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Sequential release of cellulose enzymes during germination of *Trichoderma reesei* spores

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Abstract. The pattern of release of extracellular cellulase during the germination of *Trichoderma reesei* spores has been examined. The four enzymes namely, filter paper degrading enzyme, β-1,4 endoglucanase, β-glucosidase and xylanase appear sequentially in the culture broth during germination of the spores. The order of enzyme appearance is not altered by the type of carbon source in the germination medium. Measureable quantities of filter paper degrading enzyme is detected only after the outgrowth has occurred. A possible mechanism of spore germination and induction of the enzymes by insoluble cellulose is suggested.

Keywords. *Trichoderma reesei,* cellulases; spore germination; enzyme synthesis and release.

Introduction

The degradation of crystalline cellulose to glucose involves the cooperative action of atleast three enzymes namely, β-1,4 exoglucanase (cellobiohydrolase, EC3.2.1.–) β-1,4 endoglucanase, (EC.3.2.1.21) (Wood, 1975; Petterson, 1975, Wood and McCrae, 1978). In addition, the hydrolysis of native cellulose containing hemicellulose, also needs the cooperative action of hemicellulases (Ghose and Bisaria, 1979; Wood, 1980). A variety of fungi are known to produce these enzymes extracellularly (Mandels and Andreotti, 1978) but among these, *Trichoderma reesei* QM 6A and its mutants are the best known. In this organism, the cellulase enzymes are inducible and the best inducer known todate is the insoluble substrate namely, cellulose (Mandels, 1975). In studies on cellulase production by this organism, generally spores are used as the inoculum. There is, however, no information either with regard to the early events during spore germination and outgrowth in a medium containing cellulose or as to how this insoluble polysaccharide induces the synthesis of these enzymes, especially the exoglucanase and endoglucanase. Using a thick spore suspension as the inoculum and short sampling intervals, we have tried to determine the pattern of enzyme release and its role in the early stages of spore germination and outgrowth.

Materials and methods

Culture conditions and enzyme assays: *T. reesei* QM 6A and its mutant QM 9414 used in this study were from the Army laboratory, Natick, Massachusetts USA and

were maintained on Vogel's glucose-agar slants (Vogel, 1956). For germination studies, spores of QM 9414 from 3-5 day old slants were suspended in sterile water and mixed on a vortex mixer for 5 min. The spore suspension was used to inoculate 25 ml of synthetic medium (Reese *et al.,* 1950) containing, unless otherwise stated, 1 % cellulose (Carl Schleicher and Schull, West Germany) in 150 ml conical flasks. An initial concentration of about 5-6 × 10^7 spores/ml of the medium was used. The flasks were incubated on a rotary shaker at30°C(230 rpm) and duplicate flasks were withdrawn at intervals of 6 h, filtered through a gooch crucible and the clear supernatants used for enzyme assays. Filter paper degrading enzyme (Fpase), β-1,4 endoglucanase (CMCase), β-glucosidase and xylanase were assayed using Whatman No. 1 filterpaper strips (50 mg), carboxymethyl cellulose (CMC, BDH grade), *p*-nitrophenyl β-D glucoside (B-PNG, Sigma Chemical Co., St. Louis, Missouri, USA) and xylan (Sigma), respectively, using standard procedures (Mandels, 1975; Herr, 1979; Linko *et al.,* 1978). Enzyme activity is expressed in international units defined as micromoles of product formed/min/ml of culture filtrate. Spore germination was monitored microscopically using a drop of the culture medium before filtration. The enzyme content of the spores was determined by sonicating spores (about 1-2 × 10^9 spores/ml, 10 ml) suspended in citrate buffer (0.05M, pH 4.8) for 10 min using an ultrasonic cell disintegrator (Braunsonic, 1510) with intermittant cooling. The sonicated sample was then centrifuged at 13,000 *g* for 20 min in an I.E.C. refrigerated centrifuge (4°C) and the clear supernatant was used for enzyme assays. Protein content of the spore extracts was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Results

Fungal and bacterial spores are dormant structures and the pattern of macromolecülar synthesis during germination not only represents the events that occur during the return to vegetative growth but also represents the order of gene expression during this period (Steinberg and Halvorson, 1968; Garg and Tauro, 1973).The germinating *T. reesei* spore is therefore an ideal test system to understand the sequence of gene expression and also to know the early stages of cellulose hydrolysis.

Sequence of enzyme release in cellulose medium

To determine the sequence of gene expression during germination, the order of appearance of the enzymes in the culture broth was examined (figure 1). When spores were allowed to germinate in cellulose medium, the first enzyme to be detected in the culture filtrate was β-glucosidase, which appeared between 6–12 h. The level of this enzyme thereafter remained constant till about 30-36 h when a second burst of enzyme release occurred. The two main enzymes involved in the initial stages of cellulose hydrolysis, namely filter paper degrading enzyme and endoglucanase appeared between 36-42 h and 24-36 h, respectively. Xylanase, which is presumed to have a role in the hydrolysis of native cellulose, appeared between 12 and 18 h. Thus, the release of the enzymes from the germinating spores was not simultaneous but sequential. Microscopic examination of the culture broths indicated that the spore germination and outgrowth was complete between

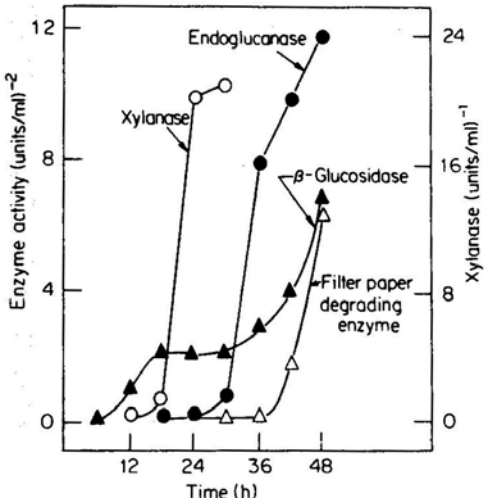

Figure 1. Sequence of enzyme release during the germination of *T. reesei* spores in cellulose medium.

24–30 h while measurable quantities of filter paper degrading enzyme appeared much later in the culture medium.

The early appearance of the β-glucosidase and the late release of endoglucanase and filter paper degrading enzyme during the germination of the spores raised questions regarding the role of this enzyme in the initial stages of cellulose hydrolysis. To examine this, two approaches were used. First, the pattern of enzyme release during the germination of spores in a medium containing glucose as the carbon source was examined. In this organism, glucose is known to repress the synthesis of β-glucosidase (Mandels, 1975). Secondly, the spores were also analyzed for their enzyme composition.

Enzyme release in glucose medium

In glucose medium, the only extracellular enzyme seen during the first 48 h was β-glucosidase, which appeared between 6-12 h as before in the cellulose medium (figure 2). However, unlike in the cellulose medium, the level of this enzyme gradually decreased and there was no second burst of enzyme release. It therefore

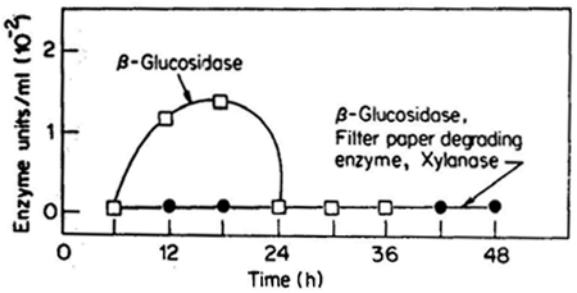

Figure 2. Release of β-glucosidase during the germination of *T. reesei* spores in glucosidase medium.

appears that this first burst of β-glucosidase corresponds to the release of the spore enzyme, while the second burst represents the release of newly synthesized enzyme.

Enzyme content of spores

Further, the enzyme composition of the dormant spores was examined (table 1). It was found that the spores contain a high level of β-glucosidase but relatively lower levels of endoglucanase and xylanase. Filter paper degrading enzyme could not be

Table 1. Enzyme composition of *T. reesei* QM 9414 spores.

Enzyme	IU/ml extract	Sp. activity*
β-Glucosidase	0.51	2.00
Endoglucanase	0.009	0.036
Filter paper degrading enzyme	Not detectable	Not detectable
Xylanase	0.01	0.04

About 1-2 × 10^{10} spores in 10 ml of citrate buffer (pH 4.8,0.05 M) were sonicated for 10 min using a ultrasonic cell disintegrator, centrifuged and the supernatant was used for enzyme and protein determination.

* IU/mg soluble protein.

detected in these extracts by the method used for its assay. From these studies, the true order of release of the new enzymes,· as determined by the time of their appearance during the germination of *T. reesei* spores, appears to be xylanase, endoglucanase, β-glucosidase and filter paper degrading enzyme.

Sequence of enzyme release in phosphocellulose or xylan medium.

If the order of enzyme appearance during spore germination in cellulose medium reflects the order of gene expression, then one would expect that this order should be similar and independent of the carbon source in the growth medium. To test this, the time of enzyme release during spore germination in media containing either phosphocellulose or xylan was examined (figure 3). In this organism,

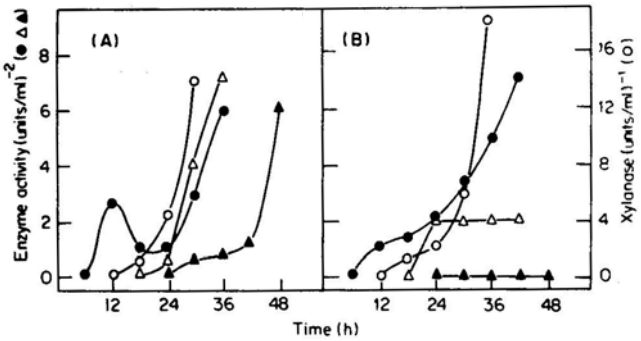

Figure 3. Pattern of enzyme release during the germination of *T. reesei*spores in phosphor-cellulose (A) and in Xylan (B) media.

The germination mediumcontained either 1% phosphocellulose or 0.5% xylanas the carbon source. (O): Xylase(▲): Filter paper degrading enzyme (△): Endoglucanase and (●):β-glucosidase.

phosphocellulose and xylan specifically elevate the levels of endoglucanase and β-glucosidase, respectively (Chaudhary, 1981). However, in media containing these substrates as carbon sources, the order of appearance of these enzymes is similar to that in cellulose medium. Thus, although the level of induction is altered, the time and order of appearance remains similar. It is therefore concluded that the order of appearance of enzyme in cellulose medium represents the true order of gene expression during the germination of the *T. reesei* spores.

Order of enzyme release in QM 6A

T. reesei QM 9414 is a mutant of strain 6A and produces a higher level of cellulose enzymes in cellulose medium (Mandels, 1975). To test whether the order of enzyme release was similar to that in the parent or whether it was altered, spores of both cultures were allowed to germinate in cellulose medium separately and the pattern of enzyme release determined as before. The sequence of enzyme release was identical in both the cultures although in mutant QM 9414, the release of these enzymes occurred slightly earlier than in the parent culture.

Discussion

Sequential synthesis of enzymes during the germination of bacterial and fungal spores has been well documented and since the germinating spores represent a synchronous system, the time of enzyme synthesis is presumed to approximate the time of gene expression (Steinberg and Halvorson, 1968). However enzyme synthesis and release are two different phenomena and it is difficult to conclude that the time of appearance of enzymes in the culture broth represents the exact time of gene expression. Although the time of appearance may not represent the exact time of synthesis, the order of appearance can be taken to represent approximately the order of gene expression during the germination of *T. reesei* spores. The four enzymes examined in this paper appear in a definite sequence irrespective of the carbon source in the growth medium.

The sequence in which the enzymes appear in the culture broth suggests that the presence of new enzymes is not required for the early stages of germination and growth. The new enzymes increase in amount only after outgrowth has occurred. This also suggests that the two spore enzymes namely the endoglucanase and β-glucosidase are adequate to generate enough soluble sugars to allow early stages of growth. Commercial cellulose contains substantial amounts of amorphous cellulose and it is likely that the small amount of spore endoglucanase released during the early stages of germination initiates its hydrolysis and it is then converted into glucose by the spore β-glucosidase. The requirement of exoglucanase enzyme is therefore necessary only when the crystalline regions of the cellulose are to be hydrolysed. These conclusions are consistent with the current predictions for cellulose hydrolysis by microbial cellulases (Wood and McCrae, 1978).

The mechanism of induction of filter paper degrading enzyme and endoglucanases by insoluble cellulose during the early stages of spore growth is at present not clear. It is likely that during the early stages of germination, the spore endoglucanase is responsible for the production of soluble cellodextrins which

then enter the germinating spores and induce both these enzymes. Alternatively, it is also likely that cellulose interacts with a specific cell surface receptor and triggers the induction of these enzymes, a mechanism similar to that described in Eucaryotic cells (Davidson and Britten, 1979). However, both these mechanisms need to be investigated.

The synthesis and release of xylanase very early during the germination process is consistent with its predicted role in native cellulose hydrolysis. Wood (1980) has reported that the extent of native cellulose hydrolysis can be increased by prior treatment with hemicellulases. Apparently, this fungus has evolved such a mechanism to enable it to first digest the hemicelluloses before it digests other regions of the native cellulose using the other enzymes.

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