Temperature sensitive mutant of *Trichoderma reesei* defective in secretion of cellulase

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Summary. Temperature sensitive mutants of *Trichoderma reesei* derived from hypersecretory strain RL-P37 were isolated and characterized. Compared to the parent strain, one mutant (LU-ts 1) grew well in the mycelial phase at both permissive (25 °C) and non-permissive (37 °C) temperatures. However, the secretion of overall protein and active cellulases was significantly reduced in the mutant at the higher temperature. No accumulation of active cellulases or intracellular proteins was observed in the mycelia of LU-ts 1 at 37 °C. The inhibitory effects of temperature on cellulase secretion in LU-ts 1 were reversible. Isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoretic analyses confirmed that the secretion of the major cellulases was greatly reduced in LU-ts 1 at 37 °C. Molecular characterization of the various temperature sensitive secretion mutants of *T. reesei* should help elucidate the crucial aspects of the secretory pathway of this cellulolytic fungus.

Introduction

The cellulase complex of the mesophilic fungus *Trichoderma reesei* is capable of efficiently degrading crystalline cellulose to glucose and provides a potential application in the conversion and recycling of renewable cellulosic biomass to fermentable sugars. *Trichoderma reesei* is currently touted as the most potent cellulolytic enzyme producer (Mandels 1982). The group of cellulolytic enzymes from *T. reesei* consists of three different hydrolytic enzymes, endo-1,4-β-glucanase (EC 3.2.1.4), exo-1,4-β-glucanase (cellobiohydrolase, EC 3.2.1.91) and an associated enzyme, β-glucosidase (cellobiase, EC 3.2.1.21). These three enzymes are known to act synergistically to bring about the conversion of crystalline cellulose to cellobiose and glucose. Although detailed biochemical studies have been carried out on the *Trichoderma* cellulase enzymes and several of the genes have been cloned and characterized (Shoemaker et al. 1983; Teeri et al. 1983), little is known about the physiological mechanisms involved in the secretion of these enzymes.

Protein secretion by eukaryotic cells is mediated by a complex, highly organized series of membranous structures. Of all eukaryotic microorganisms, the protein secretory pathway is best understood in the yeast *Saccharomyces cerevisiae*. Much of the information concerning these processes in yeast have been obtained by isolating and characterizing temperature sensitive (ts) mutants which do not secrete proteins at the non-permissive temperature (Schekman and Novick 1982). We have adapted this rationale for studies of the secretion of cellulases in *Trichoderma*. We have chosen the hypersecretory strain RL-P37 as the parent for our work because the high yield of cellulases (Sheir-Neiss and Montenecourt 1984) should facilitate localization of internal enzymes. We report here the properties of ts mutants of *T. reesei* derived from this hypersecretory strain.

Materials and methods

Stock cultures and media

*T. reesei* hypersecretory strain RL-P37 and ts mutants derived from RL-P37 were grown on potato dextrose agar (Difco).
Complete medium contained 0.5% yeast extract (Difco), 0.5% tryptone (Difco), 1% glucose and trace element in Vogel’s medium (Vogel 1956). Casein plates consisted of 1% casein (Pfanstiehl Chemical Co., Waukegan, Ill.), 1% gelatin (Difco) in Vogel’s medium. Carboxymethylcellulose (CMC), ball beaten cellulose (BBC) and starch plates contained 0.5% CMC (4 M, Hercules Inc., Wilmington, Del.), 0.5% cellulose (Solka Floc) and 0.5% soluble starch (Sigma), respectively, in Mandels’ salt medium (Mandels and Andreetti 1978).

Isolation of temperature sensitive mutants

A spore suspension of RL-P37 was mutagenized with 0.05% nitrosoguanidine (NTG) in 0.05 M citrate buffer, pH 4.8, for 2 h at 25°C (~15% survival rate). The mutagenized spores were subjected to either Ludox gradient separation as described by Novick et al. (1980) or filtration enrichment. Following these procedures, the diluted samples were spread onto agar plates containing complete medium. After 3 days at 25°C, the colonies were replica-plated onto the complete medium and incubated overnight at 37°C. The colonies which did not grow or grew poorly were designated as potential temperature sensitive mutants.

Secretion screening procedure

The ts mutants were grown in liquid medium at 25°C in 5 ml vials containing potato dextrose broth, 0.5% yeast extract and 0.5% tryptone. The vials were inoculated with a loopful of conidia and were incubated without agitation for 7 days until a mycelial mat formed. The mat was removed with sterile forceps, washed in sterile water, placed on plates containing one of the following, CMC, BBC, casein or starch and incubated at 37°C for 2–3 days. A ring of precipitation on the casein plate and a clearing zone on the starch plate after staining with Grams iodine indicate protease and amylase activity, respectively. The CMC and BBC plates were further incubated overnight at 50°C followed by staining with Congo red (CMC) according to Teather and Wood (1982) or examination for clear zone formation (BBC) to detect the presence of secreted enzymes.

Sample culture conditions and preparation

Submerged fermentation studies were carried out in Vogel's medium containing 1% lactose (cellulase inducer) as the carbon source. Cultures were inoculated with 14-day old spores of the ts mutants or RL-P37 at a final concentration of 2 × 10⁸ spores/ml and incubated with shaking (250 rpm) at 25°C. After 48 h incubation at 25°C, the mycelia were collected by centrifugation, suspended in fresh lactose medium and incubated at 25 or 37°C for an additional 72 h. At the end of incubation, the mycelia were harvested and the supernatants were assayed for secreted enzymes and protein. Dry weight was determined by washing the mycelium with distilled water and drying to constant weight at 80°C.

For preparation of cytoplasmic and particle bound samples, the mycelia were collected by centrifugation at the final time point. The washed mycelia were suspended in 0.05 M citrate buffer, pH 4.8, and were disrupted in a Bead-Beater (Bio spec Products, Bartlesville, Okla.) with glass beads (0.45–0.50 mm; time 3 min). The ruptured cell mass was centrifuged at 40,000g for 30 min to separate the particulate matter from the cell sap. The resulting 40,000g pellets were homogenized with 0.05 M citrate buffer, pH 4.8. Particle bound and cytoplasmic enzyme activity as well as protein content were determined on these fractions.

Protein determination

The protein contents of the culture filtrate and cytoplasm were precipitated with 0.15% deoxycholate and 72% trichloroacetic acid (TCA) (Pettersen 1983) and the precipitates were assayed by the method of Lowry et al. (1951), with bovine serum albumin as a standard. The protein concentration of the particle bound sample was determined by the same method following extraction of cell proteins in hot 1 N NaOH for 10 min.

Enzyme assays

The cellulase enzymes were assayed by the methods of Mandels et al. (1976) in 0.05 M citrate buffer, pH 4.8, at 50°C. The substrates for the endoglucanase and filter paper assays were CMC (7L, Hercules Inc.) and filter paper (1 × 6 cm, Whatman at # 1, Maidstone, England), respectively. Reducing sugars were analyzed according to the dinitrosalicylic acid method (Miller 1969). β-glucosidase (cellobiase) activity was measured with cellulobiose (Sigma) as a substrate and glucose was measured enzymatically by the glucose oxidase/peroxidase assay.

Xylanase or amylase was assayed using 0.5% xylan (Ristroph 1981) or 1% starch (Whelan 1964) as substrate in 0.05 M citrate buffer, pH 4.8, at 50°C. The reducing sugars released were monitored by the dinitrosalicylic acid method. Proteolytic activity employing azocoll as a substrate was measured by incubating 0.2 ml of enzyme preparation with 1 ml of a suspension of 2.4% azocoll (Sigma) in 0.1 M potassium phosphate, pH 7.0, at 37°C overnight (Saheki and Holzer 1974). The reaction was terminated by the addition of 1 ml 10% TCA. After the centrifugation, the absorbance of the supernatant was determined at 520 nm.

Electrophoresis

Culture filtrates were concentrated by ultrafiltration using PM-10 membranes (Amicon, Denver, Mass.) for gel electrophoretic analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 11% slab gels according to Merivuori et al. (1985). Apparent molecular weights were calculated as described by Weber and Osborn (1969) using Sigma high molecular weight standards. Isoelectric focusing (IEF) was performed according to Sheir-Neiss and Montene court (1984).

The gels were stained for protein with Coomassie brilliant blue R-250 (Bio-Rad Lab., Richmond, Calif.) and for glycoprotein with periodic acid/Schiff’s reagent (Sigma). For localization of enzymatic activities in the IEF gels, the gels were overlayed with agarose containing ball beaten Avicel as described by Sheir-Neiss and Montene court (1984).

Results

Growth and secretion screening for ts mutants

*Trichoderma reesei* wild type and hypersecretory strains isolated to date grow equally well at 25