Isolation and analysis of two cellulase cDNAs from Orpinomyces joyonii
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Abstract
Two cellulase cDNAs, celB29 and celB2, were isolated from a cDNA library derived from mRNA extracted from the anaerobic fungus, Orpinomyces joyonii strain SG4. The nucleotide sequences of celB2 and celB29 and the primary structures of the proteins encoded by these cDNAs were determined. The larger celB29 cDNA was 1966 bp long and encoded a 477 amino acid polypeptide with a molecular weight of 54 kDa. Analysis of the 1451 bp celB2 cDNA revealed an 1164 bp open reading frame coding for a 44 kDa protein consisting of 388 amino acids. Both deduced proteins had a high sequence similarity in central regions containing putative catalytic domains. Primary structure analysis revealed that CelB29 contained a Thr/Pro-rich sequence that separated the N-terminal catalytic domain from a C-terminal reiterated region of unknown function. Homology analysis showed that both enzymes belong to glycosyl hydrolase family 5 and were most closely related to endoglucanases from the anaerobic fungi Neocallimastigomycetes oryctawecki, Neocallimastigomycetes frontalis and Orpinomyces sp. The classification of CelB29 and CelB2 as endoglucanases was supported by enzyme assays. The cloned enzymes had high activities towards barley β-glucan, lichenan and carboxymethylcellulose (CMC), but not Avicel, laminarin, pachyman, xylan and pullulan. In addition, CelB29 and CelB2 showed activity against p-nitrophenyl-β-D-cellotrioside (pNP-G3) to p-nitrophenyl-β-D-cellobiose (pNP-G2) but not p-nitrophenyl-β-D-glucopyranoside (pNP-G1) with preferential activity against p-nitrophenyl-β-D-glucopyranoside (pNP-G1). Based on these results, we proposed that CelB29 and CelB2 are endoglucanases with broad substrate specificities for short- and long-chain β-1,4-glucans. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: cDNA sequence; Cellulose degradation; Cloning; Rumen fungus

1. Introduction
Cellulose is the most abundant source of carbon found in nature. This linear β-glucan chain, consisting of β-glucose monomers linked by β-1,4-glycosidic bonds, is the principal structural component of plant cell walls. Individual cellulose molecules are formed into insoluble cellulose microfibrils through numerous intra- and intermolecular hydrogen bonds, thereby creating the very complex and recalcitrant physical structure of cellulose found in plant cell walls (Delmer and Amor, 1995; Carpita, 1996).

The value of cellulose as a renewable source of energy and carbon has made cellulose hydrolysis the subject of scientific research and industrial interest for many years. Over the past decades, a large amount of information has been accumulated on the hydrolysis of cellulose by cellulases. Many cellulolytic systems from a wide array of organisms, including higher flowering plants, protozoa, fungi and bacteria, have been studied. Conversion of cellulose to glucose involves at least three types of cellulases: (1) endoglucanases (EC 3.2.1.4), which randomly cleave internal β-1,4-glucosidic linkages, (2) cel-
also being developed for the production of recombinant carbonate-treated water. 

Poly(A) Neocallimastix patriciarum (Selinger et al., 1996). Despite the information available on these enzyme systems and the structure of plant cell walls (Gilbert and Hazelwood, 1983; Beguin and Lemaire, 1996), our application of this knowledge to cellulose degradation has met with limited success. The lack of success may be attributed to at least two factors: the inherent complexity and heterogeneity of native cellulose, and our lack of a thorough understanding of the basic processes of the hydrolysis (Lechine, 1995). Therefore, an understanding of the molecular mechanisms underlying cellulose degradation in combination with new and superior enzymes will certainly facilitate increased usage of this valuable renewable resource.

Plant fibre is a major component of ruminant diets, and the anaerobic microorganisms, including the bacteria, fungi and protozoa, inhabiting the rumen ecosystem play a vital role in its digestion. The high cellulolytic activities of rumen fungal isolates, in the genera Anaeromyces, Caecomyces, Neocallimastix, Orpinomyces and Piroromyces (Trinci et al., 1994; Wallace, 1994), have recently attracted much scientific attention. High-specific-activity cellulases and xylanases from rumen fungi (Gilbert et al., 1992; Xue et al., 1992a,b; Trinci et al., 1994; Chen et al., 1997) are potential new enzyme supplements for the livestock industry. Cellulases, xylanases, and β-glucanases have been shown to increase the efficiency of feedstuff digestion in monogastric animals by enhancing degradation of plant cell-wall polymers (Campbell and Bedford, 1982). Plant cell-wall-degrading enzymes are also being used or considered for use by pulp and paper, textiles, detergent, and food and beverage industries. Recent developments in recombinant DNA technology have enabled researchers to develop cost-effective means of producing selected enzymes and unique enzyme combinations. Highly active fibrolytic enzymes from fastidious microorganisms, such as the ruminal fungi, may now be produced in large quantities using biotechnological methods. These methods have been used to produce 20 g of glucoamylase per litre in recombinant strains of Aspergillus niger (Hintz et al. 1995). Plant and animal expression systems are by 80% efficient of feedstu

Materials and methods

2.1. Microbial strains and culture media

Orpinomyces joyonii strain SG4, a sheep ruminal isolate, was obtained from the Lethbridge Research Centre Culture Collection and grown in modified semi-defined medium (Lowe et al., 1985) with either Whatman No. 1 filter paper or straw as a carbon source. The fungus was cultivated using the anaerobic technique of Hungate (1950) as modified by Bryant and Burkey (1953). Escherichia coli XL1-Blue MRF’ (Stratagene Cloning Systems, La Jolla, CA), the host for cDNA library construction and vector propagation, was grown in LB medium (Sambrook et al., 1989). Ampicillin (100 µg/ml) was incorporated into media to culture plasmid-bearing E. coli strains.

2.2. RNA isolation

Fungal mycelia were harvested from culture media and lyophilized. The dried mycelia were ground under liquid nitrogen into powder with a mortar and pestle. Total RNA was isolated from powdered mycelia according to Qiu and Erickson (1994) with some modifications. Briefly, following homogenization of the mycelia in extraction buffer (100 mM Tris–Cl, pH 8.0, 50 mM EDTA, 500 mM NaCl, 2% SDS, 1% β-mercapto-ethanol), an equal volume of phenol:chloroform (1:1) was added. The suspension was agitated for 1 min with a vortex mixer. The mixture was centrifuged, and the resulting aqueous phase was removed to a clean centrifuge tube, extracted with phenol-chloroform and precipitated with one-third volume of 7 M lithium chloride. The RNA pellet was washed with 2 M LiCl, followed by 80% ethanol, and then resuspended in diethyl pyrocarbonate-treated water. Poly(A)+ RNA was isolated from total RNA using oligo(dT)-cellulose chromatography following standard protocols (Sambrook et al., 1989).

2.3. Construction and screening of an O. joyonii cDNA library

An O. joyonii cDNA library was constructed with the aid of a Stratagene cDNA synthesis kit, Lambda...
ZAP® II vector and Gigapack® packaging extracts, according to the manufacturer's instructions. Recombinant phages were screened for cellulolytic activity by plating in 0.5% (w/v) top agarose containing 0.2% (w/v) CMC and barley β-glucan buffered with 50 mM sodium phosphate (pH 6.6). Cellulose hydrolytic activity was detected by Congo red staining (Teather and Wood, 1982). Plasmid derivatives of the positive phage clones were prepared by in-vivo excision according to the manufacturer's instructions (Stratagene).

2.4. Enzyme assays

Preliminary qualitative plate assays were conducted on LB agar plates containing different glycogen substrates [0.2% (w/v)]. Hydrolytic activity was detected with Congo Red staining (Teather and Wood, 1982). For quantitative assays, enzyme extracts prepared by sonication were incubated with a 1% substrate solution in 20 mM phosphate buffer (pH 6.8) at 37°C for 0.5 h. Released reducing sugars were quantified as described by Kawai et al. (1987). One unit of enzyme activity was defined as the amount of enzyme catalysing the formation of 1 μmol of product per minute. Cellulose binding assays were performed according to Ali et al. (1995).

For enzymatic assays of β-glucosidase, cellobiohydrolase and β-cellobextrinase, 0.1 ml of enzyme solution was mixed with 0.1 ml of 10 mM arylglycoside substrates in 20 mM phosphate buffer (pH 6.8). The mixture was incubated at 37°C for 15 min. The reaction was stopped with the addition of 0.1 ml 1 M Na2CO3. The resulting mixture was measured spectrophotometrically at 405 nm. One unit of enzymatic activity was defined as that forming 1 nmol of p-nitrophenol per minute.

Temperature and pH optima were determined according to the procedure of Denman et al. (1996).

3. Results and discussion

3.1. Isolation of cellulase cDNA clones

In order to identify the genes encoding cellulolytic enzymes, a cDNA library was constructed using poly(A)+ RNA from O. joyonii grown on a medium with either straw or filter paper as the sole carbon source. The resulting library contained a total of 5 x 1010 original plaque-forming units. Screening with mixed CMC/barley β-glucan overlays detected 69 plaques with hydrolytic activity.

To avoid analysing duplicate clones, restriction mapping and substrate plate assays were used to classify the positive clones into several preliminary groups. Two cDNAs from two separate groups, celB29 and celB2, were further analysed.

3.2. Nucleotide sequence analysis

CelB29 is 1966 bp in length and contains a coding region of 1431 bp with a start codon at position 167 and stop codon at position 1598 (Fig. 1). The 5′ untranslated leading sequence is rich in A/T residues (94%). The 3′ untranslated region is also A/T-rich (91%), and contains six potential eukaryotic polyadenylation signals (AATAAA) and a 19 bp poly(A) tail. Similar AT rich non-coding flanking regions have been reported for other sequences cloned from ruminal fungi (Gilbert et al., 1992; Zhou et al., 1994). The translational start site was established as a result of the following observations. There are no start codons and several stop codons present upstream of the proposed translation start site, and the first 20 amino acids of the deduced CelB29 polypeptide constitute a typical signal peptide (i.e. it is generally located at the N-terminus of the proprotein; von Heijne, 1986). The deduced CelB29 polypeptide contains 477 amino acids and has a molecular weight of 54.7 kDa and a predicted isoelectric point of 6.3. A series of 21 amino acids, rich in Thr and Pro, define a typical signal sequence (i.e. it is rich in Pro-rich sequences have also been reported in other microbial cellulases. They are hypothesized to function as hinges or linkers between two functional domains (Gilks et al., 1991). Following the Thr/Pro-rich sequence, there are two different amino acid direct repeats in the C-terminal region: (CF/WST/ERLGYN/PCC) and (VEYTDN-DGK/RWGVENGNWCGI), and both repeat twice in tandem. Tandem repeats of amino acid sequences are common features for structural proteins such as extensins that are proposed to support the plant cell-wall assembly (Kielszewski and Lamport, 1994). Several reiterated sequences have been described for microbial cellulases. The short tandem repeats of CelB (Zhou et al., 1994) and Xyla (Gilbert et al., 1992) from X. patriciarum showed significant homologies to the CelB29 repeats. This reiterated feature of cellulases has now been proposed to function as a docking domain to anchor the enzyme to a scaffolding protein to form the cellulosome complex (Fanutti et al., 1995; Beguin and Lemaire, 1996).

The cDNA celB2 is 1451 bp long. Sequence translation revealed that only the first reading frame would encode a polypeptide of sufficient length and significant similarity to other cellulases. The start codon of the celB2 was tentatively assigned at position 263 based on alignments of celB2 with homologous cellulases. The proposed ORF has a short 3′-untranslated region without a poly(A) tail. This is surprising since the synthesis of the first-strand cDNA was primed with oligo(dT) primers. The deduced CelB2 polypeptide contains 388 amino acids and has a molecular weight of 44 kDa and
Fig. 1. Nucleotide and deduced protein sequences of CelB29. The putative catalytic region is indicated in bold italics. The signal peptide, a linker sequence and putative polyadenylation signals are shown in bold. Amino acid direct repeats are underlined.


3.3. Homology analyses of the polypeptide sequences

A comparison of CelB29 and CelB2 primary sequences revealed that CelB2 is about 80 amino acids shorter than CelB29 due to about 40 residues missing from the N- and C-terminal regions. The overall identity of the two sequences is 57%. However, the highest degree of homology (66%) was found in the putative catalytic region (Fig. 2).

Database searches revealed that CelB2 and CelB29 exhibited a high degree of amino acid sequence similarity to CelA from N. frontalis (Accession No. AAC63094, Fujino et al., 1998), CelB from N. patriciarum (Accession No. Q12647, Zhou, et al., 1994) and CelB from Orpinomyces sp. (Accession No. AAD04193, Li et al., 1997). All of these enzymes belong to the cellulase family (Henrissat et al., 1989) or glycosyl hydrolase family 5 (Henrissat, 1991), which is characterized by an amino acid signature L–I–F–E–G–x–N–E–P–R with a conserved glutamic acid residue potentially involved in catalytic degradation of cellulose. A comparison of CelB29 and CelB2 primary sequences revealed that CelB2 is about 80 amino acids shorter than CelB29 due to about 40 residues missing from the N- and C-terminal regions. The overall identity of the two sequences is 57%. However, the highest degree of homology (66%) was found in the putative catalytic region (Fig. 2).

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contain hydroxyl amino acid linker sequences in their central region. While CelB29 is more closely related to CelB from *Orpinomyces* sp. and CelB from *N. patri- ciarum*, they all have two distinct domains separated by a Thr/Pro-rich linker sequence.

### 3.4 Enzymatic properties of cloned enzymes

Native celluloses are complex and heterogeneous in nature. Therefore, it could not be used in characterizing the cellulases. Instead, we utilized a range of different model substrates (e.g. CMC, Avicel, barley β-glucan, lichenan, laminarin, pachyman, and xylan) to characterize the substrate specificity. A preliminary qualitative assessment of enzymatic activities was performed by inoculating *E. coli* strains bearing the cellulase cDNAs onto agar media containing oat spelt xylan, CMC, lichenan, barley β-glucan, and pachyman or pullulan. The results indicated that both enzymes have very similar enzymatic properties, although there are distinct differences in the primary structure of the two deduced protein sequences. Except for strong activities on CMC, lichenan and barley β-glucan, neither of the cloned enzymes showed any activity to the rest of the substrates, suggesting that both enzymes are β-1,4-endoglucanases. CMC is a water-soluble long chain cellulose with carboxymethyl substitu- tions that is commonly used as a model substrate for detecting β-1,4-endoglucanases (Teeri, 1997). Cleavage between the substituent groups can result in a rapid decrease in the degree of polymerization and solution viscosity. Digestion of lichenan and barley β-glucan (mixtures of β-1,3-1,4 linkages) might be mainly due to random cleavage of β-1,4-glycosidic linkage in the substrates since both enzymes were unable to digest laminarin and pachyman, and their major components are β-1,3-glucans.

The activities of CelB2 and CelB29 on different substrates were also measured quantitatively. These results (Table 1) coincided with the plate assays. When incubated at 37 °C for 30 min, both enzymes could produce detectable reducing sugars on CMC, barley β-glucan and lichenan but not Avicel, laminarin, pachyman and pullulan. CelB29 and CelB2 exhibited the highest activity towards barley β-glucan, then lichenan, and followed by CMC. Unlike the plate assays, CelB2 had some activities towards oat spelt xylan. The difference between the plate and quantitative assays regarding enzymatic properties, although there are distinct differences in the primary structure of the two deduced protein sequences. Except for strong activities on CMC, lichenan and barley β-glucan, neither of the cloned enzymes showed any activity to the rest of the substrates, suggesting that both enzymes are β-1,4-endoglucanases. CMC is a water-soluble long chain cellulose with carboxymethyl substitu- tions that is commonly used as a model substrate for detecting β-1,4-endoglucanases (Teeri, 1997). Cleavage between the substituent groups can result in a rapid decrease in the degree of polymerization and solution viscosity. Digestion of lichenan and barley β-glucan (mixtures of β-1,3-1,4 linkages) might be mainly due to random cleavage of β-1,4-glycosidic linkage in the substrates since both enzymes were unable to digest laminarin and pachyman, and their major components are β-1,3-glucans.

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Fig. 3. Alignment of CelB29 and CelB2 with homologous cellulase CelA from Neocallimastix frontalis (Accession No. AAC63094, Fujino et al., 1998), CelB from Neocallimastix patriciarum (Accession No. Q12647, Zhou et al., 1994) and CelB from Orpinomyces sp. (Accession No. AAD04193, Li et al., 1997). "•": perfectly conserved; ": well conserved. The conserved signature sequence of glycosyl hydrolase family 5 and the threonine-rich linker sequence are framed in a box. Conserved glutamic acids and tryptophan residues are highlighted.

Artificial substrates such as methyllumellibiofuranosyl cellobiose (MUC) and p-nitrophenyl-β-α-cellulobioside (pNP-G3) may also be used to measure the cellobiohydrolase activity. The results showed that while both enzymes lacked β-glucosidase activity (i.e. they did not cleave p-nitrophenyl-β-α-glucopyranoside), they did show a broad range of substrate specificity for cellodextrins (i.e. p-nitrophenyl-β-α-celllobioside to p-nitrophenyl-β-α-cellpentaoside). The highest activities were towards pNP-G2, then pNP-G3.
followed by pNP-G₃ and pNP-G₄, indicating that CelB29 and CelB2 possess β-cellodextrinase and β-cellobiohydrolase. Cleavage of pNP-G₃ might be due to β-cellodextrinase (cellotriohydrolase), but not the combined action of cellobiohydrolase and β-glucosidase since both cloned enzymes lack the activity of β-glucosidase. Based on the same rationale, cleavage of pNP-G₄ should be the result of either cellodextrinohydrolase or the combination of cellobiohydrolase and cellotriohydrolase. Compared to CelB29, CelB2 had a higher activity towards all arylglycoside substrates, indicating that a C-terminal shortage of a number of amino acids in CelB2 did not affect the enzymatic capacity. Based on these results, we propose that CelB29 and CelB2 are endoglucanases with broad substrate specificities for short- and long-chain β-1,4-glucans, and these enzymes showed catalytic activities including endoglucanase, cellobextrinase and cellobiohydrolase. However, it is worth noting that due to the overlapping specificity on several substrates supplied, the clear-cut classification of the cloned enzymes into a specific group is difficult. A similar phenomenon has also been found in celD, a polysaccharide hydrolase cDNA from N. patriciarum encoding three multi-functional catalytic domains with high endoglucanase, cellobiohydrolase and xylanase activities (Xue et al., 1992b).

In view of the findings that many characterized cellobiohydrolases consist of two distinct domains, a cellulose-binding domain (CBD) and a catalytic domain (Gilkes et al., 1991), a search for CBD sequences was conducted. No distinct CBDs were found in either CelB29 or CelB2. This result was confirmed with a cellulose-binding assay that showed that neither CelB29 nor CelB2 was able to bind Avicel, a microcrystalline cellulose (data not shown). The absence of CBDs and the presence of reiterated scaffold binding sequences in CelB29 and CelB2 suggested that these enzymes may be immobilized to the cellulosome, a cellulose hydrolytic complex. More than 80% of extracellular cellulase activities of Orpinomyces are associated with a cellulose-binding complex (Ali et al., 1995). As such, the enzymes may function more effectively.

The effect of pH and temperature of both cloned cellulases on CMC as a substrate was measured in 20 mM sodium phosphate buffer over a pH range of 4.2–7.8 with an interval of 0.4 and a temperature range of 20–100 °C with 5 °C intervals. Results revealed that both cloned enzymes were active over a broad pH range between 5.4 and 7.4. CelB29 had the highest activity at pH 5.8, while CelB2 was most active at pH 6.6. Temperature optima for CelB2 and CelB29 were 45 and 50 °C, respectively. Both enzymes retained at least 65% of their activity over the temperature range of 30–60 °C.

In conclusion, we have isolated two cDNA celB29 and celB2 from O. joyonii that encode endoglucanases. CelB29 and CelB2 share a high amino acid identity (37%) throughout the sequence and belong to the same group of glycanases: cellulase A or glycosyl hydrolase (57% similarity in the primary structure of the two deduced polypeptide sequences, both enzymes appeared to have very similar enzymatic properties. Both could hydrolyse barley β-glucan, lichenan, CMC, but not Avicel, laminarin, pachymann, and pullulan, indicating their β-1,4-endoglucanase activity. In addition, both enzymes were able to cleave pNP-G₅, pNP-G₄, pNP-G₃, and pNP-G₂, but not pNP-G₁, indicating their cellodextrinase and cellobiohydrolase but not β-glucosidase activities.

Table 1

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<th>Substrates</th>
<th>Major components</th>
<th>CelB29 (units/ml)</th>
<th>CelB29 (units/mg)</th>
<th>CelB2 (units/ml)</th>
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*Crude cell lysate was used for the assay of the enzyme activities. One unit of enzyme activity was defined as the amount of enzyme catalysing the formation of 1 nmol of p-nitrophenol per minute.

Table 2

<table>
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