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PURIFICATION AND CHARACTERIZATION OF A LOW MOLECULAR WEIGHT 1,4-β-GLUCAN GLUCANOHYDROLASE FROM THE CELLULOLYTIC FUNGUS *TRICHODERMA VIRIDE* QM 9414

ULF HÅKANSSON, LARS FÄGERSTAM, GÖRAN PETTERSSON and LENNART ANDERSSON

Institute of Biochemistry, Biomedical Center, University of Uppsala, Box 576, S-751 23 Uppsala (Sweden)

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Summary

A low molecular weight 1,4-β-glucan glucanohydrolase (endoglucanase) (1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4) has been isolated from culture filtrates of the fungus *Trichoderma viride* QM 9414 by a two-step procedure of gel filtration and ion-exchange chromatography. The isolated enzyme appeared homogeneous upon polyacrylamide gel electrophoresis at pH 2.9, isoelectric focusing in a polyacrylamide gel slab, sedimentation equilibrium analysis and chromatography of the reduced and alkylated enzyme on a column of Sepharose 6B in 6 M guanidine · HCl. A molecular weight was calculated at approx. 20 000 and the isoelectric point was determined at pH 7.52. The purified enzyme was not a carbohydrate-containing protein.

Introduction

Culture filtrates of the fungus *Trichoderma viride* are known to be strongly cellulolytic and are capable of solubilizing native cellulose. Fractionation studies have revealed that at least three different types of enzymes, which act synergistically, are involved in the degradation. The present hypothesis involves a 1,4-β-glucan cellobiohydrolase (exoglucanase) which splits off cellobiose from the non-reducing chain ends, a 1,4-β-glucan glucanohydrolase (endoglucanase) (1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4) which creates new free chain ends by hydrolyzing internal glucosidic bonds and a cellobiase (β-glucosidase) which hydrolyzes cellobiose to the end product, glucose [1].

Both high molecular weight and low molecular weight enzymes of the endoglucanase type have been isolated and characterized from *T. viride* [2]. The function in the over-all degradation process of the high molecular weight

enzymes is presumed to be the breaking of internal bonds in less ordered regions of the cellulose fibre, thus creating new free chain ends which can be attacked by the exoglucanase. The role of the low molecular weight enzymes in this process is not known.

Selby and Maitland [3] found their partly purified low molecular weight enzyme from *T. viride* strain 92027 to be "non-essential" in the breakdown of native cellulose of cotton fibres. However, a low molecular weight endoglucanase purified to physicochemical homogeneity from a commercial *T. viride* extract (Cellulase Onozuka SS) by Berghem et al. [2] was very active in releasing free fibres from filter paper, but no studies on its synergistic effect in the degradation process were performed. The object of the present work has been to isolate the low molecular weight endoglucanase from culture filtrates of *T. viride* QM 9414 to physicochemical homogeneity.

Materials and Methods

The crude enzyme was a gift from Dr. M. Mandels, U.S. Army Laboratories, Natick, Mass., U.S.A. It consisted of freeze-dried culture filtrates of the fungus *T. viride* QM 9414. 10 g crude enzyme powder was extracted at 4°C with 100 ml 0.05 M ammonium acetate buffer (pH 4.0) with slow stirring for 60 min. The solution was then centrifuged at 4°C at 8000 $\times g$ for 30 min to remove non-soluble materials. The crude extract was fractionated by molecular sieve chromatography on a column (7.0 × 60.3 cm) packed with Bio-Gel P-30, 100—200 mesh (Bio-Rad Laboratories, Richmond, Calif. U.S.A.). The column was equilibrated with 0.05 M ammonium acetate buffer (pH 4.0) and eluted at 4°C with the same buffer. Samples of 100 ml were applied to the column. 15.4-ml fractions were collected; flow rate 240 ml/h. Fractions containing endoglucanase activity were pooled.

Further purification of the pooled material was achieved by ion-exchange chromatography on SP-Sephadex C-25 (Pharmacia Fine Chemicals, Uppsala, Sweden). The column (3.2 × 7.7 cm) equilibrated with 0.05 M ammonium acetate buffer (pH 4.0) was run at 23°C. Usually samples of about 400 ml were applied to the column at a flow rate of 50 ml/h. The column was washed with 125 ml of the equilibrating buffer and eluted with a continuous pH gradient of 0.05 M ammonium acetate (pH 5.6) and 0.05 M ammonium acetate (pH 4.0). 4.2-ml fractions were collected.

Enzyme assays. The reaction mixture consisted of 2 ml of a 1% solution of sodium carboxymethyl cellulose (Uddeholm AB, Skoghall, Sweden; with 0.4 degree of substitution and a degree of polymerization of 300, or Avicel (American Viscose Co., Marcus Hook, Pa., U.S.A.) in 0.05 M sodium acetate buffer (pH 5.0) and 50 or 200 μl enzyme solution, respectively. The incubation conditions were 40°C for 10 min, and 30°C for 2 h for CM-cellulose and Avicel, respectively, and analysis for reducing sugars according to Somogyi [4] and Nelson [5] was then done. 1 unit of enzyme activity was defined as the amount of enzyme needed to liberate reducing sugar corresponding to 1 μmol glucose per min.

The absorbance of the eluates at 280 nm was used to estimate protein concentration.

Electrophoresis in polyacrylamide gels was performed as described by Hjertén et al. [6] Cylindrical gel rods were used and the gel composition was $T = 7\%$ and $C = 3\%$ (T is the total concentration of monomer and C the fraction of monomer acting as crosslinker, respectively). Samples were run in 0.1 M acetic acid (pH 2.9). The current was 5 mA/gel and running time 1 h. The gels were stained with 0.1% Coomassie Brilliant Blue in 25% isopropanol and destained in the solvent alone.

Isoelectric focusing in polyacrylamide gel was carried out using the LKB 2117 Multiphor apparatus (LKB-Produkter AB, Bromma, Sweden). Commercial polyacrylamide gel for electrofocusing in pH 3.5—9.5 (Ampholine® PAG plates) was obtained from the same company. The sample was placed directly on the surface of the gel plate. The gel was run at 10°C for 90 min at a constant power of 1 W/cm. Fixation and staining was done according to Vesterberg [7] using 0.1% Coomassie Brilliant Blue.

The method of Andrews [8] was used for calculating the molecular weight of the native enzyme from the elution volume on a Bio-Gel P-30 column (1.4 × 88.3 cm). The column was calibrated with the following standard proteins: bovine serum albumin, $M_r = 66\,000$ (Miles-Servac Ltd., Berkshire, U.K.), chymotrypsinogen A, $M_r = 23\,200$ (bovine pancreas, Worthington Biochemical Corp., Freehold, N.J., U.S.A.) and [^{3}H]neurotoxin, $M_r = 7800$ (*Naja naja siamensis*, a gift from Dr. E. Karlsson). The flow rate was 12 ml/h, and fractions of about 1 g were collected by weighing each fraction.

Molecular weight estimation by molecular sieve chromatography in the presence of 6 M guanidine · HCl was made according to the method of Fish et al. [9]. Approx. 1 mg lyophilized enzyme was reduced (under N_2) with 8 μmol dithiothreitol in 300 μl 6 M guanidine · HCl/0.1 M Tris · HCl buffer (pH 7.9).

The mixture was shaken for 6 h and 5 mg sodium iodoacetate was added. After 30 min, the sample was applied to a Sepharose 6B column (1.03 × 97.8 cm) equilibrated in the same buffer. The column was calibrated with serum albumin, chymotrypsinogen A, ribonuclease A, $M_r = 13\,700$ (bovine pancreas, Sigma Chemical Co., St. Louis, Mo., U.S.A.) and cobra neurotoxin (reduced and alkylated with dithiothreitol and sodium iodo[^{14}C]acetate, respectively). The flow rate was 2 ml/h and fractions of 1 g were collected. The absorbance at 280 nm and radioactivity of the fractions were determined.

Sedimentation equilibrium analysis was made in a Spinco model E analytical ultracentrifuge in 0.05 M sodium acetate buffer (pH 5.0) at 83 500 × g for 16 h at 20°C. The initial protein concentration was 0.5 mg/ml. The partial specific volume, $\bar{v}$, was calculated from the amino acid composition [10] to be 0.70 cm^3/g.

Amino acid analyses were carried out with a Durrum D-500 analyzer. Two samples were hydrolyzed in 6 M HCl at 110°C for 24 and 72 h, respectively. The content of half-cystine was determined as cysteic acid after oxidation of a third sample with performic acid. The tryptophan content was determined both by amino acid analysis after hydrolysis in 3 M p-toluenesulfonic acid [11] and by spectrophotometric determination in 6 M guanidine · HCl [12].

Sugars were analyzed as alditol acetate derivatives by gas-liquid chromatography [13].

Isoelectric focusing experiments were performed in the LKB apparatus type

8100-1 according to the manufacturer's instructions, using a glycerol density gradient. The choice of glycerol rather than sucrose was made because the acidic anode solution causes inversion of the disaccharide producing glucose, which interferes with the enzyme assay. The electrodes were positioned so that the anode was at the bottom of the column. The ampholyte concentration was 1% (w/v) consisting of equal amounts of Ampholine pH 6—8 and Ampholine pH 8—9.5 (LKB-Produkter, Bromma, Sweden) and columns were run at 10°C for 48 h with a final voltage of 1000 V. The column was drained from the bottom in 1-ml fractions. The pH values of the fractions were quickly determined at 20°C.

Results

Fig. 1 shows the result of molecular sieve chromatography of the crude extract on Bio-Gel P-30. Most of the cellulolytic activities were associated with the first protein peak and the low molecular weight enzyme with activity against CM-cellulose was well separated from these and also from most of the low molecular material in the extract.

By chromatography on SP-Sephadex of the pooled low molecular enzyme from the previous fractionation step the separation of the enzyme from other low molecular proteins was achieved (Fig. 2). The yields and the specific activities after successive stages of purification are presented in Table I.

The isolated enzyme (70 μg/gel) gave a single band after polyacrylamide gel electrophoresis at pH 2.9, indicating a homogeneous protein. Analytical isoelectric focusing of approx. 50 μg of enzyme in a carrier gradient of pH 3.5—9.5 showed only a single band and its position was compatible with the isoelectric point determined in the LKB apparatus type 8101.

Gel filtration on Sepharose 6B in guanidine · HCl of the reduced and alkylated enzyme produced a single symmetrical peak (Fig. 3). The results of sedi-

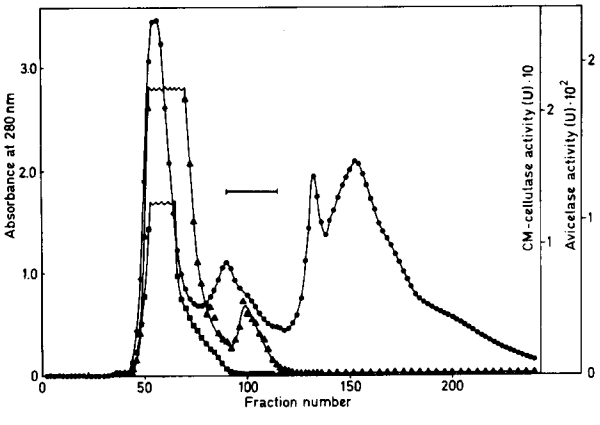

Fig. 1. Molecular sieve chromatography of a crude extract from *T. viride* QM 9414 on Bio-Gel P-30. Sample: 10 g freeze-dried culture filtrate (100 ml). Column dimension: 7.0 × 60.3 cm. Eluant: 0.05 M ammonium acetate buffer, pH 4.0. ●———●, Absorbance at 280 nm; ▲———▲, CM-cellulose activity; ■———■, avicelase activity; ⊢———⊣, pooled fractions.

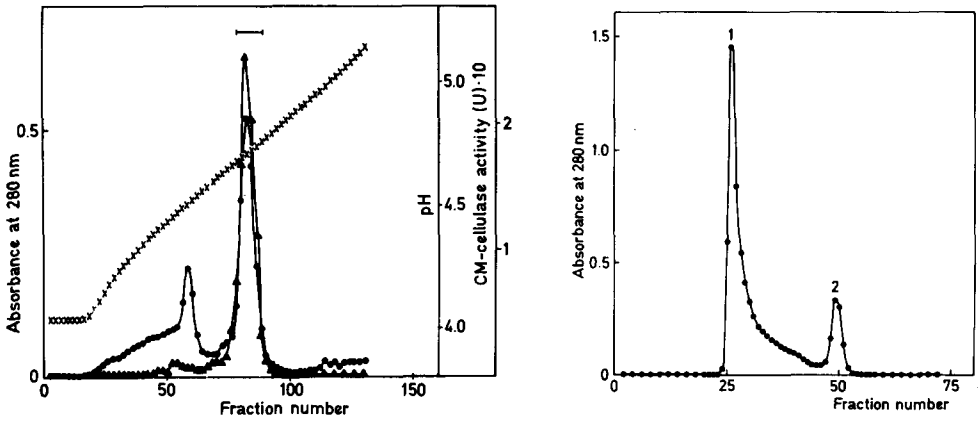

Fig. 2. Ion-exchange chromatography on SP-Sephadex C-25 of pooled material from the previous molecular sieve chromatography. Sample: 0.29 g protein (400 ml). Column dimension: 3.2 × 7.7 cm. Elution: pH gradient generated by continuously adding 400 ml 0.05 M ammonium acetate, pH 5.6, to 400 ml 0.05 M ammonium acetate, pH 4.0. ●———●, Absorbance at 280 nm; ▲———▲, CM-cellulose activity; X, pH of the fractions; ⊢——⊣, pooled fractions.

Fig. 3. Molecular sieve chromatography of a reduced and alkylated sample of the purified enzyme on a calibrated column of Sepharose 6B. Sample: 1 mg protein (reduced and alkylated). Column dimension: 1.03 × 97.8 cm. Eluant: 6 M guanidine · HCl, 0.1 M Tris · HCl buffer, pH 7.9. Fractions of 1.0 g were collected. 1 and 2 indicate the elution positions of added blue dextran and the purified enzyme, respectively.

mentation equilibrium analysis of the purified enzyme show a linear relation between log (fringe displacement) and r^2, which is additional evidence for homogeneity.

The molecular weight of the enzyme was estimated to be 8000 from a run on a previously calibrated Bio-Gel P-30 column. Molecular sieve chromatography of reduced and alkylated enzyme under denaturing conditions on Sepharose 6B indicated a single polypeptide chain with a molecular weight of 20 200 (Fig. 3). By sedimentation equilibrium centrifugation, the molecular weight was determined to be 20 400.

Table II shows the amino acid composition of the purified endoglucanase, with 35 acidic amino acids and 30 glycine residues per 197 calculated/molecule. Only traces of mannose and *N*-acetylglucosamine were detected by gas-liquid chromatography. As no glucosamine was observed in the amino acid

TABLE I

ISOLATION OF A LOW MOLECULAR WEIGHT ENDOGLUCANASE FROM *T. VIRIDE* QM 9414

Starting sample was 10 g freeze-dried extract in 100 ml buffer.

Step	Total protein (mg)	Total CM-cellulose activity (units)	Specific activity (units/mg)	Purification factor	Yield of CM-cellulose activity (%)
Bio-Gel P-30	289	240	0.83	1	100
SP-Sephadex C-25	15	143	9.6	11.4	60

TABLE II

AMINO ACID COMPOSITION OF A LOW MOLECULAR WEIGHT ENDOGLUCANASE FROM *T. VIRIDE* QM 9414

Amino acid	Residues/molecule	
	Determined	Nearest integer
Tryptophan	4.9 * (5—6) **	5
Lysine	5.2	5
Histidine	2.6	3
Arginine	4.0	4
Aspartic acid + asparagine	22.4	22
Threonine	18.3 ***	18
Serine	23.0 †	23
Glutamic acid + glutamine	14.6	15
Proline	9.0	9
Glycine	29,4	29
Alanine	16.6	17
Half-cysteine	3.6 ††	4
Valine	11.0 †††	11
Methionine	2.2 ‡	2
Isoleucine	6.1	6
Leucine	7.5	8
Tyrosine	11.0	11
Phenylalanine	4.9	5
Total residues		197
Formula weight		20 530

* Determined after hydrolysis with 3 M *P*-toluenesulfonic acid [11].
** Spectrophotometric determination [11].
*** Extrapolated linearly to zero time of hydrolysis.
† First-order extrapolation to zero time of hydrolysis.
†† Determined as cysteic acid.
††† 72 h value only.
‡ Determined as methionine sulfone.

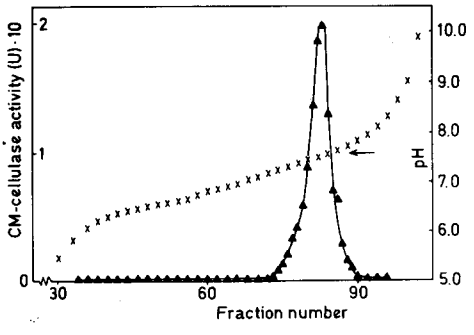

Fig. 4. Isoelectric point determination of the purified endoglucanase. Sample: 0.2 mg protein. Column: LKB-electrofocusing column (110 ml). Carrier ampholytes: Ampholine pH 6—9.5. Ampholyte concentration: 1% (w/v). Density gradient: glycerol, 0—60% (w/v). Final voltage: 1000 V. Duration of the run: 48 h. Emptying of the column: from the bottom in 1-ml fractions at a flow rate of 40 ml/h. ▲———▲, CM-cellulose activity; X, pH of the fractions at 20°C. The arrow indicates the p*I* of the endoglucanase.

analysis, we assume that the endoglucanase is not a glycoprotein.

From isoelectric focusing, the isoelectric point of the enzyme was determined to be pH 7.52 (Fig. 4).

The molar absorption coefficient of the enzyme at 280 nm calculated for a molecular weight of 20 500 was determined from ultraviolet spectra in conjunction with amino acid analysis to be $2.02 \cdot 10^4\ M^{-1} \cdot cm^{-1}$.

Discussion

When we started our study of the low molecular weight endoglucanase in *T. viride* we soon found that the enzyme content of the commercial preparation used previously (Onozuka SS) varied considerably. More disappointing was the fact that, in the last batches, the amount seemed to be very low. Something in the production of the crude extract must be non-reproducible as regards the content of the low molecular weight enzyme. Therefore, we started the fractionation with freeze-dried culture filtrates from *T. viride* QM 9414, which is probably the most efficient cellulose-degrading organism known. Culture filtrates from this strain are also very easy to produce in a reproducible way in the laboratory. We soon found that even this strain produced only a small amount of the low molecular enzyme compared to the total cellulolytic activity.

A procedure has been worked out for the preparation of a homogeneous enzyme. High molecular weight proteins containing most of the cellulolytic activity in the crude extract were removed, along with low molecular weight substances in the first purification step (molecular sieve chromatography on Bio-Gel P-30). Further purification by ion-exchange chromatography on SP-Sephadex resulted in an apparently homogeneous enzyme preparation. The results of the purification are summarized in Table I. The yield of pure endoglucanase from 10 g crude extract was about 15 mg. This low molecular weight endoglucanase is homogeneous as evidenced by polyacrylamide gel electrophoresis, by isoelectric focusing in a polyacrylamide gel slab, by analytical ultracentrifugation and by molecular sieving under denaturing conditions.

A molecular weight of 20 200 was determined for the endoglucanase by molecular sieve chromatography in 6 M guanidine · HCl. This value is in good agreement with that of 20 400 obtained by sedimentation equilibrium centrifugation. In contrast, molecular sieve chromatography under native conditions on Bio-Gel P-30 in both 0.05 and 0.5 M sodium acetate buffer (pH 5.0) indicated a molecular weight of about 8000. On the basis of amino acid analysis the value of 8000 is improbable. The reason for the peculiar behaviour on Bio-Gel is unknown. The same phenomenon has been observed on Sephadex gels. From these results, we conclude that the isolated enzyme consists of a single polypeptide chain with a molecular weight of about 20 000.

Low molecular endoglucanases have been isolated before from culture filtrates of *T. viride and Myrothecium verrucaria* [2,3,14,15], but we must emphasize that the previously determined molecular weights were estimated by molecular sieve chromatography. In the view of our observation that endoglucanases can shown affinity for both polyacrylamide and dextran gels, it seems highly probable that the molecular weights reported earlier are too low.

The pure enzyme contains no carbohydrate. It is a neutral protein with an isoelectric point of 7.52. This value is 2—3 pH units higher than other values reported for cellulolytic enzymes from *T. viride*. From these results we conclude that the enzyme studied here is not identical to the enzyme described by Berghem et al. [2].

Preliminary experiments on the synergistic action on native cellulose of the isolated low molecular weight endoglucanase and a purified 1,4-β-glucan glucanohydrolase from the same source (Fägerstam, L., Håkansson, U., Pettersson, G. and Andersson, L., unpublished data) indicates that the two enzymes are mutually interchangeable in the degradation process.

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