

Full Length Research Paper

Overexpression, purification and characterization of the *Aspergillus niger* endoglucanase, *EgIA*, in *Pichia pastoris*

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Cellulases are industrially important hydrolytic enzymes applicable in the bioconversion of cellulosic biomass to simple sugars. In this work, an endoglucanase from *Aspergillus niger* ATCC 10574, *EgIA*, was expressed in the methylotrophic yeast *Pichia pastoris* and the properties of the recombinant protein were characterized. The full length cDNA of *egIA* has been cloned into a pPICZαC expression vector and expressed extracellularly as a ~30 kDa recombinant protein in *P. pastoris* X-33. Pure *EgIA* displayed optimum activity at 50°C and was stable between 30 and 55°C. The pH stability of this enzyme was shown to be in the range of pH 2.0 to 7.0 and optimum at pH 4.0. *EgIA* showed the highest affinity toward β-glucan followed by carboxymethyl cellulose (CMC) with a specific activity of 63.83 and 9.47 U/mg, respectively. Very low or no detectable hydrolysis of cellobiose, laminarin, filter paper and avicel were observed. Metal ions such as Mn²⁺, Co²⁺, Zn²⁺, Mg²⁺, Ba²⁺, Fe²⁺, Ca²⁺ and K⁺ showed significant augmentation of endoglucanase activity, with manganese ions causing the highest increase in activity to about 2.7 fold when compared with the control assay, whereas Pd²⁺, Cu²⁺, SDS and EDTA showed inhibition of *EgIA* activity.

Key words: Cellulase, endoglucanase, recombinant, *Aspergillus niger*, *Pichia pastoris*.

INTRODUCTION

Cellulose is the principle structural polysaccharide in plants and the most abundant biomass on earth. It is composed of β-1,4-linked glucose units and contains both highly crystalline and amorphous (non-crystalline) regions (Zhang and Lynd, 2004). Cellulases are enzymes that effectively degrade cellulose into glucose. Three types of cellulase enzymes, endoglucanases (EC 3.2.1.4, endo-1,4-β-D-glucanases), cellobiohydrolases (EC 3.2.1.91)

and β-glucosidases (EC 3.2.1.21) are needed in concert to degrade crystalline cellulose to glucose effectively (Lynd et al., 2002). Endoglucanases are believed to initiate random attacks at multiple sites in the amorphous regions of the cellulose fiber and open up sites for subsequent attack by cellobiohydrolase. Cellobiohydrolases, in turn, processively cleave cellulose chains at the ends and release cellobiose and glucose. Finally, β-glucosidases hydrolyze the glucose dimers to glucose to eliminate cellobiose inhibition.

Cellulases are produced by a broad range of organisms including fungi, bacteria, plants and insects. Among these organisms, fungal cellulases have been widely

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studied and some have been produced commercially and used in the food, textile and pharmaceutical industries (de Vries and Visser, 2001; Coleman et al., 2007). In addition, current efforts have been focusing on fungal cellulases to transform lignocellulosic biomass into fermentable sugars which could be converted to ethanol by fermentation. This process will allow the production of renewable fuel from cellulosic biomass (Mielenz, 2001).

One of the most well known and efficient producers of cellulolytic enzymes is the filamentous fungus, *Aspergillus niger*. This fungus secretes a complex array of degradative enzymes to hydrolyze cellulose efficiently and it is an important commercial source of cellulase, especially in the food, textile and pharmaceutical industries (de Vries and Visser, 2001; Coleman et al., 2007). Endo- β -1,4-glucanase is the main component of cellulose degradation by *A. niger* and has been classified as a member of the glycosyl hydrolase family 12 (Khademi et al., 2002). Genes encoding endoglucanases from *A. niger*, such as *eglA*, *eglB* and *eglC*, have been cloned and characterized (van Peij et al., 1998; Hasper et al., 2002). *EglA* and *EglB* lack a cellulose binding domain (CBD) and its associated linker region, whereas, *EglC* contains CBD. These genes are regulated by a transcriptional activator, XlnR, which controls the expression of genes encoding enzymes required for the hydrolysis of plant polysaccharides (van Peij et al., 1998). All the three endoglucanases have been produced as recombinant enzymes to understand their substrate specificities (Hasper et al., 2002). *EglA*, which was expressed in *Kluyveromyces lactis*, displayed the greatest activity towards β -glucan compared to *EglB* and *EglC* (Hasper et al., 2002). However, the biochemical characterizations and enzyme kinetics of these endoglucanases were not reported. Thus, for this work *EglA* was selected to be cloned, overexpressed in the methylotrophic yeast *Pichia pastoris* and characterized. The long term objective is to utilize the recombinant enzyme and test its activity against the enzymatic hydrolysis of locally produced lignocellulosic biomass.

MATERIALS AND METHODS

Fungal culture conditions and total RNA extraction

A. niger ATCC 10574 was obtained from American type culture collection (ATCC). The fungus was maintained on PDA agar and subcultured twice monthly. To obtain fungal mycelium for RNA extraction, an approximately 1×10^6 spore suspension was subcultured in 100 ml potato dextrose yeast extract (PDYE) at 30°C and incubated with shaking at 180 rpm for 1 day. Fungal mycelium was harvested and frozen with liquid nitrogen.

A. niger mycelia were kept frozen in liquid nitrogen before ground up in an ice-cold mortar to produce powder. TRIzol[®] reagent (Invitrogen, USA) was used for total RNA extraction from the powdered mycelia, following the manufacturer's instructions. The quality and integrity of RNA was determined by gel electrophoresis in 1% agarose containing 3.5% formaldehyde as described by Sambrook and Russell (2001).

Reverse transcription-PCR (RT-PCR) amplification and cloning

RT-PCR was performed using the access RT-PCR system (Promega, USA) using the total RNA as template. The specific primers used were designed based on complete mRNA sequences of *A. niger* CBS 120.49 endoglucanase (*eglA*) available from GenBank (accession no. AJ224451). The sequence of the forward primer *eglA*-F was 5'-ATGAAGCTCCCTGTGACTTGGCTA-3' and the sequence of the reverse primer *eglA*-R was 5'-CTAGTTGACTGGCGGTCCAGTTG-3'. First-strand cDNA synthesis was carried out at 45°C for 45 min and 94°C for 2 min for 1 cycle. Second strand cDNA synthesis and PCR amplification cycles were as follows: 94°C for 30 s, 60°C for 1 min and 68°C for 2 min for 40 cycles, with a final extension at 68°C for 20 min. The PCR product of the expected size was purified from an agarose gel and cloned into the pGEM-T easy vector (Promega, USA) followed by transformation into *Escherichia coli* DH5 α . The cDNA was sequenced using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) and analyzed. The DNA sequence obtained was translated into the amino acid sequence using Translate program (<http://www.expasy.ch/tools/dna.html>). The nucleotide and the deduced amino acid sequences were aligned against the published *EglA* sequence using Boxshade version 3.21 software (www.ch.embnet.org/software/Box_form.html). The software SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>; Bendtsen et al., 2004) was used to predict the signal peptide. Motif analysis was performed using motif scan software (http://myhits.isb-sib.ch/cgi-bin/motif_scan). N-glycosylation and O-glycosylation sites were predicted using NetNGlyc 1.0 server (<http://www.cbs.dtu.dk/services/NetNGlyc>) and OGPET 1.0 (<http://ogpet.utep.edu/OGPET>).

Construction of the expression plasmids

To express the cDNA in *P. pastoris*, the cDNA with its native signal peptide was amplified, cloned and sequenced. The *Saccharomyces cerevisiae* α -factor secretion signal was used for the secretion of the recombinant enzyme. The primers to amplify the cDNA fragment encoding mature *EglA* were designed to carry *Clal* and *XbaI* restriction sites at the 5' and 3' ends, respectively. The sequence of the forward primer *eglA*-*Clal*-F was 5'-ATCGATA CAGACGATGTGCTCTCAA-3' and the sequence of the reverse primer *eglA*-*XbaI*-R was 5'-TCTAGAGCGTTGACTGCGGTC CAGTTG-3'. The PCR amplification cycles performed were 94°C for initial denaturation for 10 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension step of 72°C for 20 min. Subsequently, the PCR fragment was digested with *Clal* and *XbaI* and ligated at the *Clal*/*XbaI* sites of the *Pichia* expression vector pPICZ α C to yield the expression cassette pPICZ α C-*eglA*. The sequence of the gene was confirmed by DNA sequencing.

Transformation of *P. pastoris* and screening of recombinant colonies

Transformation of the expression cassette into *P. pastoris* X-33 (Invitrogen, USA) was performed by electroporation according to the instruction manual for the EasySelect[™] *Pichia* expression system (Invitrogen, USA). Before transformation, the expression cassette pPICZ α C-*eglA* was linearized with *PmeI* endonuclease to target the integration of the expression cassette into the *AOX1* locus of *P. pastoris* strain X-33. Transformants of *P. pastoris* containing the *eglA* cDNA were selected using yeast extract-peptone-dextrose (YPD) agar plates containing 100 μ g/ml Zeocin (Invitrogen, USA). All Zeocin-resistant colonies were replica-plated onto YPD plates containing different concentrations (1000 and 2000 μ g/ml) of Zeocin antibiotic to detect multicopy transformants and

also on minimal methanol (MM) plates which contained 1.34% yeast nitrogen base (with ammonium sulfate; without amino acids), 0.5% methanol, 0.4 µg/ml D-biotin, 1% agar and minimal dextrose (MD) plates, which have the same composition as MM but with 2% glucose instead of methanol to determine the methanol utilizing phenotypes. After 2 to 3 days of incubation, the multicopy transformants grew on 2000 µg/ml Zeocin plates and the Mut⁺ phenotypes grew normally on both MM and MD plates, whereas, the Mut⁻ phenotypes grew slowly on MM plates. PCR amplification was carried out using the 5'AOX1 primer 5'-GACTGGTTCCAATTGA CAAGC-3' and the 3'AOX1 primer 5'-GCAAATGGCATTCTGA CATCC-3' to identify the integration of the targeted gene at the AOX1 locus in the *P. pastoris* genome.

Expression of recombinant *EglA* in *P. pastoris*

The expression in *P. pastoris* was carried out by growing recombinant cultures harboring multiple copies of the expression cassette in 50 ml BMGY (buffered glycerol complex medium) containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5} % biotin and 1% glycerol at 30°C, 250 rpm for 16 to 24 h until the cell density reached an OD₆₀₀ of between 2 to 6. Yeast cells from a culture suspension at the final OD₆₀₀ value of 1 were harvested by centrifugation and resuspended in 100 ml of BMMY (buffered minimal methanol medium) containing 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5} % biotin and 0.5% methanol. Cultures were grown under the stated conditions for an additional 72 h with addition of absolute methanol to a final concentration of 0.5% every 24 h to maintain induction. The culture supernatant was harvested by centrifugation at 4°C for 5 min at 4000 rpm and concentrated using Vivaspin, a 10 kDa cut-off Centriprep device (Millipore, USA). Protein concentration was determined by the method of Bradford (Amresco, USA) using bovine serum albumin (BSA) as standard and the secreted proteins were analyzed using SDS-PAGE (Laemmli, 1970) and western blotting.

Agar plate diffusion assay of endoglucanase activity

A qualitative activity assay of partially purified *EglA* was monitored using the agar diffusion method. The concentrated crude proteins were loaded onto the wells of an azurinecrosslinked-hydroxyethylcellulose (AZCL-HE) plate (1.5% bacteriology agar and 0.1% AZCL-HE cellulose) (Megazyme, Ireland) and incubated at 30°C for 16 h. The clear zone surrounding the well indicated endoglucanase activity.

Purification of recombinant *EglA*

The concentrated supernatant was applied to a HisTrapTM column (GE Healthcare, USA), which was pre-equilibrated with binding buffer (pH 7.4) containing 20 mM NaH₂PO₄, 1 M NaCl and 20 mM imidazole by using an automated AKTA prime system (GE Healthcare, USA). The bound recombinant *EglA* was then eluted with a linear gradient of elution buffer (pH 7.4) containing 20 mM NaH₂PO₄, 1 M NaCl and 300 mM imidazole. Fractions containing the bound protein were pooled, concentrated and buffer exchanged with 50 mM sodium citrate buffer (pH 4.0) using Vivaspin with a molecular cut-off weight of 10,000 (MWCO 10,000) (Millipore, USA). The purity of recombinant *EglA* was analyzed by SDS-PAGE combined with Coomassie blue staining and western blot analyses.

Enzyme assays

The endoglucanase activity (CMCase activity) was measured

quantitatively by a colorimetric method using a 3,5-dinitrosalicylic acid (DNS) assay with carboxymethyl cellulose (CMC) as the substrate. The reaction mixture containing 0.2 ml of enzyme solution, 0.3 ml of 1% CMC and 0.5 ml of 50 mM sodium citrate buffer at pH 4.0 was incubated at 50°C for 30 min. The reaction was terminated by boiling at 100°C for 5 min and centrifuged at 13,200 rpm for 1 min. Aliquots of 100 µl supernatant were mixed with 100 µl of DNS reagent followed by boiling at 100°C for 15 min. The absorption of the reaction mixture was measured at 540 nm using a UV spectrophotometer. One unit (U) of enzyme activity is defined as the amount of enzyme that produced 1 µmol of D-glucose in one minute under the assay conditions. Specific activity is defined as the number of units per milligram of protein.

Effect of temperature and stability on purified *EglA*

The optimum temperature for the purified *EglA* was evaluated by incubating 0.2 ml of purified *EglA*, 0.3 ml of 1% CMC and 0.5 ml of 50 mM sodium citrate buffer at pH 4.0, as described previously, at the following temperatures for 30 min: 30, 35, 40, 45, 50, 55 and 60°C. The temperature stability of the enzyme was determined by measuring the residual activity after pre-incubation of the enzyme in the absence of substrate at various temperatures between 30 and 60°C for 30 min. Subsequently, the residual activity of the enzyme was assayed by the standard endoglucanase assay method. The stability of *EglA* at 4, 25 and 50°C was determined by incubating the reaction mixture for 2, 5, 24 and 30 h. The residual activity of the enzyme was subsequently assayed using the endoglucanase assay method.

Effect of pH and stability on purified *EglA*

The optimum pH for the activity of the purified *EglA* was carried out by incubating 0.2 ml purified enzyme and 1% CMC in 50 mM buffer solution with different pH values ranging from 2.0 to 8.0 at the predetermined optimal temperature. The buffers used were glycine-HCl for pH 2.0 to pH 3.0, sodium citrate for pH 3.0 to pH 6.0 and potassium phosphate for pH 6.0 to pH 8.0. pH stability of *EglA* was studied by pre-incubating the enzyme in the absence of substrate at pH's ranging from pH 2.0 to 8.0 at 50°C for 30 min and assaying the remaining enzyme activity using the standard endoglucanase assay.

Determination of purified *EglA* substrate specificity

The substrate specificity of the purified *EglA* was compared across several cellulose derived substrates such as CMC (1%), β-glucan(1%), avicel (1%) and filter paper (50 mg). The reaction mixture contained 0.2 ml pure enzyme in 0.8 ml of 50 mM sodium citrate buffer, at pH 4.0 and the different substrates was incubated at 50°C for 30 min. Enzyme activity was assessed using the standard endoglucanase assay.

Enzyme kinetics analysis

The Michaelis-Menten constant (K_m) and maximum velocity of substrate hydrolysis (V_{max}) values were determined from a Lineweaver-Burk plot. The reactions were performed by incubating 0.2 ml of purified *EglA* with 0.8 ml 50 mM sodium citrate buffer, at pH 4.0 containing different substrates at various concentrations at 50°C for 30 min. The substrates used were 1 to 20 mg/ml β-glucan and 5 to 70 mg/ml CMC. Enzyme activity was assessed using the standard method. The catalytic constant k_{cat} and the specificity constant k_{cat}/K_m were calculated.

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AJ224451      1  MKLPVSLAMLAATAMGQTMCSQYDSASSPPYSVNQNWLWGEYQGTGSQCQVYVDKLSSSGAS
cDNA          1  MKLPVTLAMLAATAMGQTMCSQYDSASSPPYSVNQNWLWGEYQGTGSQCQVYVDKLSSSGAS
consensus     1  *****

AJ224451     61  WHTETWWSGGEGTVKSYNSGVTFNKKLVSDVSSIPTSVEWKQDNTNVNADVAYDLFTAA
cDNA          61  WHTGWTWSGGEGTVKSYNSGVTFNKKLVSDVSSIPTSVEWKQDNTNVNADVAYDLFTAA
consensus     61  *** *****

AJ224451    121  NVDHATSSGDYELMIWLARYGNIQPIGKQIATATVGGKSWEVWYGSTTQAGAEQRTYSFV
cDNA          121  NVDHATSSGDYELMIWLARYGNIQPIGKQIATATVGGKSWEVWYGSTTQAGAEQRTYSFV
consensus     121  *****

AJ224451    181  SESPINSYSGDINAFFSYLTQNOGFPASSQYLINLQFGTEAF TGGPATFTVDNWTASVN
cDNA          181  SESPINSYSGDINAFFSYLTQNOGFPASSQYLINLQFGTEAF TGGPATFTVDNWTASVN
consensus     181  *****

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Figure 1. Amino acid sequence alignment of *EgIA A. niger* strain ATCC 10574 and *EgIA A. niger* strain CBS 120.49. AJ224451 refers to the amino acid sequence of *EgIA A. niger* CBS 120.49 (Accession no.: AJ224451); cDNA refers to the amino acid sequence of *EgIA A. niger* strain ATCC 10574 (this study). Black boxes show identical amino acids while white boxes show the different amino acids between both sequences.

Effect of metal ions and reagents on purified *EgIA*

The effects of metal ions and some chemicals were assessed by incubating 0.2 ml of the pure enzyme with 0.4 ml of 50 mM sodium citrate buffer, at pH 4.0, 0.3 ml of 1% CMC and 0.1 ml of solution containing different metal ions (Ca^{2+} , K^+ , Na^+ , Zn^{2+} , Co^{2+} , Ba^{2+} , Cu^{2+} , Mg^{2+} , Mn^{2+} , Fe^{2+} , Pd^{2+}) at 10 mM or different reagents such as SDS (1%), EDTA (1 mM) and urea (1 M). The reaction mixture was incubated at 50°C for 30 min and the enzyme activity was determined. To verify the enhancer or inhibitor activity of a compound towards *EgIA* activity on CMC, EDTA was mixed in the *EgIA* assay together with the enhancer or the inhibitor.

RESULTS

Isolation and cloning of the full-length cDNA of *egIA* gene from *A. niger* ATCC 10574

A full-length cDNA of *A. niger* ATCC 10574 *egIA*, with the size of 720 bp, was amplified by reverse-transcription PCR (RT-PCR) using total RNA as the template. This sequence was submitted to the GenBank with accession number GU724764. The *egIA* cDNA sequence obtained was aligned with the *A. niger* CBS 120.49 *egIA* sequence available in the GenBank (Accession no.: AJ224451). Sequence alignment using BOXSHADE 3.21 software showed that the amplified *egIA* cDNA sequence had eight nucleotide differences when compared with the *egIA* sequence of *A. niger* CBS 120.49. These nucleotide differences caused two amino acid changes at the amino acids 6 and 64 (Figure 1). The amino acid changed at position 6 was located in the signal peptide region that was removed during the cloning of mature *egIA* gene, whereas the changed amino acid at position 64, which switched from a glutamic acid residue to a glycine residue, was located outside the glycosyl hydrolase do-

main 12 (amino acids 82 to 239). Nevertheless, the differences in the sequence might be due to the strain variation as the *A. niger* strain used in this study was different from the strain for the reported cDNA sequence in the GenBank. Furthermore, the validity of the *egIA* sequence generated in this study was confirmed because the synthesis of the cDNA had been carried out twice and sequenced.

Overexpression and purification of *EgIA* in *P. pastoris*

The *egIA* cDNA from *A. niger* ATCC 10574 had been successfully cloned and the protein was expressed as an active endoglucanase in *P. pastoris*. Based on the SDS-PAGE profile and western blot analyses (Figure 2), protein expression using BMGY medium as the biomass generating medium and BMMY medium containing 0.5% absolute methanol as the protein induction medium for 3 days resulted in an overexpression of the recombinant protein with a molecular weight of ~30 kDa. This result is in agreement with the calculated mass of 27.72 kDa predicted based on the obtained amino acid sequence information. This result also indicated that no glycosylation occurred on *EgIA*, which is in accord with the predicted glycosylation sites using NetNGlyc 1.0 and OGPET 1.0 software where no glycosylation site was detected on *EgIA*. Approximately 40 mg/l of crude *EgIA* was produced by *P. pastoris*. This result is in close proximity to the expression level of *Volvariella volvacea* V14 endoglucanase in *P. pastoris* which was reported at 65 to 100 mg/l (Ding et al., 2002). Partially purified endoglucanase activity was monitored by the agar plate diffusion assay and the hydrolysis zone formed indicates

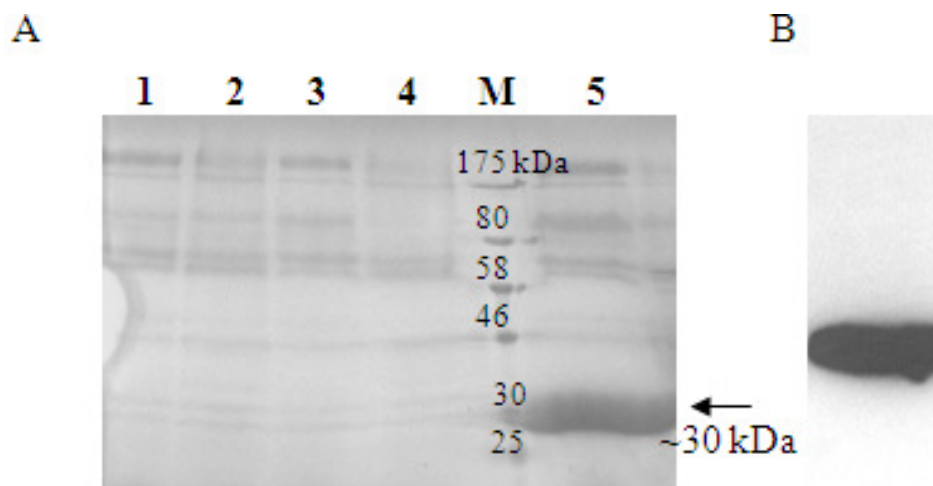


Figure 2. SDS-PAGE and western blotting analyses of the expression of EglA in *P. pastoris*, X-33. (A), Lanes 1 and 2, culture supernatant of *P. pastoris* strain X-33 uninduced and induced with 0.5% methanol; Lanes 3 and 4, culture supernatant of *P. pastoris* containing pPICZαC empty vector uninduced and induced with 0.5% methanol; M, protein marker; Lane 5, culture supernatant of integrant carrying *eglA* induced with 0.5% methanol; (B), Western blotting analyses of the recombinant EglA.

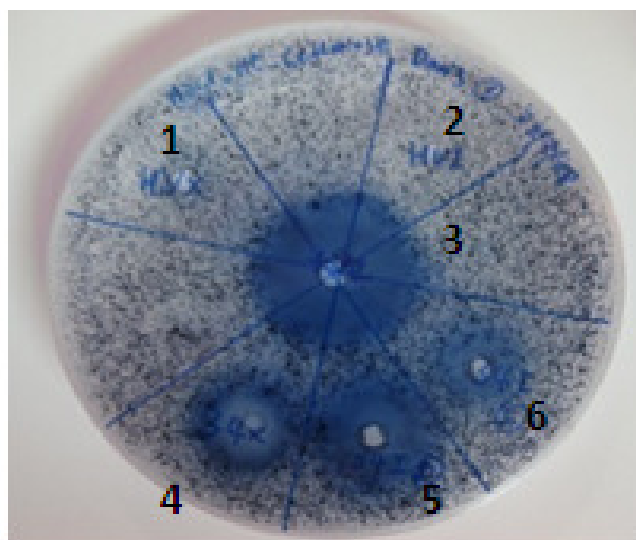


Figure 3. Agar plate diffusion assay of the endoglucanase activity using AZCL-HE cellulose agar plate. Well 1, *P. pastoris* host protein; Well 2, host protein containing an empty vector; Well 3, positive control containing celluclast (Sigma Aldrich, USA); Wells 4, 5 and 6, recombinant endoglucanase EglA. Approximately 40 µg of protein was loaded in each well.

that, the recombinant enzyme was produced in an active form and demonstrates endoglucanase activity towards CMC (Figure 3).

Recombinant EglA expressed in *P. pastoris* was fused with an affinity polyhistidine tag to simplify the purification process to obtain the purified protein for characterization. The recombinant endoglucanase was purified to homogeneity using an immobilized metal affinity chromatography

(IMAC) column. The purified enzyme showed a single band with a molecular weight of 30 kDa on SDS-PAGE and was verified by western blotting analyses (Figure 4). Purified EglA was obtained after two steps of protein purification, which were ultrafiltration with MWCO 10 kDa and IMAC and yielded a 60% purified protein recovery and a purification factor of 2.73 fold (Table 1). The electrophoretic homogeneity of the purified EglA obtained after IMAC protein purification was approximately 9 mg/l.

Enzymatic properties of purified EglA

To characterize the recombinant EglA protein, the effect of temperature (30 to 60°C) and pH (pH 2 to 8) on the enzyme activity was evaluated. The purified enzyme exhibited optimum activity at 50°C and it retained over 60% activity at 55°C (Figure 5a). The enzyme was stable at temperatures between 30 to 50°C for 30 min in the absence of substrate but gradually lost activity with increasing temperature, though over 70% of the activity remained after heat treatment at 60°C for 30 min (Figure 5b). EglA activity was not affected when stored for 30 h at room temperature (25°C) or in a cool environment (4°C). However, its activity decreased to 60% when stored at 50°C for 24 h (Figure 5c).

To determine the effect of pH on the recombinant endoglucanase activity and stability, several buffers of different pH were used, for instance, glycine-HCl buffer (pH 2.0 to 3.0), sodium citrate buffer (pH 3.0 to 6.0) and potassium phosphate buffer (pH 6.0 to 8.0). The optimal pH for EglA activity was pH 4.0 and it was stable from pH 2.0 to pH 7.0 with more than 80% residual activity, though there was a drastic drop of activity at pH 8.0

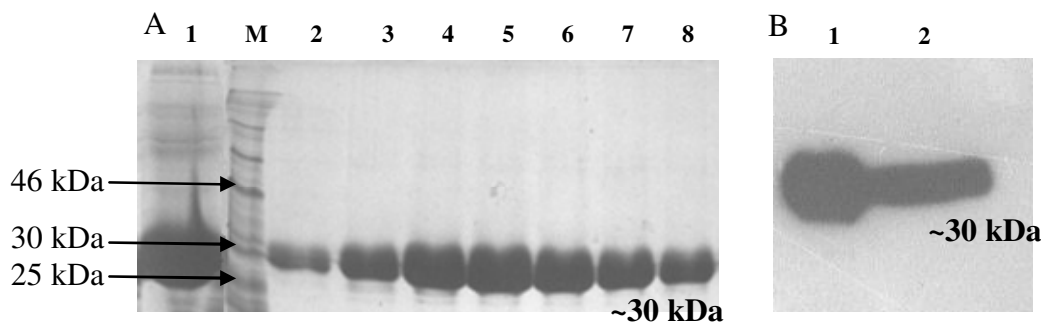


Figure 4. EglA protein purification profile using IMAC. (a) SDS-PAGE analyses for EglA protein purification fractions. Lane 1, EglA crude extract; Lane M, protein marker (New England Biolabs); Lanes 2 to 8, EglA targeted protein elution fractions. (b) Western blotting analyses for detection of purified EglA using anti-His antibody. Lane 1, EglA crude extract; Lane 2, purified EglA protein.

Table 1. Summary of recombinant EglA purification.

Purification step	Volume (ml)	Activity (U/ml) ^a	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%) ^b	Purification factor ^c
Crude extract protein	600	0.14	83.4	24.0	3.48	100	1
Ultrafiltration (MWCO 10 kDa)	5	13.02	65.12	9.5	6.85	78.08	2.00
IMAC	1	50.19	50.19	5.3	9.47	60.18	2.73

^aU, Rate of enzyme hydrolysis of 1 μ mol of reducing sugar per minute under specific conditions; ^bYield, total activity of purified sample / total activity of crude extract sample \times 100%; ^cPurification factor, specific activity of purified sample/specific activity of crude extract sample.

(Figure 5d,e). This result suggests that this purified recombinant endoglucanase requires an acidic pH near neutral pH range for optimal enzymatic activity.

Substrate specificity and kinetic analysis

The substrate specificity of the purified EglA was determined by assaying its activity on several cellulose derivative substrates such as β -glucan, CMC, cellobiose, laminarin, filter paper and avicel. The enzyme was most active towards β -glucan with a maximum activity of 63.83 U/mg, followed by CMC with 9.47 U/mg and low or no detectable activity towards cellobiose, laminarin, filter paper and avicel (Figure 6). This result suggests that EglA had no activity on shorter cellulose (dimers) and crystalline cellulose.

Enzyme kinetics analyses were subsequently carried out by measuring the initial reaction rates of the purified endoglucanase at various concentrations of β -glucan and CMC. The initial reaction rates were analyzed by a Lineweaver-Burke plot. The Michaelis-Menten constant, K_m , values for β -glucan and CMC as substrates were 92.68 and 100.3 mg/ml, respectively (Table 2). This indicates that, pure EglA showed higher affinity toward hydrolyzing β -glucan as its substrate than CMC. Furthermore, the catalytic constant k_{cat} and specificity constant k_{cat}/K_m of EglA also had higher values when β -glucan was used as

the substrate (Table 2). These results demonstrate that, the EglA enzyme had a higher affinity and kinetic specificity for β -glucan and it is capable of converting 6973 β -glucan molecules into simple sugars in one second. The turnover value (k_{cat}) of EglA was higher than the endoglucanase *Aspergillus oryzae* cmc-1 (Javed et al., 2009), which shows that the EglA recombinant enzyme had a better hydrolytic performance.

Effects of metal ions and other reagents

The effect of metal ions and some reagents on enzyme activity is summarized in Figure 7. It is interesting to observe that the addition of metal ions Mn^{2+} , Co^{2+} , Zn^{2+} , Mg^{2+} , Ba^{2+} , Fe^{2+} , Ca^{2+} and K^+ , showed a significant increase on the purified EglA activity with manganese ions causing the highest activity increase to about 2.7 fold when compared with the control assay. Co^{2+} and Zn^{2+} at the same concentration increased the enzyme activity to about 1.6 and 1.5 fold, respectively, of the activity in the absence of a metal ion. In contrast, significant reduction of reducing sugars was observed with addition of Pd^{2+} and Cu^{2+} . Palladium ions at 10 mM lowered the reducing sugars level to 90%, an almost complete inhibition of the enzyme activity. Exposure to the chemical reagents SDS and EDTA reduced the activity to 51 and 10%, respectively. No effect on EglA activity was observed with

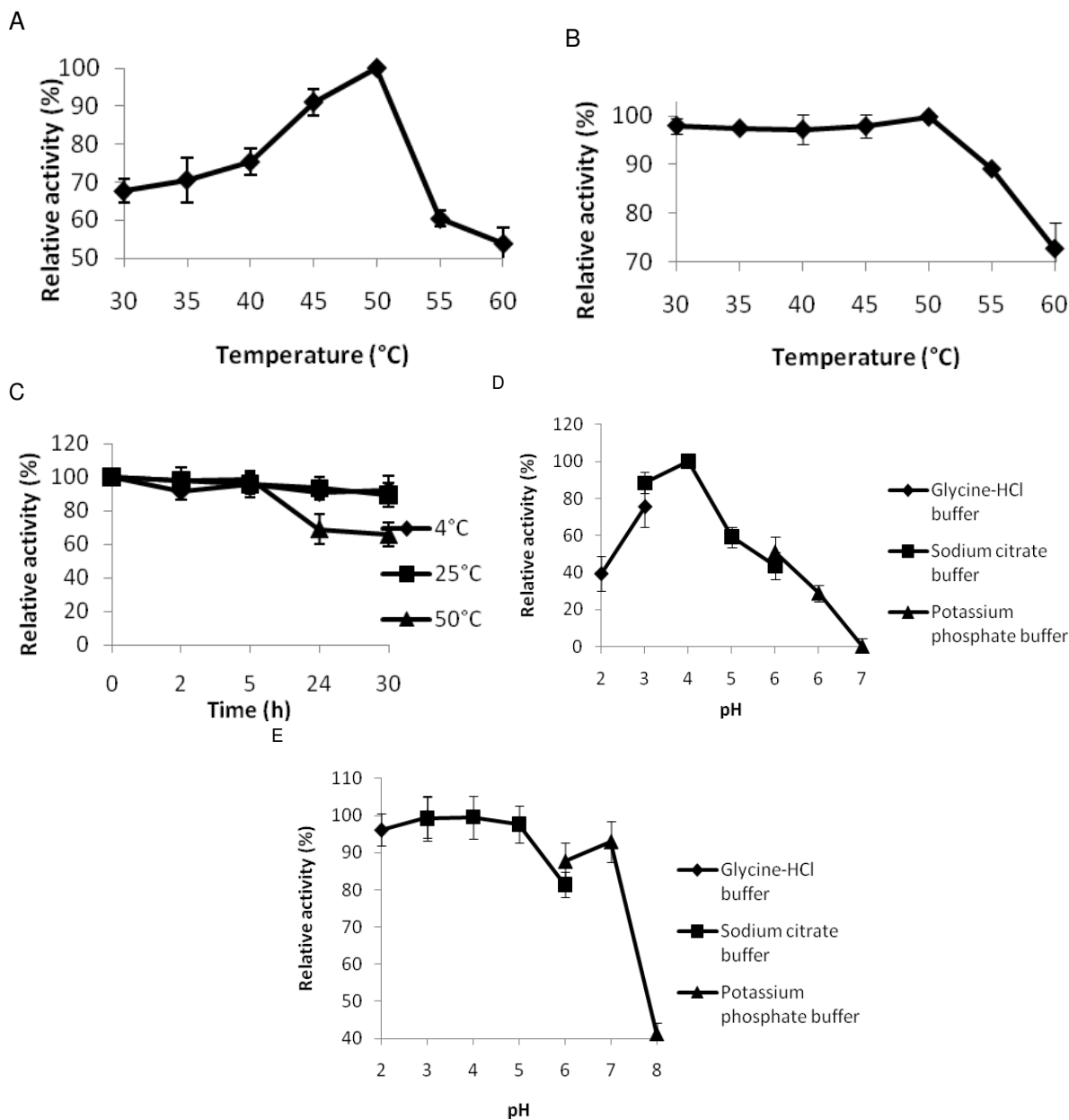


Figure 5. Biochemical properties of purified recombinant *EglA*. (a) Effects of temperature on *EglA* activity; (b) Temperature stability of *EglA*; (c) Enzyme stability at various storage temperatures and longer period; (d) Effects of pH on *EglA* activity; (e) pH stability of *EglA*.

the addition of Na⁺ or urea in the endoglucanase assay.

DISCUSSION

A. niger CBS 120.49 endoglucanase *EglA*, had been previously expressed in *K. lactis* to compare substrate specificity among several *A. niger* endoglucanases (Hasper et al., 2002). However, no biochemical characterization of *EglA* had been reported. In this work, *EglA* was produced as a recombinant enzyme and its biochemical properties

were characterized. The endoglucanase *eglA* cDNA from *A. niger* ATCC 10574 was isolated, cloned into pPICZαC expression vector and expressed in *P. pastoris* X33. The recombinant protein was overexpressed as an active 30 kDa enzyme. The specific activity of recombinant *EglA* produced in *P. pastoris* (63.83 ± 4.68 U/mg) was slightly higher than the reported values for *EglA* produced in *K. lactis* (59 ± 5 U/mg). This might be due to the different hosts used in the expression of both proteins. The presence of the Histag and Myc epitope amino acid sequence at the C-terminal region of recombinant *EglA*

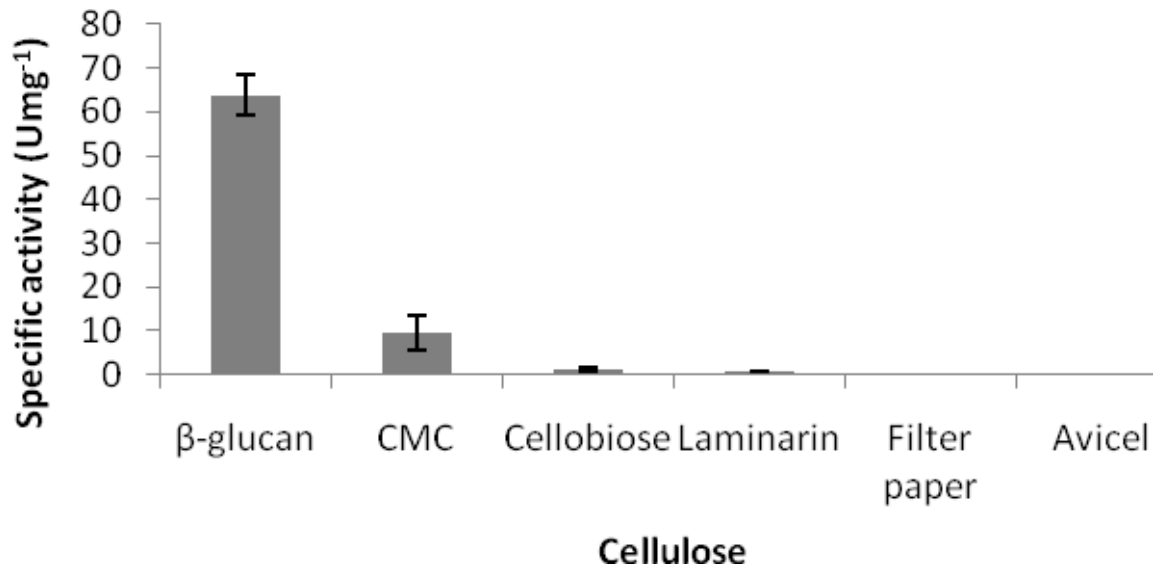


Figure 6. Substrate specificity of purified *EgIA* on different types of cellulose.

Table 2. Kinetic constants of recombinant purified *EgIA* toward different substrates.

Substrate	Michaelis-Menten constant, K_m (mg/ml)	Maximum velocity, V_{max} ($\mu\text{mol}/\text{min}$)	Catalytic constant, k_{cat} (s^{-1})	Specificity constant, k_{cat}/K_m (ml/mg/s)
β-glucan	92.68	30.96	6973	75.24
Carboxymethyl cellulose (CMC)	100.30	5.93	1336	13.32

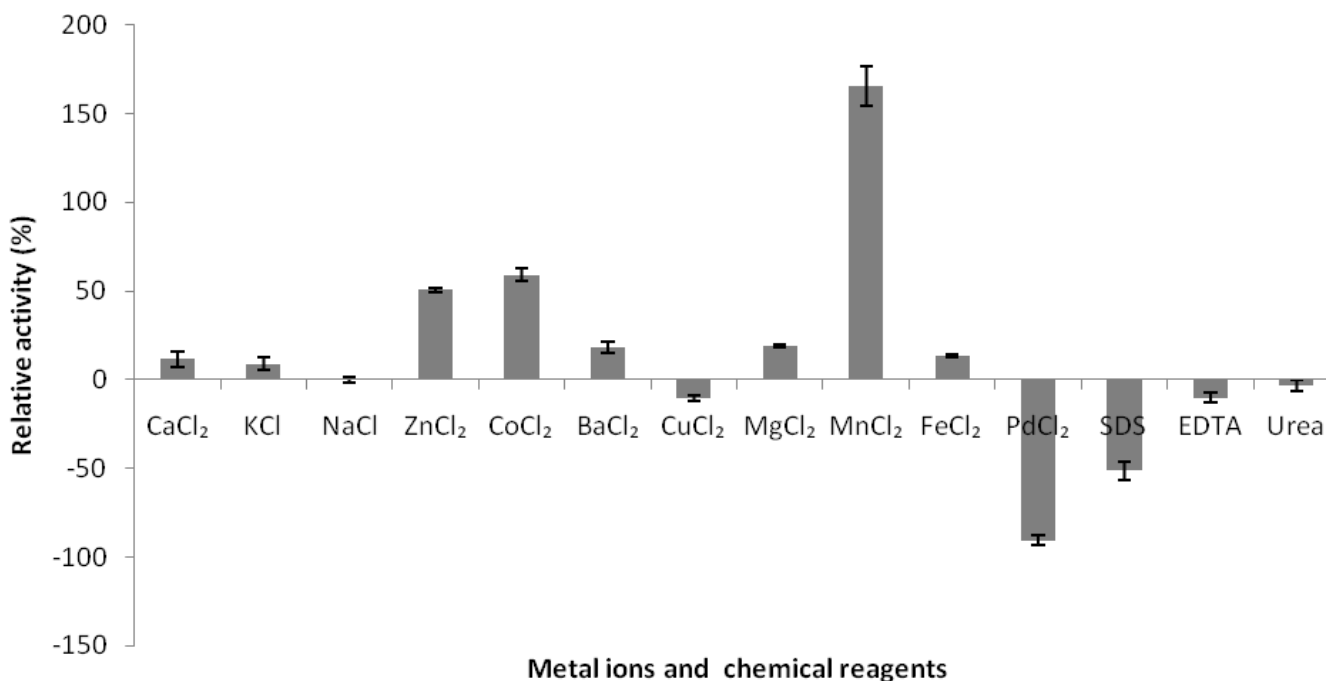


Figure 7. Effects of metal ions and chemical reagents on purified *EgIA* in relative to the activity of *EgIA* without metal ions and chemical reagents.

Table 3. Comparison of biochemical characterization of endoglucanase (*EglA*) from *A. niger* ATCC 10574 with other fungal strains.

Endoglucanase	Expression host	Protein size	Specific activity	Optimum temperature	Optimum pH	Substrate	Reference	
<i>EglA</i> <i>A. niger</i> strain ATCC 10574	<i>P. pastoris</i>	30 kDa	63.83 U/mg (β -glucan); U/mg (CMC)	9.47	50	pH 4.0	β -glucan; CMC	This study
<i>EglA</i> <i>A. niger</i> strain CBS 120.49	<i>K. lactis</i>	30 kDa	59 \pm 5 U/mg (β -glucan); U/mg (CMC)	3 \pm 0.4	ND	ND	β -glucan; CMC	Hasper et al. (2002)
<i>EglB</i> <i>A. niger</i> strain CBS 120.49	<i>K.lactis</i>	ND	22 \pm 4 U/mg (β -glucan); U/mg (CMC)	22 \pm 4	ND	ND	β -glucan; CMC	Hasper et al. (2002)
<i>EglC</i> <i>A. niger</i> strain CBS 120.49	<i>A. niger</i>	ND	19 \pm 1 U/mg		55	pH 4.5	xyloglucan	Hasper et al. (2002)
Eng1 <i>A. niger</i> IFO31125	<i>S. cerevisiae</i>	43 kDa	204 U/mg		70	pH 6.0	CMC	Hong et al. (2001)
Eg1 <i>V. voluacea</i> strain V14	<i>P. pastoris</i>	45 kDa	344 U/mg		55	pH 7.5	CMC	Ding et al. (2002)
Endoglucanase <i>Syncephalastrum racemosum</i> strain BCC18080	<i>P. pastoris</i>	55 kDa	57 U/mg		70	pH 5.0-6.0	CMC	Wonganu et al.(2007)

produced in *P. pastoris* might also have affected the activity of the recombinant enzymes. In addition, the gene encoding *EglA* originated from different fungal strains for both studies and this might contribute to the differences in specific activity. Genes obtained from different organism strains might have variations at the nucleotide and amino acid levels (Aigner et al., 2000).

High expression levels and secretion of active recombinant proteins was achieved using the methylotrophic yeast *P. pastoris* as host. This expression system has been used successfully for the production of a wide range of mammalian, bacterial and fungal proteins (Romanos, 1995). Overexpression of *EglA* yielded 40 mg/l of recombinant protein, which is in agreement with the work described by Ding et al. (2002), where 65 to 100 mg/l of *V. voluacea* V14 endoglucanase in *P. pastoris* was produced. Nevertheless, the *P. pastoris* expression system was reported to be capable of producing heterologous protein up to a few grams per liter (Sreerikshna et al., 1997). There are several factors that drastically influence protein production in this system (Sreerikshna et

al., 1997). To increase the yield of the desired recombinant protein, a protease-deficient host strain such as SMD-1163, SMD1165 and SMD1168 could be used to reduce protein degradation (Li et al., 2007). In addition, protein expression could be carried out in a bioreactor where important parameters such as pH, aeration and the carbon source feed rate could be controlled (Cregg et al., 2000; Cereghino et al., 2002; Li et al., 2007).

Biochemical characterization of the purified *EglA* revealed that, this enzyme had an optimum temperature of 50°C and optimum pH of 4.0 with a high stability at temperatures between 30 and 50°C and pH between 2.0 and 7.0. Comparison of the optimum temperature and pH showed that most of the fungal endoglucanases had optimum activity at an acidic pH and a temperature around 50 to 55°C, with a few exceptions in the case of thermophilic endoglucanases (Table 3). Endoglucanases with optimal activity between 55 and 80°C are thermostable enzymes (Maheshwari et al., 2000). Several endoglucanases from the glycosyl hydrolase family 12 have an optimal temperature at 50 to 70°C and an optimal pH at 4

to 5 (Baldrian and Valaskova, 2008; Cantarel et al., 2009; Dashtban et al., 2009). In fact, most fungal endoglucanases have acidic or neutral pH optima (pH 2.5 to 7.0) (Ding et al., 2001). The high thermostability of *EglA* at 50°C makes *EglA* a suitable candidate to be used in industry, especially in agricultural waste cellulose hydrolysis, which requires longer incubation periods in the degradation processes. Enzymes with acidic pH stability properties are suitable to be used in the animal feed industry as the alimentary tracts of the livestock are around pH 4.8 (Collins et al., 2005). Acidic cellulase could also be applied in acidic deinking of printed papers or old magazines (Xia et al., 1996).

β -glucan is a cellulose that consists of glucose units with mixed linkages of β -1,4 and β -1,3 (Edney et al., 1991), whereas CMC is a general substrate used for endoglucanase (CMCase) activity determination (Zhang and Lynd, 2004). Both β -glucan and CMC are soluble amorphous cellulose. Hence, the results indicate that *EglA* is an endoglucanase that has no hydrolytic activity on shorter cellulose chains (cellobiose) or crystallin

cellulose (filter paper and avicel) but preferentially acts on longer chain amorphous cellulose, which is the typical characteristic of an endoglucanase enzyme. Laminarin is a polysaccharide that consists β -1,3 - 1,6 linkages. Higher hydrolytic activity of *EgIA* on β -glucan than laminarin demonstrates the specificity of *EgIA* enzyme toward hydrolysis of cellulose with β -1,4 linkages.

Metal ions may influence the maximal enzyme catalytic activity by acting as enzyme cofactors or inhibitors (Schiffmann et al., 2005; Jaabar et al., 2008). The increase in enzyme activity with the addition of manganese ions in the standard endoglucanase assay was observed for an endoglucanase from *Penicillium chrysogenum*, whereby a more than 3-fold stimulatory effect occurred in the enzyme hydrolytic activity (Chinedu et al., 2008). Endoglucanases from *Cellulomonas uda* (Shankaranand and Lonsane, 1994) and *Chalara paradoxa* (Lucas et al., 2001) also showed activation by Mn^{2+} . With the addition of the cation chelator EDTA in the assay containing manganese ions, the enhancement of the activity were removed (data not shown). This result proved that the increase in the enzyme activity was solely based on the interaction between manganese ions and *EgIA* enzyme. In general, EDTA might chelate ions from the assay buffer and thus, decrease *EgIA* activity. Inhibition of *EgIA* activity by palladium ions resulting in an approximately 90% decrease from the control without addition of metal ions was in agreement with the result published by Khademi et al. (2002), whereby structural studies showed that palladium ions directly bind to and block the active sites of *EgIA* thus, inactivating the enzyme. Ca^{2+} and Mg^{2+} ions have been reported to be required by cellulose enzymes to enhance the substrate binding affinity of the enzyme and stabilizing the conformation of the catalytic site (Manfield et al., 1998), therefore, it is not surprising that these two metals stimulate the enzymes. *EgIA* activity was greatly reduced in the presence of SDS, which indicates that it is sensitive to anionic detergent. The increase in *EgIA* activity with Zn^{2+} and Co^{2+} was in agreement with the results from the recombinant EGVIII *Trichoderma viride* (Huang et al., 2009) and endoglucanase (EGt) of *Fusarium oxysporum* (Liu et al., 2006). Copper ions have shown extreme inhibition on CMCase activity of endoglucanases from *Rhizopus oryzae* (Murashima et al., 2002).

In summary, the *EgIA* endoglucanase from *A. niger* ATCC 10574 was successfully cloned, expressed and characterized using the *P. pastoris* expression system. Because of the advantageous properties demonstrated by the *EgIA* enzyme, this unique enzyme will be further exploited for enzyme hydrolysis of locally produced agriculture biomass.

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