Journal of Chromatography, 347 (1985) 310–315
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 023

Note

Isoelectric focusing separation of *Gliocladium* enzyme components

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(Received July 9th, 1985)

Cellulase is a complex of enzymes containing mainly exoglucanases and endoglucanases plus cellobiase[1,2]. The cellulase components are often present as isoenzymes, differing only slightly in isoelectric pH, and are difficult to separate. Fractionation studies reported on cellulases of different fungal origin have involved the use of standard protein separation techniques such as gel filtration, ion-exchange chromatography, affinity chromatography and isoelectric focusing[2–6], high-performance liquid chromatography (HPLC)[7] and polyacrylamide gel electrophoresis (PAGE)[8].

This work was concerned with the separation and characterization of isoenzymes from a culture filtrate obtained by fermentation of the fungus *Gliocladium* sp.

EXPERIMENTAL

Enzyme source and enzyme activity determination

A culture filtrate of the cellulolytic fungus *Gliocladium* sp. was used as the enzyme source. The culture filtrate was obtained by courtesy of the Institute of Technical Chemistry of the Academy of Science of the G.D.R., Leipzig[9].

Filter-paper degrading activity (FPA) was assayed according to the method of Mandels *et al.*[10] and the cotton-hydrolysing C_1 activity by a slight modification of the method of Mandels and Weber[11], using dinitrosalicylic acid (Merck, Darmstadt, F.R.G.)[12]. Carboxymethylcellulose (CMC) (Sigma, St. Louis, MO, U.S.A.) degrading C_x activity and β-glucosidase activity were measured by the release of glucose from a CMC solution[13] and from a cellobiose solution[14], respectively.

Proteolytic activity was determined by the Anson[15] method, using haemoglobin as substrate.

Protein content and amino acid composition

Soluble protein was measured by the phenol procedure[16] after precipitating with trichloroacetic acid (5%, w/v) using bovine plasma albumin as standard (Browning Chemical Corp., New York, NY, U.S.A.).

Amino acid composition was determined with the Biotronik LC 2000 Amino Acid Analyzer.

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Methods of separation

The culture filtrate was prepared for isoelectric focusing by concentration (10-fold) in a rotational evaporator and dialysis through a 4-22/32 in. membrane (Medicell, London, U.K.). Slab gels for analytical isoelectric focusing (IF) were prepared according to the Pharmacia manual on IF[17], using Ampholine (LKB, Bromma, Sweden) in the pH range 3–10. Samples, containing 2 mg of protein, were focused at 50, 100, 200, 600 and 1000 V each for 30 min in the Pharmacia flat-bed electrophoresis system. After completion of focusing, proteins were fixed and stained for proteins with Servablau G (Serva, Heidelberg, F.R.G.) and for glycoproteins with Schiff's reagent[17].

Preparative IF was performed on 5-mm Sephadex IEF layers (Pharmacia, Uppsala, Sweden) according to the Pharmacia manual[17].

The Ampholine was the same as used in analytical IF. The separated proteins were recovered with the fractionation grid, pressing 27 strips into the gel at the positions determined by the paper replica. The gel strips, containing proteins of interest, were removed, the pH values were determined and the proteins eluted with distilled water.

The homogeneity of the separated components was monitored by analytical IF. The pH of the fractions was determined using Protein Test Mixture 9 (Serva, Heidelberg, F.R.G.).

Determination of molecular weight

The molecular weights of the separated fractions were determined by SDS-PAGE using the Weber and Osborn[18] SDS–phosphate continuous buffer system system and 125 mm wide × 110 mm long × 1.0 mm thick gel slabs. The samples, containing 1% (w/v) protein, 0.01 *M* sodium phosphate buffer (pH 7.2), 3% (w/v) SDS (Serva) and 5% (w/v) β-mercaptoethanol (Reanal, Budapest, Hungary) were boiled for 2 min and mixed with 36% (w/v) urea (Serva) and 20% (w/v) saccharose. The electrophoresis was pre-run at 50 V for 1 h at room temperature and developed at 100 V for 4 h at 4°C. The gel was stained with silver nitrate stain[19].

RESULTS AND DISCUSSION

The isoelectric points of the cellulolytic enzyme components determined by analytical flat-bed IF were found to cover the pH range 9.3–3.0 (Fig. 1). A total of 28 protein fractions was obtained. The fractions in the pH range 4.1–6.1 and two fractions of pH 8.6 and 9.0 gave the strongest protein stains. Most of these proved to be glycoproteins.

Preparative IF confirmed the above results (Fig. 2). Most (85%) of the soluble protein content was found in fractions 14–28 with p*I* values in the range 5.9–9.8.

The C_1 activity was mainly located in the acidic p*I* range, while C_x covered the p*I* range 4.1–6.6. Two fractions of considerable cellobiase activity were on the cathodic side (p*I* 9.0 and 9.3). The remainder of the cellobiase fractions more or less coincided with the endoglucanase activities. The FPA activity spread practically over the whole p*I* range, which was to be expected as this substrate is acted on by several cellulolytic activities.

While the C_x and cellobiase activities could be recovered almost quantitatively

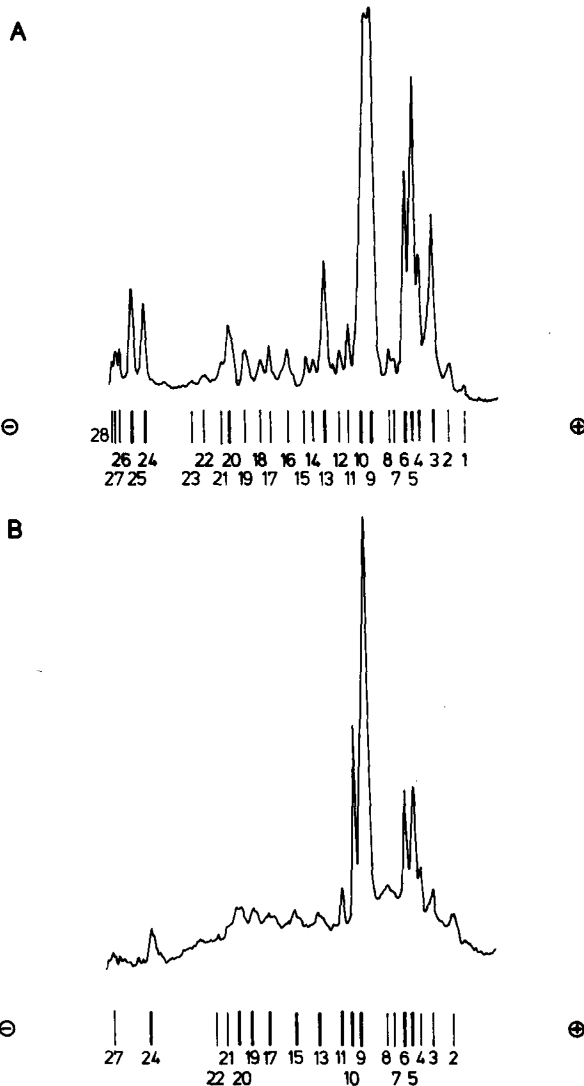

Fig. 1. Isoelectric points (p*I*) of the cellulase enzyme components. A = *Gliocladium* enzyme components; protein staining with Servablau G (Serva). B = *Gliocladium* enzyme components; glycoprotein staining with Schiff's reagent. Separation was performed by analytical IF of the concentrated and dialysed culture filtrate on slab gels according to the Pharmacia Manual[17] using Ampholine, pH 3–10 (LKB). Slab gels were evaluated with a Shimadzu Model CS-930 dual-wavelength thin-layer chromato-scanner. Enzyme component (p*I*): 1 (3.0), 2 (3.2), 3 (3.5), 4 (3.8), 5 (4.0), 6 (4.1), 7 (4.4), 8 (4.7), 9 (4.8), 10 (5.0), 11 (5.3), 12 (5.5), 13 (5.8), 14 (6.1), 15 (6.6), 16 (7.1), 17 (7.3), 18 (7.4), 19 (7.5), 20 (7.6), 21 (7.7), 22 (8.0), 23 (8.3), 24 (8.4), 25 (8.6), 26 (9.0), 27 (9.1), 28 (9.3).

(94% and 99%, respectively) after the purification procedures, only low levels of FPA and C_1 activities (41.9% and 5.7%, respectively) could be detected in the fractions. The protein recovery was 77%. The recombination of the 28 fractions resulted in the recovery of the total FPA and C_1 activities.

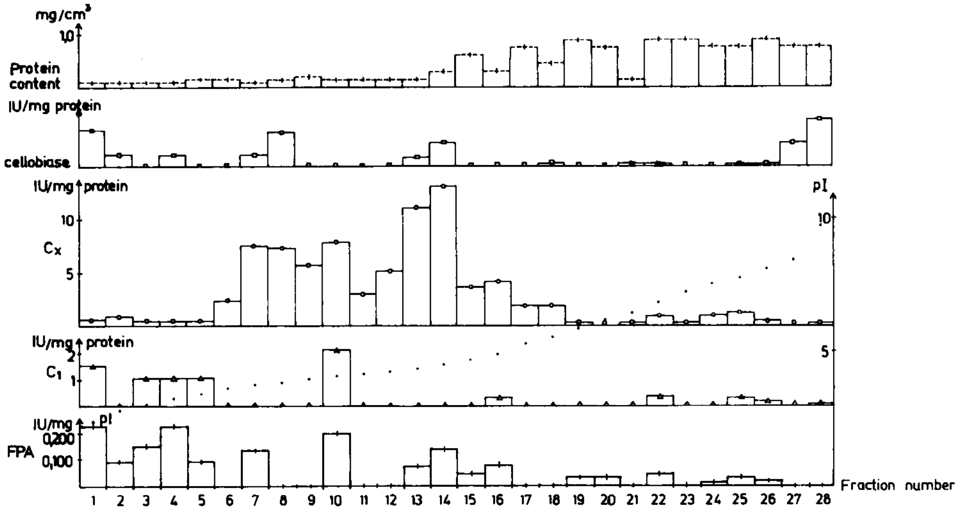

Fig. 2. Activities of the cellulase components separated by preparative isoelectric focusing (IF). Protein content according to Lowry *et al.*[16]; cellobiase activity according to Sternberg[14]; C_x = carboxymethylcellulose-degrading activity; C_1 = cotton-hydrolysing activity according to Mandels and Weber[11]; FPA = filter-paper degrading activity according to Mandels *et al.*[10]; 1 IU = released glucose equivalent, mg from 100 mg of cellulose in 24 h at pH 4.8 and 50°C.

Consequently, the separation losses are only apparent and might be due to the fact that the FPA and C_1 activities are the result of the complex action of endo- and exoglucanases plus cellobiase. These results seem to support the cellulase multienzyme theory, which means that the C_1 and FPA components (mainly cellobiohydrolase) play important initial roles in the overall process of enzymatic cellulose degradation, but for total hydrolysis the other enzyme components are also required[20].

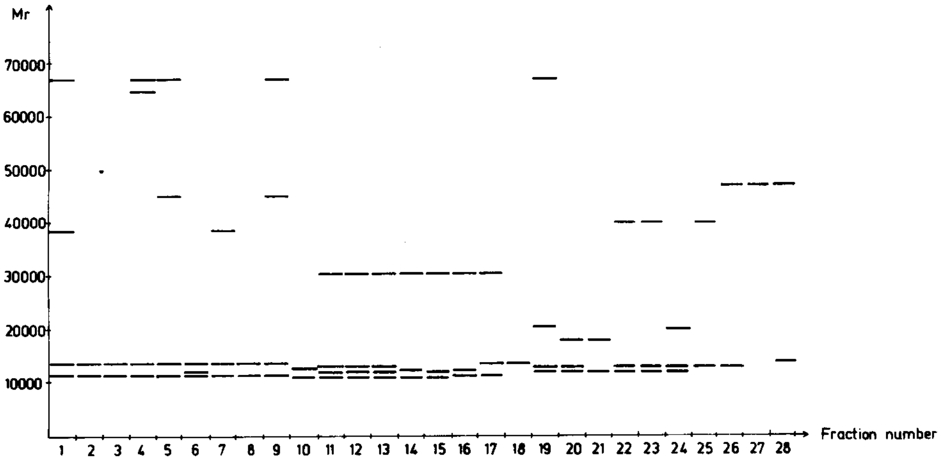

Fig. 3. Molecular weights (M_r) of the fractions separated by preparative isoelectric focusing. The determinations were carried out by SDS vertical slab electrophoresis according to Weber and Osborn[18]; proteins were stained with the silver nitrate method[19].

The presence of more than ten enzymes (or isoenzymes) was reported for some *Trichoderma* cellulases[2]. The great number of active fractions in our enzyme complex suggested this to be the result of proteolytic fragmentation. However, no proteolytic activity could be detected in the culture filtrate by the Anson method.

The molecular weights of the fractions were found to range from 10000 to 70000 (Fig. 3). The yellow colour obtained with the silver stain for 19 fractions indicate these to be glycoproteins. This is in agreement with IF findings.

The amino acid analysis of the separated 28 fractions can be seen in Fig. 4. All the fractions contained glutamic acid and aspartic acid (except fractions 18 and 25), serine (except fractions 1 and 3) and threonine (except fractions 1-4). Only a few fractions contained cysteine (1, 11 and 26), methionine (2, 11 and 26), lysine (1, 3, 6, 17, 18, 25 and 28) and tyrosine (2, 3, 4, 11, 16, 21 and 27).

Components with similar molecular weights, amino acid contents and isoelectric points were found to have similar activities.

The relatively large number of isoenzymes of similar activities might be the result of a proteolytic activity which splits the cellulase enzyme components from proenzymes of higher molecular weight during the fermentation process. As no proteolytic activity could be detected in the culture filtrate, such proteases must be endocellular. Investigations into this problem are in progress.

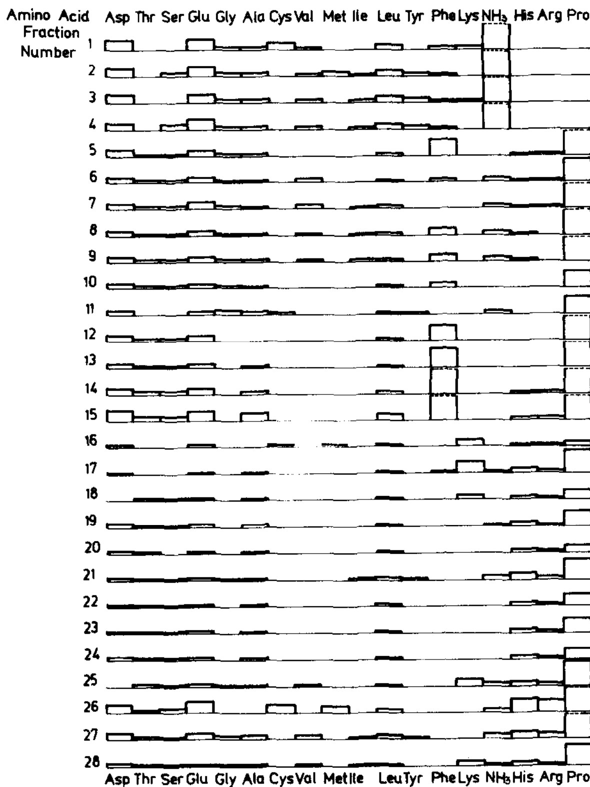

Fig. 4. Amino acid analysis of the fractions separated by preparative IF. Analysis was performed after acid hydrolysis in a Biotronik LC 2000 Amino Acid Analyzer.

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