Cloning, sequence analysis and yeast expression of the egll gene from *Trichoderma longibrachiatum*

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**Abstract.** A gene (egll) encoding an endoglucanase (EGL1) from *Trichoderma longibrachiatum* has been cloned and sequenced. This gene, homologous to the *T. reesei* egll gene, differs from it in the length of the introns (particularly the first one) and encoded protein. A cDNA fragment obtained by the rapid amplification of cDNA ends method, which takes advantage of the polymerase chain reaction, has been expressed in yeast under control of the cyc-gal inducible promoter and yeast clones able to secrete active enzyme have been obtained.

**Introduction**

Cellulose is the most abundant organic polymer in nature. It can be used as a renewable source of food, fuels and chemicals. Many organisms cooperate in the natural biodegradation of lignocellulose, the form of cellulose in nature, in a complex association with other organic polymers. The mesophilic deuteromycete *Trichoderma reesei* possesses one of the most powerful and studied cellulase enzyme complexes. Extracellular endo-β-(1,4)-glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) contribute to the degradation of cellulose to glucose in this species. Some mutants secrete more than 40 g/l of extracellular protein, cellobiohydrolase I being the major product (about 50% of the total protein) (Mandels 1985). There are other known cellulolytic members in the genus *Trichoderma* (Wood and McCrae 1972, 1982; Halliwell and Vincent 1981; Saddler et al. 1985). However, to date only isolation and characterization of *T. reesei* cellulolytic genes have been reported. The genes encoding cellobiohydrolase I (Shoemaker et al. 1983), cellobiohydrolase II (Chen et al. 1987; Teeri et al. 1987), endoglucanase I (Penttilä et al. 1986; van Arsddell et al. 1987), endoglucanase II (Saloheimo et al. 1988) and an extracellular β-glucosidase (Barnett et al. 1991) have been cloned and sequenced.

Many efforts have been made to construct a cellulolytic yeast that could solve problems in or improve processes such as brewing, baking and alcohol production. The *T. reesei* endoglucanase and cellobiohydrolase genes have been expressed in *Saccharomyces cerevisiae* (Penttilä et al. 1987; van Arsddell et al. 1987; Knowles et al. 1987b). These enzymes even hyperglycosylated by the yeast do not lose their activity on the appropriate cellulosic substrates. The fungal protein signal sequences were maintained in all cases and secretion of active enzymes were detected, although large amounts of the enzymes remained cell-bound.

We report here the cloning and sequence of a *T. longibrachiatum* gene encoding for an endoglucanase enzyme homologous to endoglucanase I of *T. reesei*. A cDNA copy of this gene has been cloned in yeast and yeast clones secreting active enzyme have been obtained.

**Materials and methods**

**Strains and media.** _Escherichia coli_ DH5α [F–, endA1, hsdR17, gyrA96, thi-1, recA1, relA1, supE44, Δ lacU169 (φ80 lacZΔM15)] was grown as described in Sambrook et al. (1989). _S. cerevisiae_ S-150-2B (MATα, leu2-3, leu2-112, his3-1, trpl-289, ura3-52) was grown on YPD (1% yeast extract, 1.5% peptone, 2% glucose, 2% agar) or MM (0.7% yeast nitrogen base without amino acids, 2% glucose or galactose, 2% agar) supplemented with 20 μg/ml of appropriate requirements. The fungal strains _T. reesei_ ATCC 26921 and _T. longibrachiatum_ CECT 2606 were grown in minimal medium (Pontecorvo et al. 1953) with glucose or carboxymethylcellulose (CMC) as the carbon source.

**Enzyme activity determination and plate assay.** For endoglucanase activity determinations, CMC or azo-barley-β-glucan were used as substrates. In the first case, the assay samples (1 ml) contained 8 mg CMC/ml and 50 μl crude enzyme solution in 50 mM sodium acetate, pH 4.5. The reaction mixtures were incubated at 40°C during 1 h and reducing sugars liberated were determined according to Somogyi (1952) and Nelson (1984). When the sugar content of the samples was too high we assayed the endoglucanase activity on azo-barley-β-glucan using a commercial kit from Biocen (Vic...
T. longibrachiatum mRNA extraction. Mycelia from a 500-ml culture incubated at 30°C for 36 h was recovered by filtration, lyophilized and ground with a glass rod. Then 5 ml of solution A (6 M guanidine hydrochloride, 20 mM sodium acetate, pH 5.0, 0.5 M dithiothreitol) was added per gram of mycelia. Then 3 vol. of 4 M sodium acetate, pH 6.0, and 20 mM EDTA were added and mixed gently. Afterwards 5 ml phenol were mixed by inversion and the mixture centrifuged at 12000g for 10 min. Extractions with phenol/chloroform/isoamyl alcohol (24:1:1) extraction was made. Five hundred microlitres of 3 M sodium acetate and 5 ml isopropanol were added to the remaining aqueous phase and the mixture stored at room temperature for 2 h. Then it was centrifuged at 12000g for 10 min and the pellet washed with ethanol/TE buffer (10 mM TRIS-HCl, pH 8.0, and 1 mM EDTA) (70:30) and desiccated. Then it was dissolved in 2 ml TE buffer and RNase to a final concentration of 20 μg/ml was added. The solution was incubated at 37°C for 1 h and afterwards proteinase K to 20 μg/ml final concentration was added and incubation was carried out as above. The solution was treated with 1 vol. phenol and extracted with chloroform. The DNA was precipitated with 200 μl of 3 M sodium acetate and 1.2 ml isopropanol at 40 min at room temperature. After centrifugation at 12000g for 10 min the pellet was washed with ethanol/TE buffer, desiccated and dissolved in TE buffer.

T. longibrachiatum cDNA cloning. Lyophilized mycelia (0.05-0.20 g) were ground with a sterile glass rod, and then 2.5 ml of HSE solution (10 mM HEPES, pH 6.9, 0.5 M sucrose and 20 mM EDTA) and 250 μl of 10% sodium dodecyl sulphate (SDS) were added and the mixture incubated at 65°C for 15 min. Then 2.5 ml of 50 mM TRIS-HCl, pH 8.0, and 20 mM EDTA were added and mixed gently. Afterwards 5 ml phenol were mixed by inversion and the mixture centrifuged at 12000g for 10 min. Extractions with phenol/chloroform/isoamyl alcohol (25:24:1) were made until the interphase was clean. Then a chloroform/isoamyl alcohol (24:1) extraction was made. Five hundred microlitres of 3 M sodium acetate and 5 ml isopropanol were added to the remaining aqueous phase and the mixture stored at room temperature for 2 h. Then it was centrifuged at 12000g for 10 min and the pellet washed with ethanol/TE buffer (10 mM TRIS-HCl, pH 8.0, and 1 mM EDTA) (70:30) and desiccated. Then it was dissolved in 2 ml TE buffer and RNase to a final concentration of 20 μg/ml was added. The solution was incubated at 37°C for 1 h and afterwards proteinase K to 20 μg/ml final concentration was added and incubation was carried out as above. The solution was treated with 1 vol. phenol and extracted with chloroform. The DNA was precipitated with 200 μl of 3 M sodium acetate and 1.2 ml isopropanol at 40 min at room temperature. After centrifugation at 12000g for 10 min the pellet was washed with ethanol/TE buffer, desiccated and dissolved in TE buffer.

Polymerase chain reaction (PCR) amplification. A DNA fragment containing the T. reesei endo β(1-3)glucanase gene, eggl, was amplified from total DNA using the PCR method with Taq polymerase (Promega, Madison, Wis., USA). The oligonucleotide primers used were 5'-GGGCGCGCCCTCAGTAC-3' for the encoding DNA strand and 5'-AAGGCGATTGGCAGTGT-3' for its complementary strand. The amplification consisted of 30 cycles as follows for each one: 30 s at 95°C for denaturation, 1 min at 52°C for annealing and 1 min at 72°C for polymerization. The amplified fragment was digested with KpnI and SacI and cloned in pBluescript II SK+ (Strategene, USA) digested with the same enzymes.

Nucleotide sequence determination. DNA was sequenced by the dideoxynucleotide chain termination procedure (Sanger et al. 1977) using commercial kits from Pharmacia on denatured double-stranded DNA templates. A series of nested deletions were obtained from larger cloned DNA fragments by the exonuclease III-S1 nucleic acid digestion method (Henikoff 1984). Selected deletion mutants or directed subcloned fragments were used as templates for sequencing.

T. longibrachiatum cDNA cloning. A simplified rapid amplification of cDNA ends (RACE) method (Frohman et al. 1988) was used to synthesize the T. longibrachiatum eggl cDNA. The mRNA was reverse transcribed using the oligonucleotide primer 5'-CGGTGAGCTGACAGAAGCTTTTAAAAATTCTT-3', which contains the recognition sequences for SalI, PstI and HindIII restriction endonucleases and AMV reverse transcriptase (Promega).

The polymerization was carried out by incubating for 1 h at 42°C and then for 30 min at 52°C. Amplification of eggl-specific double-stranded cDNA was achieved by using an oligonucleotide (5'-TCCGGAATCTCCATCCATAGTGGCTGAGATTG-3') similar to the eggl 5' end around the ATG start codon, but modified in order to create an artificial BamHI recognition site, and the oligonucleotide 5'-GGGTGAGCTGACAGAAGCTTTTAAAAATTCTT-3' identical to the first one used in reverse transcription but without the oligo-dT tail. The amplification consisted of a cycle of denaturation of cDNA-mRNA hybrids at 95°C for 5 min, annealing at 55°C for 5 min and 40 min at 72°C for polymerization followed by 40 cycles of 40 s at 95°C for denaturation, 1 min at 55°C for annealing and 3 min at 72°C for polymerization and a last polymerization step at 72°C for 15 min. The amplified fragment was digested with BamHI and HindIII and cloned in pUC18 digested with the same enzymes.

Results

Cloning of the eggl gene from T. longibrachiatum

We have carried out the following strategy to isolate the T. longibrachiatum eggl gene. Two oligonucleotides were designed (see Materials and methods) for the amplification of a fragment containing a portion of the T. reesei eggl gene (Penttila et al. 1986; van Arsdell et al. 1987). A 1.7-kb DNA fragment was amplified and we took advantage of the location of the KpnI and SacI sites, close to the termini of the fragment, to clone in the pBluescript II SK+ vector a slightly shorter fragment. When T. longibrachiatum DNA was utilized instead of T. reesei DNA, a fragment of the same length was amplified but it lacked the KpnI site (data not shown).