Degradation of cellulose by the bean-pathogenic fungus *Colletotrichum lindemuthianum*. Production of extracellular cellulolytic enzymes by cellulose induction

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Abstract

*Colletotrichum lindemuthianum* was able to grow and produce extracellular cellulolytic activity in a defined medium containing cellulose as the main carbon substrate. As measured either by the hydrolysis of 4-methylumbelliferonyl-β-D-cellotrioside or the release of glucose from carboxymethylcellulose, activity reached a peak after 13 days of incubation and then declined whereas growth markedly increased afterwards. Detection of glucose in carboxymethylcellulose hydrolysates suggested the concerted operation of endo-1,4-β-glucanase, cellobiohydrolase (exo-1,4-β-glucanase) and β-glucosidase activities. The highest levels of cellulolytic activity were obtained in media supplemented with cellulose and glutamate. Other carbon and nitrogen sources markedly influenced growth and enzyme production. Oligonucleotides homologous to specific regions of the cellobiohydrolase-encoding *cbhII* gene from *Trichoderma reesei* were used to isolate a *C. lindemuthianum cbhII*-DNA fragment whose sequence revealed homologies of 98% and 92% with the nucleotide and the deduced amino acid sequences of the corresponding *cbhII*-DNA of *T. reesei*, respectively. RT-PCR and Southern blot analyses of total RNA samples obtained from cellulose-grown but not from glucose-grown mycelium revealed the expression of the corresponding *cbhII* transcript. The *cbhII*-cDNA fragment was cloned and sequenced.

Abbreviations: *cbhII* – cellobiohydrolase gene; CMC – carboxymethylcellulose; 4-MU – 4-methylumbelliferone; 4-MU(Ch)₃ – 4-methylumbelliferonyl-β-D-cellotrioside; PDA – potato dextrose agar

Introduction

The ascomycete fungus *Colletotrichum lindemuthianum* is the etiological agent of anthracnose, one of the main diseases of bean (O’Connell et al. 1985). In addition to its relevance as an economically important phytopathogen, *C. lindemuthianum* and its host represent a convenient model to study the physiological and molecular bases of plant–pathogen interaction (Perfect et al. 1999).

*C. lindemuthianum* is an intracellular hemibiotrophic pathogen. Following cell penetration,
hyphae develop between the plasma membrane and the cell wall without penetrating the protoplast. Once a large area of the plant tissue has been colonized, necrotrophic hyphae develop (O’Connell and Bailey 1988) and this step closely correlates with the production of a set of host cell wall-degrading enzymes (Wijesundera et al. 1989; Knogge 1998). These include cutinase (Dickman and Patil 1986; Stahl and Shafer 1992), proteases (Ries and Albersheim 1973; Wijesundera et al. 1989), polygalacturonases (Anderson 1978; Keon et al. 1990), pectin and pectate lyases (Wijesundera et al. 1989), chitin deacetylase (Kauss and Bauch 1988; Tsigos and Bouriotis 1995), laccase (Anderson and Nicholson 1996) and 1,3-ß-glucanases (Daugrois et al. 1992). However, no information is presently available on enzymes that degrade cellulose, the main component of the plant cell wall, and their role in pathogenesis of the anthracnose-producing fungus. These studies might provide useful insights into the mechanism and dynamics of plant invasion by the pathogen. Cellulolytic enzymes have been studied in several fungi but the best characterized are the inducible cellulases of the saprophytic fungus Trichoderma reesei, where at least two cellobiohydrolases: CbhI and CbhII (ß-D-glucan cellobiohydrolase, EC 3.2.1.91); five endo-1,4-ß-glucanases: EgI through EgV (1,4-ß-D-glucan glucanohydrolase, EC 3.2.1.4.); and two ß-glucosidases synergistically operate in the complete degradation of cellulose (Teeri et al. 1987; Saloheimo et al. 1988, 1997, 2002; Kubicek et al. 1993; Kleman-Leyer et al. 1996; Carle-Urioste et al. 1997; Okada et al. 1998; Zeilinger et al. 1998). In T. reesei, cellobiohydrolases are considered as the key cellulases as they make 80% of the total cellulolytic protein and can achieve complete digestion of cellulose even in the absence of endo-1,4-ß-glucanases (Teeri 1997). Moreover, evidence for a determinant role of CbhII in the ability of the fungus to start growth on cellulose have been forwarded (Kubicek-Pranz et al. 1991; Seiboth et al. 1992).

Here we report the production of extracellular cellulolytic enzymes by C. lindemuthianum grown under different culture conditions and the isolation of a partial cbhII-DNA fragment and the corresponding cDNA from cellulose-induced mycelium.

Materials and methods

Strain and culture conditions

Colletotrichum lindemuthianum, race 1472, kindly provided by Dr. June Simpson (CINVESTAV-IPN, Unidad Irapuato, México), was maintained on potato dextrose agar (PDA, Difco) at 20 °C. For propagation, 200 ml-Erlenmeyer flasks containing 50 ml of modified Mathur’s medium: MgSO4·7H2O, 2.5 g; KH2PO4, 2.72 g; L-glutamic acid, 5.28 g; distilled water up to 1 l; final pH, 5.5 (Tu 1985) supplemented with 2.5% (w/v) cellulose (Sigmacell, type 101, Sigma) or other carbon sources (see below) were inoculated with 1.6 mg dry weight (about 5 cm2) of a 9 day-old colony growing on PDA and incubated at 20 °C with continuous shaking (150 rpm). After different periods of time, cultures were filtered and the filtrates were saved and used to determine protein and cellulolytic activity. Fungal growth was measured as the amount of mycelium protein since residual, undegraded cellulose in the medium interfered with dry weight quantitation. To this purpose, mycelium was filtered through Whatman No. 1 filter paper. After exhaustive washing with distilled water, it was quantitatively transferred to an assay tube, mixed with 1 ml of 1 N NaOH and incubated at room temperature. After 24–48 h, the sample was centrifuged at low speed, the supernatant was aspirated with a Pasteur pipette and used to measure protein as described below. In gene induction experiments, mycelium was pregrown for 8 days in potato dextrose medium, collected by careful decantation of medium, washed thrice with sterile distilled water and used to inoculate 500 ml-Erlenmeyer flasks containing 250 ml of modified Mathur’s medium supplemented with 2.5% cellulose. Flasks were incubated at 20 °C with continuous shaking and after different periods of time, mycelium was harvested by filtration, washed with water, stored at −85 °C and used for extraction of total RNA.

Assay of cellulolytic activity

Enzyme activity was measured by a fluorogenic and a chromogenic method using 4-methylumbelliferyl-ß-D-cellotrioside [4-MU(Ch)3, Sigma]