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# Purification and Characterization of Two Low Molecular Weight Endoglucanases Produced by *Penicillium occitanis* Mutant Pol 6

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## Abstract

Two endoglucanases (EGs), EG A and EG B, were purified to homogeneity from *Penicillium occitanis* mutant Pol 6 culture medium. The molecular weights of EG A and EG B were 31,000 and 28,000 kDa, respectively. The p*I* was about 3 for EG A and 7.5 for EG B. Optimal activity was obtained at pH 3.5 for both endoglucanases. Optimal temperature for enzyme activity was 60°C for EG A and 50°C for EG B. EG A was thermostable at 60°C and remained active after 1 h at 70°C. EGs hydrolyzed carboxymethylcellulose, phosphoric acid swollen cellulose, and β-glucan efficiently, whereas microcrystalline cellulose (Avicel) and laminarin were poorly hydrolyzed. Only EG B showed xylanase activity. Furthermore, these EGs were insensitive to the action of glucose and cellobiose but were inhibited by the divalent cations $Hg^{2+}$, $Co^{2+}$, and $Mn^{2+}$.

**Index Entries:** *Penicillium occitanis*; mutant Pol 6; cellulase; endoglucanases; purification.

## Introduction

Cellulolytic enzymes are involved in the hydrolysis of cellulosic materials. They have many applications in various industrial areas including

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conversion of cellulosic wastes into fuels, chemicals, and feeds *(1,2)*. Cellulases are also used in the textile, pulping, and detergent industries *(3–5)*.

Several microorganisms such as fungi, actinomycetes, mycoxobacteria, true bacteria, and plants produce cellulases. Fungal enzyme systems are composed of at least one β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21), two cellobiohydrolases (CBHs) (1,4-β-D-glucan CBH; EC 3.2.1.91), and several endoglucanases (EGs) (1,4-β-D-glucan glucanohydrolase; EC 3.2.1.4). Most of their encoding genes have been manipulated to produce new potent cellulasic systems.

Our research has focused on mutant Pol 6 isolated from a *Penicillium occitanis* strain, which secretes a cellulase complex rich in β-glucosidase activity *(6,7)*. Previous studies showed the existence of two β-glucosidases, two CBHs, and several EGs. Factors involved in the control of Pol 6 cellulase activity and cellulase production have been investigated *(8,9)*. Purification and characterization of two CBHs have also been achieved *(10)*.

Recently, our attention has been focused on small EGs. Because of their low molecular weight, these enzymes penetrate easily within the cellulose fibrils and then efficiently cleave the crystalline cellulose. Therefore, they act as a defibrillation agent of cellulose *(11)*, and as softeners in detergents *(12)*.

Several small EGs have been isolated from various microorganisms, such as *Trichoderma viride (13)*, *Irpex lacteus (14)*, *Fusarium lini (15)*, *Trichoderma koningii (16)*, *Trichoderma reesei (17)*, *Penicillium pinophilum (18)*, *Cellulomonas flavigena (19)*, *Humicola insolens (20)*, and *Fusarium oxysporium (21)*.

These studies showed that small EGs are only induced by crystalline cellulose *(22)* and act preferably inside the cellulosic chain (endo-type mechanism) *(14)*. Most of these EG enzymes were deficient of cellulose-binding domain *(23,24)* and were classified in glycosyl hydrolase family 12 (see Carbohydrate-Active enzymes server: http://afmb.cnrs-mrs.fr/CAZY/).

The present work was devoted to the identification and purification of small extracellular EGs of *P. occitanis* mutant Pol 6 using classic physicochemical techniques such as native electrophoresis, ion-exchange chromatography, chromatofocusing, and gel filtration. Biochemical properties of the purified enzymes were also investigated.

## Materials and Methods

### Culture and Growth

Cayla (Toulouse, France) supplied the *P. occitanis* mutant Pol 6. This microorganism was grown in Mandel's medium containing cellulose Avicel with the pH and temperature controlled at 4.8 and 28°C, respectively, in a 7-L fermentor (Chemap, Basel, Switzerland). After 7 d of fed-batch fermentation, the culture supernatant was separated from the mycelium by centrifuging at 8000*g* for 15 min and filtered through a 0.45-μm nitrocellulose membrane. The crude filtrate was used as the source of cellulases.

### *Extraction of Protein*

The protein extract was prepared from crude filtrate by $(NH_4)_2SO_4$ precipitation (50% saturation) at 4°C. After centrifuging at 8000*g* for 20 min, the precipitate was resuspended in 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 m*M* phenylmethylsulfonyl fluoride and 50 m*M* NaCl.

### *Protein Determination*

Protein content was estimated by Bradford's method *(25)*, using Bio-Rad protein reagent (Bio-Rad, France) with bovine serum albumin as protein standard.

### *Enzyme Assays*

Activities against carboxymethylcellulose (CMC) (1% [w/v]), phosphoric acid swollen cellulose (PASC) (1% [w/v]), microcrystalline cellulose (Avicel) (10 mg/mL), laminarin (1% [w/v]), β-glucan (1% [w/v]), and xylan (1% [w/v]) were determined as described by Mandels et al. *(26)*. Filter paper activity was determined as described by Montenecourt and Eveleigh *(27)*.

Released reducing sugar was estimated by the 3,5-dinitrosalicylic acid method *(28)*. The absorbancy was measured at 540 nm. One unit of EG activity was defined as the amount of enzyme required to liberate 1 µmol/min of reducing sugar expressed as glucose equivalents.

Activities against *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), *p*-nitrophenyl-β-cellobioside (*p*NPC), or *p*-nitrophenyl-β-lactoside (*p*NPL) were determined by the procedure of Wood and Bhat *(29)*. The initial concentration of substrates was 2 m*M*. The amount of *p*-nitrophenol released from the respective substrate was measured spectrophotometrically at 430 nm. One unit of enzyme was defined as the amount of the enzyme releasing 1 µmol/min of *p*-nitrophenol under the assay conditions.

### *Purification of Two EG Activities on Gel Filtration Chromatography*

Protein extract was loaded onto a column (4 × 100 cm) of Biogel P-100 (Bio-Rad) previously equilibrated in 20 m*M* Tris-HCl buffer (pH 8.0) containing 1 m*M* EDTA. Proteins were eluted with starting buffer and monitored at 280 nm. Fractions of 10 mL were collected at a flow rate of 20 mL/h. Two EG activities were eluted and isolated by pooling fractions 26–32 (Fig. 1A, peak I) and 60–70 (Fig. 1A, peak II).

#### Purification of EG A

Pooled fractions 26–32 were dialyzed overnight against 25 m*M* histidine-HCl (pH 7.0) start buffer and applied to the chromatofocusing column. The column (1.6 × 25 cm) was previously packed with polybuffer exchanger PBE 97TM gel (Pharmacia, France) and equilibrated in the start buffer. Fractions of 2 mL were collected at a flow rate of 10 mL/h. After extensive washing with start buffer, two EGs were eluted using succes-

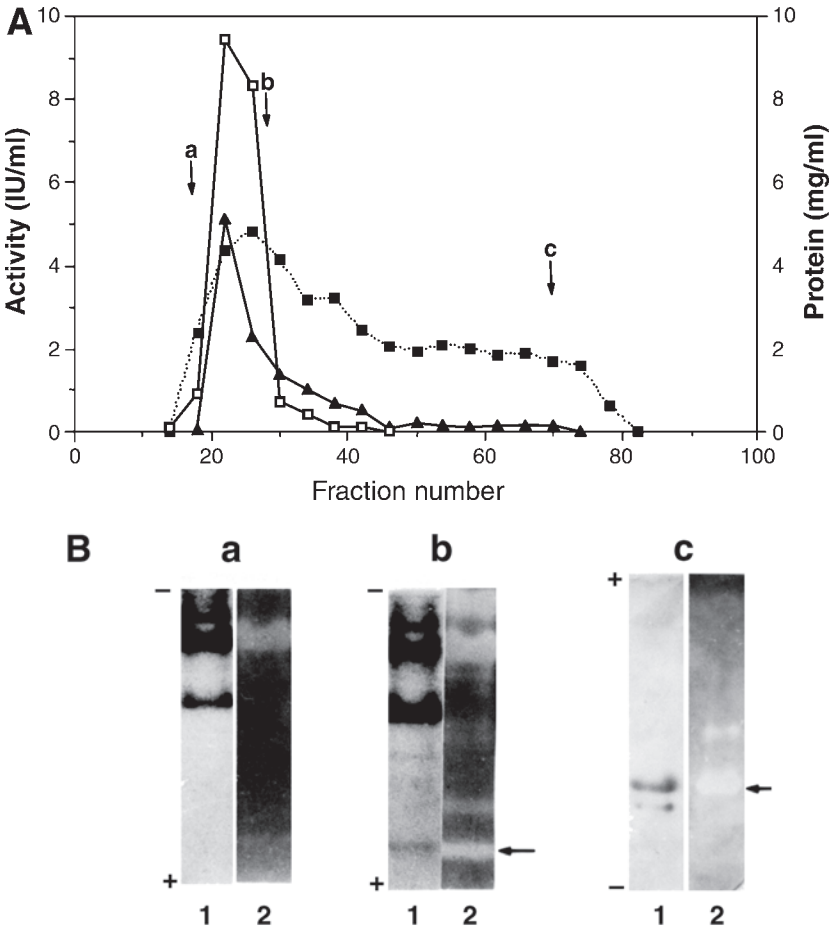


Fig. 1. **(A)** Gel filtration chromatography of enzymes extracted from *P. occitanis* mutant Pol 6 grown on cellulose Avicel. After ammonium sulfate precipitation, proteins were purified on a Biogel P-100 column (4 × 100 cm) previously equilibrated with 20 m*M* Tris-HCl buffer (pH 8.0) containing 1 m*M* EDTA. Fractions of 10 mL were collected with this buffer at a flow rate of 20 mL/h. (▲) Protein, (□) β-glucosidase activity, (■) EG activity. **(B)** Lane 1, native polyacrylamide gel electrophoresis (PAGE) stained with Coomassie brilliant blue G-250; lane 2, carboxymethylcellulase (CMCase) activity overlaps, as reported in Materials and Methods. Anodic PAGE resolved fractions 15 (a) and 28 (b) whereas cathodic PAGE resolved fraction 70 (c). Arrows show EG A and EG B in (b) and (c), respectively.

sively polybuffer 74 (pH 4.0) and citrate buffer (pH 3.0). The more retained enzyme was referred to as EG A. Fractions containing EG A activity were pooled and enzyme purification was achieved on preparative anodic PAGE.

After electrophoresis, the gel slice was stained for CMCase activity as described under Detection of EG Activity in Gel. The remaining part of unstained gel was further used to elute EG A. The gel slice was homogenized in 20 m*M* Tris-HCl buffer (pH 7.0) containing 50 m*M* NaCl, left

overnight at 4°C, filtered on glass cotton, and centrifuged at 8000*g*, and the supernatant was recovered.

### Purification of EG B

Following gel filtration with Biogel P-100, active fractions 60–70 were pooled, dialyzed against 20 m*M* sodium acetate buffer (pH 5.0), and fractionated by cation-exchange chromatography on a column (1 × 2 cm) of Bio-Rex 70 Resin (Bio-Rad). The column, previously equilibrated in 20 m*M* Sodium acetate buffer (pH 5.0), was extensively washed with the same buffer to remove unbound material. One active peak, referred to as EG B, was eluted with a linear gradient of NaCl (0–500 m*M*) in 20 m*M* sodium acetate buffer (pH 5.0).

Fractions of 1 mL were collected with a flow rate of 10 mL/h. EG B activity was eluted at 350 m*M* NaCl. Active fractions were pooled and dialyzed against 20 m*M* sodium acetate buffer (pH 5.0) containing 50 m*M* NaCl. Finally, the enzyme was purified by preparative cathodic PAGE. EG B activity was visualized on a gel slice, whereas the remaining part was used to recover EG B activity throughout the gel.

Both EG A and EG B were checked for CMCase activity and then stored at 20°C.

## *Preparative Electrophoresis Under Nondenaturing Conditions*

### Anodic PAGE

Anodic PAGE *(30)* was performed on a system containing 5% stacking gel and 10% separating gel. Electrophoresis was performed at 120 V for 3 h at 4°C in Tris-HCl (89 m*M*), borate (89 m*M*), and EDTA (2 m*M*) buffer (pH 8.3). Protein electrophoresis was carried out toward the anode until the Bromophenol blue dye migrated at the bottom of the gel.

### Cathodic PAGE

Cathodic PAGE *(31)* was performed using a 5% stacking gel and 10% separating gel in 100 m*M* sodium acetate-KOH buffer (pH 5.0). Electrophoresis was performed at 120 V for 3 h at 4°C in 40 m*M* glycine-HCl buffer (pH 4.0). In this case, the polarity of the leads was reversed in the gel electrophoresis apparatus, so protein electrophoresis was carried out toward the cathode and only neutral and basic proteins were separated. The migration was run until the Methyl green dye reached the bottom of the gel.

## *Detection of EG Activity in Gel*

Congo red staining visualized proteins associated with CMCase activity *(32)*. Following native electrophoresis, a slab gel was applied on top of an overlay agar sheet (5 mm thick) and incubated at 37°C for 10 min. The overlay agar sheet was composed of 2% (w/v) agar, 1% (w/v) CMC, and 3% (w/v) NaCl in 100 m*M* sodium acetate buffer (pH 4.0). The sheet was flooded with Congo red (1 mg/mL) for 15 min, rinsed off with water, and

then washed with 1 *M* NaCl solution at room temperature. Hydrolysis bands of CMC appeared as clear areas on the red background of the sheet.

### *Sodium Dodecyl Sulfate PAGE*

Denaturing electrophoresis *(33)* was performed on a 10% polyacrylamide gel with sodium dodecyl sulfate (SDS) (0.1% [w/v]) using a Mini-Protein II cell (Bio-Rad). Low molecular weight standards were used for calibration. Proteins were subsequently stained with Coomassie brilliant blue G-250.

### *High-Performance Liquid Chromatography Analysis of PASC Hydrolysis Products*

Purified enzyme (50 µg) and PASC (1% [w/v]) were incubated in 50 m*M* sodium acetate buffer (pH 4.0) for 2 h at 50°C. The reaction was stopped by heating the sample at 100°C for 2 min. Then, the reaction mixture was centrifuged at 10,000 rpm, and the proteins in the supernatant were removed with a SepPack cartridge C18. The resulting filtrate was lyophilized and dissolved in 50 mL of ultrapure $H_2O$. High-performance liquid chromatography (HPLC) analysis of the samples was performed using an HPX-42A carbohydrate column (300 × 7.8 mm; Bio-Rad). Elution was carried out using ultrapure $H_2O$ at 85°C; the flow rate was 0.6 mL/min. Sugar products were detected with a differential refractometer (Waters R401).

## Results and Discussion

### *Purification of EGs*

Endo-β-1,4-glucanases were characterized according to their activities toward CMC and the total cellulase toward PASC. The increase in the ratio of CMCase to PASC activity was used as an index of purification. The protein extract obtained from culture medium of *P. occitanis* mutant Pol 6 was loaded onto a Biogel P-100 column. Two EG activities were eluted and isolated by pooling fractions 26–32 (Fig. 1A, peak I) and 60–70 (Fig. 1A, peak II). Congo red staining following native electrophoresis visualized CMCase activities in each of the peak fractions. Fractions 26–32, which shared a little EG activity (Fig. 1B), were picked out for further purification. This EG was named EG A. Proteins in these collected fractions were dialyzed overnight against starting buffer (25 m*M* histidine-HCl, pH 7.0) and applied to a chromatofocusing column. Final purification of EG A was achieved by the chromatofocusing column (Fig. 2A) followed by preparative anodic PAGE (Fig. 2B). EG A was strongly bound on the chromatofocusing column, and its elution was carried out only by 25 m*M* sodium citrate buffer (pH 3.0).

The second EG (EG B) was eluted on Biogel P-100 in a broad peak with lower activity (Fig. 1B). This peak eluted from fraction 60 to 70 and was well separated from the other cellulase components. EG B was finally purified

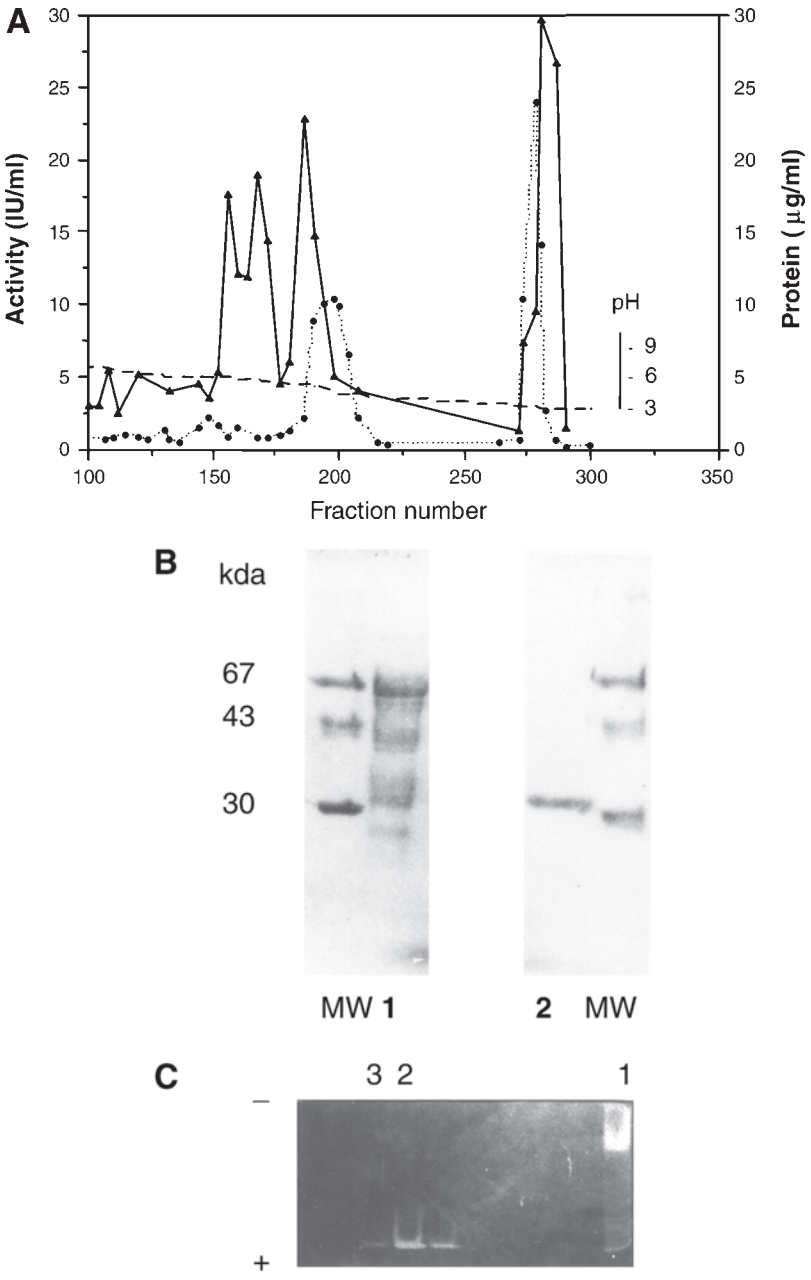


Fig. 2. **(A)** Chromatofocusing of pooled fractions (27–29) from Biogel P-100 chromatography containing EG A activity. Pooled proteins dialyzed against 25 m*M* histidine-HCl buffer (pH 7.0) were applied to a polybuffer exchanger PBE 94TM chromatofocusing column (1.6 × 25 cm). The bound EG A activity washed with polybuffer 74 (pH 4.0) following start buffer (25 m*M* histidine-HCl buffer, pH 7.0) was eluted with 25 m*M* sodium citrate buffer (pH 3.0). Fractions of 2 mL were collected at a flow rate 10 mL/h. (▲) Protein; (—●—) CMCase; (– – –) pH. **(B)** SDS-PAGE of pooled fractions (27–29) from Biogel P-100 chromatography (lane 1) and purified EG A in fraction 280 from chromatofocusing (lane 2). MW, molecular weight. Proteins were stained with Coomassie brilliant blue. **(C)** Electrophoretic characterization of EG A activity toward CMC following anodic PAGE. Pooled fractions (27–29) from a Biogel P-100 column (lane 1), fraction 280 (lane 2), and fraction 275 (lane 3) were collected from a PBE 94TM chromatofocusing column, respectively.

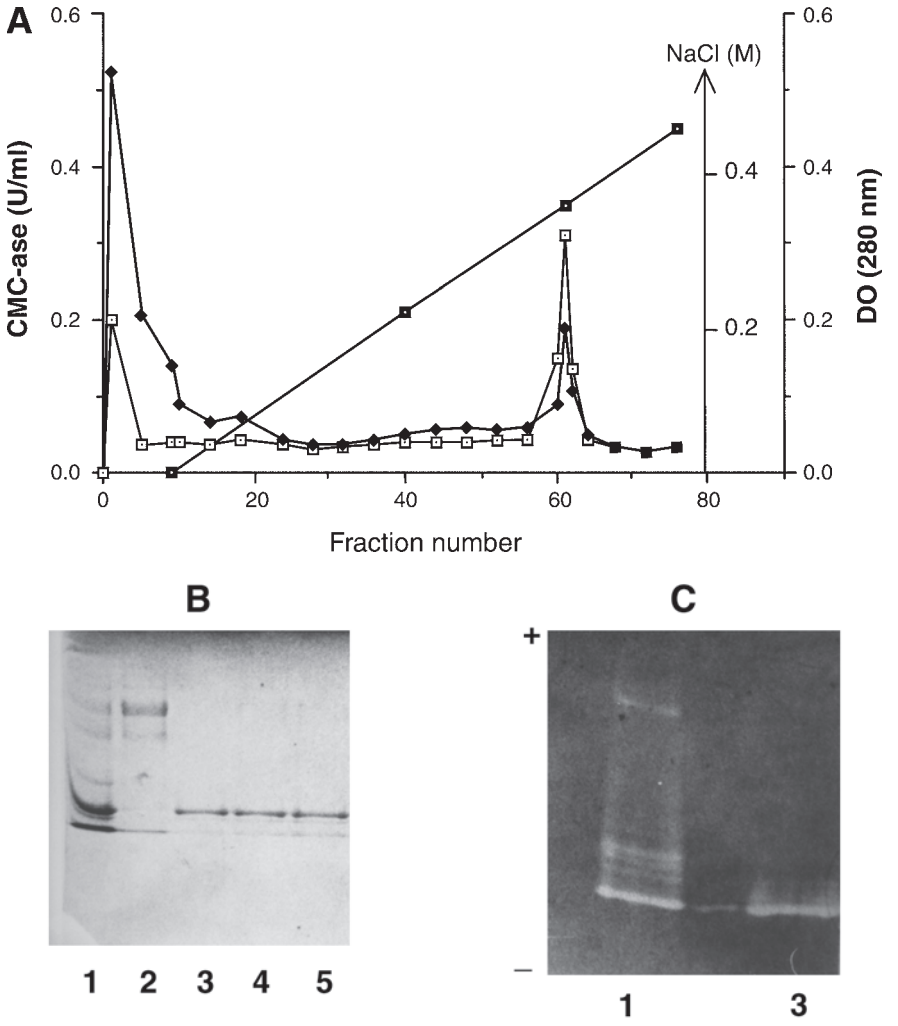


Fig. 3. **(A)** Purification of EG B using cation-exchange chromatography. Pooled fractions from Biogel P-100 (fractions 60–70) were applied to a BioRex 70 column (1 × 2 cm) previously equilibrated with 20 m*M* sodium acetate buffer (pH 5.0). Elution was carried out with a linear gradient of NaCl (0–500 m*M*) in the same buffer. Fractions of 1 mL were collected at a flow rate of 10 mL/h. (■) $OD_{280nm}$; (□) CMCase activity. **(B)** SDS-PAGE of proteins. **(C)** Electrophoretic characterization of EG B activity following cathodic native electrophoresis. Pooled fractions from Biogel P-100 (60–70) (lane 1); flow-through of BioRex 70 column (lane 2); and purified EG B present in fractions 59, 60, and 61, respectively, collected from BioRex 70 column (lanes 3–5). For characterization of EG B activity, only the endoglucanase in fraction 59 (lane 3) was analyzed because the three fractions (59 [lane 3], 60 [lane 4], and 61 [lane 5]) gave the same degree of purity.

by a Bio-Rex 70 resin column (Fig. 3), followed by preparative cathodic PAGE. Some difficulties in purifying this EG by means of gel filtration chromatography were encountered. EG B was particularly delayed not only on Biogel P-100 chromatography support but also on Sephadex.

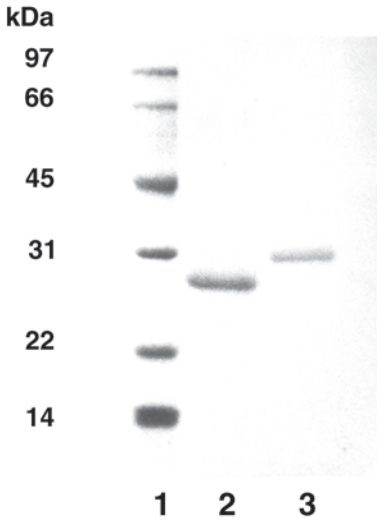


Fig. 4. SDS-PAGE of purified EG A and EG B: lane 1, molecular weight marker; lane 2, EG B; lane 3, EG A.

Similar problems have also been encountered during purification of low molecular weight EGs from *T. koningii (16)*, *F. oxysporum (21)*, *P. pinophilum (18)*, and *Cellulomonas fermentans (34)*. It has been suggested that their interactions with gel filtration matrix cause the low mobility of these enzymes.

### *Enzyme Purity*

Analytical PAGE followed by staining activity against CMC substrate was used to examine the purity of the small EGs. The patterns displayed a single protein band for each enzyme. The purity was also monitored by SDS-PAGE, and a single protein band was observed, indicating that both EGs were homogeneous proteins (Fig. 4).

### *Molecular Weight and p*I

The molecular weights of purified EG A and EG B, determined by SDS-PAGE, were about 31 and 28 kDa, respectively (Fig. 4). The p*I* values of these enzymes were estimated at about 3 for EG A and 7.5 for EG B by chromatofocusing.

### *Effect of pH on Enzyme Activity and Stability*

The optimum pH was determined by measuring the activity against CMC substrate at 50°C for 1 h over a pH range of 2.0–9.0 using the following buffers: 50 m*M* sodium citrate buffer for pH 2.0–6.0 and Tris-HCl buffer for pH 7.0–9.0. Optimal activity occurred at pH 3.5 for both enzymes (EG A and EG B).

Enzyme stability was also studied at various pH values by incubating an aliquot of each purified protein in the absence of the substrate at 4°C for

Table 1
Specificity of EG A and EG B Activities Against Various Substrates[a]

| Substrate specificity | Specific activity (μmol/[min·mg]) EG A | EG B |
|---|---|---|
| CMC | 4.67 | 3.33 |
| PASC | 3.06 | 23.89 |
| Barley β-glucan | 0.15 | 0.48 |
| Xylan | Not detected | 3.68 |
| Filter paper | 0.031 | 0.062 |
| Microcrystalline cellulose (Avicel) | 0.018 | 0.032 |
| Laminarin | <0.01 | <0.01 |

[a]Assays were performed in 50 m*M* sodium acetate buffer (pH 4.0) under optimal conditions. The results are expressed in micromoles of glucose equivalents released per minute per milligram of protein. Xylanase activity is expressed in micromoles of xylose equivalents released per minute per milligram of protein.

24 h; the remaining activity was then measured under optimal assay conditions. Both enzymes were very stable from pH 2.0 to 9.0.

### *Effect of Temperature on Enzyme Activity and Stability*

The optimum temperature was determined for both EGs. Each enzyme was incubated in 50 m*M* sodium acetate buffer (pH 4.0) in the presence of CMC substrate at various temperatures ranging from 20 to 80°C for 1 h. Optimal activity was obtained at 60°C for EG A and 50°C for EG B. Moreover, EG A still remained active at 80°C and was more active than EG B at higher temperature.

The thermal stability of both EGs was tested in 50 m*M* sodium acetate buffer (pH 4.0) after incubating without substrate for 1 h at 20–80°C. Residual activity was measured under optimal assay conditions. EG B lost 20% of its activity at 60°C and became completely inactive at 70°C. By contrast, the EG A showed an extensive thermal stability, losing only 30% of its activity at 70°C and still remaining active at 80°C. The thermal stability of EG A was comparable with that of some cellulases of thermophylic species *(35,36)*. These results render EG A suitable in some industrial processes occurring at low pH and high temperature.

### *Substrate Specificity*

Investigation of the specificity of EG A and EG B on hydrolysis of various cellulosic or hemicellulosic substrates was performed as reported in Table 1. Purified EG A was active on CMC, PASC, and barley β-glucan. It displayed negligible activity toward microcrystalline cellulose (Avicel), filter paper, laminarin, and xylan. EG B strongly hydrolyzed the PASC, but it did not hydrolyze much of the CMC and barley β-glucan, and it showed significant xylanase activity, which was not detected with EG A. This multifunctional property was also mentioned for other EGs such as that of

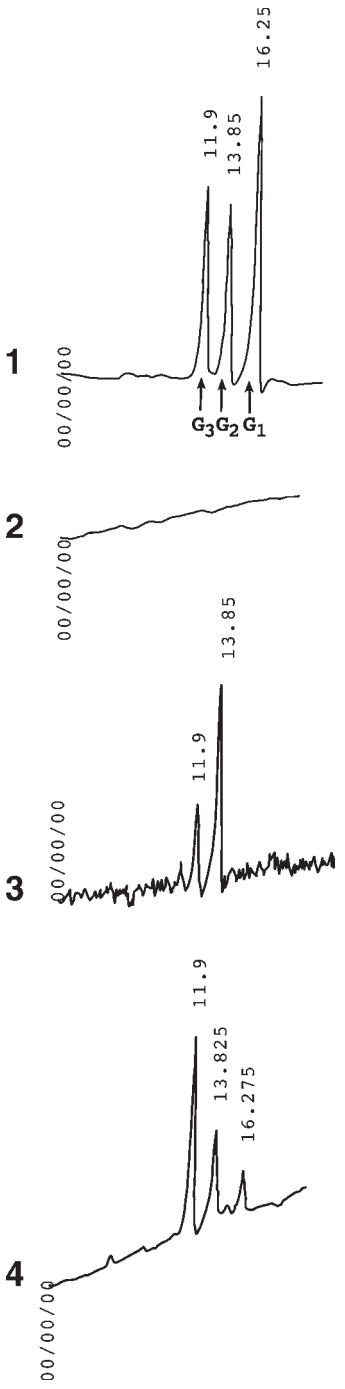


Fig. 5. HPLC analysis of PASC hydrolysis by EG A and EG B. The substrate (1%) in 20 m*M* sodium acetate (pH 4.8) was incubated at 60°C for 2 h, in the absence of enzyme (2) or in the presence of EG A (3) or EG B (4); and in a mixture of glucose ($G_1$), cellobiose ($G_2$), and cellotriose ($G_3$) (1). All the samples were treated under the same conditions and analyzed by HPLC as described in Materials and Methods.

Table 2
Effect of Divalent Cation at 2 m*M* on Activity of EG A and EG B Toward CMC

| | Residual activity (%) | |
|---|---|---|
| Divalent cation | EG A | EG B |
| $Hg^{2+}$ | 52 | 39 |
| $Co^{2+}$ | 60 | 47 |
| $Mn^{2+}$ | 40 | 51 |

*Fusarium lini (15)*, EG E1 of *Irpex lacteus (14)*, EG A and B of *Ruminococcus flavefaciens* FD-1 *(37)*, and EG I of *T. reesei (38)*. The two enzymes did not hydrolyze the synthetic substrates *p*NPG, *p*NPC, and *p*NPL.

The effect of end products of cellulose hydrolysis such as glucose and cellobiose on EG activities was studied. No inhibition was noticed at 100 m*M* glucose or cellobiose.

### *HPLC Analysis of PASC Hydrolysis Products by EGs*

EG A and EG B were incubated separately in 50 m*M* sodium acetate buffer (pH 4.0) containing PASC at 50°C for 2 h. The hydrolysis products were then analyzed by HPLC (Fig. 5). EG A produced cellotriose, cellobiose (22 and 78%, respectively), and traces of glucose. Similar results have been obtained with other EGs such as $E_{3a}$ isolated from *T. koningii (39)* and F II from *Aspergillus aculeatus (40)*. By contrast, EG B generated 65% cellotriose, 25% cellobiose, and 10% glucose. This enzyme acted the same as EG III of *T. reesei (17)* and *H. insolens (20)* and that of *F. oxysporum (21)*.

### *Action of Divalent Ions*

The effect of several divalent cations on EG A and EG B activity was also determined. $Hg^{2+}$, $Mn^{2+}$, and $Co^{2+}$ inhibited both enzymes at 2 m*M* (Table 2). $Mg^{2+}$, $Ca^{2+}$, $Fe^{2+}$, and $Zn^{2+}$ had no effect on CMCase activity of these enzymes at 2 m*M*.

## Conclusion

Two EGs, EG A and EG B, were purified to homogeneity. Their basic characteristics, such as molecular weight, p*I*, optimal pH and temperature activity, stability, and specificity, were established. The results showed that EG A is a thermostable enzyme that acts differently on cellulose than EG B. The features of the latter are similar to those of family 12 EGs *(23,24)*.

It would be important to classify these enzymes by determining the sequence of the N-terminal amino acids and/or the proteolysis peptides. These sequences will be further used for preparing the corresponding oligonucleotides and then cloning their genes. This work would provide interesting information concerning their structure and expression.

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