Production, purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*☆

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Abstract

A newly isolated strain of the fungus, *Mucor circinelloides* (NRRL 26519), when grown on lactose, cellobiose, or Sigmacell 50 produces complete cellulase (endoglucanase, cellobiohydrolase, and 3-glucosidase) system. The extracellular endoglucanase (EG) was purified to homogeneity from the culture supernatant by ethanol precipitation (75%, v/v), CM Bio-Gel A column chromatography, and Bio-Gel A-0.5 m gel filtration. The purified EG (specific activity 43.33 U/mg protein) was a monomeric protein with a molecular weight of 27 000. The optimum temperature and pH for the action of the enzyme were at 55°C and 4.0-6.0, respectively. The purified enzyme was fully stable at pH 4.0-7.0 and temperature up to 60°C. It hydrolysed carboxymethyl cellulose and insoluble cellulose substrates (Avicel, Solka-floc, and Sigmacell 50) to soluble celodextrins. No glucose, cellobiose, and short chain cellobiosaccharides were formed from these substrates. The purified EG could not degrade oat spelt xylan and larch wood xylan. It bound to Avicell, Solka-floc, and Sigmacell 50 at pH 5.0 and the bound enzyme was released by changing the pH to 8.0. The enzyme activity was enhanced by 27 ± 5 and 44 ± 14% by the addition of 5 mM MgCl₂ and 0.5 mM CoCl₂, respectively, to the reaction mixture. Comparative properties of this enzyme with other fungal EGs are presented. Published by Elsevier Ltd.

Keywords: Cellulase; Endoglucanase; *Mucor circinelloides*; Cellulose; Carboxymethyl cellulose; Carboxymethyl cellulase

1. Introduction

Cellulose, a major polysaccharide constituent of plant cell walls, is a β-1,4 linked linear polymer of 8000–12 000 glucose units. Three major enzymes are involved in the degradation of cellulose to glucose: endoglucanase (endo-1,4-β-d-glucanase, EG, EC 3.2.1.4), cellobiohydrolase (exo-1,4-β-d-glucanase, CBH, EC 3.2.1.91), and 3-glucosidase (1,4-β-d-glucosidase, BG, EC 3.2.1.21). EG acts in random fashion, cleaving 3-linked bonds within the cellulose molecule; CBH removes cellobiose units from the nonreducing ends of the cellulose chain and BG degrades cellobiose and cellobiosaccharides to glucose. The *Trichoderma reesei* cellulase system has been the most widely studied among the cellulolytic fungi [1].

In the USA, the production of fuel alcohol from corn starch reached about 2.1 billion gallons in 2002. Research emphasis is to use various waste and under-utilised agricultural residues as a source of low-cost feedstock for production of fuel alcohol. One constraint is the high cost and low effectiveness of commercial cellulases. In previous research, it was determined that solubilised corn fibre heteroxylan is very resistant to degradation by commercially available hemicellulases [2]. The author then isolated three fungal cultures (*Fusarium proliferatum*, *F. verticilloides*, and *Mucor circinelloides*) the enzyme system of which degrades corn fibre xylan well [3,4]. However, only one strain (*M. circinelloides*) was able to produce a complete cellulase enzyme system (EG, CBH, and BG) which converts various cellulose substrates to glucose. The other two strains produce little cellulase. In this paper, the author reports the production, purification, and properties of EG from this newly isolated *M. circinelloides* strain.

☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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2. Materials and methods

2.1. Materials

Carboxymethyl cellulose (CMC), Avicel, Sigmacell Type 50 (50 \(\mu\)m), lactose, cellobiose, all aryl-glycosides, and molecular weight (MW) markers for gel filtration were purchased from Sigma Chemical Co., St. Louis, MO. MW markers and precast gels for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), CM Bio-Gel A, Aminex HPX-87C column for high pressure liquid chromatography (HPLC) were obtained from Bio-Rad Laboratories, Hercules, CA. Corn steep liquor (Solulys K) was provided by Roquette America, Inc., Keokuk, IA.

2.2. Cultivation and enzyme production

The fungal strain \((M. \text{circinelloides} \text{v. Tieghem})\) used in this study was isolated by screening 132 soil samples surrounding decaying corn and wood using a procedure described previously [3]. It was identified by Centralbureau voor Schimmelcultures, Institute of the Royal Netherlands Academy of Arts and Sciences and deposited in the ARS Culture Collection, NCAUR, Peoria, IL (designated NRRL 26519).

The medium used for seed culture and enzyme production contained lactose (10 g/l) and corn steep liquor (5 g/l). Lactose was sterilised separately. The pH of the medium was adjusted to 5.0 with 1 M HCl before inoculation. A 250-ml Erlenmeyer flask containing 100 ml medium was inoculated with a loopful of cells taken from a stock slant and incubated at 30 \(^\circ\)C on a rotary shaker (200 rpm) for 2 days. Shake flasks (11 Erlenmeyer flask containing 400 ml medium) were inoculated with 20 ml of this culture and cultivated on a rotary shaker (200 rpm) at 30 \(^\circ\)C. After 4 days, the cells were removed from the culture broth by centrifugation (18000 \(\times\) g, 30 min). The resulting supernatant solution was used as the crude enzyme preparation.

2.3. Enzyme assays

EG (carboxymethyl cellulase, CMCase) activity was assayed in a reaction mixture (0.5 ml) containing 1\% (w/v) boiled CMC solution, 50 mM acetate buffer, pH 5.0, and appropriately diluted enzyme solution. After 30 min incubation at 50 \(^\circ\)C, the reducing sugar liberated in the reaction mixture was measured by the dinitrosalicylic acid method [5]. One unit (U) of EG or CMCase activity is defined as the amount of enzyme which produces 1 \(\mu\)mole reducing sugar as glucose per min in the reaction mixture under the specified conditions.

CBH and BG activities were assayed in a reaction mixture (1 ml) containing 5 mM \(p\)-nitrophenyl-\(\beta\)-D-cellubioside and 5 mM \(p\)-nitrophenyl-\(\beta\)-D-glucoside, respectively, 50 mM acetate buffer, pH 5.0, and appropriately diluted enzyme solutions. After incubation at 50 \(^\circ\)C for 30 min, the reaction was stopped by adding 1 ml of ice-cold 0.5 M Na\(_2\)CO\(_3\) and the colour that developed as a result of \(p\)-nitrophenol (\(p\)NP) liberation was measured at 405 nm. One unit (U) of each enzyme activity is defined as the amount of enzyme that releases 1 \(\mu\)mole \(p\)NP per min in the reaction mixture under these assay conditions.

2.4. Purification of endoglucanase

All purification steps were performed at 4 \(^\circ\)C, unless otherwise stated.

2.4.1. Ethanol precipitation

The culture broth (2680 ml) was concentrated by ultrafiltration with a stirred cell (Model 202, Amicon, Inc., Beverly, MA) equipped with a YM5 membrane under nitrogen pressure of 20 psi. Chilled ethanol was added drop-wise to the concentrated enzyme solution (1000 ml) while stirring to give an ethanol concentration of 75\% (v/v) and the whole broth was left overnight. The precipitate formed was collected by centrifugation at 48 000 \(\times\) g for 30 min, dissolved in 50 mM acetate buffer, pH 4.5, and dialysed overnight against the same buffer.

2.4.2. CM Bio-Gel A column chromatography

The dialysed enzyme solution (410 ml) was applied to a CM Bio-Gel A column (2.5 cm \(\times\) 26 cm) pre-equilibrated with 50 mM acetate buffer, pH 4.5. The column was washed extensively with the same buffer and eluted with a gradient of 0–0.5 M NaCl in the same buffer (300 ml each) at a flow rate of 30 ml/h. The EG activity eluted as a single enzyme peak. The active enzyme fractions (175 ml) were pooled and dialysed overnight against 50 mM acetate buffer, pH 5.0.

2.4.3. Gel filtration on Bio-Gel A-0.5 m

The EG was further purified by gel filtration on a Bio-Gel A-0.5 m column (1.5 cm \(\times\) 120 cm) pre-equilibrated with 50 mM acetate buffer, pH 5.0. The dialysed enzyme solution from CM Bio-Gel column chromatography was concentrated to about 1 ml by membrane (YM5) ultrafiltration. It was then applied to the column and eluted with the same buffer at a flow rate of 9 ml/h. The EG activity eluted as a single peak of protein. The highly active EG fractions were pooled, concentrated by ultrafiltration (YM 5 membrane), and used as purified EG for subsequent studies.

2.5. Adsorption of endoglucanase onto cellulose and elution

The EG in 50 mM acetate buffer, pH 5.0 was applied to prewashed Avicel, Solka-floc, or Sigmacell 50 and stirred occasionally at 4 \(^\circ\)C for 1 h. The liquid was removed by centrifugation (14 000 rpm; 15 min) and the residual EG activity in the supernatant solution was assayed. For the elution of the enzyme, 50 mM phosphate buffer, pH 8.0 containing 0.1 M NaCl was applied to the residue, mixed, and stirred
occasionally for 2 h at 4°C and the activity in the super­
natant solution was assayed.

2.6. Other methods

Protein was determined by the method of Lowry et al. [6]
with bovine serum albumin as the standard. Protein in the
column effluents was monitored by measuring absorbance at
280 nm. SDS-PAGE was performed on a 12% gel according
to Laemmli [7]. The MW of the native enzyme was deter­
mained by gel filtration on Bio-Gel A-0.5 m as described by
Andrews [8]. Cellulose hydrolysis product analysis was per­
formed by HPLC (Spectra-Physics, San Jose, CA) using an
ion moderated partition chromatography column (Aminex
HPX-87C). The column was maintained at 85°C, and the
sugars were eluted with Milli-Q (Millipore Corp., Bedford,
MA) water at a flow rate of 0.6 ml/min. Peaks were detected
by refractive index and identified and quantified by compari­
sion to retention times of authentic standards such as glucose
and cellobiose.

3. Results

3.1. Production of endoglucanase

The fungus produced cellulase extracellularly when
grown on lactose, cellulose, and Sigmacell 50. The ex­
tracellular enzyme system contained EG, CBH, and BG
activities and hydrolysed CMC, Avicel, Solka-floc, Sigma­
cell 50, and filter paper at the relative rates of 100, 29, 27,
38, and 22%, respectively. The final hydrolytic product of
CMC, Avicel, Solka-floc, as well as Sigmacell 50 by the
crude enzyme preparation was only glucose as analysed by
HPLC. The fungus produced 0.17–0.25 U of EG per ml of
cell-free culture broth.

3.2. Purification of endoglucanase

An extracellular EG was purified to apparent homogene­
ity from the culture filtrates of M. circinelloides grown on
lactose by ethanol precipitation (75%, v/v), CM Bio-Gel A
cation-exchange column chromatography, and gel filtration
on Bio-Gel A-0.5 m. The EG activity showed a single peak
of activity during the purification. A summary of the purifi­
cation procedures is presented in Table 1. The final purifica­
tion resulted in a yield of 2.7% of the EG activity and 0.01%
retention of total protein and a 408-fold increase in specific
activity. Upon SDS-PAGE of the purified EG, a single band
was visualised when stained with Coomassie Brilliant Blue
(Fig. 1).

3.3. Characterisation of endoglucanase

3.3.1. Molecular weight

The molecular weight of the native EG estimated by gel
filtration on Bio-Gel A-0.5 m was 25 000. By SDS-PAGE
analysis, the molecular weight of the enzyme was 27 000
(Fig. 1).

3.3.2. pH and temperature dependence

The enzyme (0.35 U/ml) was stable at pH 3.5–7.5 (1 h at
40°C). It displayed an optimum activity at pH 5.0 and re­
tained 80% activity at pH 3.0 and also at pH 8.0 (Fig. 2).
This means that the EG from M. circinelloides NRRL 26519
is fairly stable and highly active over a broad pH range.

Table 1
Purification of endoglucanase from Mucor circinelloides NRRL 26519

<table>
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<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg protein)</th>
<th>Recovery (%)</th>
<th>Purification fold</th>
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<td>53</td>
<td>10.2</td>
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<td>96</td>
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<tr>
<td>Bio-Gel A-0.5 m</td>
<td>0.28</td>
<td>12</td>
<td>43.0</td>
<td>3</td>
<td>408</td>
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</table>
3.3.1. Substrate specificity and cellulose binding

The purified enzyme in 50 mM acetate buffer, pH 5.0 (0.35 U/ml; 7.0 µg protein/ml) was quite stable up to 60 °C for 30 min and lost only 10% activity at 70 °C upon incubation for 30 min. It exhibited maximum activity at 55 °C, with 84% relative activity at 60 °C and 29% activity at 70 °C under the assay conditions used.

3.3.2. Effect of temperature on stability and activity

The thermostability and thermoactivity of the purified EG are shown in Fig. 3. The purified enzyme solution in 50 mM acetate buffer, pH 5.0 (0.35 U/ml; 7.0 µg protein/ml) was quite stable up to 60 °C for 30 min and lost only 10% activity at 70 °C upon incubation for 30 min. It exhibited maximum activity at 55 °C, with 84% relative activity at 60 °C and 29% activity at 70 °C under the assay conditions used.

3.3.3. Substrate specificity and cellulose binding

The purified EG (0.35 U/ml) hydrolysed soluble CMC and insoluble cellulolic substrates such as Avicel, Solka-floc, and Sigmacell 50 (each at 1%, w/v) and produced soluble celldextrins. It could not degrade oat spelt xylan and larch wood xylan. Also, the purified enzyme preparation did not possess any significant CBH and BG activities. HPLC analysis of the hydrolysis products from the CMC, Avicel, Solka-floc, and Sigmacell 50 indicates that the enzyme did not produce any detectable glucose, cellobiose, and short chain celldextrins from these substrates.

The purified EG (0.35 U/ml) was found to bind to Avicel and Solka-floc completely (based on no detectable activity in the supernatant solution) and 64% on Sigmacell 50 at pH 5.0, but not to oat spelt xylan under the conditions described in Section 2. The bound enzyme was partially eluted from the insoluble substrates by using 50 mM phosphate buffer, pH 8.0 containing 0.1 M NaCl (54% from Avicel, 36% from Solka-floc and 22% from Sigmacell 50).

3.3.4. Effect of metal ions and reagents on activity

The influence of certain inhibitors or activators on EG activity (0.35 U/ml, 50 °C, pH 5.0 or 6.0, 30 min reaction) was studied (Table 2). The enzyme activity was enhanced by 27 ± 5% by Mg²⁺ (5 mM) and 44 ± 14% by Co²⁺ (0.5 mM). It was not affected by ethylenediaminetetraacetate (EDTA, 10 mM), dithiothreitol (DTT, 10 mM), or by p-chloromercuribenzoic acid (pCMB, 0.2 mM) assayed at pH 6.0.

4. Discussion

This paper reports on the purification and characterisation of EG from a newly isolated M. circinelloides strain capable...
of utilising corn fibre xylan as growth substrate. The fungal strain makes complete cellulolytic enzymes (EG, CBH, and BG) necessary to convert cellulose to glucose quantitatively as is evident from the fact that only glucose was obtained after the hydrolysis of cellulosic substrates such as CMC, Avicel, and Sigmacell 50 by the crude enzyme preparation and also from enzyme assays on substrates such as CMC, pNP-β-D-celllobioside and pNP-β-D-glucoside. Like other cellulolytic fungal cultures, M. circinelloides can utilise lactose, Sigmacell, and celllobiose as sole growth substrate to produce cellulases [9].

The EG from the M. circinelloides strain was stable over a wide pH range (3.5–7.5) and at temperatures up to 65 °C, thus making the enzyme suitable for use in cellulose saccharification at moderate temperature and pH. Properties of some EGs from various fungal strains are presented in Table 3. It is evident that fungal EG often exists in multiple forms. The EG from A. aculeatus [10], D. squalens [13], M. incrassata [16], T. koningii [19], T. reesei [20], and T. viride [21] possessed multiple forms of EG with different properties. The extracellular EG from M. circinelloides NRRL 26519 exits only in one form.

The EG belongs to the family 6, 7, 8, 9, 12, and 45 based on amino acid sequence [22]. The MW of EG differs significantly from each other (Table 3). The MW (27000) of EG from M. circinelloides NRRL 26519 is similar to that of EG

<table>
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<tr>
<th>Fungus</th>
<th>Molecular weight</th>
<th>pl</th>
<th>Optimum temperature (°C)</th>
<th>Optimum pH</th>
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<td>38000</td>
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<tr>
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<td>4.0</td>
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--; not reported.
from A. aculeatus [10], A. niger [11], and M. Incrasata [16] but differs significantly from the MW of EG reported from another strain of M. circinelloides FERM BP-6890 [15]. The substrate specificity of each EG also differs significantly from each other. For example, the EG from Thermococcus aurantiacus was active on CMC, barley β-glucan, and lichenan, CM-cellulose, cellobiose, pNP-β-D-cellobioside, pNP-β-D-lactoside, salicin, and methyl-β-D-glucoside [18].

The EG from Pyrococcus furiosus had highest activity on cellobentaose and cellohexaose and can also hydrolyse shorter cellodextrins, CMC, and microcrystalline cellulose [23]. Some other EG can degrade xylan substrates well [24]. The EG from M. circinelloides can attack cellulose and makes only soluble high MW cellodextrins as was evident from HPLC analysis of products. No short chain cellodextrins such as cellobiose, cellotriose, cellotetraose, and cellopentaose were produced by the action of the purified enzyme on different cellulose substrates. The culture supernatant (crude enzyme), on the other hand, hydrolysed cellulose to glucose. This indicates that the cellulase system from the fungal strain contains all three enzyme activities needed to produce glucose from cellulose and has great potential to be used in enzymic saccharification of various lignocellulosic substrates.

The EG from M. circinelloides was found to bind strongly to insoluble Sigmacell 50, Avicel, and Solka-floc and the bound enzyme can be released by changing the pH. This indicates that the enzyme possesses a cellulose binding domain. Unlike some other EG, the EG from M. circinelloides does not have the ability to bind to xylan and cannot degrade xylan. The purified EG is active over a broad pH (4.0–6.0) and temperature up to 55°C and is thus may prove suitable for industrial application.

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References


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