Carbohydrate Research, 142 (1985) 299–314
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

PURIFICATION AND PARTIAL CHARACTERISATION OF THREE ENDO-GLUCANASES FROM *Dichomitus squalens**

XAVIER ROUAU†

Institut National Agronomique, Laboratoire de Microbiologie (Lignine), 9, Rue de l'Arbalète, 75231 Paris Cedex 05 (France)

AND MARIE-JOSÉ FOGLIETTI

Université René Descartes, Faculté des Sciences Pharmaceutiques et Biologiques, Laboratoire de Chimie Biologique, 4, Avenue de l'Observatoire, 75270 Paris Cedex 06 (France)

(Received February 15th, 1985; accepted for publication, April 17th, 1985)

ABSTRACT

Three endo-glucanases (En I–III) were obtained by chromatofocusing fractionation of a culture supernatant from the white-rot fungus *Dichomitus squalens*. They were purified further on Phenyl-Sepharose CL-4B, DEAE-Trisacryl, and Ultrogel AcA 54; ~21-, ~16-, and ~9-fold purifications were obtained for En I–III, respectively. The enzymes appeared as homogeneous proteins on disc gel electrophoresis with and without SDS (sodium dodecyl sulphate), and on isoelectric focusing; the respective mol. wts. were 42,000, 56,000, and 47,000, and the isoelectric points 4.8, 4.3, and 4.1. Optimum conditions for the hydrolysis of CM-cellulose were pH 4.8 and 55° for each enzyme, and each was stable over the pH range 4.0–8.5 but inactivated completely within 30 min at 70°. None of the purified enzymes exhibited β-D-glucosidase or cellobiohydrolase activity, but En II was weakly active towards laminaran and xylan. En I and En II acted more randomly on CM-cellulose than did En III. Cellotetraose was degraded by each endo-glucanase, whereas only En III could hydrolyse cellotriose.

INTRODUCTION

The use of enzymes active on polysaccharides from plant cell-walls is of increasing importance, not only because of bioconversion of lignocellulosics into fermentable sugars, but also because pure enzymes are powerful analytical tools for the study of cell-wall organisation or for extraction, purification, and elucidation of the structure of polysaccharides[1]. Cellulose is one of the main components

*Presented in part at the 611th Meeting of the Biochemical Society, Galway, Ireland, September 1984.
†Present address: Institut National de la Recherche Agronomique, Centre de Recherches Agro-Alimentaires, Laboratoire de Biochimie et Technologie des Glucides, Rue de la Géraudière, 44072 Nantes Cedex, France.

0008-6215/85/$ 03.30

of the cell wall from terrestrial plants and is often encountered in studies of structural polysaccharides. The hydrolysis of cellulose is achieved by at least two kinds of hydrolases, namely, endo-glucanases [(1→4)-β-D-glucan-4-glucanohydrolases, EC 3.2.1.4] which cleave, more or less randomly, the internal bonds of cellulose molecules, and exo-glucanases [(1→4)-β-D-glucan-4-cellobiohydrolases, EC 3.2.1.91] which release cellobiose units from the non-reducing ends of the glucan chains. The cooperative action of these enzymes causes the extensive degradation of native (crystalline) cellulose[2,3]. The unfractionated cellulolytic complex is usually known under the generic name "cellulase". The most powerful cellulases are of fungal origin. Enzymic preparations from the ascomycetes *Trichoderma viride*[4], *T. reesei*[5], and *T. koningii*[6] have been studied extensively. Cellulases from basidiomycetes have also been described, especially those from the white-rot fungi *Sporotrichum pulverulentum*[6–8] and *S. thermophile*[9].

The white-rot fungus *Dichomitus squalens* (syn: *Polyporus anceps*) has been studied previously for its production of polysaccharide-hydrolases especially endo-glucanases. A "crude enzyme" has been prepared from a cellulose-containing liquid culture of this fungus[10]. We now report the extensive purification and some properties of three endo-glucanases from this preparation.

RESULTS AND DISCUSSION

Purification (see Table I). — When applied to a PBE 94 chromatofocusing column at pH 7.2, most of the protein from the crude enzyme preparation from

TABLE I

PURIFICATION OF THE ENDO-GLUCANASES FROM *Dichomitus squalens*

Step		*Specific activity (nkat/mg)*	*Protein[a] (mg)*	*Total activity[b] (nkat)*	*Yield (%)*	*Purification (fold)*
Crude enzyme		230	100	23000	100	1
Chromatofocusing	En I	1666	5.40	8990	39.0	7.2
on PBE 94	En II	1310	1.72	2254	9.8	5.7
	En III	845	7.73	6532	28.4	3.7
Hydrophobic-interaction	En I	2964	1.75	5187	22.6	12.9
chromatography on	En II	2713	0.84	2195	9.5	11.8
Phenyl-Sepharose CL-4B	En III	1360	3 64	4952	21.5	5.9
Ion-exchange chromatography	En I	3694	1.36	5024	21 8	16.1
on DEAE-Trisacryl	En II	3338	0.58	1931	8.4	14.5
	En III	1357	2.72	3688	16.0	5.9
Gel-filtration on	En I	4890	0.84	4600	20.0	21.3
Ultrogel AcA 54	En II	3632	0.53	1914	8.3	15.8
	En III	2155	1.67	3600	15.6	9.4

[a]Determined by the method of Bradford. [b]Towards CM-cellulose (see Experimental).

Dichomitus squalens bound to the gel. Three main endo-glucanase peaks, as detected by their action on CM-cellulose and CMC-azure, were eluted by the pH gradient at pH 6.2 (En I), 5.1 (En II), and 4.7 (En III) (Fig. 1). An additional endo-glucanase peak was eluted by 0.5M NaCl. En I–III accounted for 80% of the total activity measured on CM-cellulose with relative ratios of 1.0:0.2:0.7. When measured on CMC-azure, 68% of the total activity was associated with the three endo-glucanases, with relative ratios of 1.0:0.7:1.0. On the basis of CM-cellulose-degrading activity, the chromatofocusing step gave ~7.2-, ~5.7-, and ~3.7-fold purifications for En I–III, respectively.

Each fraction was then subjected to hydrophobic-interaction chromatography on Phenyl-Sepharose CL-4B, in the presence of ammonium sulphate (M for En I and En II, 1.5M for En III) (Fig. 2). Nearly half of the injected protein (32, 46, and 47%) bound to the gel contained most of the injected endo-glucanase activity (52, 97, and 76%). Elution by decreasing concentrations of ammonium sulphate gave En I, and addition of ethylene glycol gave En II (0.5M ammonium sulphate, 25% ethylene glycol) and En III (0.4M ammonium sulphate, 37% ethylene glycol). The use of ethylene glycol was avoided with En I, because this fraction was inactivated during storage in the presence of this diol. Chromatography on Phenyl-Sepharose

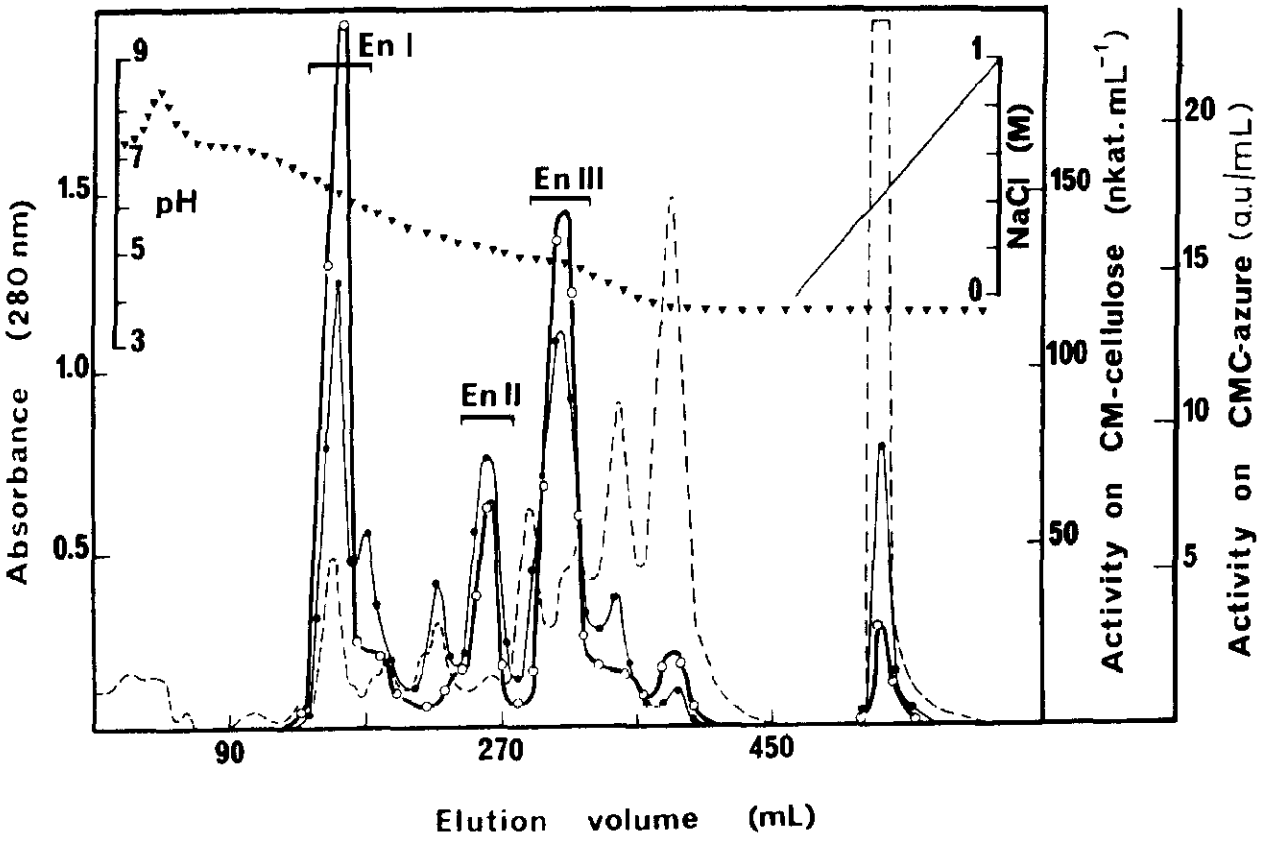

Fig. 1. Chromatofocusing of the crude enzyme preparation from *Dichomitus squalens* on PBE 94. A solution of the crude enzyme preparation (100 mg) in 25mM imidazole–HCl buffer (pH 7.2, 20 mL) was dialysed overnight in collagene tubes against the same buffer. The sample was applied to the column (50 × 1.0 cm) equilibrated with the same buffer. Proteins were eluted with 8-fold-dilution Polybuffer 74 (450 mL, Pharmacia) adjusted to pH 3.7. Bound proteins were removed by a linear gradient (100 mL) of 0→1.0M NaCl in the Polybuffer at 60 mL/h. Fractions (5 mL) were tested for pH (▼), absorbance at 280 nm (– – –), activity towards CM-cellulose (nkat/mL) (—○—), and activity towards CMC-azure (a.u./mL) (—●—).

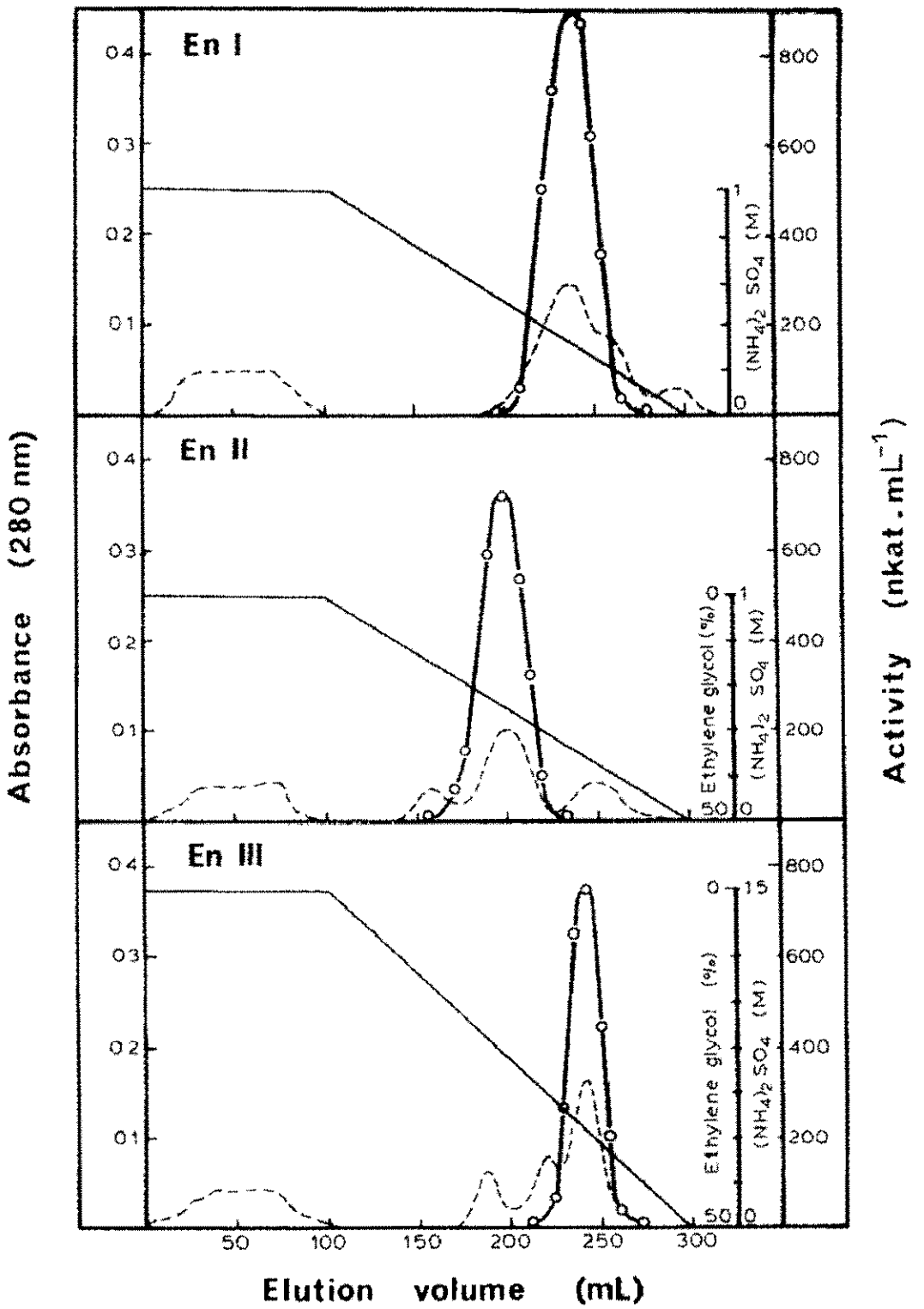

Fig. 2. Chromatography of the fractions En I–III (Fig. 1) on Phenyl-Sepharose CL-4B. Solid ammonium sulphate was added to give a final concentration of M for En I and En III, and 1.5M for En II. Samples were then applied to the column (20 × 0.8 cm) equilibrated with 25mM sodium acetate buffer (pH 5.0) containing M (En I and En II) or 1.5M (En III) ammonium sulphate. Bound proteins were eluted by a linear gradient (200 mL) of ethylene glycol (0→50%) in 25mM sodium acetate buffer (pH 5.0) at 50 mL/h. Fractions (5 mL) were tested for absorbance at 280 nm (– – –) and activity towards CM-cellulose by reducing sugar determination (—○—).

then allowed further purification of endo-glucanases (~1.8-, ~2.0-, and ~1.6-fold for En I–III, respectively), and elimination of any ampholines present after chromatofocusing.

Each endo-glucanase fraction was then submitted to ion-exchange chromatography on DEAE-Trisacryl at pH 7.5 (Fig. 3). Fractions containing endo-glucanase activity were eluted by 0.12, 0.13, and 0.12M sodium chloride for En I–III, respectively. En III was not further purified by this step, whereas En I and

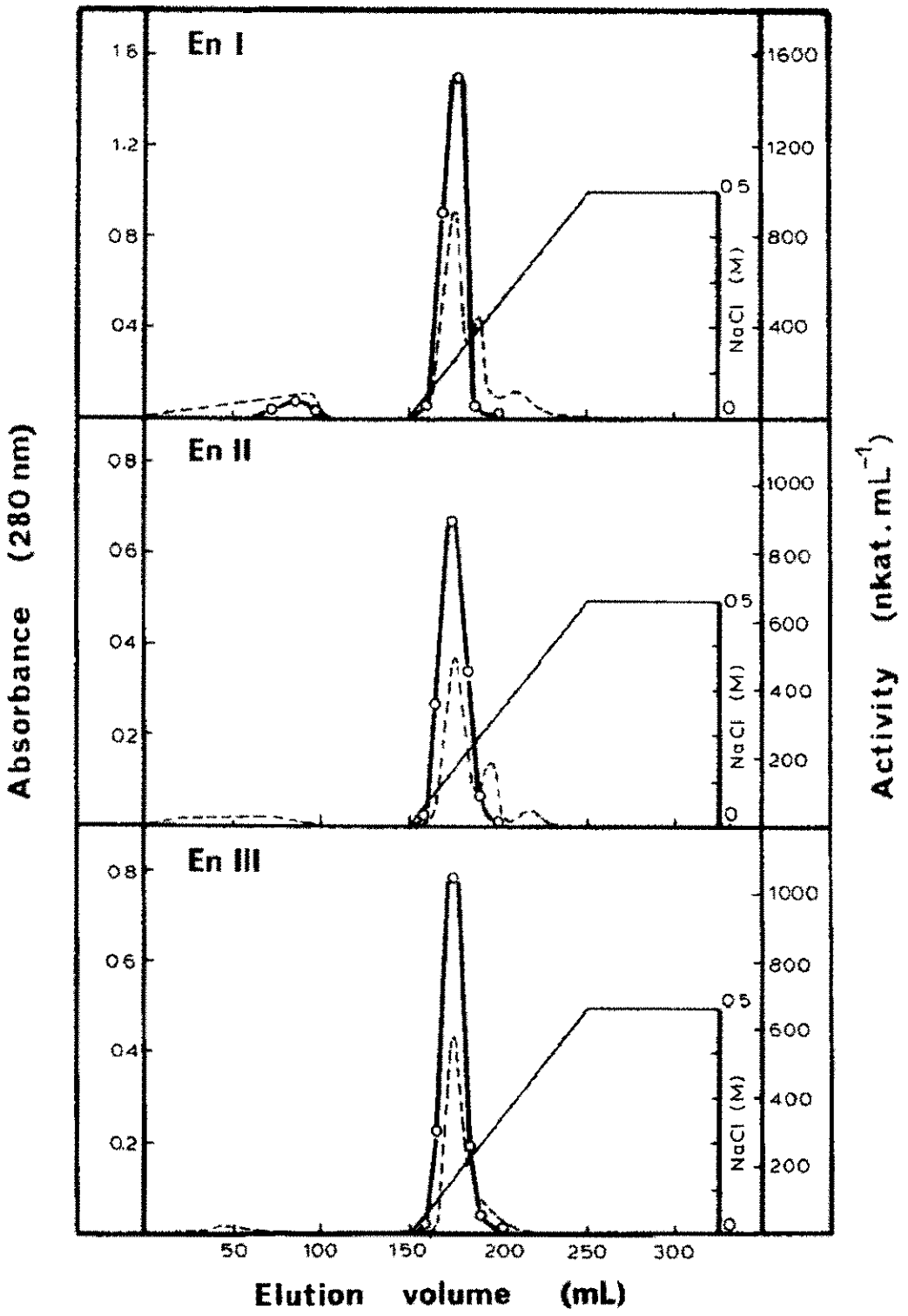

Fig. 3. Ion-exchange chromatography of En I–III fractions from Fig. 2. The fractions were dialysed in collagene tubes at 4° against 0.01M imidazole–HCl buffer (pH 7.5, 5 L) and then applied to a column (10 × 0.9 cm) of DEAE-Trisacryl equilibrated with the same buffer. Bound proteins were eluted by a linear gradient (100 mL) of 0→0.5M NaCl in starting buffer at 25 mL/h. Fractions (2.5 mL) were tested for absorbance at 280 nm (– – –) and activity towards CM-cellulose by reducing sugar determination (—○—).

En II were freed from minor protein contaminants (22% and 31% of injected proteins, respectively); they were both purified 1.2-fold.

After dialysis, the appropriate fractions were freeze-dried and subjected to gel filtration on Ultrogel AcA 54 in the presence of 0.1M NaCl to avoid interactions of gel and proteins. The three endo-glucanase activities were eluted as a single peak, at K_{av} 0.39, 0.30, and 0.32 for En I–III, respectively (Fig. 4). The recoveries of protein from the previous step were 69, 91, and 61%, and the recoveries of activity were 91.7, 98.8, and 97.5%; the purification factors obtained by this last

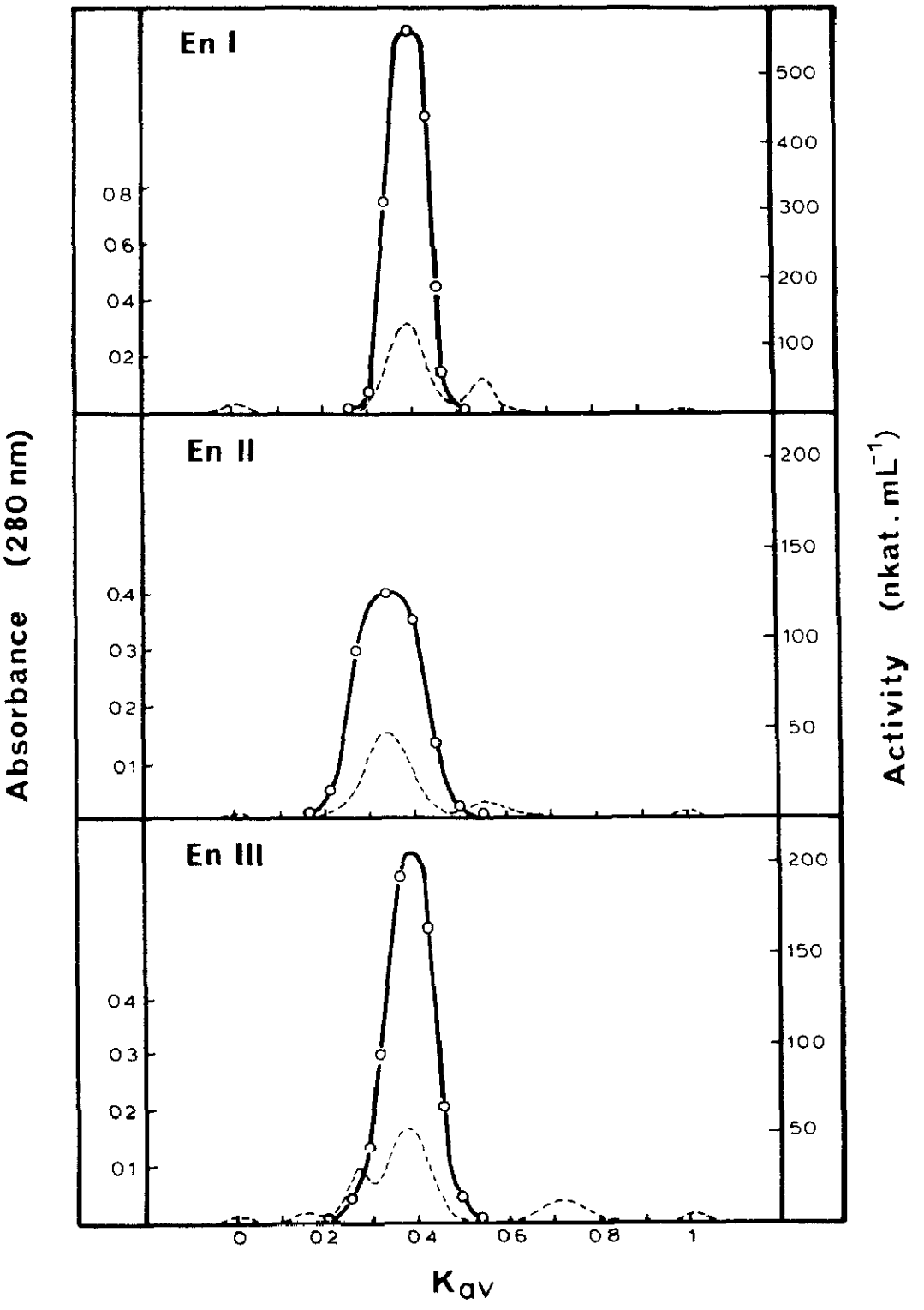

Fig. 4. Gel filtration of En I–III from Fig. 3. The fractions were dialysed overnight against 5mM sodium acetate buffer (pH 5.0) and freeze-dried. Solutions of samples in 0.05M sodium acetate buffer (pH 5.0, 1.5 mL) containing 0.1M NaCl were applied to a column (97 × 1.6 cm) of Ultrogel AcA 54 equilibrated and eluted with the same buffer at 7.5 mL/h. Fractions (2 mL) were tested for absorbance at 280 nm (– – –) and activity towards CM-cellulose by reducing sugar determination (—○—). The purified endo-glucanases were dialysed overnight against 0.05M sodium acetate buffer (pH 5.0), freeze-dried, and stored at −20°.

step were thus 1.3, 1.1, and 1.6.

The purified endo-glucanases had ~20, ~8, and ~16% of the activity of the crude enzyme, but represented only ~0.9, ~0.5, and ~1.7% of the original protein (corresponding to ~21-, ~16-, and ~9-fold purifications, respectively).

Each purified endo-glucanase gave a single protein band on disc gel electrophoresis, with and without SDS, and on agarose isoelectrofocusing.

Properties. — The main properties of the three purified endo-glucanases En

TABLE II

PROPERTIES OF THE ENDO-GLUCANASES

Property	*En I*	*En II*	*En III*
Mol. wt.	42,000	56,000	47,000
pI	4.8	4.3	4.1
pH Optimum	4.8–5.0	4.8	4.6–4.8
θ optimum (degrees)	55–60	55–60	55–60
Activation energy (kcal/mol)	5.5	5.1	6.1
Q10, 30–40°	1.3	1.4	1.7

I–III are summarised in Table II. Molecular weights, as determined by SDS/polyacrylamide gel electrophoresis (Fig. 5), were estimated to be 42,000, 56,000, and 47,000 for En I–III, respectively. These values were confirmed by gel permeation: endo-glucanase peaks were eluted respectively at appropriate K_{av} with respect to log mol. wt. Isoelectric points, determined by isoelectric focusing on agarose over the pH range 3–10, were 4.8, 4.3, and 4.1 for En I–III, respectively. These values were in good agreement with those obtained from the titration curve of activity revealed by a specific zymogram technique (Fig. 6), since the same pI values were obtained for the endo-glucanases. Only three major activity lines were visible by this technique. This could imply that the peak of activity released from the chromatofocusing column by 0.5M NaCl did not correspond to a fourth individual endo-glucanase but, rather, to part from En I, En II, or En III, not eluted by the pH gradient for unexplained reasons. The probable low density of charge of endo-glucanases explained their being eluted from the chromatofocusing at pH values less acidic than those of their isoelectric points.

Maximum activity towards CM-cellulose was exhibited at pH 4.8 (Fig. 7) and 55–60° (Fig. 8) in 30-min assays, for each endo-glucanase. The calculated activation energy was 5.1–6.1 kcal/mol. The Q10 values at 30–40° were 1.3, 1.4, and 1.7 for En I–III, respectively. The endo-glucanases were stable in the presence of substrate at pH 4.0–8.0 for 20 h at 30°. The purified enzymes were quickly inactivated at pH ~3.5 or ~8.0 for En III, and ~9.0 for En I and En II (Fig. 9). The endo-glucanases were also inactivated readily at 70° for 30 min. En III exhibited good thermostability since half of the optimal activity was retained after 30 min at 65°, whereas the same loss was observed at 57° for En I and En II (Fig. 10).

Enzyme specificity. — The ratios of "liquefying" activity measured by viscometry and "saccharifying" activity measured by reducing sugar release on the same substrate, *i.e.*, CM-cellulose, were ~1.0 for En I and En II and 0.7 for En III (*cf.* 0.6 for the unfractionated enzyme preparation). These results demonstrated that En I and En II were the most randomly acting enzymes of the cellulase complex of *Dichomitus squalens*. The low value obtained for the crude enzyme may be due to the action of osidases or exo-enzymes, or to the synergism between exo- and endo-enzymes. Variations in the degree of randomness, exhibited by endo-

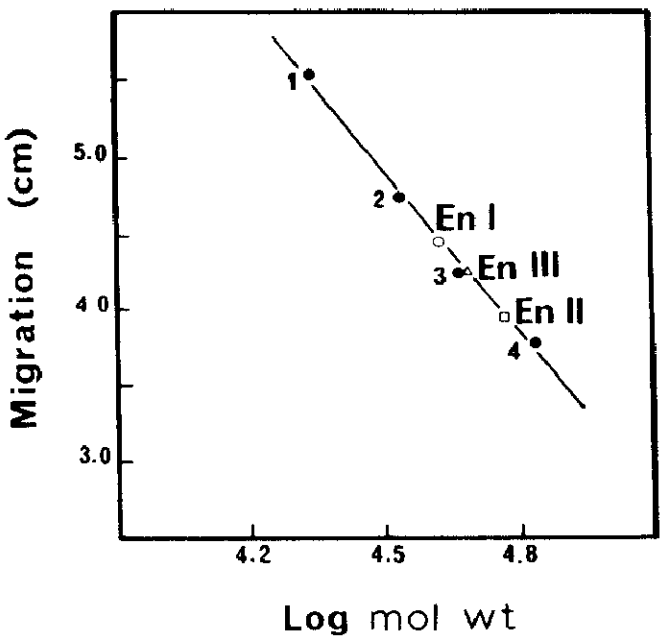

Fig. 5. Determination of molecular weights of En I–III by SDS–disc gel electrophoresis: 1, trypsin inhibitor (21,500); 2, pepsin (35,000); 3, ovalbumin (45,000); 4, bovine serum albumin (68,000).

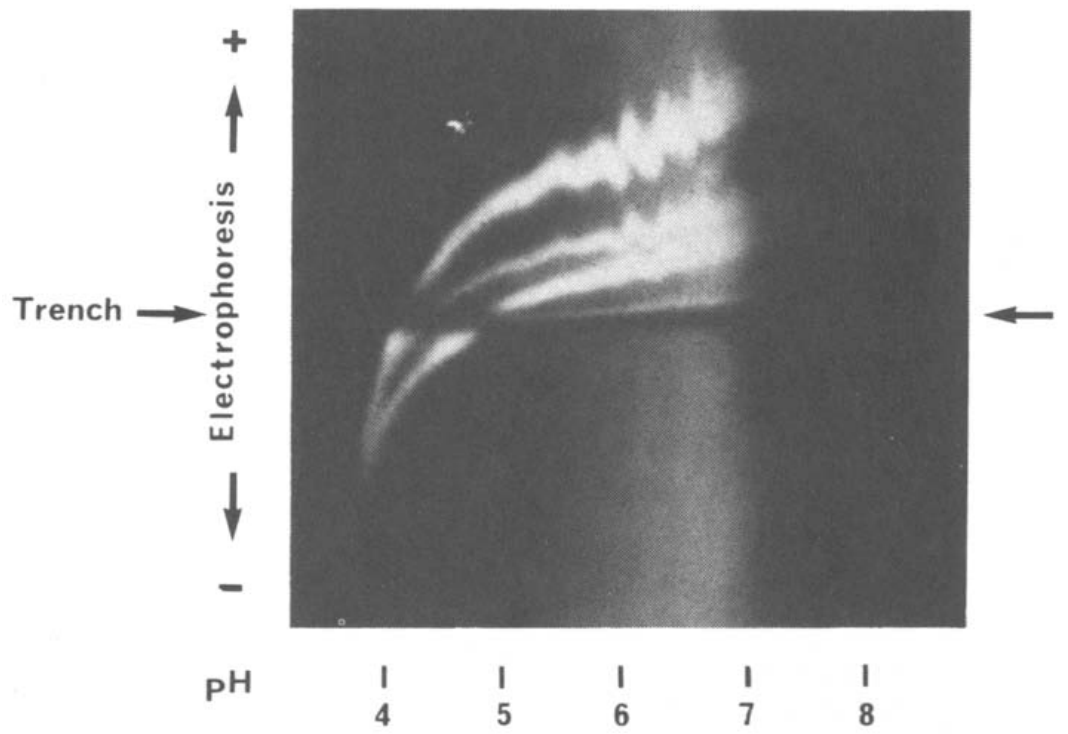

Fig. 6. Titration curve of endo-glucanase activity of the crude enzyme preparation from *Dichomitus squalens* (see Experimental).

glucanases from a single cellulase complex, have already been reported in several fungi, namely, *Trichoderma viride*[4], *Trichoderma reesei*[11], *Irpex lacteus*[12], *etc.*

The activities of the three purified endo-glucanases towards various substrates are presented in Table III. No activity was detected against inulin, dextran, starch, larch arabinogalactan, cellobiose, or *p*-nitrophenyl β-D-glucopyranoside. En I and En II exhibited similar activities on all cellulosic substrates, whereas En III was less active. The high randomness of En I and En II was confirmed by their high activities against CMC-azure, specific for the detection of endo-type enzymes[9], and their relatively lower activities on H_3PO_4-swollen cellulose, as compared to that of En III. However, purified endo-glucanases were poorly active on crystalline cellulose.

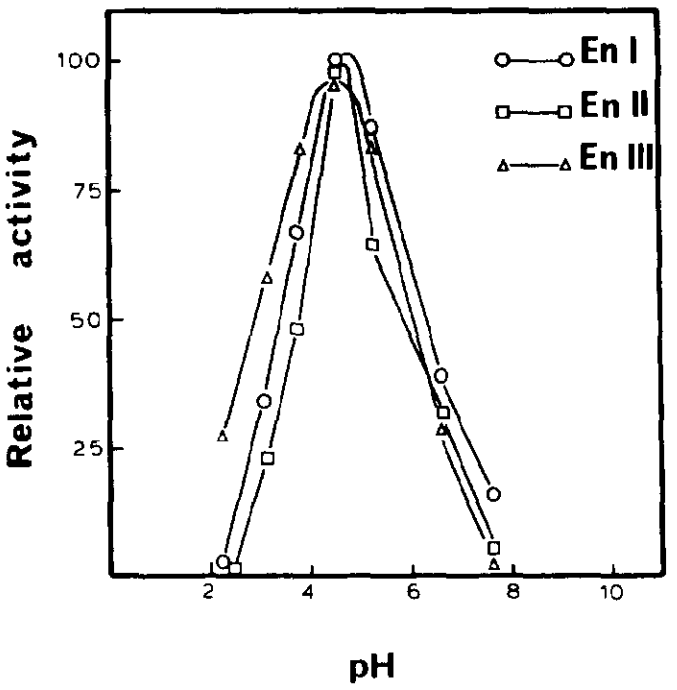

Fig. 7. Effect of pH on enzymic activities (see Experimental).

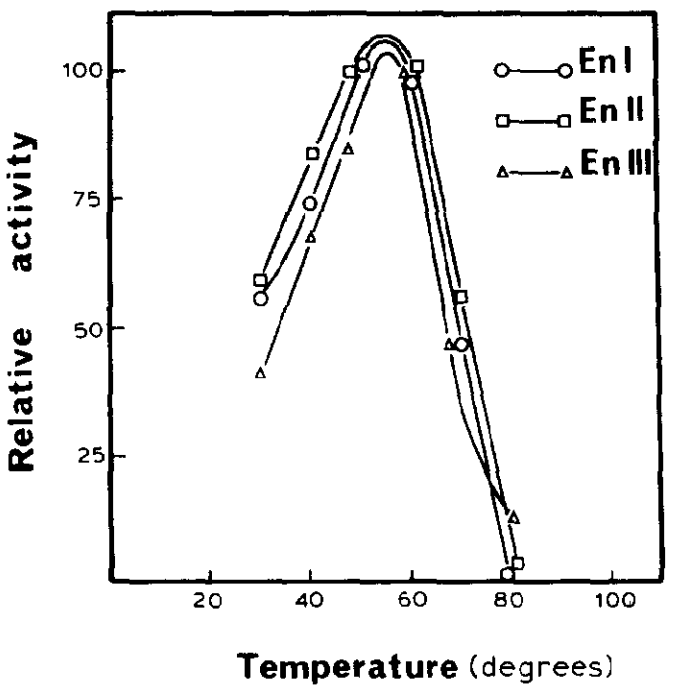

Fig. 8. Effect of temperature on enzymic activities (see Experimental).

Barley glucan and lichenan were hydrolysed by each endo-glucanase. En III, the less randomly acting enzyme, was the most active on these substrates. This finding could be explained by the need of fewer adjacent (1→4)-linked β-D-glucosyl residues for the binding and action of this enzyme than for En I and II, since barley glucan consisted of cellotriose and cellotetraose units interrupted by single (1→3)-β linkages[13], and lichenan mainly of (1→3)-β-linked cellotriose units[14]. En III was the only one of the endo-glucanases of *Dichomitus squalens* to degrade cellotriose, and it was also the most active on cellotetraose, but less active on cellopentaose. Thus, as suggested by Nisizawa[12], "less-random-type" endo-glucanases could differ from "highly-random" attacking endo-glucanases, not because they initially act on the

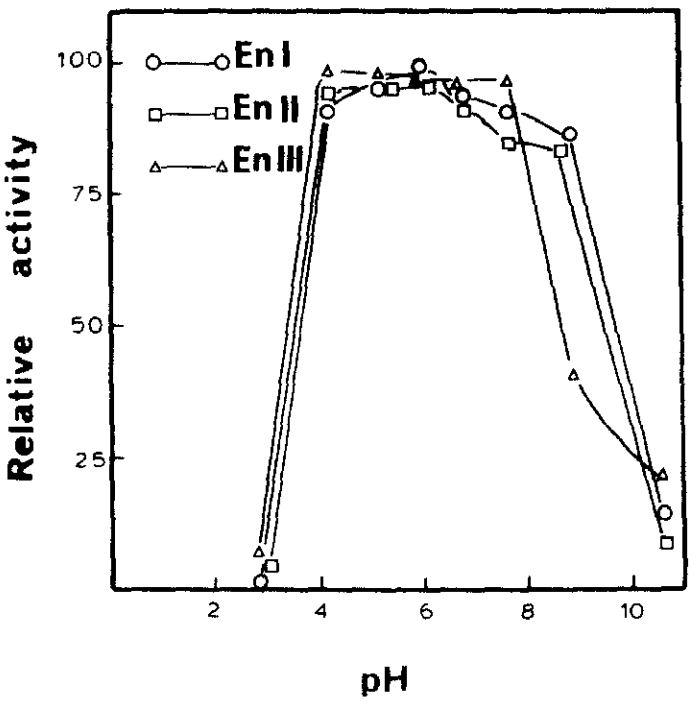

Fig. 9. Effect of pH on enzyme stability (see Experimental).

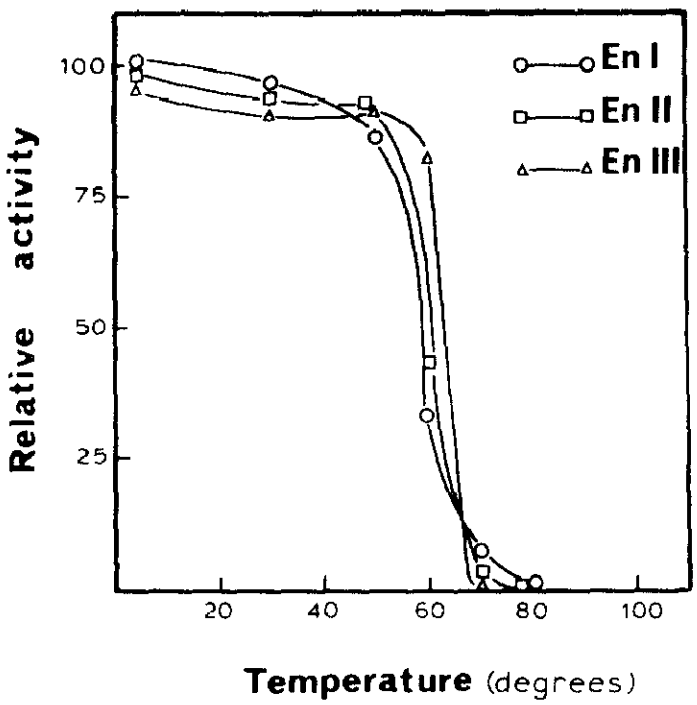

Fig. 10. Effect of temperature on enzyme stability (see Experimental)

periphery of the β-D-glucan chains but just because they degrade cello-oligosaccharides of low d.p. None of the three endo-glucanases from *Dichomitus squalens* exhibited considerable activity towards *p*-nitrophenyl β-cellobioside in contrast to some endo-cellulases from *Irpex lacteus*[15], *Sporotrichum pulverulentum*, and *Trichoderma reesei*[16]. This result could be explained with respect to results observed with soluble cello-oligosaccharides as substrates. En III was the only endo-glucanase able to hydrolyse cellotriose. Substrates of this enzyme must contain at least three glucosyl units. For En I and En II, the cello-oligosaccharide of lowest d.p. to be hydrolysed was cellotetraose. Thus, their substrates must contain at least four glucosyl units.

TABLE III

ACTIVITY OF THE ENDO-GLUCANASES TOWARDS VARIOUS SUBSTRATES

Substrate	*Specific activity[a] (nkat/mg)*		
	En I	*En II*	*En III*
CM-cellulose	959	895	565
CMC-azure[b]	117	99	46
H_3PO_4-swollen cellulose	86	78	61
Avicel	0.3	0.3	0.2
Barley glucan	34	45	68
Lichenan	36	26	44
Laminaran	0	18	0
Xylan	0	17	0
p-NPC[c]	1	2	0.5
Cellotriose	0	0	10
Cellotetraose	8	34	63
Cellopentaose	155	162	116

[a]Proteins were measured by the method of Lowry. [b]Specific activity on CMC-azure is expressed as a.u./mg. [c]*p*-Nitrophenyl cellobioside.

En II degraded laminaran and xylan. The occurrence of endo-glucanases active against non-cellulosic substrates, in particular xylan, has been reported, for example, for *Aspergillus niger*[17], *Trichoderma viride*[18], or *Irpex lacteus*[19]. This absence of absolute specificity is explained by the close structural relationship between β-D-glucose and β-D-xylose residues, and may be related to the extensive degradation of naturally associated cell-wall polymers *in situ, i.e.,* cellulose and hemicellulose, by the organism.

Mode of action. — T.l.c. of the hydrolysis products obtained using different types of cellulose as substrates is shown in Fig. 11. No influence of cellulose crystallinity was observed on the d.p. of the hydrolysis products. Indeed, the same cello-oligosaccharides were found in hydrolysates of H_3PO_4-swollen cellulose, Whatman CC41 cellulose, or avicel, but the spots were more intense since the substrate was more amorphous. En I released a series of oligosaccharides (d.p. 2–7) after reaction for 1 h, but, even after 24 h, cello-oligosaccharides of various d.p. were still present and glucose had not appeared. En II produced similar oligosaccharides but with lower d.p. This is the behaviour of a true endo-glucanase. In contrast, En III released mainly cellobiose from cellulose, even early in the incubation, and glucose appeared in the medium as the reaction proceeded. However, En III cannot be considered as an exo-glucanase of cellobiohydrolase-type since small proportions of cellotetraose and cellotriose were detected, in addition to cellobiose, at the beginning of hydrolysis. Moreover, this enzyme was highly active on CM-cellulose and CMC-azure, which are specific substrates for endo-glucanases. To some extent, the endo-glucanases of *Dichomitus squalens* resembled those of another white-rot fungus, *Sporotrichum thermophile*[9], where endo-cellulases I and III, the two less

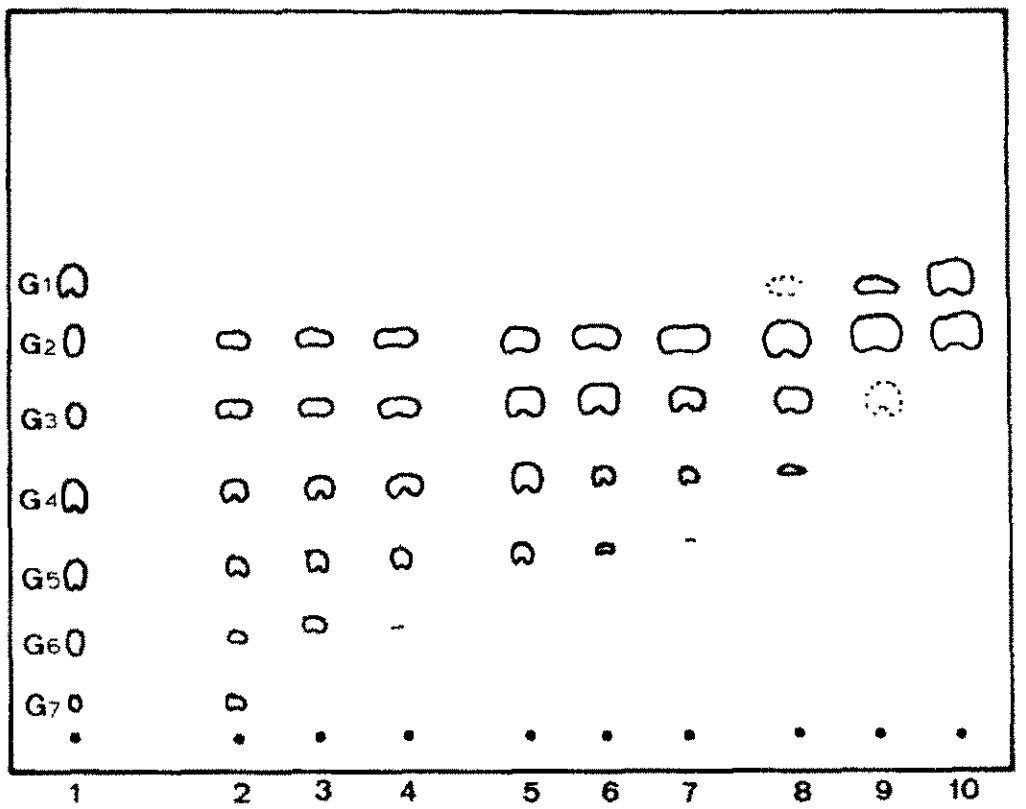

Fig. 11. Hydrolysis of H_3PO_4-swollen cellulose by En I–III. T.l.c. of the products: 1, standard cello-oligosaccharides; 2–4, hydrolysis by En I after 1, 3, and 24 h; 5–7, hydrolysis by En II after 1, 3, and 24 h; 8–10, hydrolysis by En III after 1, 3, and 24 h.

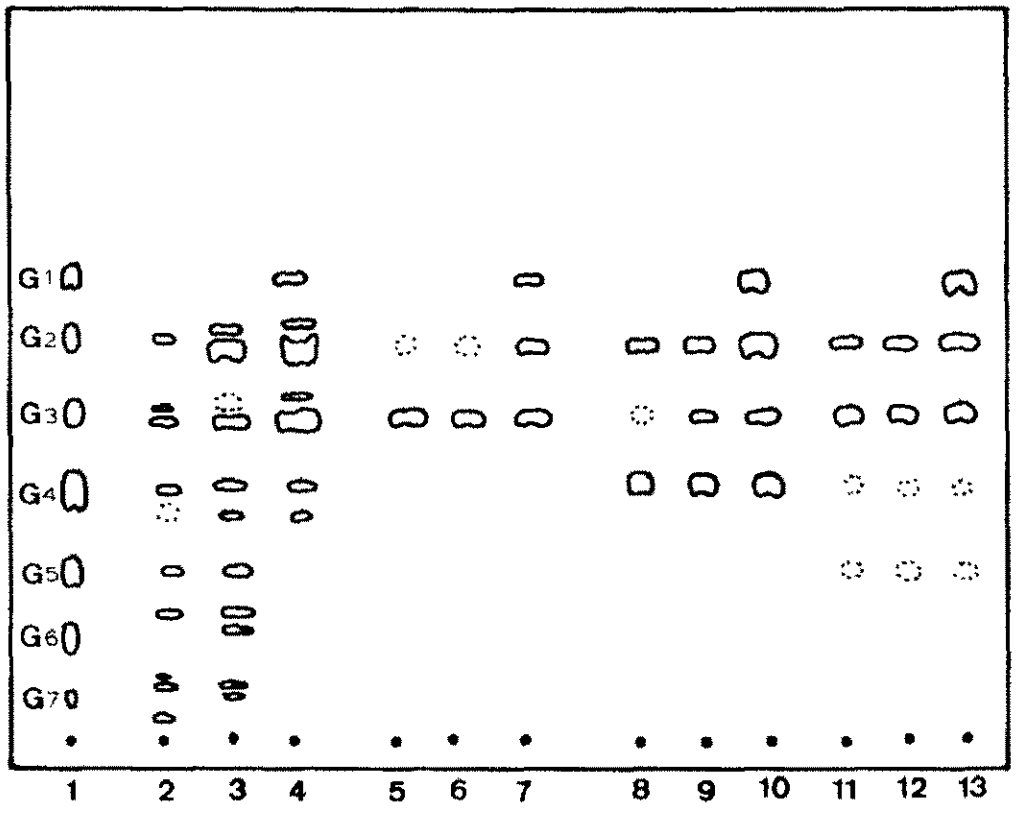

Fig. 12. Hydrolysis of barley glucan and cello-oligosaccharides by En I–III. T.l.c. of the products: 1, standard cello-oligosaccharides; 2–4, barley glucan: 5–7, cellotriose; 8–10, cellotetraose; 11–13, cello-pentaose.

acidic endo-glucanases, released cellobiose and cello-oligosaccharides from cellulose, whereas endo-cellulase II, which was more acidic, produced glucose and smaller proportions of cello-oligosaccharides.

Hydrolysis products from barley glucan (Fig. 12) were similar to those ob-

tained from cellulose except that two spots were often obtained having the same mobility, perhaps due to the presence of oligosaccharides containing (1→3) and/or (1→4) linkages. The action of purified endo-glucanases on pure cello-oligosaccharides (Fig. 12) showed that the middle linkages of cellopentaose were attacked, giving cellobiose and cellotriose; En III cleaved cellotriose to give glucose and cellobiose. Cellotetraose was the smallest available substrate for En I and En II and was split into two cellobiose units by En I, but the reason for the presence of cellotriose among the hydrolysis products formed by En II, although no glucose was detected, is unclear. Transglycosylation reactions may explain this result, and a mechanism similar to this has been described by Dekker and Richards[20] for the hydrolysis of xylotetraose by an endo-xylanase from *Ceratocystis paradoxa.* In this scheme, the tetraose is hydrolysed to give two xylobiose units, one of which remains bound to the enzyme. A tetraose unit is then transferred to this complex, and the resulting hexaose is split into two triose units.

Thus, the three enzymes purified from *Dichomitus squalens* appear to be true endo-glucanases and exhibit differences in their physico-chemical properties, specificities, and mode of action. They constitute only parts of the extracellular cellulolytic system of the organism[10] and act, under natural conditions, in combination with other enzymes. The purification and characterisation of enzymes from *Dichomitus squalens* active towards *p*-nitrophenyl β-D-cellobioside are in progress.

EXPERIMENTAL

Materials. — The following polymers were used: carboxymethylcellulose sodium salt (Sigma C-4888, medium viscosity, d.s. 0.7), avicel (Merck), Whatman CC41 cellulose, dextran (Sigma D-4751), larchwood arabinogalactan (Sigma A-2012), poly(galacturonic acid) (ICN 102 771), lichenan from *Cetraria islandica* (Sigma L-8378), laminaran from *Laminaria* spp. (Fluka 61400), waxy-maize beta-limit dextrin (gift of Dr. P. Colonna), larchwood xylan (Sigma), and barley glucan (gift of Dr. B. Stone). Polybuffer exchanger (PBE) 94, Phenyl-Sepharose CL-4B (Pharmacia), DEAE-Trisacryl (IBF), and Ultrogel AcA 54 (LKB) were used as chromatographic supports. Collagen dialysis tubes (Soussana S.A.) were used.

Enzyme. — Crude enzyme was prepared[10] from the supernatant of a 10-L liquid culture of *Dichomitus squalens* on cellulose avicel, by ammonium sulphate precipitation (80% saturation).

Preparation of substrates. — (*a*) CMC-azure was prepared as described by Leisola *et al.*[21], except that contact time between CM-cellulose and Remazol Brilliant Blue was increased to 3 h.

(*b*) H_3PO_4-swollen cellulose was prepared by suspending Whatman cellulose CC41 (40 g) in aqueous 85% (w/v) phosphoric acid, with occasional stirring for 2 h at 4°. The product was then treated as described by Wood[22].

(*c*) Cello-oligosaccharides were prepared by acetolysis of dewaxed cotton fibres[23], followed by deacetylation with sodium methoxide and fractionation by pre-

parative h.p.l.c. (R Sil C13 HL, Alltech Europe) with water as the eluant.

Assays. — (*a*) endo-*Glucanase activity*. After incubation with CM-cellulose, enzyme activity was measured either by determination of liberated reducing sugars or by a viscometric assay. Activity was also measured with CMC-azure as substrate.

(*b*) *Determination of reducing sugars*. Each incubation mixture contained 1% CM-cellulose in 0.05M sodium acetate buffer (pH 5.0, 1 mL) and appropriate enzyme dilution (0.1 mL). After incubation at 40° for 30 min, the liberated reducing sugars were determined by the Nelson procedure[24], using D-glucose as standard. Activity was expressed in nkat (1 nkat of enzyme liberated 1 nmol of glucose equivalent reducing-power per s).

(*c*) *Viscometry*. 0.5% CM-cellulose in 0.05M sodium acetate buffer (pH 5.0, 8 mL) was incubated at 40° with appropriate enzyme dilution (0.1 mL) in an Ostwald viscometer. The activity was defined as the rate of change in specific fluidity per min.

(*d*) *Assay with CMC-azure.* Each incubation mixture contained 1% CMC-azure in 0.05M sodium acetate buffer (pH 5.0, 0.5 mL), and an appropriate enzyme dilution (0.05 mL). After 30 min at 40°, the reaction was stopped by adding cold ethanol (1 mL). After centrifugation (9,000*g* for 2 min), the absorbance of the supernatant solution was estimated at 595 nm. The activity was expressed in arbitrary units (a.u.) corresponding to the amount of enzyme which increases the absorbance by 0.1 per min.

(*e*) *Activity towards various substrates*. Polysaccharide-hydrolase activities, other than those of endo-glucanases, were measured under conditions similar to those used for the determination of endo-glucanase activity by the reducing sugar method, using 0.5% suspensions or solutions of substrates in 0.05M sodium acetate buffer (pH 5.0). Activity on cello-oligosaccharides was determined by the same method, except that the concentration of the substrate was 0.02%. Activity on *p*-nitrophenyl β-D-glucopyranoside and *p*-nitrophenyl cellobioside was measured by incubating mM substrate solution in 0.05M sodium acetate buffer (pH 5.0, 1 mL) with diluted enzyme (0.1 mL) at 40° for 60 min. The reaction was stopped by the addition of M Na_2CO_3 (2 mL) and the absorbance was measured at 400 nm.

Protein determination. — Proteins were recorded from column effluents at 280 nm. Protein concentration was measured by the Coomassie Blue method of Bradford[25] during the enzyme purification steps, and by the method of Lowry *et al.*[26] for determination of the specific activity of the crude enzyme and the purified endo-glucanases. Bovine serum albumin (Sigma, A-4503) was used as the standard.

Electrophoresis, isoelectric focusing, and titration curves. — Disc gel electrophoresis was performed at pH 8.3, using 6% acrylamide gel, on a Pleuger Acrylophor apparatus. Gels were stained for protein with Coomassie Brilliant Blue. Electrophoresis in the presence of 0.1% SDS was carried out at pH 7.2, using 5% acrylamide gels. Solutions of samples (10–15 μg of protein) in 0.08M sodium phosphate buffer (pH 7.2) containing 1.7% of SDS, 3% of 2-mercaptoethanol, and 90% of glycerol were kept at 90° for 3 min. After migration, gels were stained for

protein with Coomassie Brilliant Blue. Cytochrome C (mol. wt. 12,500), tryptic inhibitor (21,500), pepsin (35,000), ovalbumin (45,000), and bovine serum albumin (68,000) were used as standards.

Analytical isoelectric focusing was performed on IEF-agarose (Pharmacia) in the pH range 3–10, with an LKB 2117 Multiphor. After focusing, gel pieces, cut from the edge of the gel, were incubated overnight at 4° in distilled water (1 mL); the pH of the mixture was then determined. Proteins were stained with Coomassie Brilliant Blue.

Titration curves were performed on polyacrylamide gel, using a Multiphor 2117 cell and a 2103 LKB power supply. The pH gradient of the first dimension was developed at 30 W (1 h). Then the gel was rotated through 90°. For the second dimension, the sample (80 μL of culture supernatant) in the trench was electrophoretically separated at 800 V (20 min). The pH gradient was determined by using a surface electrode (LKB 2117-III, Tacussel Minisis 6000 apparatus). The visualisation of endo-glucanases was made possible by the method described by Bertheau *et et al.*[27], where enzymic action is detected by the clearance zone revealed by Congo Red in CM-cellulose-containing agar gel.

Analysis of hydrolysis products. — Suspensions of H_3PO_4-swollen cellulose, Whatman CC41 cellulose, or avicel, or solutions of barley glucan or of cello-oligosaccharides (0.5%) in 10mM sodium acetate buffer (pH 5.0) were incubated at 40° with 5 nkat of purified endo-glucanase for appropriate periods of time. Reaction mixtures were then centrifuged, and the supernatant solutions were deionized with Dowex 1-X8 ($HCOO^-$) and Amberlite IR-120 (H^+) resins and freeze-dried. Solutions of the hydrolysis products in distilled water were subjected to t.l.c. on silica gel F 1500 (Schleicher and Schüll), using 1-propanol–ethyl acetate–ethanol–pyridine–acetic acid–water (7:3:3:2:2:1) with detection by charring with sulphuric acid.

Properties of purified enzymes. — (*a*) *pH optimum.* The endo-glucanase activity was measured after incubation with solutions of CM-cellulose in citrate–phosphate buffer of various pH values, instead of 0.05M sodium acetate buffer (pH 5.0).

(*b*) *pH Stability.* The endo-glucanases were incubated in universal buffer of various pH values at 30°. After 17 h, the enzymic solutions were dialysed against 0.05M sodium acetate buffer (pH 5.0) and the residual activity was measured under standard conditions.

(*c*) *Temperature optimum.* The endo-glucanase activity was measured under standard conditions in the range 20–80°.

(*d*) *Temperature stability.* A solution of each endo-glucanase in 0.05M sodium acetate buffer (pH 5.0) was maintained at various temperatures in the range 4–80° for 30 min. Each solution was then cooled at 4° for 10 min and the residual activity was measured under standard conditions.

ACKNOWLEDGMENTS

We thank R. Vedel for technical assistance, and Drs. J. M. Brillouet (INRA, Nantes), D. Robic (Faculté de Pharmacie, Paris), and Y. Bertheau (INA, Paris) for helpful discussions.

REFERENCES

1 F. M. ROMBOUTS AND W. PILNIK, in C. MERCIER AND M. RINAUDO (Eds.), *Proc. Int. Workshop on Plant Polysaccharides, Structure and Function,* Nantes, France, 1984, pp. 19–30.
2 T. M. WOOD AND S. I. MCCRAE, *Biochem. J.*, 171 (1978) 61–72.
3 A. MCHALE AND M. P. COUGHLAND, *FEBS Lett.*, 117 (1980) 319–321.
4 G. BELDMAN, M. F. SEARLE-VAN LEEUWEN, F. M. ROMBOUTS, AND F. J. G. VORAGEN, *Eur. J. Biochem.*, 42 (1984) 1–8.
5 I. LABUDOVA AND V. FARKAS, *Biochim. Biophys. Acta*, 744 (1983) 135–140.
6 K. E. ERIKSSON AND B. PETTERSSON, *Eur. J. Biochem.*, 51 (1985) 193–206.
7 M. STREAMER, K. E. ERIKSSON, AND B. PETTERSSON, *Eur. J. Biochem.*, 51 (1975) 207–213.
8 K. E. ERIKSSON AND B. PETTERSSON, *Eur. J. Biochem.*, 51 (1975) 213–218.
9 G. CANEVASCINI, D. FRACHEBOUD, AND H. MEIER, *Can. J. Microbiol.*, 29 (1983) 1071–1080.
10 X. ROUAU AND E. ODIER, *Enzyme Microbiol. Technol.*, in press.
11 S. P. SHOEMAKER AND R. D. BROWN, *Biochim. Biophys. Acta*, 492 (1978) 133–146.
12 K. NISIZAWA, *J. Ferment. Technol.*, 51 (1973) 267–304.
13 J. R. WOODWARD, G. B. FINCHER, AND B. A. STONE, *Carbohydr. Polymers*, 3 (1983) 207.
14 S. PEAT, W. J. WHELAN, AND J. G. ROBERTS, *J. Chem. Soc.*, (1957) 3916–3924.
15 K. NISIZAWA, *J. Biochem. (Tokyo)*, 42 (1955) 825–835.
16 M. V. DESHPANDE, K. E. ERIKSSON, AND L. G. PETTERSSON, *Anal. Biochem.*, 138 (1984) 481–487.
17 P. L. HURST, P. A. SULLIVAN, AND M. G. SHEPHERD, *Biochem. J.*, 169 (1978) 389–395.
18 S. TODA, H. SUZUKI, AND K. NISIZAWA, *J. Ferment. Technol.*, 49 (1971) 499–521.
19 T. KANDA, K. WAKABAYASHI, AND K. NISIZAWA, *J. Biochem. (Tokyo)*, 79 (1976) 989–995.
20 R. F. H. DEKKER AND G. N. RICHARDS, *Adv. Carbohydr. Chem. Biochem.*, 32 (1976) 277–352.
21 M. LEISOLA, M. LINKO, AND E. KARVONEN, in M. BAILEY, R. M. ENARI, AND P. M. LINKO (Eds.), *Proc. Symp. Enzymatic Hydrolysis of Cellulose,* Aulanko, Finland, 1975, pp. 297–313.
22 T. M. WOOD, *Biochem. J.*, 121 (1971) 353–362.
23 M. L. WOLFROM AND A. THOMPSON, *Methods Carbohydr. Chem.*, 3 (1963) 143–149.
24 N. NELSON, *J. Biol. Chem.*, 153 (1944) 376–380.
25 M. M. BRADFORD, *Anal. Biochem.*, 72 (1976) 248–254.
26 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
27 Y. BERTHEAU, E. MADGIDI-HERVAN, A. KOTOUJANSKY, C. NGUYEN-THE, T. ANDRO, AND A. COLENO, *Anal. Biochem.*, 139 (1984) 383–393.