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Note

Chromatofocusing of cellulolytic enzymes produced by *Trichoderma viride, Trichoderma reesei, Aspergillus niger* and *Pleurotus ostreatus*

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The degradation of cellulose with the enzymes produced by the title organisms is a complex process involving the action of several components[1]. At least three important enzyme activities are contained in these preparations: C_1-activity is responsible for the initial step (it attacks microcrystalline cellulose[1]), the most important phase of the degradation process is catalysed with C_x-cellulases[1] (both endocellulases and exocellulases) and the degradation is terminated by the action of cellobiase, which yields glucose from cellobiose[1]. It is known that these enzymes occur in multiple forms in the above-mentioned organisms[2–6]. Separations of these enzyme complexes on ion exchangers[2–6] and molecular sieves[2,6] and by isoelectric focusing[4] have been described. This paper presents the results of high-performance chromatofocusing of the title enzyme complexes. This method gives a high resolution and the results are comparable to those obtained by isoelectric focusing.

EXPERIMENTAL

Cellulase from *Trichoderma viride* (Onozuka R-10) was obtained from Serva (Heidelberg, F.R.G.), cellulase from *Aspergillus niger* from Calbiochem-Behring (La Jolla, CA, U.S.A.) and cellulase from *Pleurotus ostreatus* was a generous gift from Dr. A. Ginterová. The enzyme preparation from *Trichoderma reesei* was obtained by a submerse cultivation according to ref. 6. Tris(hydroxymethyl)aminomethane (Tris) was purchased from Merck (Darmstadt, F.R.G.), cellobiose from Koch-Light (Colnbrook, U.K.), Polybuffers 96 and 74 from Pharmacia (Uppsala, Sweden) and other chemicals from Lachema (Brno, Czechoslovakia).

The separations were carried out on a Mono P HR 5/20 column (200 × 5 mm I.D.) from Pharmacia. The column was attached to a fast protein liquid chromatography (FPLC) system (Pharmacia) described previously[6]. Approximately 15 mg of proteins dissolved in 25 m*M* Tris–HCl buffer (pH 8.0) were injected on to the column with a V-7 sample injector (Pharmacia). The elution was started with the above buffer, then the chromatofocusing mobile phase (8% Polybuffer 74–2% Polybuffer

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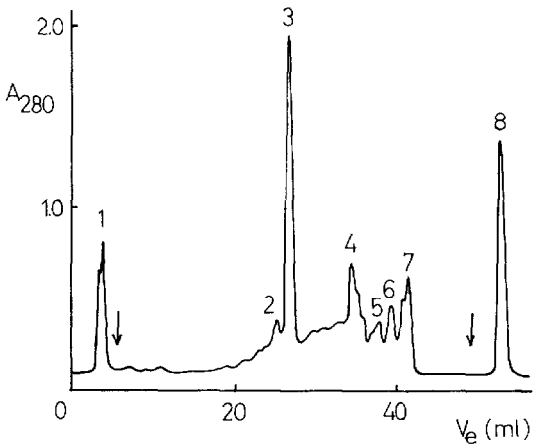

Fig. 1. Chromatofocusing of cellulolytic enzymes from *Trichoderma viride*. Solid line, absorbance at 280 nm (A_{280}); V_e, elution volume; first arrow, start of chromatofocusing (application of buffer B); second arrow, end of chromatofocusing and injection of 2 ml of 2 *M* sodium chloride solution; flow-rate, 1 ml/min; *ca.* 15 mg of proteins were injected. The following enzyme activities were detected in the peaks: 1, C_1-factor, exocellulase (pH 8.0); 2, C_1-factor, cellobiase (pH 6.2); 3, cellobiase (pH 6.0); 4, endocellulase (pH 5.3); 6, endocellulase (pH 4.3); 8, C_1-factor, endocellulase, cellobiase (pH 3.8).

96 adjusted to pH 3.8 with HCl) was applied. The separations were terminated with injections of 2 ml of 2 *M* sodium chloride solution.

The enzyme activities were determined as described[6] using a Cary 118 spectrophotometer (Varian, Palo Alto, CA, U.S.A.).

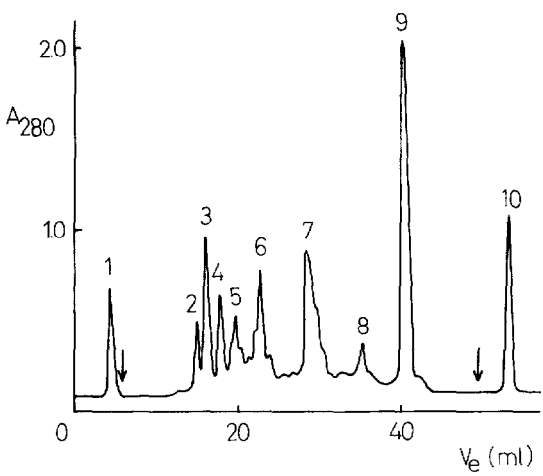

Fig. 2. Chromatofocusing of cellulolytic enzymes from *Trichoderma reesei*. Conditions as in Fig. 1. Peaks: 1, endocellulase, cellobiase (pH 8.0); 4, exocellulase (pH 6.1); 5, C_1-factor, endocellulase (pH 5.7); 6, C_1-factor, endocellulase (pH 5.4); 7, C_1-factor, exocellulase (pH 5.0); 8, endocellulase (pH 4.5); 9, C_1-factor (pH 3.9); 10, exocellulase, cellobiase (pH 3.8).

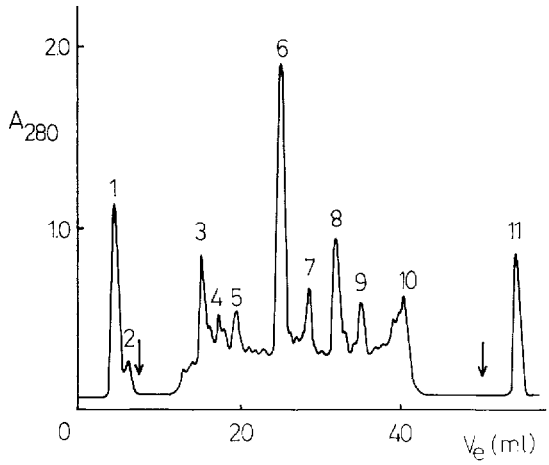

Fig. 3. Chromatofocusing of cellulolytic enzymes from *Pleurotus ostreatus*. Conditions as in Fig. 1. Peaks: 1, exocellulase (pH 8.0); 4, C_1-factor, cellobiase (pH 7.2); 5, C_1-factor, cellobiase (pH 6.8); 6, cellobiase (pH 6.1); 7, C_1-factor (pH 5.6); 8, endocellulase (pH 5.3); 9, endocellulase (pH 5.1); 11, endocellulase (pH 3.8).

RESULTS AND DISCUSSION

The results of the chromatofocusing experiments are shown in Figs. 1–4. The method showed a high resolving power even for the complex materials under study; 7–11 well separated main protein fractions were obtained. Their isoelectric points (p*I*) were mostly in the pH range used (8.0–3.8), but several activities were also detected in the starting buffer (p$I \geq 8.0$) and some of them were eluted after the injec-

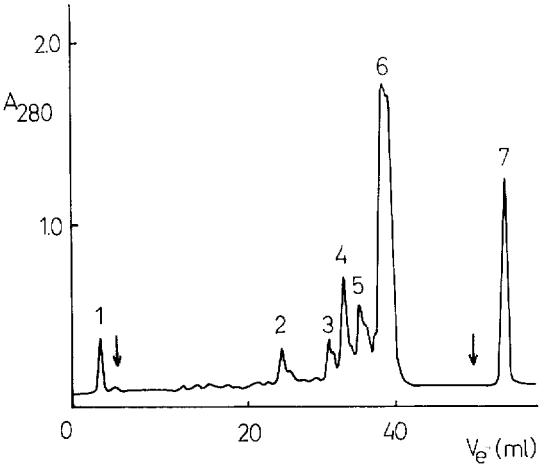

Fig. 4. Chromatofocusing of cellulolytic enzymes from *Aspergillus niger*. Conditions as in Fig. 1. Peaks: 1, exocellulase (pH 8.0); 4, C_1-factor (pH 5.0); 6, C_1-factor, endocellulase, cellobiase (pH 3.9); 7, endocellulase, cellobiase (pH 3.8).

tion of sodium chloride solution (pI < 3.8). Our results confirmed that most of the enzymes examined occurred in multiple forms in the tested preparations (see the data in refs. 2–6). The C_1-activity in the preparation from *T. viride* was distributed in three protein fractions differing in their isoelectric points (≥ 8.0, 6.2 and < 3.8) (*cf.*, Fig. 1) and at least three forms of this enzyme were present in the sample from *T. reesei* (pI = 5.4–5.7, 5.0 and 3.9) (Fig. 2). The C_1-activity in the preparation from *P. ostreatus* was distributed in several fractions eluted close to neutral pH (with pI in the range of 5.6–7.2) (Fig. 3). Two distinct C_1-activities were observed in the preparation from *A. niger* (pI = 5.0 and 3.9) (Fig. 4).

The distribution of endocellulases and exocellulases in the materials under study was also characteristic of the individual organisms (Figs. 1–4). Three endocellulases (pI = 5.3, 4.3 and < 3.8) and one measurable exocellulase (pI ≥ 8.0) occurred in the sample from *T. viride*, at least three endocellulases (pI ≥ 8.0, 5.4–5.7 and 4.5) and three exocellulases (pI = 6.1, 5.0 and < 3.8) were present in the sample from *T. reesei* and 2–3 endocellulases (pI = 5.1–5.3 and < 3.8) and one exocellulase (pI ≥ 8.0) occurred in the preparation from *P. ostreatus*. With *A. niger*, one to two endocellulases (pI = 3.9 and < 3.8) and one exocellulase (pI ≥ 8.0) were detected. The cellobiase activity stemming from *T. viride* was eluted between pH 6.2 and 6.0 and after the injection of sodium chloride solution (pI < 3.8), that from *T. reesei* was found in the starting buffer (pI ≥ 8.0) and also after the injection of sodium chloride solution (pI < 3.8), that from *P. ostreatus* was eluted in the pH range 6.1–7.2 and that from *A. niger* at pH 3.9 and after injection of 2 *M* sodium chloride solution (pI < 3.8) (see Figs. 1–4).

The observed distributions of the examined enzyme activities in the samples analysed were similar to those obtained by traditional[2–5] and high-performance[6] ion-exchange chromatography. Nearly all of the main peaks observed here (Figs. 1–4) have their counterparts in the chromatograms obtained with the same materials on a Mono Q anion-exchange column at pH 8.0 using elution with a linear sodium chloride concentration gradient[6]. Some additional peaks and the detected activities described here might be attributed to the better resolving power of the chromatofocusing technique. In general, the number of distinct protein peaks obtained by chromatofocusing on a Mono P column was about 50% higher than that obtained by chromatography on a Mono Q column[6] and three to four times higher than that attained by chromatography on DEAE-Sephadex[2] or DEAE-Trisacryl[4] columns.

The isoelectric points of endocellulases and exocellulases from *T. reesei* obtained by chromatofocusing (Fig. 2) can be compared with those determined for these enzymes by isoelectric focusing[4]. The agreement between the data is very good. The isoelectric points for endocellulases were 5.7 and 4.0–4.6 by isoelectric focusing and 5.7 and 4.5 by chromatofocusing. The values for exocellulases were 5.9 and 3.9 by isoelectric focusing and 6.1, 5.0 and < 3.8 by chromatofocusing. Chromatofocusing is superior to isoelectric focusing for the exact determination of the isoelectric points of the individual components as it allows a reliable quantification of enzyme activities.

Chromatofocusing of the crude cellulolytic complexes stemming from the title organisms proved to be not only a convenient method for the analysis of these materials and for the determination of isoelectric points of the individual enzymes, but also a suitable semi-preparative method yielding several milligrams of purified cellulolytic components in a single run.

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