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A 1,4-β-Glucan Glucanohydrolase from the Cellulolytic Fungus *Trichoderma viride* QM 9414

PURIFICATION, CHARACTERIZATION AND PREPARATION OF AN IMMUNOADSORBENT FOR THE ENZYME

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A 1,4-β-glucan glucanohydrolase (EC 3.2.1.4) was isolated from culture filtrates of the fungus *Trichoderma viride* QM 9414 by molecular-sieve chromatography on Bio-Gel P-30, ion-exchange chromatography on DEAE-Sephadex A-50 and isoelectric focusing in a density gradient. Polyacrylamide-gel electrophoresis at two different pH values, analytical isoelectric focusing in a polyacrylamide-gel slab and molecular-sieve chromatography of the reduced and alkylated enzyme in a denaturing medium indicated a homogeneous protein. The enzyme has a mol.wt. of 51000 and is not a glycoprotein. The pI was found to be 4.66 at 23°C. Antiserum against the purified enzyme was prepared and the amount of enzyme in the original filtrate was determined by rocket immunoelectrophoresis to be about 50mg/litre. An immunoadsorbent made from CNBr-activated Sepharose 4B and antiserum affords a rapid and highly specific purification of the enzyme.

Many laboratories have attempted to obtain strains of highly active cellulolytic micro-organisms for the enzymic conversion of cellulose into glucose. One of the most potent organisms available is the fungus *Trichoderma viride* QM 9414 (Mandels *et al.*, 1971, 1975).

Culture filtrates of *Trichoderma viride* contain three cellulolytic activities, namely, 1,4-β-glucan glucanohydrolase (EC 3.2.1.4), 1,4-β-glucan cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Pettersson *et al.*, 1972). The 1,4-β-glucan glucanohydrolase attacks internal bonds in less-ordered regions of cellulose fibrils, thereby increasing the number of chain ends that can be attacked by the 1,4-β-glucan cellobiohydrolase. The cellobiose produced by this enzyme is further degraded to glucose by β-glucosidase. Since the experimental support for this hypothesis is not complete, it might be an over-simplification.

There are several molecular species of 1,4-β-glucan glucanohydrolase in *Trichoderma viride* (Berghem *et al.*, 1976; Håkansson *et al.*, 1978; Okada & Nisizawa, 1968), *Trichoderma koningi* (Wood, 1968; Halliwell & Riaz, 1970) and *Sporotrichum pulverulentum* (Eriksson & Pettersson, 1975). The aim of the present work has been to isolate and characterize the main 1,4-β-glucan glucanohydrolase of *Trichoderma viride* QM 9414.

Materials and Methods

The fungus *Trichoderma viride* QM 9414 and freeze-dried culture filtrates were a gift from Dr. M. Mandels, U.S. Army Natick Research and Development Command, Natick, MA, U.S.A. Filtrates were also produced in our laboratory by culturing the fungus in 10-litre shaker flasks at 30°C for 7 days. The basal medium was prepared as described by Mandels *et al.* (1975). To 2 litres of this medium 15g of α-cellulose (Sigma Chemical Co., St Louis, MO, U.S.A.), 1.5g of Neopeptone (Difco Laboratories, Detroit, MI, U.S.A.) and 0.4ml of Tween 80 (Kebo AB, Stockholm, Sweden) were added. CNBr-activated Sepharose 4B and DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. A 110ml Ampholine electrofocusing column (LKB 8100), standard Ampholine pH3.5–5.0 and ready-made polyacrylamide-gel plates (LKB PAG plates) for electrofocusing in the pH range 3.5–9.5 and an LKB Multiphor apparatus were obtained from LKB-Produkter AB, Bromma, Sweden. Bio-Gel P-30 was a product of Bio-Rad Laboratories, Richmond, CA, U.S.A. All other chemicals were of analytical grade.

Activity towards Avicel

The reaction mixture consisted of 2.0ml of a 1% suspension of Avicel (microcrystalline cellulose powder; Kebo AB) in 0.05 M-sodium acetate buffer, pH5.0, and 100μl of enzyme solution. After incubation at 40°C for 2h the mixture was filtered and analysed for reducing sugars (Somogyi, 1952; Nelson, 1944). One unit of activity was defined as the amount of enzyme needed to liberate reducing sugars corresponding to 1μmol of glucose/min under the assay conditions.

Activity towards CM-cellulose

The assay was essentially performed as above but with a 1% solution of CM-cellulose (degree of substitution 0.4, degree of polymerization 300) with an incubation time of (10min. One unit of enzyme activity was defined as above.

Polyacrylamide-gel electrophoresis

The experiments were performed as described by Hjertén *et al.* (1965). Gels (10cm long; composition T = 5%, C = 3%; Hjertén *et al.*, 1965) were cast in quartz tubes (0.7cm × 12cm). A load of 75μg of protein/gel (calculated from A_{280}) was used at both pH8.0 (0.05M-Tris/HCl) and pH2.9 (0.1M-acetic acid). The pH8 gels were cast with *NNN'N'*-tetramethylethylenediamine as a catalyst, and the pH2.9 gels were cast with the system ascorbic acid/$FeSO_4$/H_2O_2 as a catalyst (Jordan & Raymonds, 1969). After the runs the gels were scanned at 280 and 310nm in a gel scanner attached to a Zeiss spectrophotometer monochromator M4QII connected to a recorder (Fries & Hjertén, 1975). The gels were then removed from the tubes and stained overnight with 0.05% Coomassie Brilliant Blue R-250 in 25% (v/v) propan-2-ol and destained in 7% (v/v) acetic acid.

Analytical isoelectric focusing

Ready-made polyacrylamide-gel plates containing carrier ampholytes in the range pH3.5–9.5 were used together with a LKB Multiphor electrophoresis apparatus and a LKB 2103 constant power supply. Samples were applied directly on the gel surface and run for 90min at 10°C at a load of 1W/cm gel width. Fixation and staining was with 0.1% Coomassie Brilliant Blue as described by Vesterberg (1972).

Molecular-sieve chromatography in 6M-guanidine hydrochloride

The method of Fish *et al.* (1969) was used to estimate the molecular weight of the purified enzyme. About 1mg of freeze-dried enzyme was reduced under N_2 with 8μmol of dithiothreitol in 300μl of 6M-guanidine hydrochloride buffered at pH7.9 with 0.1M-Tris/HCl. After 6h, 5mg of sodium iodoacetate was added to alkylate all thiol groups present. Then 30min later the sample was applied to a column (1.0cm × 98cm) of Sepharose 6B equilibrated in 6M-guanidine hydrochloride / 0.1M-Tris/HCl, pH7.9. The column had previously been calibrated with reduced and alkylated proteins of known molecular weight: bovine serum albumin, mol.wt. 66000 (Miles-Seravac, Maidenhead, Berks., U.K.); bovine pancreatic chymotrypsinogen A, mol.wt. 23000 (Worthington Biochemical Corp., Freehold, NJ, U.S.A.); bovine pancreatic ribonuclease, mol.wt. 13700 (Sigma Chemical Co., St Louis, MO, U.S.A.); ^{3}H-labelled neurotoxin from *Naja naja siamensis*, mol.wt. 7800 (a gift from Dr. E. Karlsson, Institute of Biochemistry, University of Uppsala, Sweden). The flow rate was 2ml/h, and 1g fractions were collected. The protein content of the fractions was monitored by measurement of the A_{280} and by liquid-scintillation counting (Insta-Gel, made by Packard Instrument International SA, Zürich, Switzerland).

Amino acid analysis

Amino acid analysis was performed in a Durrum D-500 analyser after hydrolysis for 24 and 72h in 6M-HCl at 110°C. Cysteine and methionine were determined on the analyser as cysteic acid and methionine sulphone respectively, after performic acid oxidation. Serine and threonine were calculated by linear (threonine) or first-order (serine) extrapolation to zero time of the values observed after 24h and 72h hydrolysis. Tryptophan was determined after hydrolysis with 4M-mercaptoethanesulphonic acid.

Carbohydrate analysis

Sugars were analysed as alditol acetate derivatives by g.l.c. (Sawardeker *et al.*, 1965).

Preparation of antiserum

Freeze-dried enzyme (60μg) was mixed with 75μl of Freund's incomplete adjuvant and 75μl of sterile water. Two rabbits were given four injections each, at intervals of 14 days, in the thicker part of the skin above the scapula. Beginning 10 days after the last injection, blood was drawn from the ear vein every sixth week. A dose of antigen was given 10 days before each bleeding. The sera were stored at −20°C until used.

Preparation of the immunoadsorbent

Dry CNBr-activated Sepharose 4B (3g) was swollen and washed with 200ml of 1mM-HCl and water on a glass filter. Antiserum (10ml portions) was treated with $(NH_4)_2SO_4$ (2.5g/10ml of serum for 20h), and the precipitates were dialysed against 0.1M-$NaHCO_3$ containing 0.25M-NaCl and mixed with the washed gel. After coupling at room temperature (23°C) under mild stirring for 20h the non-bound material was washed away with 500ml of the coupling buffer. Remaining reactive groups were blocked by reaction with 1M-glycine solution, adjusted to pH8 with NaOH, for 2h. After washing with 200ml of each of the following solutions: coupling buffer, 0.1M-sodium acetate, pH7.5 (con-

taining 1M-NaCl) and 0.05M-Tris/acetate buffer, pH7.5, the gel was stored at 4°C until used.

Rocket immunoelectrophoresis

The rocket immunoelectrophoresis experiments were performed as described by Weeke (1972). To 11.4ml of a 1% agarose solution in 24mM-barbital buffer, pH8.6, 0.125ml of antiserum was added and the gels were cast on 8.4cm×9.4cm glass plates. After congealing, 5μl wells were punched in the gel and 5μl of enzyme standard solution or filtrate was added and the electrophoresis started. Runs were made for 16h at 10°C with an electric field strength of 2V/cm.

Preparative methods

Crude extract. Freeze-dried culture filtrate (10g) was extracted with 100ml of 0.05M-ammonium acetate buffer, pH4.0, by gentle stirring in the cold (6°C) for 2h. Insoluble material was removed by centrifugation at 10000*g* for 15min.

Molecular-sieve chromatography. The crude extract was applied to a column (7.0cm×60cm) packed with Bio-Gel P-30 (100–200 mesh) equilibrated with the buffer used for extraction. Fractions (15.4ml) were collected at a flow rate of 240ml/h. The fractions containing high-molecular-weight enzyme activity towards CM-cellulose were pooled and freeze-dried.

Ion-exchange chromatography. A column (5.0cm× 17.0cm) was packed with DEAE-Sephadex A-50 and equilibrated with 0.05M-ammonium acetate buffer, pH4.9. Usually 2g of the freeze-dried material from the Bio-Gel P-30 run was dissolved in 30ml of the equilibrating buffer and after centrifugation (10000*g*, 15min) the supernatant was applied to the column. Fractions (10ml) were collected at a flow rate of 35ml/h. Stepwise elution was done with ammonium acetate buffers of increasing ionic strength, but with a constant pH of 4.9. Fractions containing active material were pooled and re-run on a second column (3cm×65cm) of DEAE-Sephadex A-50 equilibrated in 0.12M-ammonium acetate buffer, pH4.9. Some of the fractions were analysed by isoelectric focusing to determine the distribution of the different components in the effluent, and fractions were pooled on this basis.

Isoelectric focusing. The experiments were performed essentially as described by Vesterberg & Svensson (1966). A 100ml electrofocusing column was used at 10°C. The pH gradient (standard Ampholine pH3.5–5.0, 1%, v/v) was stabilized with a 0–60% (v/v) glycerol gradient, formed with a gradient

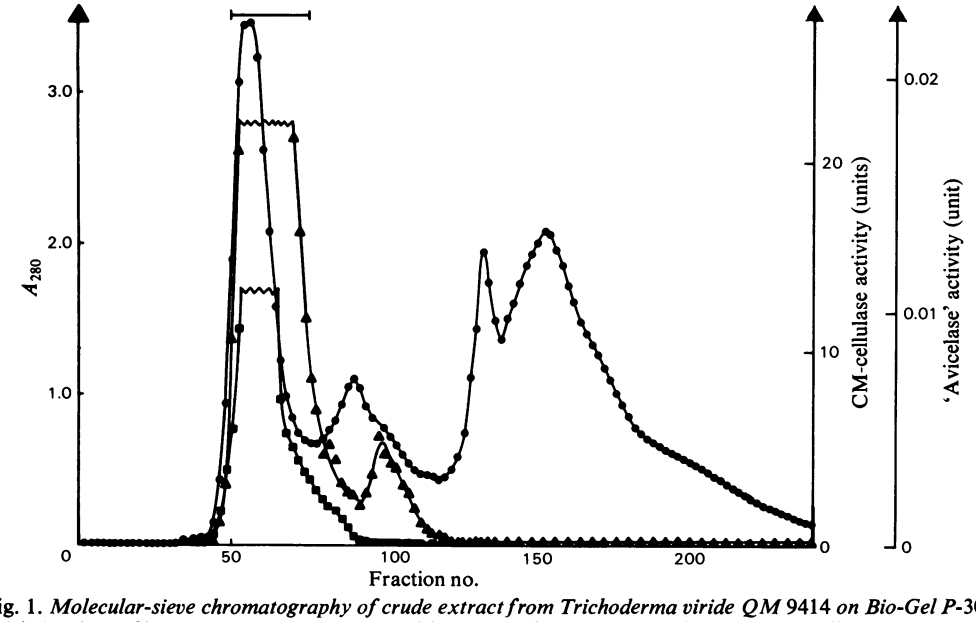

Fig. 1. *Molecular-sieve chromatography of crude extract from Trichoderma viride QM* 9414 *on Bio-Gel P-30*
Freeze-dried culture filtrate (10g) was extracted with 100ml of 0.05M-ammonium acetate buffer, pH4.0, and after centrifugation the supernatant was applied to a column (7.0cm×60.3cm) of Bio-Gel P-30 equilibrated and subsequently eluted with the same buffer. Fractions of volume 15.4ml were collected at a flow rate of 240ml/h. Fractions containing high-molecular-weight CM-cellulase were pooled and freeze-dried. ●, A_{280}; ■, 'Avicelase' activity; ▲, CM-cellulase activity; pooled fractions are indicated by the bar.

mixer (LKB 8121). Owing to a tendency of the focused enzyme to precipitate in the column, the polarity configuration was chosen so that the enzyme would focus in the denser part of the gradient, i.e. with the anode at the top of the column. The sample was dissolved in the denser gradient solution and runs were made at 10°C for 70h at a constant load of 3W until the limiting voltage of 1200V was reached. The pH of each fraction (1.1ml), removed by pumping out the solution from the bottom of the column, was measured at 23°C. The protein content was measured as the difference between A_{280} and A_{310} to compensate for the high u.v. absorption of the glycerol. The carrier ampholytes and the glycerol were removed by molecular-sieve chromatography on a column (1.4cm×90cm) of Bio-Gel P-30.

Adsorption experiments

A crude filtrate from *Trichoderma viride* QM 9414 was dialysed against 0.05M-Tris/acetate buffer, pH7.5, in a hollow-fibre concentrator (model DC 2; Amicon Corp., Lexington, MA, U.S.A.) and was applied to an immunoadsorbent column (1.4cm× 3cm) equilibrated with the same buffer. Fractions (5.2ml) were collected at a flow rate of 30ml/h, and desorption was carried out with the Tris/acetate buffer adjusted to pH2.5 with conc. HCl.

Results

By molecular-sieve chromatography of the crude extract on a column of Bio-Gel P-30 (Fig. 1), the CM-cellulase activity can be separated into two peaks. The first peak, near the void volume of the column, contains both CM-cellulase activity and activity against Avicel. This material was used for the isolation of a high-molecular-weight 1,4-β-glucan glucanohydrolase. The second peak contains a low-molecular-weight component, which also has been purified and characterized (Håkansson *et al.*, 1978). Each run, starting with 10g of freeze-dried filtrate, gave about 1.3g of dry material when pooled as indicated in Fig. 1.

Further fractionation was achieved by ion-exchange chromatography on DEAE-Sephadex A-50 (Fig. 2). The peak containing the main part of the CM-cellulase activity is eluted near the total volume of the column, which indicates weak interaction with the ion-exchanger. Therefore the pooled

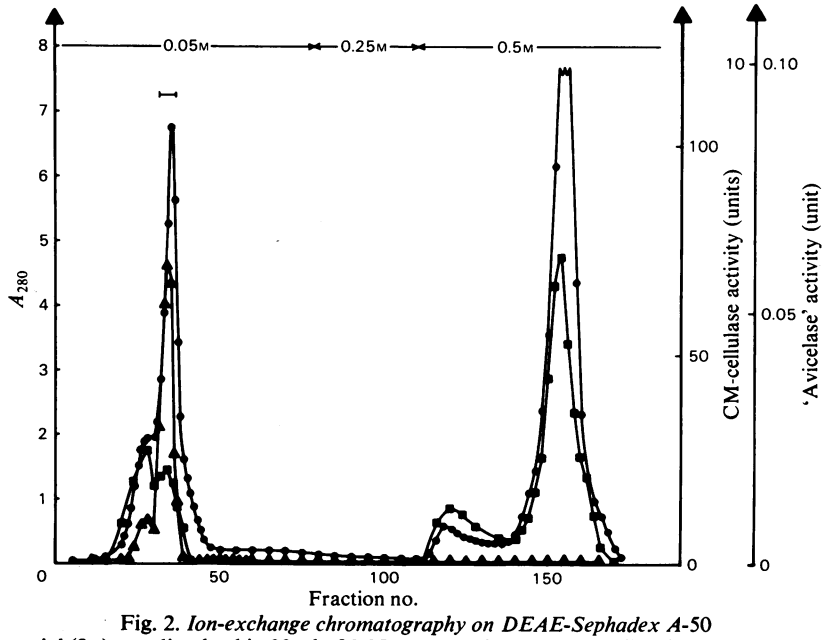

Fig. 2. *Ion-exchange chromatography on DEAE-Sephadex A-50*

Freeze-dried material (2g) was dissolved in 30ml of 0.05M-ammonium acetate buffer, (pH4.9) and applied to a column (5.0cm×17.0cm) equilibrated in the same buffer. The column was eluted with ammonium acetate buffers with a constant pH of 4.9, but with stepwise increasing ionic strength. Fractions (10.0ml) were collected at a flow rate of 35.0ml/h. The peak containing the CM-cellulase activity was pooled and freeze-dried. ●, A_{280}; ■, 'Avicelase' activity; ▲, CM-cellulase activity; pooled fractions are indicated by the bar.

material was subjected to elution chromatography on a column of the same ion-exchanger, but with a different geometry. The elution profile (Fig. 3) from this column indicates the presence of at least two superimposed components with CM-cellulase activity. Analytical isoelectric focusing (inset in Fig. 3)

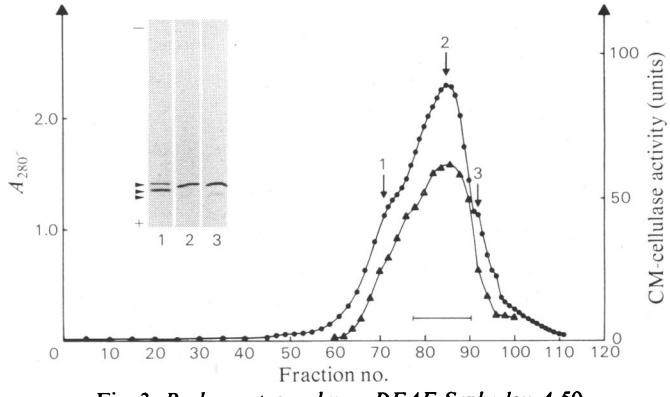

Fig. 3. *Rechromatography on DEAE-Sephadex A-50*

Freeze-dried material (280mg) from the first DEAE-Sepharose column was dissolved in 23ml of 0.12M-ammonium acetate buffer, pH4.9, and applied to a column (3.0cm×65.0cm) of DEAE-Sepharose A-50 equilibrated in the same buffer. The column was eluted with the equilibrating buffer at a flow rate of 23ml/h, and fractions of volume 6.0ml were collected. Some fractions (indicated with arrows) were analysed by isoelectric focusing, as shown in the inset, and the fractions containing the main component were pooled and freeze-dried. ●, A_{280}; ▲, CM-cellulase activity; pooled fractions are indicated by the bar.

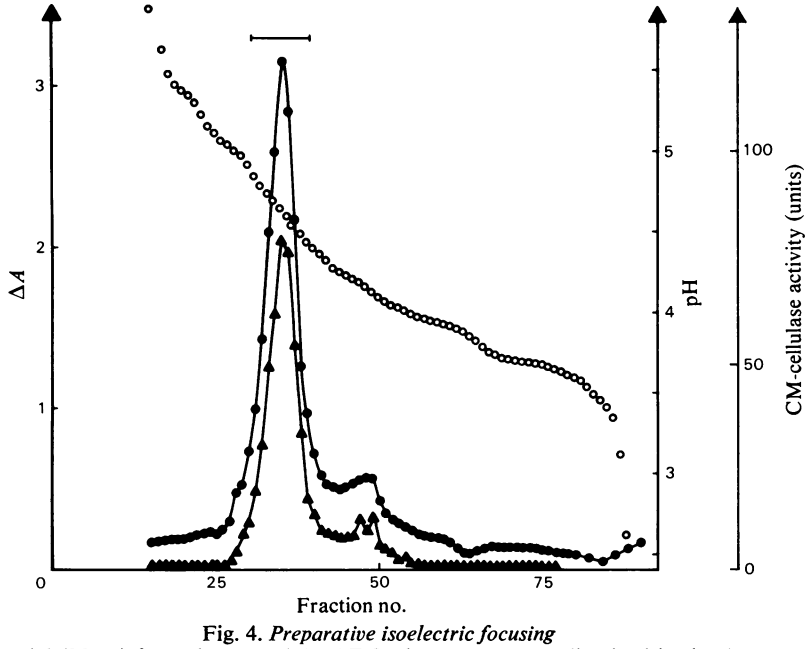

Fig. 4. *Preparative isoelectric focusing*

Freeze-dried material (25mg) from the second DEAE-Sepharose step was dissolved in the denser gradient (0–60%, v/v, glycerol) solution before loading of the column (110ml LKB 8100 Ampholine electrofocusing column). Standard Ampholine pH3.5–5.0 was used in 1% concentration. The electrofocusing was done at 10°C for 70h at a constant power of 3W, with the anode at the top of the column. Fractions (1.1ml) were removed by pumping out the solution from the bottom of the column. ●, $\Delta A = A_{280} - A_{310}$; ○, pH; ▲, CM-cellulase activity; pooled fractions are indicated by bar.

reveals that the shoulder at the front of the peak (arrow no. 1, fraction 71) contains predominantly a component with a pI lower than that of the main component (arrow no. 2, fraction 85). At the trailing edge (arrow no. 3, fraction 92) there is also some contamination by a minor component with an even lower pI.

By preparative isoelectric focusing (Fig. 4) the main component (pI 4.66; 23°C) can be isolated. This material was tested for homogeneity as described below.

Criteria of homogeneity

After polyacrylamide-gel electrophoresis at two different pH values, higher and lower than the pI, only a single component could be detected by scanning the gel at 280nm in a u.v.-scanner or by staining with Coomassie Brilliant Blue.

Only one band could be detected by analytical isoelectric focusing.

Molecular-sieve chromatography of the reduced and alkylated protein on a column of Sepharose 6B in the presence of 6M-guanidine hydrochloride gave rise to one peak, indicating a single homogeneous polypeptide chain.

Physicochemical and chemical characterization of the enzyme

By molecular-sieve chromatography of the reduced and alkylated enzyme on a previously calibrated column of Sepharose 6B in the presence of 6M-guanidine hydrochloride, the mol.wt. was estimated as 51000.

Table 1. *Amino acid composition*

Amino acid	Residues/molecule: Determined	Residues/molecule: Nearest integer
Asparagine/aspartate	62.4	62
Threonine	49.8*	50
Serine	64.6†	65
Glutamine/glutamate	33.2	33
Proline	25.5	26
Glycine	54.0	54
Alanine	28.0	28
Half-cystine	25.0‡	25
Valine	20.9§	21
Methionine	11.4‖	11
Isoleucine	14.2	14
Leucine	25.9	26
Tyrosine	25.3	25
Phenylalanine	9.7	10
Lysine	11.0	11
Tryptophan	6.9¶	7
Arginine	9.2	9
Total residues		484
Formula weight		50958

* Extrapolated linearly to zero time of hydrolysis.
† Extrapolated by first-order extrapolation to zero time of hydrolysis.
‡ Determined as cysteic acid.
§ 72h value only.
‖ Determined as methionine sulphone.
¶ Determined after hydrolysis with 4M-mercaptoethanesulphonic acid.

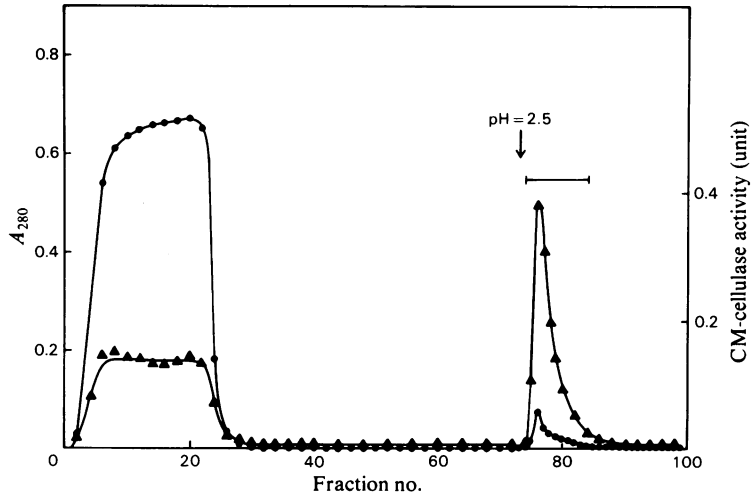

Fig. 5. *Chromatography of crude filtrate on an immunoadsorbent column*
On a column (1.4cm × 3.0cm), packed with the immunoadsorbent and equilibrated with 0.05M-Tris/acetate, pH7.5, 100ml of four times water-diluted filtrate was applied. After washing off the column with the equilibrating buffer, desorption was done by lowering the pH to 2.5 (vertical arrow, equilibrating buffer adjusted to pH2.5 by addition of 1M-HCl). Fractions of volume 5.2ml were collected at a flow rate of 30ml/h. ●, A_{280}; ▲, CM-cellulase activity.

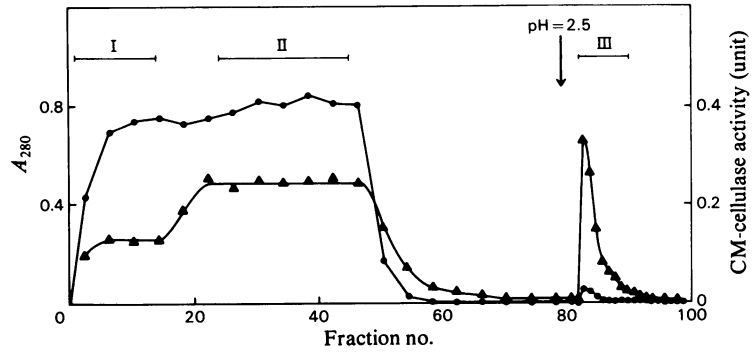

Fig. 6. *Saturation of the immunoadsorbent column*
To saturate the immunoadsorbent column 235 ml of four times water-diluted filtrate was applied. Running and desorption conditions were as described in the legend to Fig. 5. Fractions were pooled as indicated by bars with roman numbers. ●, A_{280}; ▲, CM-cellulase activity.

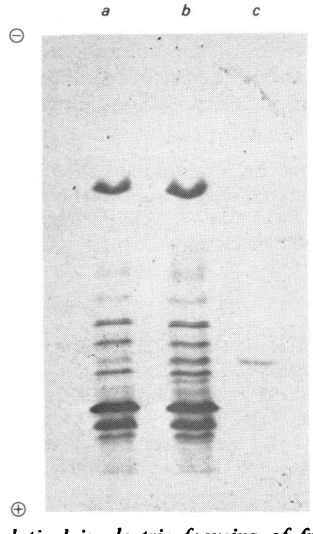

Fig. 7. *Analytical isoelectric focusing of fractions from immunoadsorbent chromatography*
Pools I (*a*), II (*b*) and III (*c*) from the immunoadsorbent saturation experiment were analysed by isoelectric focusing on a polyacrylamide-gel plate (LKB PAG plate) containing ampholytes in the pH range 3.5–9.5. Sample *c* shows the desorbed CM-cellulose.

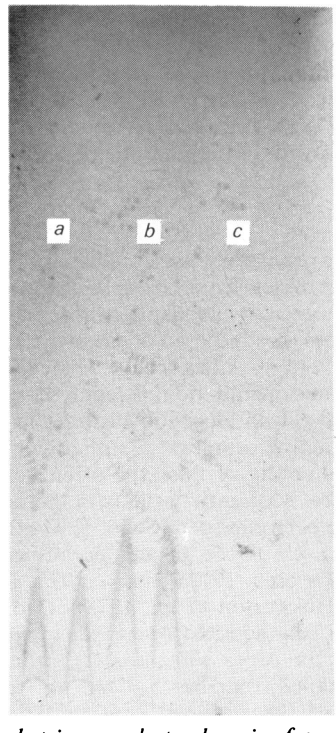

Fig. 8. *Rocket immunoelectrophoresis of pooled fractions from saturation experiment*
Pools I (*c*), II (*a*) and III (*b*) were analysed by immunoelectrophoresis in an anti-glucanohydrolase antibody-containing agarose gel.

The amino acid composition of the purified enzyme is shown in Table 1. To establish whether the enzyme was a glycoprotein the carbohydrate content was determined by g.l.c. Since only traces of mannose and glucose were detected, we conclude that the enzyme is not a glycoprotein.

From absorbance measurements on an enzyme solution with known concentration (determined by amino acid analysis) the molar absorption coefficient was calculated to be $6.15 \times 10^4\ M^{-1} \cdot cm^{-1}$ at 280 nm. Correction for unspecific absorption was made by linear extrapolation from the values at 370, 360, 350, 340 and 330 nm.

By column isoelectric focusing the pI was found to be 4.66 (mean value of five determinations) at 23°C.

The amount of enzyme in the original filtrate was determined by rocket immunoelectrophoresis. In two filtrates produced at different times the amount of enzyme was found to be 48 and 52 mg/litre respectively.

Purification by an immunoadsorbent

Fig. 5 shows the result of chromatography of the crude filtrate on the immunoadsorbent column. Desorption was tried with a number of buffers at pH 2–4, but 0.05M-Tris/acetate buffer, pH 7.5, adjusted to pH 2.5 with conc. HCl gave the best yield. The adsorbent gave a 26-fold increase in specific activity, but only 47% of the total CM-cellulase activity was adsorbed on the column. To determine the basis for this phenomenon a saturation experiment (Fig. 6) was carried out. The result shows that at least two immunologically different high-molecular-weight CM-cellulases exist, as the low-molecular-weight CM-cellulase contributes only about 5% of the total activity. Analytical isoelectric focusing (Fig. 7) and rocket immunoelectrophoresis (Fig. 8) testify to the high specificity of the adsorbent.

Discussion

Many investigators have isolated 1,4-β-glucan glucanohydrolases from *Trichoderma viride*, either from commercially available 'crude cellulase' or from filtrates produced in their own laboratories. Though a lot of work has been dedicated to the study of the enzymic degradation of cellulose, the function of the 1,4-β-glucan glucanohydrolases in the degradation of native cellulose is still obscure. Also, a confusing diversity of this type of enzyme exists in the literature. Molecular weights in the range 5300–65000 have been reported (Selby & Maitland, 1965, 1967; Li *et al.*, 1965; Okada & Nisizawa, 1968; Ogawa & Toyama, 1972; Okada, 1975; Berghem *et al.*, 1976; Håkansson *et al.*, 1978). Two classes of enzymes can be detected, one with mol.wts. below 20000 and the other with mol.wts. around 50000. A more detailed comparison of the physicochemical properties of the enzymes is generally difficult to make, owing to the sparse information available. The great apparent diversity among the glucanohydrolases, even if they come from the same organism, can be illustrated by the work of Berghem *et al.* (1976). From commercial crude *Trichoderma viride* cellulase they isolated a glucanohydrolase with mol.wt. 50000, similar to the enzyme studied here, but their enzyme had a different pI and was apparently a glycoprotein. Most of the glucanohydrolases must be present in very minute amounts in the filtrates, since very few components can be detected by analytical isoelectric focusing of whole filtrates of, for example, the highly cellulolytic mutant strain QM 9414. The predominant extracellular protein is the cellobiohydrolase, of which three components can be resolved (Fägerstam *et al.*, 1977). The glucanohydrolase activity can be subdivided into two components of mol.wts. 20000 and 51000.

The immunochemical experiments show that specific antibodies against the high-molecular-weight glucanohydrolase can be prepared. The antibodies do not cross-react with the cellobiohydrolase or with the low-molecular-weight glucanohydrolase. The results also indicate that high-molecular-weight glucanohydrolases, which do not cross-react with the antibodies, are present. The high specificity of the antibodies is further demonstrated with immunoadsorption experiments. This method will make it easier to isolate the other glucanohydrolase forms so that direct comparisons can be made. It is also shown that the antibodies can easily be used for the quantitative determination of the glucanohydrolase. The potentialities of the immunochemical technique in this field should be further studied, especially for the investigation of the relations between the different glucanohydrolases.

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