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# EglC, a New Endoglucanase from *Aspergillus niger* with Major Activity towards Xyloglucan

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**A novel gene, *eglC*, encoding an endoglucanase, was cloned from *Aspergillus niger*. Transcription of *eglC* is regulated by XlnR, a transcriptional activator that controls the degradation of polysaccharides in plant cell walls. EglC is an 858-amino-acid protein and contains a conserved C-terminal cellulose-binding domain. EglC can be classified in glycoside hydrolase family 74. No homology to any of the endoglucanases from *Trichoderma reesei* was found. In the plant cell wall xyloglucan is closely linked to cellulose fibrils. We hypothesize that the EglC cellulose-binding domain anchors the enzyme to the cellulose chains while it is cleaving the xyloglucan backbone. By this action it may contribute to the degradation of the plant cell wall structure together with other enzymes, including hemicellulases and cellulases. EglC is most active towards xyloglucan and therefore is functionally different from the other two endoglucanases from *A. niger*, EglA and EglB, which exhibit the greatest activity towards β-glucan. Although the mode of action of EglC is not known, this enzyme represents a new enzyme function involved in plant cell wall polysaccharide degradation by *A. niger*.**

Plant cell walls are composed predominantly of structural polysaccharides, which are associated in a matrix of cellulose microfibrils, hemicellulose polymers, and pectin. Bacteria and filamentous fungi, including *Aspergillus* and *Trichoderma* species, can degrade plant cell wall polysaccharides efficiently by producing a mixture of extracellular hydrolytic enzymes.

The major component of plant cell walls is the β-1,4-glucan cellulose. Cellulose can be degraded by the coordinated action of cellulolytic enzymes, such as endoglucanase, cellobiohydrolase, and β-glucosidase. Most cellulolytic enzymes consist of a catalytic domain linked to a cellulose-binding domain (CBD) by a Pro/Ser/Thr-rich linker peptide. The cellulolytic enzyme system of *Trichoderma reesei* is the best-studied fungal example. This system contains five genes encoding endoglucanases, *egl1* to *egl5* (14), two genes encoding cellobiohydrolases, *cbh1* and *cbh2* (2, 19), and two β-glucosidase-encoding genes, *bgl1* and *bgl2* (1, 21). Endoglucanases cleave internal β-1,4-glucosidic bonds, while cellobiohydrolases cleave the disaccharide cellobiose from either the nonreducing or reducing end of the cellulose polymer chain (22). β-Glucosidases hydrolyze cellooligosaccharides and cellobiose to D-glucose.

Expression of cellulases is controlled at the transcriptional level in both *T. reesei* and *Aspergillus niger*. In the presence of D-glucose transcription is repressed, while in the absence of D-glucose and in the presence of cellulose certain oligosaccharide and/or disaccharide (e.g., sophorose) transcription is strongly induced. In *T. reesei* D-glucose repression of transcription is mediated by Cre1, which also mediates repression of genes coding for enzymes involved in the degradation of hemicellulose (10, 12).

In *A. niger* two genes encoding endoglucanases, *eglA* and *eglB* (25), and two cellobiohydrolase-encoding genes, *cbhA* and *cbhB* (6), have been isolated and characterized. Both EglA and EglB lack a CBD and the associated linker region. CbhB has a catalytic domain and a CBD separated by a Ser/Pro/Thr-rich linker peptide, while CbhA has only the catalytic domain. Both enzymes release cellobiose upon incubation with carboxymethyl cellulose (CMC) (6).

We cloned the *xlnR* gene, encoding the transcriptional activator XlnR, in *A. niger* based on its ability to regulate the expression of genes encoding xylanolytic enzymes. However, XlnR controls both the transcription of genes encoding principal and accessory xylanolytic enzymes and the transcription of the cellulolytic genes *eglA*, *eglB*, *cbhA*, and *cbhB* (25).

In this paper we describe a new endoglucanase, EglC, from *A. niger*. This enzyme is unique among the *A. niger* endoglucanases because it exhibits its greatest activity towards xyloglucan.

## MATERIALS AND METHODS

***A. niger* strains and growth conditions.** All of the *A. niger* strains used were derived from wild-type strain N400 (= CBS 120.49). The strains used were NW188 (*goxC17 cspA1 pyrA6 leuA1 prtF28*), NW219 (*cspA1 pyrA6 leuA1 nicA1*), NW199 (*fwnA6 goxC17 pyrA6 ΔxlnR::argB leuA5*), NW283 (*fwnA1 cspA1 pyrA6 lysA7 creA$^{d}$4*), and NW188::pGW635::pIM4496-22 containing an EglC expression construct.

All media had a pH of 6 and were based on *Aspergillus* minimal medium (MM) (15); the media were supplemented with carbon sources as indicated. Spores were inoculated at a concentration of $10^6$ spores ml$^{-1}$. In transfer experiments precultures grown with D-fructose were supplemented with 0.1% (wt/vol) Casamino Acids and 0.1% (wt/vol) yeast extract. After 18 h of growth, mycelia were recovered by filtration with a Büchner funnel and were washed with MM without a carbon source. These mycelia were transferred to MM containing various carbon sources.

**Cloning and characterization of *eglC*.** While screening a differential cDNA library, constructed by subtracting the cDNA of a D-xylose-induced *A. niger* XlnR

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loss-of-function mutant from the cDNA of a D-xylose-induced wild type (9), we isolated a 300-bp *Rsa*I fragment that encoded the C-terminal portion of *eglC*. The complete *A. niger eglC* gene was recovered after we screened an *A. niger* N400 genomic library in λEMBL4 (7). We used standard methods for other DNA manipulations, including Southern blotting, subcloning, DNA digestion, and λ phage and plasmid DNA isolation (18). Sequencing reactions were performed by using a Thermo-Sequenase fluorescently labeled primer cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and universal sequencing primers. The sequencing reaction mixtures were analyzed with an ALF *express* sequencer (Amersham Pharmacia Biotech).

**Expression vector for *eglC* in *A. niger*.** The *eglC* gene was fused to the promoter of the *Aspergillus tubingensis xlnA* gene (3) at its start codon using splicing by overlap extension as described by van Peij et al. (24), which resulted in pIM4496. This plasmid included the coding region and a 3′ noncoding flanking region of the *eglC* gene. Transformation of *A. niger* was performed as previously described (11).

**Purification and characterization of *A. niger* EglC.** *A. niger* strain NW128::pIM4496-28 was precultured overnight in 2 liters of MM containing 3% D-fructose and then was transferred to 2 liters of MM containing 50 mM D-xylose and incubated for 8 h. The mycelia were removed by filtration over a Büchner funnel, the culture was diluted with water so that the volume was 7.5 liters in order to decrease the salt concentration (<10 mM), and the pH was adjusted to pH 6 with 1 M NaOH. The culture filtrate was incubated in a batch preparation overnight at 4°C with 20 ml of Streamline DEAE (Amersham Pharmacia Biotech) that had been preequilibrated in 10 mM piperazine (pH 6) to bind protein. The supernatant was decanted, the Streamline DEAE was poured into a column, and EglC was eluted in eight 5-ml fractions with 1 M NaCl. The concentrated enzyme preparation was loaded onto a 15.5-ml Source 30 Q column (Amersham Pharmacia Biotech) equilibrated with 10 mM piperazine (pH 6) and was eluted with a linear 0 to 1 M NaCl gradient. EglC eluted at an NaCl concentration of 0.3 M. The Source 30 Q enzyme pool was desalted and was purified further by using a 313-ml Superdex 200 prep grade column (gel permeation; Amersham Pharmacia Biotech) equilibrated with 10 mM piperazine and 100 mM NaCl.

The optimum pH of the purified EglC (3.7 ng) was determined by using 175-μl (final volume) portions of McIlvaine buffer at pHs ranging from 2.9 to 7.5 (ionic strength equivalent to 0.5 M) with 200 μl of azo-CMC (Megazyme, Wicklow, Ireland) and incubation for 10 min at 40°C. The optimum temperature was determined by using 175-μl portions of 100 mM sodium acetate buffer at the optimum pH (pH 4.5) with 200 μl of azo-CMC and incubation for 10 min at temperatures ranging from 25 to 80°C.

We tested EglC activity towards CMC (Sigma, St. Louis, Mo.), β-glucan (Megazyme), and tamarind xyloglucan (Megazyme) by incubating 2 μl of purified EglC (0.54 mg/ml) in preparations containing 200 μl of 0.5% substrate and 400 μl of 20 mM sodium acetate buffer (pH 4.5) for 10 min at 40°C. Release of reducing end groups was measured at least two times in duplicate as previously described by Nelson and Somogyi (13). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing end groups per min.

**Purification of *A. niger* EglA and EglB.** Endoglucanases EglA and EglB were purified from culture filtrates of *Kluyveromyces lactis* CBS 2359 containing *eglA* and *eglB* expression constructs. The culture filtrates were diluted 1:10 in 10 mM piperazine (pH 6) and loaded on a 1-ml Mono Q column (Amersham Pharmacia Biotech) equilibrated with 10 mM piperazine (pH 6). Both enzymes were eluted by using a linear 0 to 1 M NaCl gradient. EglA eluted at an NaCl concentration of 0.2 M, and EglB eluted at an NaCl concentration of 0.5 M. The presence of EglA or EglB in the fractions was determined by screening for β-glucanase activity on plates containing azurin-dyed and cross-linked (AZCL) β-glucan (Megazyme). The purified enzymes were desalted and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining.

We determined the activities of 1 μl of EglA (0.5 mg/ml) and 2 μl of EglB (0.44 mg/ml) towards CMC, beechwood xylan (Sigma), β-glucan, and tamarind xyloglucan; we also determined the activities of these enzymes towards β-glucan and tamarind xyloglucan in combination with 2 μl of EglC (0.504 mg/ml). Activity was determined by incubating the enzymes with 200 μl of 0.5% substrate in 400 μl of 20 mM sodium acetate buffer (pH 4.5) for 10 min at 40°C. The release of reducing end groups was measured as previously described by Nelson and Somogyi (13).

**Northern blot analysis.** To verify that XlnR-controlled transcription of *eglC* occurred and to examine the effect of CreA repression on *eglC*, Northern blot analyses were performed. RNA was isolated from *A. niger* NW219 (wild type), from *A. niger* NW199 (Δ*xlnR*), from *A. niger* N902::230-25.12 with 10 copies of the *xlnR* gene (25), and from *A. niger* NW283, a mutant (*creA*$^{d}$*4*) relieved of carbon repression (17). All strains were precultured in MM with 100 mM D-fructose as a carbon source and were then transferred to MM with 1% beechwood xylan and incubated for 3, 8, and 24 h.

Total RNA was isolated from powdered mycelia by using TRIzol reagent (Life Technologies, Rockville, Md.) according to the manufacturer's instructions. For Northern blot analysis, 10 μg of total RNA was glyoxylated and separated on a 1.6% (wt/vol) agarose gel. After capillary blotting to a Hybond-N membrane (Amersham Pharmacia Biotech), the transfer and the amount of RNA were verified by staining the RNA on the Hybond filter with 0.2% (wt/vol) methylene blue. Filters were hybridized at 42°C in a solution containing 50% (vol/vol) formamide, 10% (wt/vol) dextran sulfate, 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.2% (wt/vol) Ficoll, 0.2% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) bovine serum albumin, 0.1% (wt/vol) SDS, and 100 μg of single-stranded herring sperm DNA (Life Technologies) per ml. The filters were washed in 0.2× SSC–0.1% (wt/vol) SDS at 65°C.

**Nucleotide sequence accession number.** The *eglC* sequence has been deposited in the GenBank and EMBL sequence databases under accession no. AY040839.

## RESULTS

**Isolation and analysis of the *A. niger eglC* gene.** We cloned a 3,959-bp *Cla*I genomic DNA fragment containing the *eglC* gene (accession no. AY040839) into pIM4490. This *Cla*I fragment was sequenced and contained 1,037 bp of the 5′ noncoding region and 118 bp of the 3′ noncoding region. In the promoter region, one putative XlnR binding site (5′-GGCTA A-3′) (24) was found 395 bp upstream of the ATG translation start codon. The coding region of *eglC* is 2,574 bp long and is interrupted by five introns. All five introns are located in the first 1,000 bp of the coding region, and they range from 43 to 49 bp long. The *eglC* open reading frame encodes an 858-amino-acid protein. EglC contains a putative N-terminal signal sequence (amino acids 1 to 19), a Ser/Thr-rich linker peptide (amino acids 744 to 814), and a C-terminal CBD (amino acids 814 to 846). Based on the deduced amino acid sequence, the molecular mass of EglC was estimated to be 90.5 kDa, and the isoelectric point was estimated to be 4.1. A Blast search with the Swissprot database showed that EglC exhibited a high level of sequence homology (75% identity) with AviIII (accession no. AB015511) of *Aspergillus aculeatus* (20) (Fig. 1).

**Purification and characterization of EglC.** We purified EglC from an overproducing *A. niger* strain. This strain was obtained by transforming *A. niger* NW188 (3) with an *xlnA*-promoter-*eglC* gene fusion (pIM4496) to increase *eglC* transcription. The purified protein was analyzed for activity with azo-CMC.

Maximum EglC activity towards azo-CMC occurred between pH 3.5 and 5, and the optimum pH was pH 4.5. The optimum temperature, at which the enzyme had the greatest activity, was 55°C, as determined in 20 mM sodium acetate (pH 4.5). The stability of the enzyme was examined at the optimum pH (pH 4.5) in 20 mM sodium acetate buffer at 30 and 55°C. After incubation for 145 h at 30°C, 90% of the activity remained. The enzyme was less stable at the optimum temperature (55°C) and optimum pH (pH 4.5); 50% of the activity remained after incubation for 48 h, and 20% of the activity remained after incubation for 145 h.

**EglC is an endoglucanase.** EglC has endoglucanase activity, since it exhibited activity towards CMC, azo-cellulose, β-glucan, and tamarind xyloglucan, but no activity was found towards xylan. EglC released oligosaccharides from xyloglucan (Fig. 2). Xyloglucan consists of a β-1,4-glucan backbone with substitutions of α-1,6-linked D-Xyl *p* residues that are in turn variably replaced by β-1,2-linked Gal *p* residues (27). In tamarind xyloglucan, the Glc/Xyl/Gal/Ara ratio is 11:9:5:1

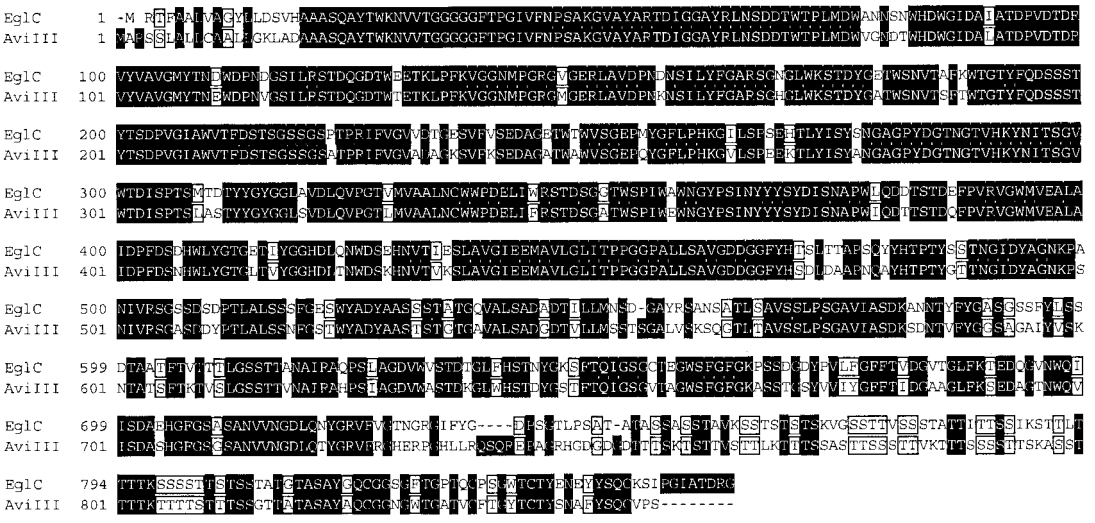

FIG. 1. Alignment of the amino acid sequences of EglC from *A. niger* (accession no. AY040839) and AviIII from *A. aculeatus* (accession no. AB015511) obtained by using ClustalW (http://www.ebi.ac.uk/clustalw) and Boxshade (http://www.ch.embnet.org). Identical amino acids at conserved positions are indicated by black boxes; similar residues are indicated by open boxes.

(Megazyme). However, the precise mode of action of EglC could not be determined based on the high-performance liquid chromatography (HPLC) patterns. EglC was most active towards xyloglucan (Table 1) (activity, 19 U/mg).

**Transcription of *eglC* is induced by XlnR and repressed by CreA.** We compared the patterns of transcription of *eglC*, *eglA*, and *cbhA* (Fig. 3). The *xlnB* gene was used as a reference. The levels of the *eglC* and *cbhA* transcripts were low in the wild-type strain. The level of the *eglC* transcript was much higher in the strain containing multiple copies of *xlnR* than in the wild type. No *eglC* transcription was observed in the *xlnR* knockout mutant.

CreA represses xylanolytic gene expression in *A. niger* (3, 5). None of the genes shown in Fig. 3 were transcribed in the presence of D-fructose. In the presence of xylan *eglC* transcription increased in NW283, an *A. niger creA$^{d}$4* mutant strain relieved of carbon repression; this was also the case for transcription of *cbhA* and *eglA*.

## DISCUSSION

We screened a differential library containing cDNA fragments derived from XlnR-regulated genes and isolated the *eglC* gene. *eglC* encodes an 858-amino-acid protein containing a C-terminal CBD attached to the catalytic domain via a Ser/Thr-rich linker peptide.

EglC is the third endoglucanase isolated from *A. niger* that is regulated by XlnR. The CBD of EglC (as well as the CBD of *A. niger* CbhB and *T. reesei* CbhI) can be grouped into carbohydrate-binding module 1, previously known as CBD family I (carbohydrate-binding module classification; http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html). This region contains approximately 40 amino acid residues and is found almost exclusively in fungi. The presence or absence of a CBD affects the activities of the different cellulases in *T. reesei* (22). Removal or mutagenesis of the CBD from CbhI or CbhII reduces the activities of these enzymes with crystalline cellulose but not with soluble substrates (16, 23).

Based on its derived amino acid sequence, EglC can be placed in glycosyl hydrolase family 74. A Blast search with the Swissprot database showed that no EglC homologue could be found in *T. reesei* or any other well-known cellulose-degrading fungus. However, a high level of sequence homology (75% identity) with *A. aculeatus* AviIII (accession no. AB015511) was found. It has been proposed that AviIII exhibits exoglu-

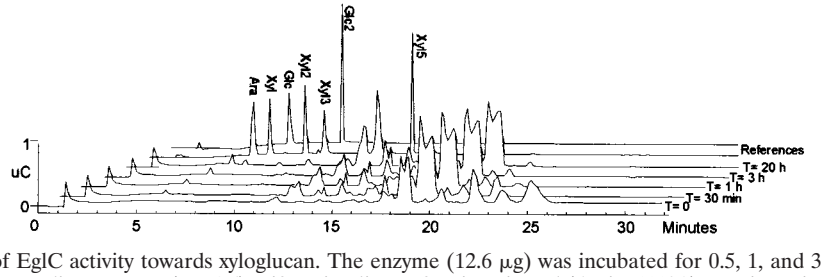


FIG. 2. HPLC analysis of EglC activity towards xyloglucan. The enzyme (12.6 μg) was incubated for 0.5, 1, and 3 h and overnight with 0.2% xyloglucan in 500 μl of 20 mM sodium acetate (pH 4.5). Fifty microliters of an inactivated (5 min, 100°C) reaction mixture that was diluted 20-fold in water was analyzed by high-performance anion-exchange chromatography by using a Dionex system with a Carbopac PA-100 column and pulsed amperometric detection with a 0.05 to 0.90 M NaOH gradient suitable for glucose oligosaccharide separation. The standards contained D-glucose (Glc), cellobiose (Glc2), L-arabinose (Ara), D-xylose (Xyl), xylobiose (Xyl2), xylotriose (Xyl3), and xylopentaose (Xyl5).

TABLE 1. Specific activities of EglA, EglB, and EglC towards CMC,β-glucan, and xyloglucan

| Enzyme | Sp act (U/mg of protein) towards the following substrates[a] | | |
|---|---|---|---|
| | CMC | β-Glucan | Xyloglucan |
| EglA | 3 ± 0.4 | 59 ± 5 | 0 |
| EglB | 8 ± 0.6 | 22 ± 4 | 0 |
| EglC | 1 ± 0.2 | 1 ± 0.1 | 19 ± 1 |

[a] Specific activities were determined by using the reducing end group method (13) in triplicate.

canase activity (20). Based on our data, EglC is an endoglucanase, and this enzyme is active towards azo-CMC, AZCL-cellulose, CMC, β-glucan, and xyloglucan. The HPLC analysis showed that this enzyme degrades xyloglucan by an endo type of reaction.

The substrate specificities of EglA, EglB, and EglC towards β-glucan, CMC, and xyloglucan have been determined. EglA and EglB exhibit the greatest activity towards β-glucan and minor activity towards CMC. No activity of EglA and EglB towards xyloglucan was found. The substrate specificity of EglC was different because the highest level of activity was towards xyloglucan and the lowest level of activity was towards β-glucan (Table 1). However, no enzyme activity synergy was found when EglA, EglB, and EglC were combined. *Trichoderma viride* produces an endoglucanase, endoIV, that has activity towards xyloglucan (26). Although *T. viride* endoIV and EglC both have xyloglucanase activity, we detected no significant sequence similarity between these proteins.

*eglC* transcription is regulated by XlnR, the transcriptional activator that controls genes encoding xylanolytic and cellulolytic enzymes in *A. niger*. Transcription of *eglC* in xylan-grown cultures is the same as transcription of *xlnB*, *cbhA*, and *eglA*. In the wild type, the level of *eglC* transcription is relatively low, but it increases in a strain containing multiple copies of *xlnR*.

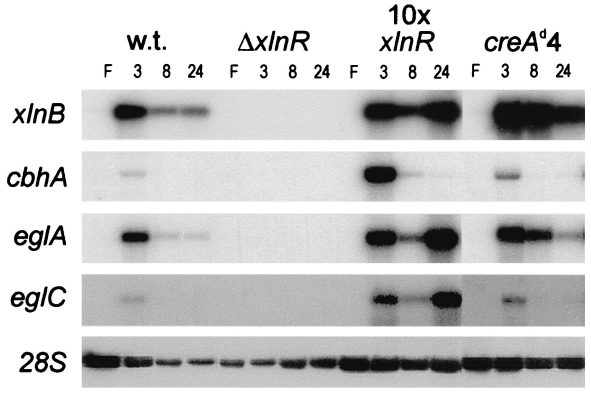


FIG. 3. Patterns of transcription of *xlnB*, *cbhA*, *eglA*, and *eglC* in *A. niger* strains NW219 (w.t.), NW199 (Δ*xlnR*), N902::230-25.12 (10x *xlnR*) (25), and NW283 (*creA*$^d$4). All of the strains used were precultured in MM containing 100 mM D-fructose (lanes F) as a carbon source. For induction, mycelium was transferred to MM containing 1% beechwood xylan and grown for 3, 8, and 24 h (lanes 3, 8, and 24, respectively), after which the total RNA was isolated. The fragments used as probes for *xlnB*, *cbhA*, and *eglA* were the fragments described by van Peij et al. (25) and Gielkens et al. (6). For *eglC* an 850-bp *Sal*I-*Nco*I fragment was used as the probe. The 28S rRNA serves as a loading control.

TABLE 2. Activities of EglA (62.5 ng), EglB (109 ng), and EglC (135 ng) towards β-glucan and xyloglucan

| Enzyme(s) | Activity (nmol of reducing end groups/min) towards[a]: | |
|---|---|---|
| | β-Glucan | Xyloglucan |
| EglA | 3.7 ± 0.3 | 0 |
| EglB | 2.4 ± 0.3 | 0 |
| EglC | 0.4 ± 0.1 | 2.6 ± 0.1 |
| EglA + EglC | 3.2 ± 0.4 | 2.7 ± 0.3 |
| EglB + EglC | 2.2 ± 0.1 | 2.9 ± 0.1 |
| EglA + EglB + EglC | 4.7 ± 0.2 | 2.8 ± 0.2 |

[a] Activities were determined by using the reducing end group method (13) in triplicate.

However, no transcripts are found in an *xlnR*-disrupted strain. Based on these results, we concluded that XlnR regulates transcription of *eglC*. Although we found no putative CreA binding motifs in the *eglC* promoter, we did observe a slight increase in transcription of *eglC* in the *creA*$^d$*4* mutant. This result may indicate that CreA is involved in carbon catabolite repression of *eglC* since previous studies have shown that CreA-mediated carbon catabolite repression also plays a role under xylan-induced conditions (3, 4). Although transcription of all three endoglucanases of *A. niger* is regulated by XlnR, no synergy between the enzymes for degradation of β-glucan or xyloglucan was found (Table 2).

In plant cell walls, xyloglucan is closely linked to the cellulose fibrils. The *eglC* gene codes for an endoglucanase that has a high relative activity towards xyloglucan. EglC can cleave β-1,4 bonds between D-glucose units based on its activity towards CMC and β-glucan. This enzyme is probably more active on bonds that have D-xylose-containing side chains nearby in the chain. We assume that EglC binds to the cellulose chain by using its CBD, which enables it to efficiently degrade xyloglucan. Although no synergy was found towards EglA and EglB, EglC has a specific role in plant cell wall degradation. It is the first enzyme from *A. niger* described having activity towards xyloglucan.

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