Kinetic Study and Characterization of 1,4-β-Endoglucanase of Aspergillus niger ANL301

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ABSTRACT

Submerged fermentation of Aspergillus niger ANL301 in basal medium containing cellulose as sole carbon source, yielded crude extracellular proteins with 0.54 ± 0.02 units mg protein−1 of 1,4-β-endoglucanase activity. Partial purification by ammonium sulphate precipitation (80% saturation) and gel filtration on Sephadex 25-300 gave two active fractions of 1,4-β-endoglucanase, which exhibited close activity towards carboxymethyl-cellulose (CMC). The pH profile of the pooled enzyme fractions showed three activity peaks at pH 3.5, 5.5 and 7.0. The enzyme was most active at pH 5.5 and showed optimal activity at 50°C. V_max of 4.4 ± 0.4 μmol min−1 mg protein−1 and K_m of 12.5 ± 0.4 gL−1 was obtained with CMC for the enzyme. Different divalent metal ions and EDTA affected the enzyme activity. Mg2+, Ca2+, Cu2+, and Zn2+ inhibited the enzyme by between 22.3 and 29.4%, whereas 75.0 and 71.3% inhibition were obtained with Hg2+ and EDTA, respectively. Manganese ion showed an exceptional activation of the 1,4-β-endoglucanase. The organism produced two types of 1,4-β-endoglucanase with different molecular weights.

Keywords: Aspergillus niger ANL301, carbon source, carboxymethyl-cellulose (CMC), 1,4-β-endoglucanase activity, kinetics, manganese ion

INTRODUCTION

Cellulase is the generic name for the family of enzymes which hydrolyze the β-glycosidic bonds of native cellulose and related cello-oligosaccharides (Chellapandi and Jani 2008). It is a key enzyme for the bioconversion of cellulosic biomass into simple sugars which can subsequently feed-stock for the production of different chemicals and fuels via anaerobic fermentation (Zhang and Lynd 2004).

Filamentous microfungi are the most studied and best known cellulase-producing organisms because of their rapid growth, high enzyme yield and capacity to secrete active and separable cellulase enzyme types (Berry and Paterson 1990). Studies on hyperscretory microfungi such as Trichoderma reesei, Penicillium pinophilum, Penicillium funiculosum, Fusarium oxysporum, Aspergillus niger, Sclerotium rolfsii and Humicola sp. show that the enzyme is adaptive and also regulated by catabolite repression in most fungi (Reese and Levinson 1952; Berry and Paterson 1990; Howard et al. 2003). Cellulose and other substrates such as sophorose, sawdust, and sugarcane pulp are known to stimulate the enzyme production (Mandels and Reese 1956; Nwodo-chinedu et al. 2007). Complete hydrolysis of native cellulose by most fungi requires the synergistic action of three types of cellulase, namely, 1,4-β-endoglucanase [EC 3.2.1.4], also known as 1,4-β-glucan glucohydrolase, endo-(1,4)-D glucanase, endoglucanase, endocellulase or CMCase, 1,4-β-exoglucanase [EC 3.2.1.91], also called exo-(1,4)-β glucanase, cellubiohydrolase, exocellulase, microcrystalline cellulase or avicelase, and β-glucosidase [EC 3.2.1.21], also called cellobiase (Khan 1980; Bhat and Bhat 1997). 1,4-β-endoglucanase generally initiate cellulase action within the cellulose chain by cleaving at random the β-1,4-glucosidic bonds, commonly in the amorphous parts of the cellulose chain, thereby generating glucose and cello-oligosaccharides of various lengths, and consequently new chain ends. Aspergillus niger group is noted for the secretion of extracellular enzymes which hydrolyze the β-glycosidic bonds of native cellulose and associated hemicelluloses (de Vries and Visser 2001). Several strains of the fungus have been isolated and studied to assess their potential for commercial cellulase production using different fermentation substrates and methods (Acharya et al. 2008; Chinedu et al. 2008a; Omojasola and Jilani 2008; Bjorn et al. 2009; Chinedu et al. 2010; Gamara et al. 2010). Aspergillus niger ANL301 was isolated from a wood-waste dump in Lagos, Nigeria (Nwodo-Chinedu et al. 2005). The organism grows effectively in basal medium supplemented with sawdust or sugarcane as sole carbon sources (Nwodo-Chinedu et al. 2007) and produces cellulases (Chinedu et al. 2008a) and xylanases (Okafor et al. 2007; Chinedu et al. 2008b) in media containing cellulose or agrowastes as sole carbon sources. In the present study, 1,4-β-endoglucanase of A. niger ANL301 was partially purified and characterized. The kinetic properties and effect of metal ions on the enzyme activity were investigated.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents were of analytical grade. Potato dextrose agar (PDA) and crystalline cellulose (Avicel) were obtained from Merck, Germany. Carboxymethyl-Cellulose (CM52) was obtained from Whatman Ltd., UK. All other chemicals and reagents were obtained from Sigma Chemicals Co. Ltd., UK.

Organism

The strain of Aspergillus niger ANL301 was isolated from wood-wastes in Lagos, Nigeria and identified as described previously.
Cultivation and enzyme production

Fresh culture of the organism was made on sterile PDA plates and incubated at 30°C for 72 h. Colonies on the plates were covered with 10 mL of 0.1% (v/v) Tween 80. The spores were harvested using sterile cotton swab and transferred into a sterile test tube. The spore suspension served as inoculum for enzyme production. The extracellular enzymes were produced through submerged fermentation. The organism was grown on basal medium containing (per liter of distilled water): NaNO₃, 3.0 g; KCl, 0.5 g; KH₂PO₄, 1.0 g; MnSO₄·7H₂O, 0.5 g; FeSO₄·7H₂O, 0.01 g; and 10.0 g cellulose. One liter (1 L) of the media was supplemented with 1.0 g of trace solution containing (per L of distilled water): ZnSO₄, 1.0 g; and CuSO₄·5H₂O, 0.5 g. The pH of each media was adjusted to 5.6. Conical flasks (250 mL) containing 100 mL of respective media were autoclaved at 121°C for 15 min, cooled and inoculated with 1.0 mL of spore suspension (2-4 × 10⁶ spores per mL) of the pure fungal isolate. The cultures were incubated for 72 h with continuous agitation at 100 rpm using a Griffin flask shaker. Cells were harvested by centrifugation at 6000 × g for 15 min at 4°C using an ultra centrifuge (Super-speed RC-B, USA). The cell-free culture supernatant was used as source of crude extracellular enzyme.

Protein assay

Protein content of the culture supernatant was determined by the Folin-Ciocalteau method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard. The protein concentration of the chromatographic fractions was determined by extinction at 280 nm wavelength with a spectrophotometer (Thermospectronic Genesys 4001/1, USA).

1,4-β-Endoglucanase assay

A modification of the reducing sugar method described by Khan (1980) was used for the assay of 1,4-β-endoglucanase (EC 3.2.1.4). Activity. Carboxymethyl-cellulose (CMC) was used as enzyme substrate. The reaction mixture contained 2.0 mL of 0.1% (w/v) CMC in 0.1M sodium acetate buffer (pH 5.0) and 2.0 mL of cell-free culture supernatant (or 0.5 mL of partially purified enzyme). The mixture was incubated at 40°C in a water bath with shaking for 30 min. The reducing sugar released was measured using 3, 5-dinitrosalicylic acid and read at 540 nm using a spectrophotometer (Miller 1959). The released reducing sugar was expressed in glucose equivalent and expressed in Units mL⁻¹. A unit of activity was defined as amount of enzyme required to liberate 1 μmol of glucose per minute under the assay conditions.

Partial purification of 1,4-β-endoglucanase

Partial purification of the crude extracellular enzyme was achieved by the process of freeze drying, ammonium sulphate precipitation/dialysis, and gel chromatography. These were carried out in duplicates. Two hundred milliliters of the crude extracellular enzyme was freeze dried at -4°C. The enzyme was redissolved in 20 mL acetate buffer (0.05 M, pH 5.0) and precipitated with (NH₄)₂SO₄ at 80% (w/v) saturation. This was centrifuged at 1000 × g for 15 min. The supernatant was carefully decanted and the precipitate was redissolved in the acetate buffer and dialyzed over-night at 4°C against the buffer.

Gel chromatography (molecular sieving) was done using Sephadex G25-300 (Sigma). Ten-grains of Sephadex (Sigma) was suspended in 50 mL sodium acetate buffer (0.05 M, pH 5.0) and packed into a glass column (12 × 400 mm) at room temperature. The gel in the column was further washed with the same buffer. Five-mL of the concentrated dialyzed enzyme was then introduced to the top of the sephadex-packed column. After the enzyme had been absorbed, the same buffer was passed through the column at a constant flow rate of 1 mL min⁻¹. Five-mL fractions were collected and assayed for their protein and 1,4-β-endoglucanase activity. Enzymatically active fraction were pooled and concentrated by freeze drying.

Properties of 1, 4-β-endoglucanase

1. pH

The pH profile of the enzyme was determined by varying the pH of the reacting mixtures between 3.0 and 9.0. These substrates were prepared in two buffer solutions: 0.1 M acetate-NaOH (pH 3.0 to 7.0) and 0.1 M Tris-HCl buffer (pH 8.0 to 9.0).

2. Temperature

The temperature profile of the enzyme was determined by incubating the enzyme with substrates for 30 min at various temperatures between 30-80°C. The enzyme activity values were determined.

3. Time course

The time course of the enzyme was determined by measuring the enzyme activity at different period of incubation under the above standard assay conditions of pH 5.5 and 50°C. Total reducing sugars (glucose equivalent) released by the enzyme per time was monitored at 10-min intervals for 60 min.

4. Effect of substrate concentration

The effect of various substrate (CMC) concentrations (2.0 to 20 g/L) on the enzyme activity was studied under the above standard assay conditions of pH (5.0) and temperature (40°C). The Michaelis-Menten constant (Kₘ) and maximum velocity (Vₘₐₓ) of the enzyme were obtained using the reciprocal plot (Line-weaver–Burk plot).

5. Effect of metal ions and EDTA

The effect of divalent cations and ethylene diamine tetraacetic acid (EDTA) on the enzyme activity was determined by incubating the standard enzyme-substrate (CMC) mixture containing 2.0 mM salts of the respective cations at the standard assay conditions for 30 min. The salts included MgSO₄·7H₂O, MnSO₄·5H₂O, CuSO₄·7H₂O, CaSO₄, HgCl₂, FeSO₄·7H₂O. Effect of cation-chelator, EDTA, on the enzyme activity was also determined by including 10.0 mM of EDTA in enzyme-substrate mixture.

Data analysis

All assays were in triplicates, unless otherwise stated, and the results are expressed as Mean ± SEM (Standard Error of Mean).

RESULTS

Partial purification of 1,4-β-endoglucanase

Table 1 shows the purification steps for the 1,4-β-endoglucanases of A. niger ANL301. The enzyme was purified by 1.5-fold using ammonium sulphate at 80% saturation and 8.6-fold through gel filtration with Sephadex G25-300.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (Unit)</th>
<th>Total protein (Mg)</th>
<th>Specific activity (Units Mg protein⁻¹)</th>
<th>Enzyme yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>64.8 ± 2.4</td>
<td>120.0 ± 10.0</td>
<td>0.54 ± 0.02</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ 10 g/L</td>
<td>5.32 ± 1.8</td>
<td>42.6 ± 3.2</td>
<td>0.80 ± 0.03</td>
<td>52.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Sephadex G25-300</td>
<td>23.7 ± 0.8</td>
<td>5.1 ± 0.2</td>
<td>4.65 ± 0.30</td>
<td>36.6</td>
<td>8.6</td>
</tr>
</tbody>
</table>
Endoglucanase of Aspergillus niger ANL301. Chinedu et al

The elution profile of the 1,4-β-endoglucanase is shown in Fig. 1. Two different active enzyme peaks were obtained. The 1,4-β-endoglucanase fractions A and B gave the activity of 0.049 ± 0.002 and 0.051 ± 0.002 units mL⁻¹, respectively.

Properties of 1,4-β-endoglucanase

The effects of different pH, temperatures, metal ions and substrate concentrations on the partially purified 1,4-β-endoglucanases of A. niger ANL301 were determined.

Effect of pH

Fig. 2 shows the effect of different pH (3.0-9.0) on the activity of the 1,4-β-endoglucanases of A. niger ANL301. Three activity peaks (pH 3.5, 5.5 and 7.0), with the highest peak at pH 5.5, were obtained for the enzyme.

Temperature profile

The effects of different temperatures (30-80°C) on the 1,4-β-endoglucanases of A. niger ANL301 is shown in Fig. 3. The enzyme showed optimal activity at 50°C, with over 50% activity at 70°C.

Time course of 1,4-β-endoglucanases

Fig. 4 shows the total reducing sugars released by the 1,4-β-endoglucanases of A. niger ANL301 at the different time of incubation. Total reducing sugars released increased with incubation time, but at disproportionate rates. There was a very rapid release of glucose in the first 30 min followed by a decline thereafter up to 60 min. The average rate of the release of glucose in the first 30 min was 1.25 μmol glucose mg protein⁻¹ min⁻¹.

Effect of substrate concentration on 1,4-β-endoglucanases activity

Plots of endoglucanase activities versus substrate concentrations showed normal hyperbola curve (Fig. 5). The cellulase activity increased rapidly as the substrate concentration increased between 2.0–10.0 gL⁻¹. Thereafter, subsequent increases in the substrate concentration had very small effect on the rate of enzyme activity. The Line Weaver-Burk plot (Fig. 6) was used to determine the maximum velocities (Vₘₐₓ) and Michaelis-Menten constants (Kₘ) of the enzyme. Maximum velocity (Vₘₐₓ) of 4.4 μmol min⁻¹ mg protein⁻¹ was obtained for the endoglucanase Aspergillus niger. The Kₘ value was 12.5 gL⁻¹.

Effects of metal ions and EDTA on the 1,4-β-endoglucanases activity

The effects of the metal ions and EDTA on 1,4-β-endoglucanase activity is shown in Table 2. Manganese (Mn²⁺) and iron (Fe²⁺) increased the enzyme activity by 253.4 and
lytic fungi are known to produce isoenzymic forms of the enzyme activity of the organism produces two different types of endoglucanases. The two fractions therefore indicated the potential of the enzyme for industrial processes and for the efficient utilization of cellulosic biomass. The expanding uses of cellulase and the need for high performance cellulase preparation from a strain of Aspergillus niger ANL301, compared favorably with the activity of a wild-type strain of A. niger. The specific cellulase activity of 0.54 units mg protein⁻¹ (97.2 μg glucose min⁻¹ mg protein⁻¹) obtained for the crude extracellular enzyme preparation of A. niger ANL301 compared favorably with the activity of 109.0 μg glucose min⁻¹ mg protein⁻¹ reported for a wild type strain of A. niger. The production of 1,4-β-endoglucanases and xylanases (Okafor et al. 2002) reported the isolation of a novel endoglucanase, EglC, which is most active towards xyloglucan and functionally different from two other endoglucanases, EglA and EglB, from A. niger. The two enzyme fractions obtained in this study may therefore represent two isoenzymic forms of endoglucanase produced by A. niger (ANL301).

**DISCUSSION**

Cellulase is recognized as a potential tool for the industrial saccharification of cellulosic biomass; its production is regarded as crucial for the successful utilization of cellulosic materials (Wu and Lee 1997; Solomon et al. 1999; Zhang and Lynd 2004). The expanding uses of cellulase and need for its viable commercial production have continued to propel the search for new strains of cellulase-producing organisms as well as cost-effective means for the enzyme production. (Coral et al. 2002; Ojumu et al. 2003). Aspergillus species are major sources of commercial cellulases (Berry and Paterson 1990). The specific cellulase activity of 0.54 units mg protein⁻¹ (97.2 μg glucose min⁻¹ mg protein⁻¹) obtained for the crude extracellular enzyme preparation of A. niger ANL301 compared favorably with the activity of 109.0 μg glucose min⁻¹ mg protein⁻¹ reported for a commercial cellulase preparation from a strain of A. niger (Khan 1980). The production of 1,4-β-endoglucanases and xylanases (Okafor et al. 2007; Chinedu et al. 2008b) by this strain of A. niger is indicative of its potential as a source of enzymes for industrial saccharification of cellulosic biomass. Xylanase is also required for the hydrolysis of native cellulose (Khan 1980).

The endoglucanase of A. niger (ANL301) showed two different activity peaks on Sephadex G 25-300 gel chromatography. Gel chromatography separates proteins according to their molecular weights. The two fractions therefore imply the organism produces two different types of endoglucanases which differ in their molecular weights. Most cellulolytic fungi are known to produce isoenzymic forms of the endoglucanase having different molecular weights (Beldman et al. 1985; Berry and Paterson 1990). For instance, the Trichoderma reesei cellulase mixture consists of many catalytically active proteins; at least five endoglucanases (EG 1–5), two cellobiohydrolases (CBH 1-2), β-glucosidases, and hemicellulases have been identified by 2-dimentional electrophoresis (Beldman et al. 1985). Coral et al. (2002) obtained two protein bands with molecular weights of about 83,000 and 50,000 Da for the carboxymethyl-cellulase enzyme of a wild-type strain of A. niger Z10. Two protein bands have also been obtained respectively for the crude enzymes of A. niger and A. fumigatus with SDS-polyacrylamide gel electrophoresis (Immanuel et al. 2007). Hasper et al. (2002) reported the isolation of a novel endoglucanase, EglC, which is most active towards xyloglucan and functionally different from two other endoglucanases, EglA and EglB, from A. niger. The two enzyme fractions obtained in this study may therefore represent two isoenzymic forms of endoglucanase produced by A. niger (ANL301).

The optimum temperature of the enzyme was 50°C; it also retained over 50% of its activity at 70°C under the assay condition. This may be as a result of adaptation to the hot-humid climate from where the organisms were obtained. Coral et al. (2002) reported an optimal temperature of 40°C for the carboxymethyl-cellulase enzyme of a wild-type strain of A. niger Z10. The higher optimal temperature obtained for the endoglucanase of the organism implies better heat stability. This is desirable, especially in industrial processes where thermal treatment may be inevitable. An optimum temperature of 50°C has been reported for the endoglucanase from A. glaucus XC9 (Tao et al. 2010). Some commercial cellulase enzymes have also been reported to be stable at 60°C (Immanuel et al. 2007).

The cellulase enzyme of A. niger ANL301 has a broad pH range of between 3.5 and 7.5 with three major activity peaks at 3.5, 5.5 and 7.0. A broad pH range between 3 to 9 was reported for a wild type strain of A. niger Z10 (Coral et al. 2002). This is probably due to the isoenzymes or sub-
units of the endoglucanase enzyme. The implication is that the endoglucanase can tolerate variations in pH and function effectively at both acidic and neutral environments. With an optimum pH of the enzyme is 5.5, the cellulase enzyme is well suited for the acidic environment of most fermentation processes, especially for simultaneous saccharification and fermentation (SSF) of lignocelluloses. TheSSF of sawdust and sugarcane pulp by *A. niger* ANL301 and the brown-rot fungus *Trametes versicolor* were carried out in our laboratory using yielded appreciable amounts of ethanol (unpublished data).

A hyperbolic curve was obtained for the activities of the celllulase enzyme. The Line Weaver-Burk plot gave a $V_{\text{max}}$ of 4.4 μmoll n mg protein $^{-1}$. The $V_{\text{max}}$ of the endoglucanase of *A. niger* was less than 10.0 μmol n mg protein $^{-1}$ obtained for the endoglucanase of *Aspergillus* PCL 501 in our laboratory.

Metal ions and EDTA were found to have profound effects on the activity of the endoglucanases of the organism. Manganese (Mn$^{2+}$) and iron (Fe$^{3+}$) increased the enzyme activity by 253.4% and 24.0%, respectively. The endoglucanase activity of *A. glaucus* XC9 was reported to be stimulated by Mn$^{2+}$ and Fe$^{3+}$ (Tao et al. 2010). However, the stimulatory effect of manganese ions on the endoglucanase of *A. niger* ANL301 is quite phenomenal. The metal ion exhibited 253.4% stimulation of the activity of cellulase enzymes of the organism. This implies a significant increase in the yield of hydrolytic products. Fukimoto and Kishi (1952) had reported a phenomenal increase of cellulase activity by manganese stimulation, but their finding was largely ignored because, as at the time, no cellulase enzyme was shown to have any prosthetic group or coenzyme, or to require any metal ions for its activity (Mandels and Reese 1956). Fahraeus (1947) in an earlier work, observed that *Cytophaga* cannot grow on cotton wool unless calcium and manganese were added to the medium. Manganese ion was shown to exert considerable stimulatory effect on 1,4-β-endoglucanases of the fungus, *Penicillium chrysogenum* PCL501 (Chinedu et al. 2008c). This work clearly indicates that manganese certainly has stimulatory effect on the activity of the endoglucanase of *A. niger* ANL301. There is the possibility that the manganese ion may indeed be a requirement for the enzyme activity and may even be an integral component of the enzyme complex. The potent inhibitory effect of EDTA, a chelating agent of divalent metal ions, on the enzyme activity may be connected with the removal of the manganese ions from the enzyme mixture. There is the need to further examine and establish the mechanism of manganese stimulation of the cellulase enzyme (Singh et al. 1990) from their studies on the kinetic properties of endoglucanase of *A. niger* AS-101 concluded that the enzyme was a metallo-protein or it required certain metal ions for activation. There is need for further investigations. Mercury ions on the other hand strongly inhibited the enzyme activity. This is normal since heavy metals are known to inhibit the activity of most enzymes. The activity of endoglucanase from *A. glaucus* XC9 was reported to be inhibited by the heavy metals, Cd$^{2+}$, Pb$^{2+}$ and Cu$^{2+}$ (Tao et al. 2010).

**CONCLUSIONS**

*A. niger* ANL301 produces extracellular proteins with cellulase activity comparable to commercially available cellulase preparation. Our data shows that the organism produces two types of 1,4-β-endoglucanase (EC 3.2.1.4) which differs in their molecular weight; they may be two isoenzymic forms of the enzyme. Manganese ion has been found to be an effective activator of the 1,4-β-endoglucanases of *A. niger* ANL301, with over 3-fold stimulatory effect on the hydrolytic activity of the enzyme. Further work is required to establish the mechanisms by which manganese ions stimulate the 1,4-β-endoglucanase of *A. niger* ANL301. The metal ion can be used to improve cellulase activity for industrial purposes.

**REFERENCES**


Omugasa PF, Jilani OP (2008) Cellulase production in *Trichoderma longi,*
Aspergillus niger and Saccharomyces cerevisiae cultured on waste materials from orange. *Pakistan Journal of Biological Sciences* 11 (20), 2382-2388


Ryu DD, Mandels M (1980) Cellulases: Biosynthesis and applications. *Enzyme Microbiology and Technology* 2, 92-102


Wu Z, Lee YY (1997) Inhibition of the enzymatic hydrolysis of cellulose by ethanol. *Biotechnology Letters* 19, 977-979