Journal of Chromatography, 521 (1990) 301–310
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1985

Separation of the cellulolytic and xylanolytic enzymes of *Clostridium stercorarium*

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ABSTRACT

Exoenzymes of the thermophilic, anaerobic bacterium *Clostridium stercorarium* involved in cellulose and arabinoxylan degradation were resolved by a combination of fast protein liquid chromatographic (FPLC) chromatofocusing and FPLC anion-exchange chromatography. The purified enzymes include an *endo*-β-1,4-glucanase (Avicelase I), an *exo*-β-1,4-glucanase (Avicelase II), a β-D-glucosidase, a β-D-xylosidase, an α-L-arabinofuranosidase and multiple xylanases. Two celloxylanases capable of hydrolyzing both cellodextrins and xylans were also purified.

INTRODUCTION

Cellulose and xylans are the major polysaccharide components of plant biomass and constitute the most abundant renewable resources for the production of fermentable sugars by enzymatic saccharification. Cellulose is a linear polymer of D-glucose residues linked by β-1,4-glucosidic bonds. Its enzymatic hydrolysis involves at least three types of enzymes: *endo*-β-1,4-glucanases (1,4,-β-D-glucanohydrolase; E.C. 3.2.1.4), *exo*-β-1,4-glucanases (1,4,-β-D-glucan cellobiohydrolase; E.C. 3.2.1.91) and β-D-glucosidase (β-D-glucoside glucohydrolase; E.C. 3.2.1.21) [1]. A synergistic interaction of these enzymes is necessary for the complete hydrolysis of crystalline cellulose.

Xylans are branched heteroglycans with a backbone of β-1,4-linked D-xylopyranosyl residues. Branches consist commonly of α-1,3-linked L-arabinofuranose and α-1,2-linked D-glucuronic acid residues [2]. The frequency and composition of branches in xylans varies depending on the source. Thus, xylans from grasses and softwoods are generally arabinoxylans. Enzymatic hydrolysis of the xylan backbone involves *endo*-β-1,4-xylanases (1,4-β-D-xylan xylanohydrolase; E.C. 3.2.1.8) and β-D-xylosidases (1,4-β-D-xylan xylohydrolase; E.C. 3.2.1.37) [3]. With arabinoxylans, removal of L-arabinosyl side-groups is catalysed by α-L-arabinofuranosidases (E.C. 3.2.1.55).

Thermophilic bacteria have received considerable attention as sources of highly active and thermostable cellulolytic and xylanolytic enzymes. The cellulase complex of *Clostridium thermocellum* has been studied most extensively. This complex contains 14–18 different polypeptides forming a stable extracellular structure termed cellulo-

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some [4]. The thermophilic anaerobe *Clostridium stercorarium* differs from *C. thermocellum* by being able to ferment a wide variety of carbohydrates in addition to cellulose [5]. Growth on either cellulose or xylan was found to induce the production of both cellulotytic and xylanolytic enzymes [6]. The cellulase of *C. stercorarium* is of low complexity and consists of single *endo*-β-1,4-glucanase, *exo*-β-1,4-glucanase and β-D-glucosidase activities [7–11]. On the other hand three distinct endoxylanases [6] and two β-D-cellobiosidases of unknown function [9] have been purified from *C. stercorarium* culture supernatants.

In this paper we report the complete resolution of the various *C. stercorarium* exoenzymes involved in cellulose and arabinoxylan hydrolysis employing a combination of fast protein liquid chromatographic (FPLC) chromatofocusing and FPLC anion-exchange chromatography. It is shown that the purified β-D-cellosidases possess xylanase activity and might be considered as celloxylanases. In addition to the previously identified *C. stercorarium* enzymes, the isolation of a β-D-xylosidase and an α-L-arabinofuranosidase is described.

EXPERIMENTAL

Enzyme preparation

C. stercorarium NCIB 11745 was grown under anaerobic conditions at 60°C in prereduced GS-2 medium [12] with 1% xylan as carbon source. Culture supernatant (8.3 l) was concentrated by tangential flow ultrafiltration in a Minitan system (Millipore) using polysulphone filter sheets with a nominal molecular mass limit 10000. The retentate was desalted by five cycles of ultrafiltration following a two-fold dilution with 20 m*M* Tris–HCl (pH 8.0). Protein concentration was determined by the method of Sedmark and Grossberg [13].

Chromatography and chromatofocusing

The chromatographic system consisted of a Pharmacia FPLC apparatus. Anion-exchange chromatography on Q Sepharose was carried out in a Pharmacia HR 16/10 column (10 × 1.6 cm I.D.) packed with Q Sepharose FF. Pooled fractions were concentrated and desalted by tangential flow ultrafiltration. FPLC chromatofocusing was performed on a Pharmacia Mono P HR 5/20 column (20 × 0.5 cm I.D.) with a pH gradient from 6.0 to 3.0 formed with Polybuffer 74. Pooled fractions were concentrated by Centriprep-30 ultrafiltration (Amicon). Change of buffer was effected by three cycles of dilution and ultrafiltration. Anion-exchange FPLC was carried out on a Pharmacia Mono Q HR 5/5 column (5 × 0.5 cm I.D.) or on an HR 10/10 column (10 × 1.0 cm I.D.). Gel filtration FPLC was performed on a Pharmacia Superose 12 HR 10/30 column as described previously [10].

Enzyme assays

Avicelase and carboxymethylcellulase (CMCase) activities were determined as described previously [9]. One unit of activity corresponds to the formation of 1 μmol of glucose equivalent per minute. Xylanase was assayed by incubation for 60 min at 60 °C in a 1% (w/v) solution of oat spelts xylan in 0.1 *M* succinate buffer (pH 6.0). Reducing sugars released from the substrate were determined with 3,5-dinitrosalicylic acid reagent [14]. One enzyme unit corresponds to the release of 1 μmol of xylose equivalent per minute.

β-D-Glucosidase, β-D-cellobiosidase, β-D-xylosidase and α-L-arabinofuranosidase activities were determined by measuring the release of *p*-nitrophenol from the corresponding *p*-nitrophenylglycoside. Assay mixtures (1 ml) containing 2 m*M* substrate in 40 m*M* citrate–phosphate buffer (pH 6.0) were incubated for 30 min at 60°C. Reactions were stopped by addition of 2 volumes of 1 *M* sodium carbonate solution. The absorbance of the liberated *p*-nitrophenol was measured at 395 nm. One unit of activity is defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per minute.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate–Polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% polyacrylamide gels in the presence of 0.1% SDS according to Laemmli [15] using a Pharmacia Midget electrophoresis unit. Protein bands were detected by staining with Coomassie Brilliant Blue R-250. Xylanase activity was detected by *in situ* staining of polyacrylamide gels containing 0.1% xylan as described previously [16]. Glycosidases were identified by fluorescence on incubating the gels with the corresponding 4-methylumbelliferyl glycoside [16].

Chemicals

Avicel TG 104 and CMC (extent of carboxymethylation 0.7%) and chemicals for SDS-PAGE were obtained from Serva (Heidelberg, F.R.G.) and xylan (oat spelts), *p*-nitrophenylglycosides, 4-methylumbelliferyl glycosides and molecular mass markers for SDS-PAGE from Sigma (St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

Fractionation by Q Sepharose chromatography

A prerequisite for the characterization of the *C. stercorarium* exoenzyme was the concentration of the culture supernatant with minimum loss of enzyme activities. This was achieved by ultrafiltration employing a Minitan tangential flow system equipped with polysulphone filters. The concentrated enzyme preparation was then fractionated on a Q Sepharose column connected to a Pharmacia FPLC system. The protein fractions were assayed for various enzymes involved in cellulose and hemicellulose degradation.

Fig. 1 shows the elution profile of the enzymes related to cellulose degradation. With microcrystalline cellulose (Avicel) as substrate, two peaks of activity were observed. The first peak eluting at 0.27 *M* NaCl also exhibited CMCase activity and corresponds to Avicelase I [9,11], which has been previously characterized as an *endo*-β-1,4-glucanase [7]. The smaller Avicelase peak eluting at 0.33 *M* NaCl has been designated Avicelase II [9] and is presumably identical with the *exo*-β-1,4-glucanase activity detected by Creuzet *et al.* [8].

With the substrate *p*-nitrophenyl-β-D-cellobioside three peaks of activity were detected (Fig. 1B). A distinct peak eluting at 0.16 *M* NaCl was also able to hydrolyse *p*-nitrophenyl-β-D-glucoside and has previously been characterized as β-D-glucosidase [10]. At higher salt concentration two broad and overlapping peaks of β-cellobiosidase activity were observed, which were devoid of β-glucosidase activity. The corresponding enzymes have been designated β-cellobiosidase I and β-cellobiosidase II, respectively [9].

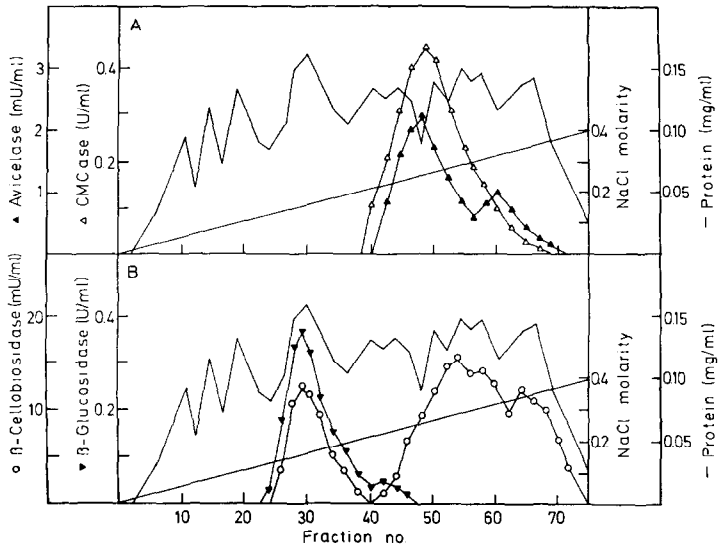

Fig. 1. Fractionation of *C. stercorarium* cellulolytic enzymes by Q Sepharose chromatography. Concentrated culture supernatant (90 mg of protein) was applied to a Q Sepharose Fast Flow HR 16/10 column equilibrated with 20 m*M* Tris–HCl (pH 8.0). Elution was performed with a 1000-ml linear gradient (0.0–0.6 *M* NaCl) in equilibration buffer at a flow-rate of 4 ml/min. Fractions (10 ml) were collected and assayed for (A) Avicelase and CMCase and (B) β-D-glucosidase and β-D-cellobiosidase.

The separation of enzymes involved in the hydrolysis of arabinoxylans is shown in Fig. 2. A major peak of xylanase activity was found to elute around 0.1 *M* NaCl (Fig. 2A). This peak corresponds to the xylanase activity described by Creuzet and Frixon

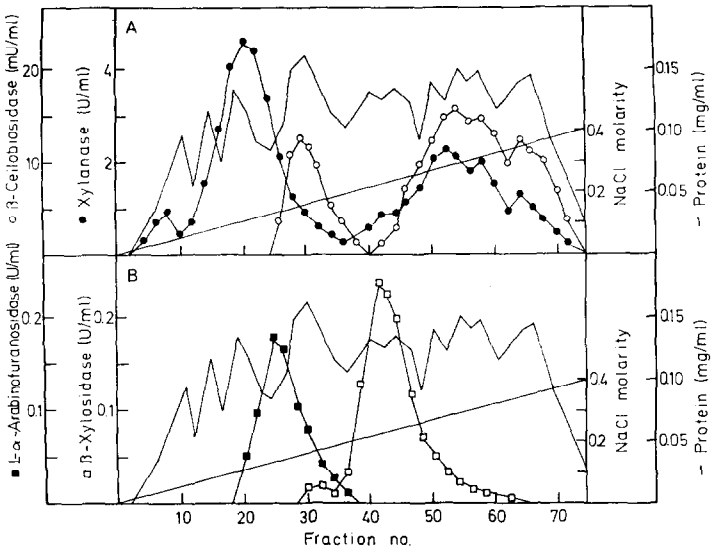

Fig. 2. Fractionation of *C. stercorarium* arabinoxylanolytic enzymes by Q Sepharose chromatography. Fractions from the Q Sepharose column described in Fig. 1 were assayed for (A) xylanase and β-D-cellobiosidase, and (B) β-D-xylosidase and α-L-arabinofuranosidase.

[7]. In contrast to the previous work, two additional xylanase peaks were detected, which coeluted with β-cellobiosidase I and II.

With *p*-nitrophenyl-α-L- arabinofuranoside as substrate one peak of activity was observed (Fig. 2B). The elution profile of the α-L-arabinofuranosidase overlapped with that of xylanase and β-D-glucosidase. Similarly, a single peak of β-D-xylosidase activity hydrolysing β-nitrophenyl-β-D-xyloside was detected. The β-D-xylosidase peak partially overlapped with that of Avicelase I (Fig. 1A).

Separation by FPLC chromatofocusing

A further separation of the individual enzymes was achieved by chromatofocusing. For that purpose, the fractions from the Q Sepharose column were combined in two pools. Pool I (fraction 5–36) contained the major xylanase activity in addition to the β-D-glucosidase and α-L-arabinofuranosidase activities. Pool II (fraction 38–70) included the β-D-xylosidase, Avicelase and β-D-cellobiosidase activities. Chromatofocusing was performed on a Pharmacia Mono P column with a pH gradient from 6.0 to 3.0.

The α-L-arabinofuranosidase eluted at pH 5.2 and could be completely separated from β-D-glucosidase and xylanase activities eluting at pH 4.8 and 4.2, respectively (Fig. 3). During gel filtration on Superose 12 this enzyme migrated as a single peak with an apparent molecular mass of 180 000 (data not shown). Analyis of the purified enzyme by SDS-PAGE revealed two proteins bands with molecular masses of 45 000 and 55 000, indicating an oligomeric structure of the enzyme.

The β-D-glucosidase was freed from residual xylanase activity and purified to homogeneity by a combination of Mono Q FPLC and gel filtration [10]. It is a monomeric protein with a molecular mass of 85 000 as determined by SDS-PAGE. Similarly, the major peak of xylanase activity was further resolved by Mono Q chromatography as described below.

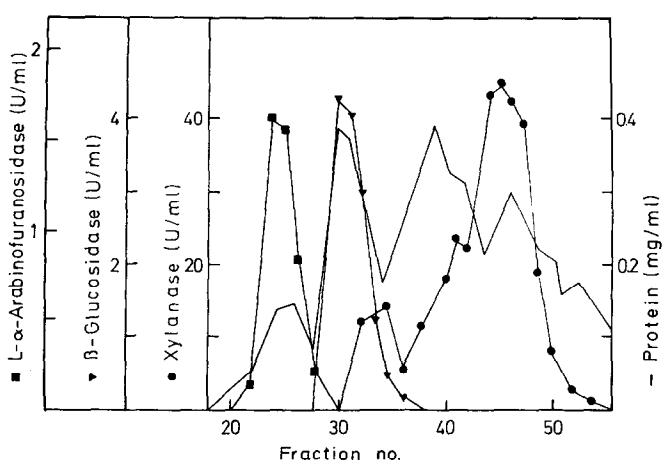

Fig. 3. Separation of α-L-arabinofuranosidase, β-D-glucosidase and xylanase by chromatofocusing. Fractions 5–36 from the Q Sepharose column (12 mg of protein) were applied to a Mono P HR 5/20 column equilibrated with 20 m*M* histidine–HCl (pH 6.0). Elution was performed with 10 ml of starting buffer followed by 45 ml of 1:10 diluted Polybuffer 74 (pH 3.0) at a flow-rate of 1 ml/min. Fractions (0.5 ml) were assayed for protein and enzyme activities.

Chromatofocusing clearly resolved the β-D-xylosidase (elution pH 5.0) from other cellulolytic and xylanolytic enzyme activites present in pool II (Fig. 4). SDS-PAGE revealed a single protein band with a molecular mass of 85000. The identity of this protein band with β-D-xylosidase was established by activity staining with 4-methylumbelliferyl-β-D-xyloside. The β-cellobiosidase activity (elution pH 4.7) again copurified with xylanase activity (Fig. 4A), indicating that both activities are properties of the same enzyme(s). This was shown by further purification as described below.

It should be noted that chromatofocusing did not result in a complete separation of the β-D-cellobiosidase and xylanase activities from Avicelase I, which eluted around pH 4.5 (Fig. 4B). However, as proteins elute from the Mono P column at a pH close to their p*I*, the Avicelase showed a strong tendency to precipitate during overnight storage at 4°C and could be removed by centrifugation. The combined Avicelase fractions could be resolved into Avicelase I and II by subsequent gel filtration on a Superose 12 column [11]. They represent monomeric proteins with molecular masses of 100000 (Avicelase I) and 87000 (Avicelase II).

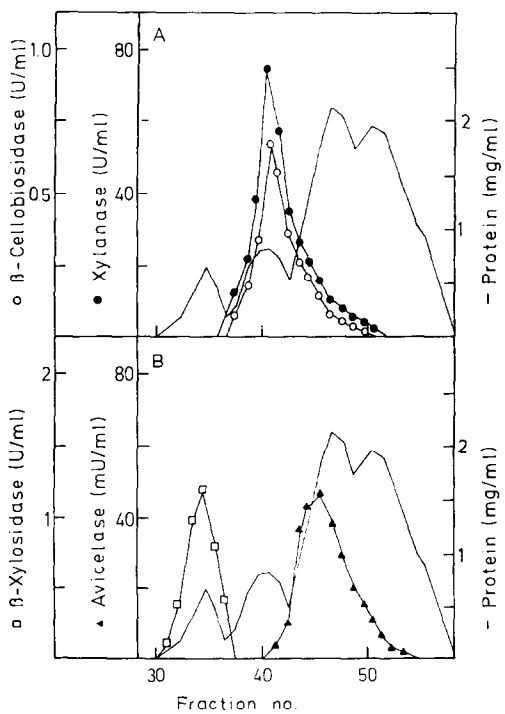

Fig. 4. Separation of β-D-xylosidase, β-D-cellobiosidase and Avicelase by chromatofocusing. Fractions 38–70 from the Q Sepharose column (19 mg of protein) were applied to a Mono P HR 5/20 column equilibrated with 20 m*M* histidine–HCl (pH 6.0). Elution was performed as described in Fig. 3. Fractions (0.5 ml) were assayed for (A) xylanase and β-D-cellobiosidase activity and (B) β-D-xylosidase and Avicelase activity.

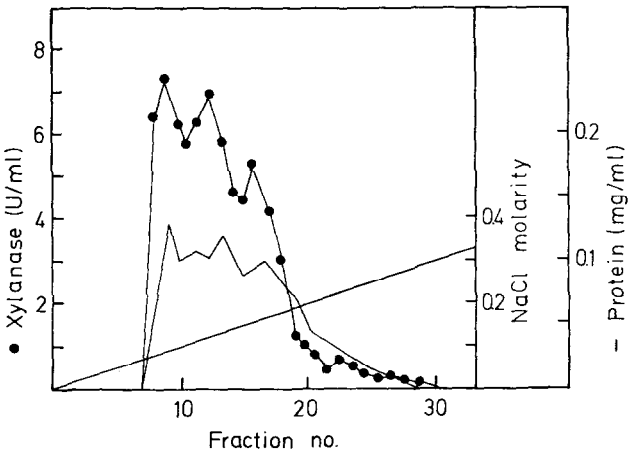

Fig. 5. Resolution of xylanases by Mono Q anion exchange chromatography. Fractions 38–50 from the Mono P column (Fig. 3) were applied to a Mono Q HR 5/5 column equilibrated with 20 m*M* Tris–HCl (pH 8.0). Proteins were eluted with a 20-ml linear gradient (0.0–0.40 *M* NaCl) in equilibration buffer at a flow-rate of 1 ml/min. Fractions (0.5 ml) were assayed for xylanase activity.

Resolution of xylanases and celloxylanases by Mono Q FPLC

The pooled xylanase fractions from the gradient shown in Fig. 3 were further fractionated by FPLC on a Mono Q column at pH 8.0 (Fig. 5). Three peaks of xylanase

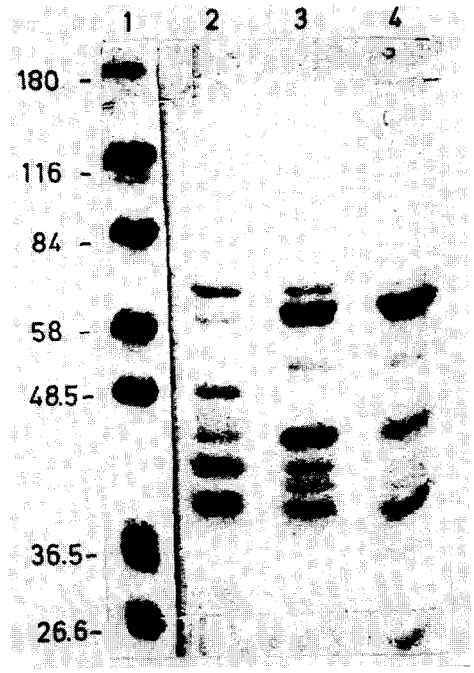

Fig. 6. SDS-PAGE of xylanase fractions, performed in a 10% polyacrylamide slab gel in the presence of 0.1% SDS [15]. Lanes: 1 = molecular mass markers; 2 = fraction 8; 3 = fraction 12; 4 = fraction 16. The numbers on the left are the molecular masses ($\times 10^{-3}$) of marker proteins.

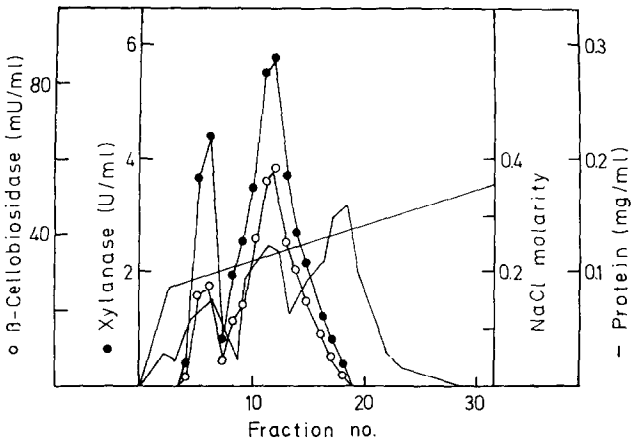

Fig. 7. Resolution of celloxylanases by Mono Q anion exchange chromatography. Fractions 38–43 from the Mono P column (Fig. 4) were applied to a Mono Q HR 10/10 column equilibrated with 20 m*M* Tris–HCl (pH 6.0). Elution was effected with an 8-ml linear gradient (0.0–0.17 *M* NaCl) followed by a 168-ml linear gradient (0.17–0.40 *M* NaCl) in equilibration buffer at a flow-rate of 4 ml/min. Fractions (4 ml) were assayed for enzyme activities.

activity could be partially resolved. Analysis of the peaks fractions by SDS-PAGE (Fig. 6) revealed the presence of several protein bands in each fraction. *In situ* activity staining for xylanase activity showed that all protein bands were enzymatically active. Prominent activity bands were detected at positions corresponding to molecular masses of 70000, 62000 and 42000. The molecular masses of these isoenzymes are nearly identical with those reported previously for xylanase A, B and C of *C.*

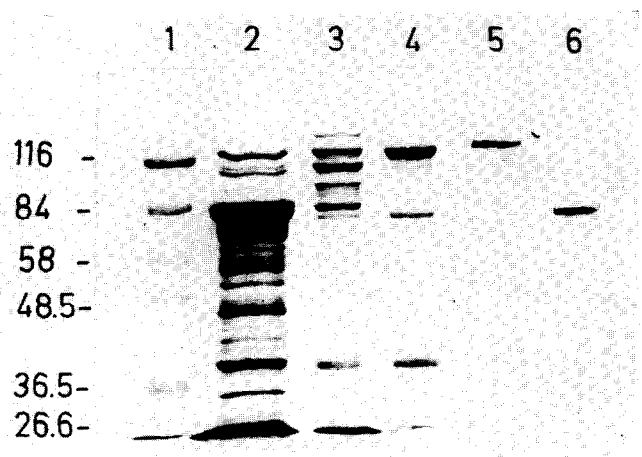

Fig. 8. SDS-PAGE of celloxylanase fractions, carried out as described in Fig. 6. Lane 1, molecular mass markers; lane 2, concentrated culture supernatant; lane 3, pooled fractions from the Q Sepharose column; lane 4, pooled and centrifuged fractions from the Mono P column; lane 5, pooled fractions 4–5 from the Mono Q column (celloxylanase II); lane 6, pooled fractions 10–13 from the Mono Q column (celloxylanase I). The numbers on the left are the molecular masses ($\times 10^{-3}$) of marker proteins.

stercorarium by Berenger *et al.* [6]. The immunological cross-reactivity of these enzymes [6] suggests that the different isoenzymes arose by partial proteolysis of a common enzyme precursor. This notion was confirmed by analysis of the gene products produced by expression of the cloned *C. stercorarium* xylanase gene *xynA* in *E. coli* [17].

The fractions from the gradient shown in Fig. 4 expressing both β-D-cellobiosidase and xylanase activity were subjected to Mono Q chromatography at pH 6.0 (Fig. 7). It can be seen that the xylanase activity copurified again with the β-cellobiosidase activity. SDS-PAGE (Fig 8) showed single protein bands with molecular masses of *ca.* 80 000 (cellobiosidase I) and 120 000 (cellobiosidase II). Activity staining for xylanase and β-cellobiosidase activity revealed that both activities reside in the same protein bands (data not shown). Both enzymes were able to hydrolyse cellodextrins and β-glucans in addition to xylan and aryl-β-cellobiosides. Enzymes displaying activity with either cellodextrins, β-glucans or xylans as substrates have been isolated from rumen bacteria [18] and *C. acetobutylicum* [19]. This novel type of enzyme has been termed celloxylanase [18]. The *C. stercorarium* β-D-cellobiosidases are therefore more appropriately designated as celloxylanase I and II, respectively.

The properties of the *C. stercorarium celX* gene product cloned in *E. coli* [20] closely resemble those of celloxylanase II with respect to substrate specificity, temperature optimum and pH profile (unpublished results). Moreover, the DNA sequence of the *celX* gene contains an open reading frame coding for a protein of $11.54 \cdot 10^5$ dalton, which is in good agreement with the molecular mass of celloxylanase II determined by SDS-PAGE (Fig. 8). These data strongly suggest that celloxylanase II is the product of the *celX* gene. It remains to be determined whether celloxylanase I arises by proteolytic processing of celloxylanase II or whether it is expressed from a different gene.

ACKNOWLEDGEMENT

This work was supported by a grant from the Bundesministerium für Forschung und Technologie (BMFT project 0319001A8).

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