of the endoglucanase activity was contained in Fraction I.

To a column (2.5 × 40 cm) of DEAE-Sephadex A-50 was added 150 mg of Fraction I protein dissolved in 5.0 mM imidazole/3.0 mM sodium azide, pH 6.0, adjusted to an ionic strength equivalent to 0.07 M NaCl. Two endoglucanases were eluted from this column when a three-step gradient in ionic strength equivalent to 0.07 M, 0.15 M, and 0.3 M NaCl was applied. Endoglucanase III was obtained in pure form upon elution with the buffer equivalent in ionic strength to 0.3 M NaCl. Contaminating proteins associated with Endoglucanase IV, which had been eluted at an ionic strength of 0.07 M, were removed by further chromatography on DEAE-Sephadex A-50 using a single step in ionic strength equivalent to an increase from 0.01 M to 0.07 M NaCl.

Purification of "buffer fraction" enzymes

Concentrated "buffer fraction" enzymes from the Avicel column were separated using the batch process [7]. Endoglucanase activity was almost entirely associated with Fraction I (eluted at pH 5.35).

To a column (2.5 × 40 cm) of DEAE-Sephadex A-50 was added 280 mg of Fraction I protein dissolved in 5.0 mM imidazole/3.0 mM sodium azide, pH 6.0, at a buffer strength of 0.01 M. Again two regions of endoglucanase activity were obtained when a three-step gradient in ionic strength equivalent to 0.07 M, 0.15 M and 0.3 M NaCl was applied. Endoglucanase II, which was eluted at an ionic strength equivalent to 0.07 M NaCl, subsequently was purified by the chromatographic method described previously for Endoglucanase IV. Similar chromatographic procedures yielded only a small (<0.2 mg) quantity of pure Endoglucanase I.

Endoglucanases II, III and IV each were concentrated, dialyzed and filtered through a 0.22 μm Millipore filter. Storage at −20°C for three months did not result in any change in activity or electrophoretic properties.

The endoglucanases are named as isozymes according to their electrophoretic mobility in polyacrylamide gels.

Ultracentrifugation

Sedimentation velocity studies were performed on a Beckman-Spinco Model E Ultracentrifuge. Solutions of Endoglucanases II, III and IV containing 2.55, 3.09 and 3.56 mg enzyme/ml, respectively, in 0.1 M KCl were centrifuged at 49 780 rev./min. For sedimentation equilibrium studies 0.1 mg samples of each enzyme containing 1.01—2.33 mg enzyme/ml in 0.1 M KCl were centrifuged at a rotor speed of 20 410 rev./min. Fringe displacements obtained by Raleigh interference optics were measured using a Nikon Shadograph. The partial specific volumes of Endoglucanases II, III and IV were calculated from the amino acid and carbohydrate composition according to the method of Cohn and Edsall [24] and found to be 0.711, 0.696 and 0.698, respectively.

pH optimum

The optimum pH for enzyme activity using CM- or phosphoric acid-swollen cellulose as substrate was determined using the viscosimetric and reducing sugar assays, respectively. CM-cellulose (0.22%, w/v) solutions or phosphoric acid swollen cellulose (1.0%, w/v) suspensions in 50 mM sodium acetate buffer which had been adjusted to specific pH values were incubated with each enzyme at 40°C for 30 min.

pH stability

The stability of Endoglucanases II, III and IV was examined over a pH range of 3—11. Buffer solutions of 0.05 M citrate, HEPES (*N*-2-hydroxyethyl piperazine-*N*-2-ethane sulfonic acid) or CAPS (cyclohexylaminopropane sulfonic acid) were used over pH ranges of 3.0—6.0, 6.0—9.0 and 9.0—11.0, respectively. Stability of the endoglucanases was measured by the viscosimetric assay at pH 4.5 after pre-incubation at 40°C for 1 h at specific pH values.

Amino acid and amino sugar analyses

Amino acid and amino sugar analyses were performed as stated previously [7] with the exception that values for methionine were based only on oxidized samples. Tryptophan content was calculated according to the method of Bencze and Schmid [25].

Neutral carbohydrate

The neutral carbohydrate components of the endoglucanases were identified as the alditol acetate derivatives [26] by gas liquid chromatography at 210°C on a 10-ft column of 1% OV-225 on Chromasorb G HP in a Varian Aerograph 2740 instrument and confirmed by use of a Varian MAT-112 Gas Chromatograph-Mass Spectrometer equipped with a Varian 620/L Computer. Total neutral carbohydrate was determined both by gas chromatography [26] and by the phenol/sulfuric acid method [27] using mannose as the standard.

Results

Purification of endoglucanases

A flow diagram of the overall procedure for endoglucanase purification is shown in Fig. 1. After enzymic components in Pancellase were separated

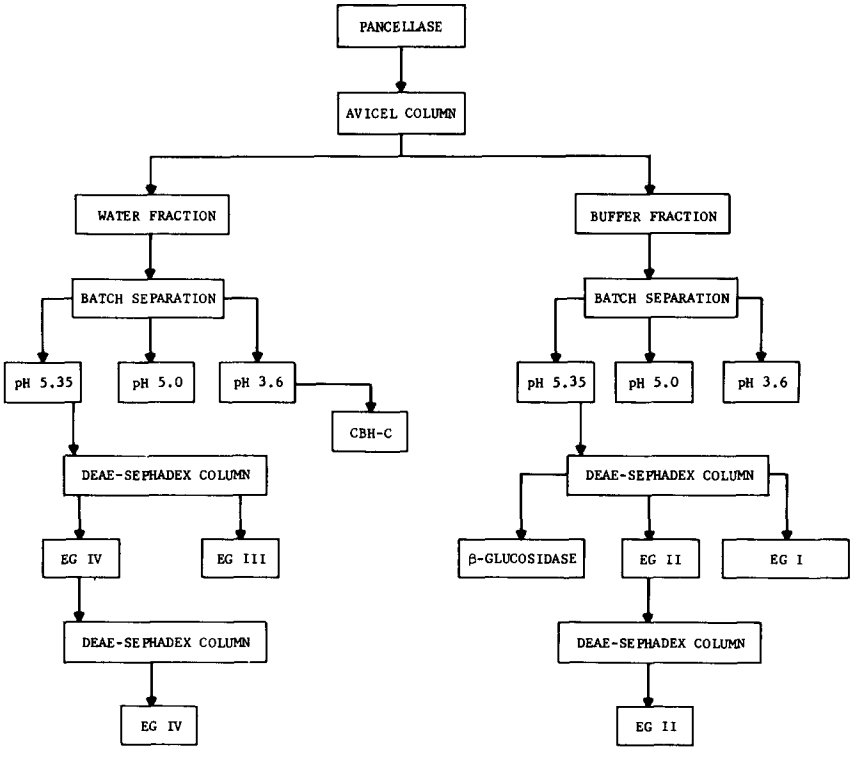

Fig. 1. Flow diagram of the general procedure used in purifying endo-1,4-β-D-glucanases from Pancellase, a commercial enzyme preparation derived from *T. viride*.

according to their affinities for Avicel, electrophoretic analysis of the buffer and water fractions showed that most of the proteins were not adsorbed. The "buffer fraction" contained β-glucosidase, whereas the enzyme present in the "water fraction" included an exo-cellobiohydrolase C.

"Water fraction" endoglucanases

The purification of "water fraction" endoglucanases is presented in Table I. These enzymes, constituting only a small fraction of the total protein and endoglucanase activity, are distinguished by their affinity for crystalline cellulose. Batch separation on DEAE-Sephadex A-50 of the "water fraction" proteins removed exo-cellobiohydrolase C. Column chromatography of fraction I proteins from batch separation resulted in the separation of two endoglucanases (Fig. 2). The protein of fractions 25—35 (Fig. 2), which contained Endoglucanase IV, had a specific activity of 240 units per mg protein (Table I). Endoglucanase III in fractions 145—155 was obtained in pure form. Electrophoretic analysis indicated that the endoglucanase activity associated with the major protein peak (fractions 110—140) was due to Endoglucanase III. Further anion exchange chromatography yielded pure Endoglucanase IV. Approx. 30 mg of each Endoglucanase (III and IV) were purified by these procedures.

TABLE I

SUMMARY OF PURIFICATION OF WATER FRACTION ENDOGLUCANASES

		Protein (g)		Total activity ($\Delta\phi_{sp} \cdot min^{-1}$)		Specific activity ($\Delta\phi_{sp} \cdot min^{-1} \cdot mg^{-1}$ protein)
Avicel column	Pancellase	12.1		128 260		10.6
	Buffer fraction	9.43	(77.9%) *	98 072	(76.5%)	10.4
	Water fraction	1.45	(12.0%)	23 345	(18.2%)	16.1
Batch separation	Water fraction	1.42		24 850		17.5
	Fraction 1, pH 5.35	0.368	(25.9%)	18 400	(74.0%)	50
	Fraction 2, pH 5.0	0.145	(10.2%)	2 900	(11.7%)	20
	Fraction 3, pH 3.6	0.178	(50.6%)	2 154	(8.7%)	3
DEAE column	Fraction 1	0.26		13 910		53.5
	Endoglucanase IV	0.02	(7.7%)	4 800	(34.5%)	240
	Peak 2	0.2	(77.0%)	5 000	(35.9%)	25
	Endoglucanase III	0.02	(7.7%)	1 200	(8.6%)	60
DEAE column	Endoglucanase IV	0.041		9 840		240
	Endoglucanase IV	0.025	(61.0%)	6 250	(63.5%)	250

* In parentheses, percent recovery of protein or activity in that step.

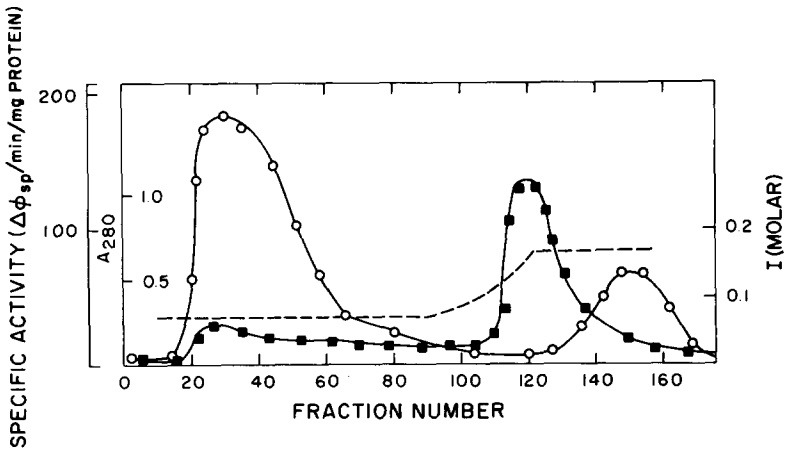

Fig. 2. Elution patterns from DEAE-Sephadex column chromatography of the proteins in the water fraction-derived fraction I retentate. The retentate from fraction I of a batch separation was applied to a DEAE-Sephadex column (2.5 × 45 cm). The proteins were eluted using a flow rate of 18—20 ml/h and a step gradient (ionic strength) from 0.07 M to 0.15 M at fraction 80 to 0.30 M at fraction 130 in pH 6.0, 5.0 mM imidazole buffer containing 3.0 mM sodium azide. Protein concentration is estimated by absorbance at 280 nm (□————□). Specific endoglucanase activity (○————○) and ionic strength (I) of effluent (- - - - -) are also given. Fractions of approx. 2.5 ml each were collected every 10 min.

"Buffer fraction" endoglucanases

Two endoglucanases were identified in the "buffer fraction" proteins and a substantial quantity (30 mg) of one of these endoglucanases (Endoglucanase II) was purified to homogeneity. Table II, which summarizes the purification of these endoglucanases, shows that most of the protein and endoglucanase activity were associated with the buffer fraction from an Avicel column. Batch elution of "buffer fraction" protein adsorbed on DEAE-Sephadex at pH 5.35 (fraction 1) increased specific endoglucanase activity but did not separate β-glucosidase and endoglucanase activities. Ultrafiltration of the "buffer fraction" enzymes provided a significant increase in specific endoglucanase activity of the retentate.

During separation of endoglucanases from other protein components in fraction I from the buffer fraction by anion exchange column chromatography (Fig. 3), the β-glucosidase was eluted isocratically (fractions 1—12). Fractions 41—45, which contained a highly active endoglucanase, yielded two protein bands when analyzed by gel electrophoresis. Whereas similar analysis of fractions 97—100 demonstrated two other protein bands, each of which was apparently slightly more anionic than the two corresponding bands observed for fractions 41—45. Additional anion exchange chromatography achieved purification of the enzyme (Endoglucanase II) of fractions 41—45 (Table II). Only a small quantity of the enzyme (Endoglucanase I) insufficient for its structural characterization, was obtained by further anion exchange chromatography.

Enzyme purity

Endoglucanases II, III and IV each yielded a single protein band when analyzed by gel electrophoresis (Fig. 4); the addition of 0.1 percent sodium dodecyl sulfate (SDS) to the gel medium also resulted in single bands for each of

TABLE II

SUMMARY OF PURIFICATION OF BUFFER FRACTION ENDOGLUCANASES

		Protein (g)	Total activity ($\Delta\phi_{sp} \cdot min^{-1}$)	Specific activity ($\Delta\phi_{sp} \cdot min^{-1} \cdot mg^{-1}$ protein)
Avicel column	Pancellase	12.1	128 260	10.6
	Buffer fraction	9.43 (77.9%) *	98 072 (76.5%)	10.4
	Water fraction	1.45 (12.0%)	23 345 (18.2%)	16.1
Batch separation	Buffer fraction	1.59	40 227	25.3
	Fraction 1, pH 5.35	0.863 (54.3%)	40 820 (101%)	47.3
	Fraction 2, pH 5.0	0.126 (7.9%)	3 591 (8.9%)	28.5
	Fraction 3, pH 3.6	0.476 (30.1%)	1 317 (3.3%)	2.75
DEAE column	Fraction 1	0.277	18 559	67
	Endoglucanase II	0.025 (9.0%)	9 000 (48.5%)	360
	Endoglucanase I	0.035 (12.6%)	2 100 (11.3%)	60
DEAE column	Endoglucanase II	0.036	11 880	330
	Endoglucanase II	0.009	9 090 (76.5%)	1010

* In parentheses, percent recovery of protein or activity in that step.

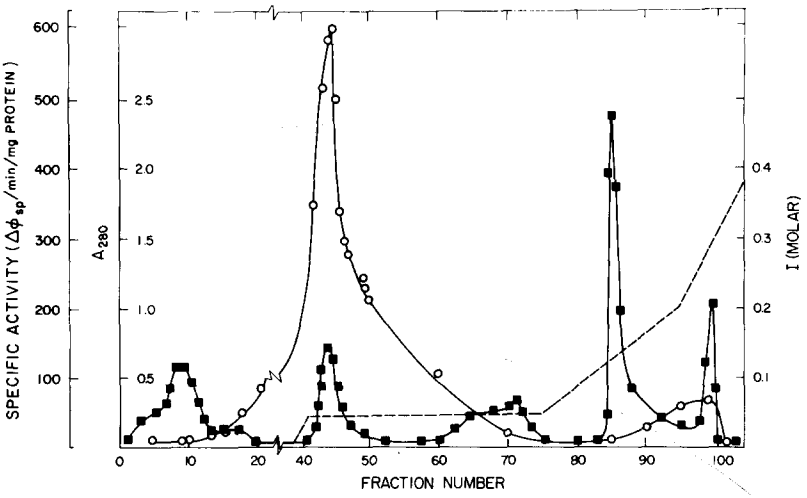

Fig. 3. Elution patterns from DEAE-Sephadex column chromatography of the proteins in the buffer fraction-derived fraction I retentate. The retentate from fraction I of a batch separation was applied to a DEAE-Sephadex column (2.5 × 45 cm). The proteins were eluted using a flow rate of 18—20 ml/h and a step gradient (ionic strength) in pH 6.0, 0.05 M imidazole buffer containing 3.0 mM sodium azide. The initial ionic strength of the eluting buffer was 0.01 M which was followed by subsequent increases in ionic strength from 0.07 M (fraction 35) to 0.15 M (fraction 72) and finally to 0.3 M (fraction 92). Protein concentration is estimated by absorbance at 280 nm (------). Specific endoglucanase activity (o———o) and ionic strength (I) of the effluent are also given. Fractions of approx. 2.5 ml each were collected every 10 min.

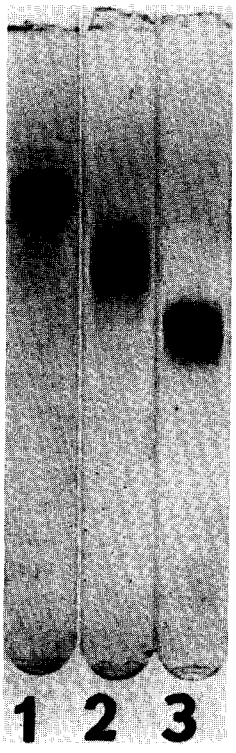

Fig. 4. Disc gel electrophoresis of three endo-1,4-β-D-glucanases purified from *T. viride*. The protein samples applied to these gels were, from left to right: 29 μg of Endoglucanase IV; 29 μg of Endoglucanase III and 33 μg of Endoglucanase II. The gels were stained for protein with Coomassie blue. The separator gels contained 7.5% acrylamide buffered at pH 8.9 and sample gels contained 2.5% acrylamide buffered at pH 6.9.

these enzymes. Staining for carbohydrate demonstrated that Endoglucanases II, III and IV are glycoproteins and that only a minute amount of carbohydrate is associated with Endoglucanase II compared with Endoglucanases III and IV.

Ultracentrifugation studies using the sedimentation velocity method revealed no macroheterogeneity since a single sedimenting boundary was observed for each enzyme. Under sedimentation equilibrium conditions, a linear relationship was observed between the logarithm of fringe displacement and the square of the distance from the center of the rotor. This indicates a monodisperse enzyme solution and taken together with the results of SDS gel electrophoresis, confirms the absence of protein aggregates.

pH optimum

The activity of each endoglucanase exhibited similar pH dependence, with optimum activities at pH 4.0—4.5 on phosphoric acid swollen cellulose. On CM-cellulose (Fig. 5), Endoglucanase III and IV again displayed optima at pH 4.0—4.5 whereas a broader pH optimum range was found for Endoglucanase II (pH 3.0—4.0). These optima observed for Endoglucanases III and IV are in fair agreement with those reported for cellulases (endoglucanases) II-A, II-B, and III [8,9]. The region below pH 2.5 was not investigated due to the effect of low pH on the viscosity of the soluble substrate. The similar optima with both substrates suggest that there is little or no effect on the enzymic activity of Endoglucanases III and IV by the ionic substituent groups in CM-cellulose.

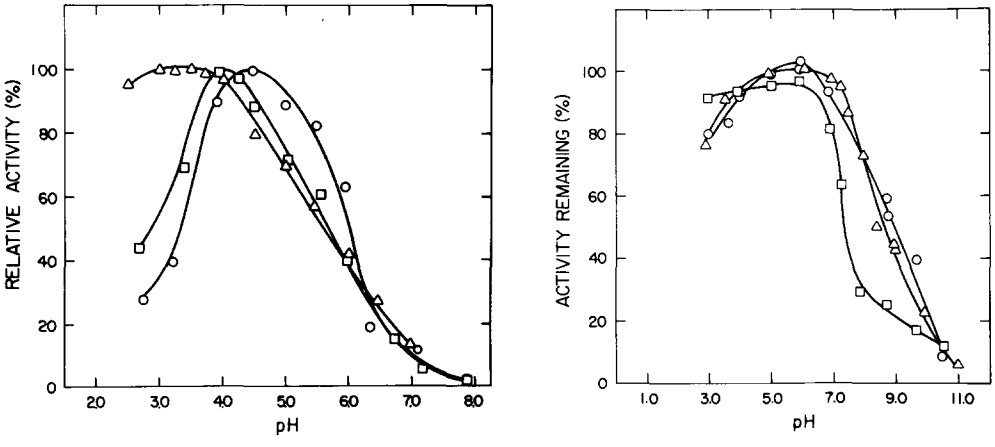

Fig. 5. pH optima for Endoglucanases II, III and IV with carboxymethylcellulose as substrate. Enzyme samples were incubated with CM-cellulose solutions at different pH values at 40°C for 30 min (see Materials and Methods) and assayed for activity using the viscosimetric assay. The curves represent Endoglucanases II (△), III (○) and IV (□).

Fig. 6. pH stability of Endoglucanases II, III and IV. Enzyme samples were incubated in different pH buffer solutions at 40°C for 1 h, and then assayed for activity using the viscosimetric assay. Buffer solutions of 0.05 M citrate, HEPES and CAPS were used over pH ranges of 3.0—6.0, 6.0—9.0 and 9.0—11.0, respectively (see Methods). At pH 6.0 and 9.0 samples were analyzed in each of two different buffers. The curves represent Endoglucanases II (△), III (○) and IV (□).

pH stability

Since endoglucanase activity in crude cellulase preparations had been observed to decrease upon exposure to pH values greater than 9.0, the effect of pH on the activity (viscosimetric) of purified endoglucanases was determined. Above pH 8.0 each of the purified endoglucanases demonstrated marked lability (Fig. 6). Thus the purified endoglucanases may be more sensitive to the effect(s) of pH than are the same enzymes in the presence of the other components of the cellulase system. These results are consistent with the demonstration by Okada of alkali lability at 45°C with cellulases II-A, II-B and III over a limited pH range [2].

Molecular weight

A weight-average molecular weight was calculated for each endoglucanase using the long-column meniscus depletion technique [23]. Computer analysis yielded weight-average molecular weights of 37 200 ± 2400, 52 000 ± 2600, and 49 500 ± 2200 for Endoglucanases II, III and IV, respectively.

Carbohydrate composition

Total carbohydrate content measured by the phenol/sulfuric acid method agreed with results from carbohydrate staining procedures. Thus Endoglucanase II possessed much less carbohydrate (3.2%) than either Endoglucanase III or IV (14.0% or 15.9%, respectively). These results were consistent with the weight percent of total carbohydrate determined by gas chromatography of the alditol acetate derivatives (Table III). Like the exo-cellobiohydrolase C [7], all three of the endoglucanases contained mannose, galactose, and glucose. Mannose was the predominant neutral sugar in each enzyme. The "water fraction" endoglucanases have nearly identical neutral sugar compositions, whereas the "buffer fraction" endoglucanase (Endoglucanase II) contained a much smaller amount of mannose. In contrast to the amino acid and amino sugar compositions, Endoglucanase III did not differ remarkably from Endoglucanase IV in its neutral carbohydrate composition.

Glucosamine, but not galactosamine, was present in each of the endoglucanases. Based on galactosamine as an internal standard and assuming glucosamine to

TABLE III

Carbohydrate composition (weight percent) of endo-1,4-β-D-glucanases. As described in Materials and Methods, *N*-acetylglucosamine was determined by ion exchange chromatography, total carbohydrate by the phenol/sulfuric acid method and neutral carbohydrates by gas chromatography of alditol acetates.

	II	III	IV
Total amino sugar			
N-acetylglucosamine	0.28	0.44	0.20
Total carbohydrate	3.2	14.0	15.9
Neutral carbohydrate			
Total	4.5	15.0	15.2
Mannose	2.49	11.4	10.9
Galactose	1.05	1.68	2.20
Glucose	0.95	1.92	2.13

be present in its *N*-acetylated form, Endoglucanase III contained twice as much *N*-acetylglucosamine as either Endoglucanase II or IV (Table III).

Composition of endoglucanases

By use of the molecular weights calculated for the enzymes from sedimentation equilibrium data, the compositions of Endoglucanases II, III and IV were expressed as mol of constituent per mol of endoglucanase (Table IV). In this determination amino acids, glucosamine and neutral sugars were assumed to constitute all of the dry weight of the enzyme. It should be noted that amino acid and carbohydrate content apparently accounted for 68.2%, 104.3% and 88.5% of the dry weight of Endoglucanases II, III and IV, respectively. Since repeated amino acid analysis yielded consistent results, the basis for the incomplete aggregate composition of Endoglucanase II is not known. It may be due, in part, to adsorbed ionic materials.

The compositions of Endoglucanases II, III and IV are presented in Table IV. These enzymes are high in acidic and hydroxylated amino acids and glycine but low in basic amino acids. Among the endoglucanases, Endoglucanase III contained the lowest percentage of arginine, glutamic acid (or amide), isoleucine and phenylalanine and the highest percentage of threonine and proline. The compositions of Endoglucanases II and IV, particularly as expressed in mol percent of constituent amino acids, are quite similar. The relative abundances of all amino acids, except threonine, proline and half cystine, are identical in these

TABLE IV

COMPOSITION OF ENDOGLUCANASES II, III AND IV

	mol/mol endoglucanases		
	II	III	IV
Lysine	6	7	7
Histidine	5	5	6
Arginine	9	6	11
Aspartic acid	46	54	49
Threonine	29	47	40
Serine	31	50	38
Glutamic acid	28	28	34
Proline	15	32	23
Glycine	37	45	47
Alanine	26	31	32
Cystine (1/2)	7	14	12
Valine	18	22	21
Methionine	4	5	3
Isoleucine	17	15	19
Leucine	23	24	21
Tyrosine	12	17	13
Phenylalanine	11	9	12
Tryptophan	8	11	10
Glucosamine	0.5	1.1	0.5
Mannose	5.9	36.6	33.2
Galactose	2.4	5.4	6.7
Glucose	2.2	6.2	6.5
Molecular weight	37 200	52 000	49 500

two enzymes. The amino acid composition of the β-glucosidase differs significantly from those of the other cellulase enzymes [28],whereas the composition of the exo-cellobiohydrolase C [7] is generally similar to those of the endoglucanases.

The composition of the water fraction enzymes (Endoglucanases III and IV) can be compared directly because of their similar molecular weights. The significantly different amounts of arginine, serine, proline and glucosamine in Endoglucanases III and IV justified naming these enzymes as isozymes.

Endoglucanase II is also named as an isozyme; however, on the basis of its composition alone this nomenclature could be questioned. This "buffer fraction" enzyme is smaller and thus, could have arisen by proteolytic modification of precursor molecules. Endoglucanase II has significantly less carbohydrate, especially mannose, but exceeds either Endoglucanase III or IV in phenylalanine, leucine, isoleucine and arginine. In addition, its lack of affinity for crystalline cellulose, yet high endoglucanase activity with CM-cellulose, could reflect unique activity and specificity.

Discussion

Four electrophoretically distinct endoglucanases, two from the water fraction (Endoglucanases III and IV) and two from the buffer fraction (Endoglucanases I and II) have been identified from a commercial preparation of *T. viride* enzymes. The specific activities of the "buffer fraction" endoglucanases (Endoglucanases I and II) were four times that of the corresponding "water fraction" endoglucanases (Endoglucanases III and IV). The endoglucanases, in general, were found to be very similar in size and composition to the exo-cellobiohydrolase C [7] and were in agreement with literature reports of molecular weight and total carbohydrate of purified endoglucanases from *T. viride* [8,10].

Multiple forms of glycoprotein endoglucanases have been reported from *S. pulverulentum* [13,29] and three endoglucanases recently have been purified from *T. viride* by Okada [8,9] and two by Berghem et al. [10]. Okada found 12% and 14% carbohydrate associated with cellulases II-A and II-B, respectively. This substantial carbohydrate content is consistent with present results on the two water fraction enzymes. Molecular weights of 30 000, 43 000, and 45 000 were determined by gel filtration for cellulases II-A, II-B and III, respectively [8,9]. However, some questions have arisen concerning use of this procedure for molecular weight determination of glycoproteins [30]. Since no other attempts to characterize cellulases II-A, II-B and III have been reported, it is impossible to compare further these endoglucanases based on structural properties. In addition, Berghem et al. [10] found carbohydrate associated with two endoglucanases purified from *T. viride*. These enzymes, although somewhat similar in molecular weight and carbohydrate composition, do not appear identical to any of the endoglucanases from our laboratory. Differences in enzyme properties may be due to culture techniques or to strain variation within the *Trichoderma* species.

Endoglucanases II, III and IV differ markedly from the purified endoglucanases of bacterial [31] or plant [32,33] origin which have little or no carbohy-

drate associated with them and function optimally at pH values of 5.5—7.0. It may be significant that the *Trichoderma* endoglucanases showing the highest affinity for cellulose (Endoglucanases III and IV) also contain the most carbohydrate. The value to fungal nutrition of a soluble enzyme activity on an insoluble substrate at a distance from the cell surface depends upon the avidity of enzyme binding to the substrate surface. The primary source of saccharifying activity, exo-cellobiohydrolase C, has the highest affinity for cellulose among the enzymes of the *T. viride* cellulase system [5]. On the other hand under normal growth conditions the *Sporocytophaga* endoglucanases may be in the slime layer or cell bound [31] and the pea enzymes may function primarily in cell wall modification [33]. The *Trichoderma* endoglucanases must act at pH values favorable for cell growth (i.e. below pH 5.5) and at values which permit activity of associated enzymes of the cellulase complex. Further comparisons between the activities of *T. viride* enzymes and those from other sources will be the subject of a subsequent paper.

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