Synthesis of Cellulase by *Mucor pusillus* and *Mucor miehei*

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SUMMARY

Strains of *Mucor pusillus* and *M. miehei* were found to synthesize β -1,4-glucan glucanohydrolase (cellulase) in a complex medium. The inducible enzyme complex hydrolysed carboxymethylcellulose, acid-swollen cellulose and unmodified cellulose.

INTRODUCTION

Several investigators have reported that thermophilic species of *Mucor* are non-cellulolytic (Chang, 1967; Rész, 1968; Eggins & Malik, 1969; Fergus, 1969; Tansey, 1971). On the other hand, *Mucor pusillus* was reported to produce cellulase (β -1,4-glucan glucanohydrolase, EC. 3.2.1.4) by Somkuti, Babel & Somkuti (1969). However, the culture used by Somkuti *et al.* was subsequently identified as *M. miehei* by J. J. Ellis (Department of Agriculture, Peoria, Illinois, U.S.A.). In view of this development and the persistence of negative data in recent literature concerning the cellulolytic ability of thermophilic *Mucor* fungi (Tansey, 1971) we have re-examined previous findings, extending the investigation to include several strains of *M. pusillus* and *M. miehei*.

METHODS

Cultures and culture maintenance. *Mucor pusillus* Cooney & Emerson, strains 6 and 8, were obtained from D. J. Cooney (University of Nevada). *Mucor pusillus* PCC410 originated from the Purdue University Culture Collection and its identity was verified by D. J. Cooney (personal communication). *Mucor miehei* Cooney & Emerson was a gift from D. J. Cooney; *Mucor miehei* NRRL2543 and NRRL3169 were obtained from J. J. Ellis. Stock cultures were propagated on 10% (w/v) potato malt agar (Difco) slants at 37 °C and transferred weekly.

Production of cellulase. A 4% (w/v) wheat-bran suspension in deionized water was kept at boiling point for 30 min. The suspension was filtered through gauze, dispensed in 1 l Erlenmeyer flasks (300 ml/flask), and autoclaved at 121 °C for 20 min. The pH before autoclaving was 6.8 and was not adjusted. Inoculum was prepared by flooding agar slants, each with 10 ml of sterile deionized water. Incubation was on a gyrotory shaker at 37 °C for 7 days. The flasks were cooled to 4 °C and the broth was filtered through four layers of sterile gauze into pre-chilled vessels. The filtrates were centrifuged at 10000 g at 4 °C for 20 min. The supernatants served as the crude cellulase preparations and were used immediately.

Assay of cellulase and β -glucosidase activity. Protein content of the various enzyme preparations was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as the standard. In the determination of cellulase activity, the

following carboxymethylcellulose (CMC) preparations, each with a different degree of substitution (d.s.) and/or viscosity range (v.r.), were employed: CMC 4H1F (d.s. 0.4, v.r. high), CMC 7LF (d.s. 0.7, v.r. low), CMC 7MF (d.s. 0.7, v.r. medium), CMC 7HF (d.s. 0.7, v.r. high), CMC 9M8F (d.s. 0.9, v.r. medium), and CMC 12M8P (d.s. 1.2, v.r., medium). All CMC preparations were obtained from Hercules Inc., Oak Brook, Illinois, U.S.A. Stock solutions of substrates (0.25 %, w/v) were prepared in 0.1 M-sodium acetate buffer, pH 5.0. In the enzyme assays, 0.5 ml enzyme was mixed with 1.5 ml substrate and incubated at 37 °C. The amount of reducing sugar produced from CMC was calculated as glucose according to a modification of Bernfeld's method (1955). One unit of enzyme activity was defined as the amount of reducing sugar, in μmol , liberated by 1 mg of enzyme protein/h at 37 °C.

In the assay of β -glucosidase activity, *p*-nitrophenyl- β -D-glycopyranoside (PNPG, Sigma Chemical Co., St Louis, Missouri, U.S.A.) was used. One ml of a 10^{-4} M-PNPG stock solution prepared in 0.1 M-sodium acetate (pH 5.0) was mixed with 1 ml crude enzyme solution and incubated for 1 h at 30 °C. The reaction was stopped by adding 2 ml of 1 M- Na_2CO_3 solution. Absorbance was read at 400 nm in a Beckman DU spectrophotometer. One unit of β -glucosidase activity was defined as the amount (μmol) of PNPG hydrolysed/h by 1 mg of enzyme protein.

Hydrolysis of acid-swollen cellulose, CMC, unmodified cellulose and cellobiose, and the identification of hydrolytic products. Acid-swollen cellulose was prepared according to the method of Walseth (1952). Other substrates were Whatman ashless cellulose powder (standard grade), CMC 4H1F, and cellobiose. To 100 mg of substrate (except for cellobiose, of which only 10 mg were used) suspended in 8 ml of 0.1 M-acetate buffer (pH 5.0), were added 2 ml of enzyme solution. The reaction mixture was covered with toluene and incubated at 37 °C for varying lengths of time. The products of hydrolysis were identified by paper chromatography. Reaction mixtures were treated with MB-3 mixed-bed ion-exchanger (Mallinckrodt) and filtered through Whatman no. 42 filter paper. The filtrates were evaporated to dryness under heat lamps. The residues were dissolved in deionized water and spotted on Whatman no. 4 chromatography paper. Paper chromatograms were developed in an isopropanol-*n*-butanol-water (140:20:40, v/v) system at 25 °C for 16 h. Carbohydrates were reacted with triphenyltetrazolium chloride or AgNO_3 as described by Trevelyan, Procter & Harrison (1950).

RESULTS

*Cellulase and β -glucosidase activity of *Mucor pusillus* and *M. miehei**

The results of a time study indicated that all six strains had the capacity to hydrolyse CMC. The total amount of reducing sugar produced after 6 h of incubation ranged from 3 to 21 μmol , indicating a large degree of fluctuation in cellulase activity among the various strains of the two species cultivated under identical conditions. It was also evident that 1 h incubation time was long enough to measure the activity of even the least active preparation (*Mucor miehei* Cooney & Emerson). The specific activity of each enzyme preparation was determined for a number of CMC preparations (Table 1). A correlation was found between the activity of enzymes and the molecular organization of the substrates. As a rule, with increasing degree of substitution in the substrate the specific cellulase activity of the crude enzyme diminished. This was true for all enzyme preparations examined, which would indicate the likely presence of a β -1,4-glucan endo-hydrolase in the enzyme complex requiring the availability of adjacent unsubstituted anhydroglucose units for activity. There was also a clear, though less significant, negative correlation between the

Table 1. *Specific activity of *Mucor pusillus* and *M. miehei* β -1,4-glucan glucanohydrolase with various carboxymethylcellulase preparations*

Source of enzyme	Glucose liberated from CMC by 1 mg of enzyme protein/h at 37 °C (μ mol)					
	4H1F	7LF	7MF	7HF	9M8F	12M8P
<i>Mucor pusillus</i> PCC410	4.05	2.70	2.48	1.99	1.48	0.70
<i>M. pusillus</i> Cooney & Emerson (strain 6)	5.60	3.94	3.40	3.15	2.02	1.10
<i>M. pusillus</i> Cooney & Emerson (strain 8)	1.80	1.65	1.47	1.32	1.02	0.63
<i>M. miehei</i> NRRL2543	2.33	2.06	1.92	1.65	1.37	0.88
<i>M. miehei</i> NRRL3169	3.85	3.85	3.08	2.88	2.14	1.32
<i>M. miehei</i> Cooney & Emerson	0.70	0.64	0.58	0.50	0.44	0.35

viscosity ranges of a particular substrate having the same degree of substitution, and cellulase activity. Apparently, the increased viscosity of the substrate presented a physical barrier to the formation of the enzyme-substrate complex.

The specific activity of β -glucosidase also varied among the cultures (from 0.23 to 0.62 enzyme units/mg protein) and there was no correlation between the respective specific activities of cellulase and β -glucosidase present in the same enzyme preparation.

Paper chromatography of hydrolytic products

A time study was carried out on the hydrolysis of carboxymethylcellulose (CMC 4H1F) and cellobiose by crude enzyme preparations of *Mucor pusillus* Cooney & Emerson (strain 6) and *M. miehei* NRRL2543. Reaction mixtures were incubated at 30 °C and samples were taken for sugar analysis at pre-determined intervals. The chromatographic evidence clearly showed that both *Mucor pusillus* and *Mucor miehei* hydrolysed CMC to glucose. Also, as the incubation time increased, more slowly-moving components appeared on the chromatograms.

The β -glucosidase activity of crude enzyme preparations was examined with cellobiose as the substrate. The chromatograms indicated the almost complete hydrolysis of cellobiose to glucose. Transient products with low R_F values were also observed, suggesting the presence of glycosyltransferase activity in the enzyme preparations. However, these transient products also appeared to be susceptible to hydrolytic attack by one or more components of the enzyme complex because they became nearly undetectable towards the end of incubation.

The hydrolysis of unmodified cellulose and acid-swollen cellulose by all six cultures was also examined (Fig. 1, 2) by using partially purified enzyme preparations devoid of β -glucosidase activity (Somkuti *et al.* 1969). Only glucose was present on chromatograms when unmodified cellulose served as the substrate, but both cellobiose and glucose were detected when the substrate was acid-swollen cellulose. Unmodified cellulose, because of its highly crystalline structure, may have been attacked by a β -1,4-glucan exo-hydrolase component of the enzyme complex, and its limited activity was probably restricted to the amorphous regions of the cellulose molecule. Acid-swollen cellulose, on the other hand, appeared to be open to attack by both β -1,4-glucan endo-hydrolase and exo-hydrolase components of the enzyme complex, giving rise to both glucose and cellobiose.

1 2 3 4 5 6 7

Fig. 1

1 2 3 4 5 6 7

Fig. 2

Fig. 1. Hydrolysis of unmodified cellulose by *Mucor pusillus* and *M. miehei*. 1. *M. pusillus* PCC410; 2. *M. pusillus* Cooney & Emerson (strain 6); 3. *M. pusillus* Cooney & Emerson (strain 8); 4. *M. miehei* NRRL2543; 5. glucose (G) and cellobiose (CB) standards; 6. *M. miehei* NRRL3169; 7. *M. miehei* Cooney & Emerson. Incubation time: 72 h; reagent: triphenyltetrazolium chloride.

Fig. 2. Hydrolysis of acid-swollen cellulose by *Mucor pusillus* and *M. miehei*. 1. *M. pusillus* PCC410; 2. *M. pusillus* Cooney & Emerson (strain 6); 3. *M. pusillus* Cooney & Emerson (strain 8); 4. *M. miehei* NRRL2543; 5. glucose (G) and cellobiose (CB) standards; 6. *M. miehei* NRRL3169; 7. *M. miehei* Cooney & Emerson. Incubation time: 48 h; reagent: triphenyltetrazolium chloride.

Growth and cellulase production in chemically defined media

Mucor pusillus PCC410 and *M. miehei* NRRL3169 were cultivated in a chemically defined medium (Gordon, Stewart & Clark-Walker, 1971) in which either the carboxymethyl-cellulose preparation CMC 4H1F (0.25 %, w/v) or glucose (2 %, w/v) was incorporated as the sole carbon source. After 7 days of incubation at 37 °C, good growth was observed in both types of medium. Cellulase activity of mycelium-free filtrates was measured with CMC 4H1F as the substrate. With glucose as the carbon source, cellulase activity was not measurable in the filtrates; however, filtrates of both cultures grown in CMC 4H1F-containing medium displayed cellulase activity, averaging 1.8 enzyme units/mg protein.

DISCUSSION

The evidence presented here supports the suggestion (Somkuti *et al.* 1969) that thermophilic species of *Mucor* possess the capacity to hydrolyse cellulose and its derivatives. The inducibility of the enzyme complex in *Mucor miehei* and *M. pusillus* was demonstrated by their failure to synthesize cellulase in a defined medium with glucose as the carbon source.

These observations are at variance with those of Chang (1967), Eggins & Malik (1969), Fergus (1969), and Tansey (1971). However, as a rule these investigators employed the most resistant forms of cellulose in their selective media and enzyme assays. Methods such as the measurement of the depth of clearing in cellulose-agar tubes (Tansey, 1971) or the disruption of filter paper, are simply not sensitive enough to detect the activity of culture filtrates of weakly cellulolytic organisms. The selective medium employed by Fergus (1969) included, in addition to a cellulose derivative (CMC), components such as urea that on their own may serve as the source of carbon for *Mucor pusillus* (G. A. Somkuti, unpublished observations). Therefore the repression of cellulase synthesis under such conditions is to be expected.

We do not suggest that either *Mucor pusillus* or *M. miehei* plays a primary role in the degradation of cellulose in nature; the experimental data indicate that not one component of the enzyme complex of these fungi would qualify as the so-called C_1 component of a cellulase complex as defined by King & Vessal (1969) that is necessary for the solubilization of native cellulose. However, it is known that the C_1 component, when freed from the β -1,4-glucan glucanohydrolases of the cellulase complex, is practically without activity; this indicates a strong synergistic action on cellulose between the two types of enzyme components. Furthermore, cross-synergism between the C_1 and β -1,4-glucan glucanohydrolase components of the cellulase complexes of different fungi has also been demonstrated (Selby, 1969). Thus, the possibility cannot be excluded that both *M. pusillus* and *M. miehei* contribute to the degradation of cellulosic materials in nature, by virtue of cross-synergism between their own β -1,4-glucan glucanohydrolases and the C_1 components of the cellulase complexes of other fungi.

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