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Cloning, Expression, Purification, and Properties of an Endoglucanase Gene (Glycosyl Hydrolase Family 12) from *Aspergillus niger* VTCC-F021 in *Pichia pastoris*

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A gene coding for an endoglucanase (EglA), of the glycosyl hydrolase family 12 and derived from *Aspergillus niger* VTCC-F021, was cloned and sequenced. The cDNA sequence, 717 bp, and its putative endoglucanase, a 238 aa protein with a predicted molecular mass of 26 kDa and a pI of 4.35, exhibited 98.3–98.7% and 98.3–98.6% identities, respectively, with cDNA sequences and their corresponding endoglucanases from *Aspergillus niger* strains from the GenBank. The cDNA was overexpressed in *Pichia pastoris* GS115 under the control of an AOX1 promoter with a level of 1.59 U/ml culture supernatant, after 72 h of growth in a YP medium induced with 1% (v/v) of methanol. The molecular mass of the purified EglA, determined by SDS–PAGE, was 33 kDa, with a specific activity of 100.16 and 19.91 U/mg toward 1% (w/v) of β-glucan and CMC, respectively. Optimal enzymatic activity was noted at a temperature of 55°C and a pH of 5. The recombinant EglA (rEglA) was stable over a temperature range of 30–37°C and at pH range of 3.5–4.5. Metal ions, detergents, and solvents tested indicated a slightly inhibitory effect on rEglA activity. Kinetic constants (K_m, V_{max}, k_{cat}, and k_{cat}/K_m) determined for rEglA with β-glucan as a substrate were 4.04 mg/ml, 102.04 U/mg, 2,040.82 min^{-1}, and 505.05, whereas they were 10.17 mg/ml, 28.99 U/mg, 571.71 min^{-1}, and 57.01 with CMC as a substrate, respectively. The results thus indicate that the rEglA obtained in this study is highly specific toward β-glucan. The biochemical properties of rEglA make it highly valuable for downstream biotechnological applications, including potential use as a feed enzyme.

Keywords: *Aspergillus niger* VTCC-F021, endoglucanase gene, cloning, expression, properties

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Carboxymethylcellulases (β-1,4-D-glucan-4-glucanohydrolase, E.C. 3.2.1.4) are members of the cellulase system, a consortium of that enzymes mainly comprises endoglucanases (E.C. 3.2.1.4), exoglucanases (E.C. 3.2.1.91), and cellobiases (E.C. 3.2.1.21), which act in synergy [13]. Endo-β-1,4-glucanases have strict enzymatic specificity for the cleavage of β-1,4-glycosidic bonds to produce shorter cellulose chains [2, 19]. Many useful applications of these enzymes have been documented, such as the biopolishing of cotton fabric, the improvement of the processing of paper pulp, the de-inking of paper, the enhancement of the efficiency of laundry detergents, and in increasing the utilization efficiency of plant materials in animal feeds [6, 7].

Endo-β-1,4-glucanases are products of a broad range of organisms, including microbes, plants, and animals [9, 11]. However, production of this enzyme is most abundant in strains of *Trichoderma* and *Aspergillus* [3]. Genes encoding endo-β-1,4-glucanases from several different fungal species have been studied, such as *A. aculeatus* [25], *A. kawachii* [31], and *A. niger* [10]. For production on an industrial scale, heterologous expression of endo-β-1,4-glucanase has also been carried out in a number of host organisms, including *E. coli* [23, 24], *S. cerevisiae* [10, 31], *A. niger* [30], and *Pichia pastoris* [26, 35]. Among these host organisms, *P. pastoris* has proved to be an excellent system for expressing secreted heterologous proteins [20]. So far, several cDNA of endo-β-1,4-glucanase A from *Aspergillus niger* strains have been deposited in the GenBank. Zhao *et al.* [35] recently reported high levels of expression of a codon-optimized and synthesized endo-β-1,4-glucanase gene, *egI* (999 bp, 332 aa), from *Aspergillus niger* IFO31125 in *P. pastoris* [35], which belongs to the glycosyl hydrolase family 5. However, there has been as yet no report in relation to the expression of the glycosyl hydrolase family 12 endo-β-1,4-glucanase A from *Aspergillus niger* in *P. pastoris*.

In this study, we have cloned and expressed a glycosyl hydrolase family 12 endoglucanase A gene (717 bp, 238 aa), from *A. niger* VTCC-F021 in *P. pastoris* GS115, under the control of an AOX1 promoter for the expression and secretion of EglA. A comparison of the sequence alignments of *egI* and *eglA* revealed a less than 3% identity between DNA sequences and a less than 6% similarity of amino acid sequences. Thus, the two genes *egI* and *eglA* were seen to be completely different. We then purified it and characterized the kinetics and influences of metal ions, detergents, and organic solvents on rEglA.

Materials and Methods

Chemicals and Reagents

The carboxymethylcellulose (CMC) used was obtained from DBH Prolabo, a brand from VWR International S.A.S. (Fontenay-sous-Bois, France). Peptone and yeast extract were purchased from Bio Basic Inc. (New York, USA). 3,5-Dinitrosalicylic acid was sourced from Fluka, a Sigma-Aldrich Co. (St. Louis, USA). Restriction enzymes, *Pfu* and *Taq* DNA polymerase, and T4 ligase were supplied by Fermentas, part of Thermo Fisher Scientific Inc. (Waltham, USA). The kit Ni^{2+}-ProBond was obtained from Invitrogen Corp. (Carlsbad, USA).

Strains and Vectors

A. niger VTCC-F021 strain, from the Vietnam Type Culture Collection (Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi, Vietnam) was used as the source for the endoglucanase gene (*eglA*). *Escherichia coli* DH5α and pJET1.2/blunt vector (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) were used for DNA manipulations and amplification. *Pichia pastoris* strain GS115 and pPICZαA (Invitrogen Corp., Carlsbad, USA) were used for expression of the endoglucanase. Luria–Bertani (LB) medium containing 1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl, at a pH of 7–7.5, was used for the cultivation of the *E. coli*. The LB agar plates additionally contained 2% (w/v) agar and 100 μg ampicillin/ml or 25 μg zeocin/ml.

DNA Manipulations

Genomic and plasmid DNA isolation was carried out by methods that have been previously described [28]. DNA fragments and PCR products were excised from a 0.8% agarose gel and purified by a gel extraction kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. DNA sequencing was performed on an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems Inc., Foster City, USA). *E. coli* DH5a was transformed using heat shock methods as previously described [28].

DNA Amplification and Plasmid Construction

Based on the nucleotide sequence of *eglA*, encoding endoglucanase A from *A. niger* CBS 513.88 (XM001400865) and *A. niger* contig An14c0110 (AM270318), two oligonucleotides gpPICF (GC GAA TTC AAG CTC YCT DTG GCA CTT) and gpPICR (GC TCT AGA GC GTT GAC ACT RGC RGT CCA) were designed as primers to amplify the gene *eglA* from *A. niger* VTCC-F021 with the introduction of the underlined *Eco*RI and *Xba*I restriction sites at 5' of forward and reverse primers, respectively. The PCR mixture contained 2 μl of 10× PCR buffer, 1.6 μl of 2.5 mM dNTP, 1.2 μl of 25 mM $MgCl_2$, 2 μl of genomic DNA (50 ng), 0.2 μl of 5 unit *Taq* polymerase, and 1 μl each primer (10 pM), supplemented with 11 μl of distillated water to a final volume of 20 μl. The thermocycler conditions were as follows: 95°C/4'; 35 cycles of 95°C/45", 55°C/45", 72°C/1'; 72°C/10'. The PCR product was inserted into the pJET1.2/blunt vector and sequenced. The obtained DNA sequence was aligned with coding sequences from *A. niger* CBS 513.88 and *A. niger* contig An14c0110 using the DNAStar program to find the introns and exons for *eglA* from *A. niger* VTCC-F021.

Total RNA Extraction

Total RNA from *A. niger* VTCC-F021 was extracted from 2 g of mycelia using a Trizol Reagents Kit (Invitrogen Corp., Carlsbad, USA). The frozen mycelia were ground to a fine powder under liquid nitrogen using a mortar and pestle, suspended in 1 ml of Trizol Reagent, and incubated at room temperature for 5 min. The homogenized suspension was supplemented with 200 μl of chloroform (per 1 ml Trizol), shaken vigorously (not vortexed) for 15 s, and incubated at room temperature for 3 min. After centrifugation at 10,000 rpm, 4°C for 15 min, the upper (clear) aqueous layer was transferred to a fresh 1.5 ml Eppendorf tube, supplemented with 0.5 ml of isopropanol, mixed thoroughly by shaking for 15 s, and incubated at room temperature for an extra 10 min. After centrifugation at 10,000 rpm, 4°C for 15 min, the supernatant was carefully removed and the RNA pellet was supplemented with 1 ml of 75% DEPC-ethanol and vortexed at low speed for 5–10 s to wash the pellet thoroughly. After centrifugation at 6,000 rpm, 4°C for 5 min, the supernatant was carefully removed and the RNA pellet was air-dried at room temperature for 5–10 min. The RNA pellet was then dissolved in DEPC-dH_2O (30–100 μl, depending on yield) by gentle pipetting and incubated at 55°C for 5–10 min.

First cDNA Strand Synthesis

Prior to cDNA synthesis, the RNA was treated with reaction reagents [8 μl of RNA, 1 μl of 10× reaction buffer with $MgCl_2$, and 1 μl of DNase I (1 unit)], and incubated at 37°C for 30 min. The reaction mixture was then supplemented with 1 μl of 50 mM EDTA and incubated at 65°C for 10 min.

The first strand of cDNA was synthesized, from the RNA prepared above, using a cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA). The reaction mixture containing 10 μl of template RNA (1 μg), 1 μl of oligo (dT)18 primer (0.5 μg), and 1 μl of water was then supplemented with 4 μl of 5× reaction buffer, 1 μl of RiboLock RNase inhibitor (20 U/μl), 2 μl of 10 mM dNTP mix, and 1 μl of RevertAid M-MuLV reverse transcriptase (200 U/μl). The mixture was then mixed gently and incubated at 37°C for 60 min. The reaction was terminated by heating at 70°C for 5 min, kept on ice, and subjected to control PCR amplification.

cDNA Synthesis and Plasmid Construction

The first-strand cDNA product was used directly for the amplification of the *eglA* gene using the two primers gpPICF and gpPICR. The PCR mixture contained 2 μl of 10× PCR buffer, 1.6 μl of 2.5 mM dNTP, 1.2 μl of 25 mM $MgCl_2$, 2 μl cDNA (50 ng) from the control RT reaction, 0.2 μl 5 unit *Taq* polymerase, and 1 μl each primer (10 pM), supplemented with 11 μl of distillated water to a final volume of 20 μl. The thermocycler conditions were as follows:

94°C/4'; 35 cycles of 94°C/45", 55°C/45", 72°C/1'; 72°C/10'. PCR products were inserted into the pJET1.2/blunt vector (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) resulting in pJEglA, and transformed into the *E. coli* DH5α. The recombinant plasmid was confirmed by restriction enzyme analysis and DNA sequencing. cDNA obtained from pJEglA digested by *Eco*RI and *Xba*I was inserted into pPICZαA, resulting in the recombinant plasmid pPEglA.

Yeast Transformation and Screening

The plasmid pPEglA linearized with *Sac*I was transformed into *P. pastoris* GS115 according to the manufacturer's instructions for the EasySelect *Pichia* Expression Kit (Invitrogen Corp., Carlsbad, USA). Transformants were screened on YPDS [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1 M sorbitol, and 2% (w/v) agar] plates containing Zeocin at a final concentration of 1,000 μg/ml. The presence of the *eglA* gene in the transformants was confirmed by PCR using yeast genomic DNA as a template and *eglA*-specific primers.

Gene Expression

P. pastoris transformants were grown in 20 ml of YP medium [1% (w/v) yeast extract, 2% (w/v) peptone, 1% (w/v) glycerol] at 30°C with agitation at 220 rpm until an $OD_{600\,nm}$ of 5–6 was reached. The cell pellet was harvested by centrifugation at 4,500 rpm for 5 min. For *AOX1* promoter-controlled expression of EglA, the cell pellet was resuspended in 25 ml of YP medium [1% (w/v) yeast extract, 2% (w/v) peptone, 1% (v/v) methanol] and methanol was added to a final concentration of 1% every 24 h to maintain induction. Cultivation was performed at 30°C and 220 rpm. The culture supernatant was collected periodically to detect for the expression of EglA.

Purification of Recombinant EglA

The culture supernatant containing EglA was applied to a ProBond Ni^{2+}-affinity column (Invitrogen Corp., Carlsbad, USA). The purification of recombinant His-tagged EglA was carried out under native conditions according to the manufacturer's instructions.

Electrophoresis Analysis and Protein Concentration

The homogeneity and molecular mass of the EglA were determined by 12.5% SDS polyacrylamide gel electrophoresis with Biometra equipment (Göttingen, Germany) [18]. Proteins were visualized by staining with 0.1% (w/v) of silver nitrate. Protein concentrations were measured by the Bradford assay [4].

Estimation of EglA Activity

The EglA activity of the culture medium from methanol-induced expression strains was assayed by the 3,5-dinitrosalicylic acid (DNS) method for reducing sugar analysis [21], using 1% (w/v) CMC as the substrate in a 100 mM potassium phosphate buffer (pH 5). The reactions were carried out at 55°C for 5 min. Glucose was used as the standard reduced sugar for concentration estimation. The enzyme activities were expressed in liberating 1 μmol reducing sugar from the substrate solution per minute under standard assay conditions.

Kinetic Parameters

The apparent kinetic parameters for K_m, V_{max}, k_{cat}, and k_{cat}/K_m ratio were determined against β-glucan and CMC as substrates using Lineweaver–Burk plots.

Temperature and pH Optima and Stability

The pH and temperature optima of rEglA were determined by measuring the activity, as described above, using a 100 mM potassium acetate buffer (pH 3–5.5) and a potassium phosphate buffer (pH 5.5–8) at 55°C, in the temperature range of 35–70°C at a pH of 5, respectively.

For the determination of temperature and pH stability, purified rEglA, 5 μg for each reaction, was preincubated at the temperatures of 30, 37, and 40°C, and under various pH conditions, with 100 mM potassium acetate at pH 3–5, and potassium phosphate at pH 5.5, at 30°C for 1–8 h. The residual activity was then determined.

Effects of Metal Ions, Organic Solvents, and Detergents

Purified rEglA, 5 μg for each reaction, was incubated with 5–15 mM of various metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, Ag^{+}, and K^{+}) and EDTA, in 10–30% (v/v) of different solvents (methanol, ethanol, isopropanol, acetone, *n*-butanol, and ethyl acetate), and in 0.5–2% (w/v) of different detergents (Tween 80, Tween 20, SDS, and Triton X-100) at 37°C for 1 h. The residual activity was then determined.

All measurements were carried out in triplicate with the resulting values being the mean of the cumulative data obtained.

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of the Endoglucanase A Gene

The recombinant plasmid pJEglA containing the insert was sequenced and the putative endoglucanase A gene was aligned with sequences from the GenBank using DNAStar. The whole nucleotide sequence of the *eglA* gene from *A. niger* VTCC-F021 had 2 introns and 3 exons, confirmed

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aagctctctttgacacttgctatgcttgcggccaccgccatgggccagacaatgtgctct   60
K  L  S  L  T  L  A  M  L  A  A  T  A  M  G  Q  T  M  C  S     20
cagtatgacagtgcctcgagcccccatactcagtgaaccagaacctctggggcgagtac  120
Q  Y  D  S  A  S  S  P  P  Y  S  V  N  Q  N  L  W  G  E  Y     40
caaggcaccggcagccagtgtgcatatgtcgacaaactctccagcagtggtgcatcctgg  180
Q  G  T  G  S  Q  C  A  Y  V  D  K  L  S  S  S  G  A  S  W     60
cacaccgaatggacctggagcggtggtgagggaacagtgaaaagctactctaactctggc  240
H  T  E  W  T  W  S  G  G  E  G  T  V  K  S  Y  S  N  S  G     80
gttacatttaacaagaagctcgtgagtgatgtatcaagcatccccacctcggtggaatgg  300
V  T  F  N  K  K  L  V  S  D  V  S  S  I  P  T  S  V  E  W    100
aagcaggacaacaccaacgtcaacgccgatgtcgcgtatgatcttttcaccgcggcgaat  360
K  Q  D  N  T  N  V  N  A  D  V  A  Y  D  L  F  T  A  A  N    120
gtggaccatgccacttctagcggcgactatgaactgatgatttggTATGTGAGCTCGCAA  420
V  D  H  A  T  S  S  G  D  Y  E  L  M  I  W                   135
AGAATCAAAGAATACAGAAATCTAACAACGCCAGGctgcccgctacggcaacatccagc  480
              Intron (406-455)       L  A  R  Y  G  N  I  Q  P    144
ccattggcaagcaaattgccacggccacagtgggaggcaagtcctgggaggtgtggtatg  540
  I  G  K  Q  I  A  T  A  T  V  G  G  K  S  W  E  V  W  Y  G    164
gcagcaccacccaggccggtgcggagcagaggacatacagctttgtgtcggaaagcccta  600
  S  T  T  Q  A  G  A  E  Q  R  T  Y  S  F  V  S  E  S  P  I    184
tcaactcatacagtggggacatcaatgcatttttcagctatctcactcagaaccaaggct  660
  N  S  Y  S  G  D  I  N  A  F  F  S  Y  L  T  Q  N  Q  G  F    204
ttcccgccagctctcagtacttgatcaGTAAGATTCCCTGATTCTACCAGCGGACGCCGC  720
  P  A  S  S  Q  Y  L  I  N                                     213
AGCGAAACAGTCACTAACAGAAGTGATGTGTAGatctgcagtttggaactgaggcgttca  780
              Intron (688-753)       L  Q  F  G  T  E  A  F  T    222
ccgggggcccggcaaccttcacggttgacaactggactgccagtgtcaac            830
  G  G  P  A  T  F  T  V  D  N  W  T*  A  S  V  N              238
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Fig. 1. Construct of the gene *eglA* from *A. niger* VTCC-F021 (2 introns and 3 exons) cloned into pPICZαA.

The initiation codon (Met) and stop codon are not shown in the construct. Bold and underlined letters are the 10 conserved amino acids of family 12 endoglucanases. The bold asterisk represents the predicted glycosylation site. DNA sequencing was performed on a ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems Inc., Foster City, USA). Sequence alignments were constructed and analyzed using the MegAlign DNAStar program.

by comparing sequences determined from both genomic DNA and cDNA that encompassed the whole EglA ORF (Fig. 1). Nucleotide sequence analysis of the cloned cDNA insert from *A. niger* VTCC-F021 showed a 717 bp open reading frame that encoded a protein of 238 amino acid residues, with a predicted molecular mass of 25.7 kDa and a pI of 4.35 (Fig. 1).

The coding sequence of *eglA* from *A. niger* VTCC-F021 exhibited 98.3–98.7% similarity with corresponding nucleotide sequences from *A. niger* strains (AJ224451, XM_001400865, and AM_270318), 56–81.9% with sequences from *A. terreus* strains (XM_0012118515, XP_001216041, and XM_001214697), and 61.1–76.5% with sequences from *A. clavatus* (XM_001273447), *A. fumigatus* (XM_743802), and *A. oryzae* (XM_001821547). The deduced amino acid sequence of EglA revealed similarities of 98.3–98.6%, 60.3–79%, and 63.4–75.6% with the corresponding amino acid sequences from the three above-mentioned *Aspergillus* groups, respectively.

Interestingly, the *eglA* gene (717 bp, 238 aa) from *A. niger* VTCC-F021 was completely different to the *egI* gene (999 bp, 332 aa), of glycosyl hydrolase family 5 [10], from *A. niger* IFO31125 reported by Zhao *et al*. [35] in 2010. This was despite the fact that the latter gene was codon-optimized, synthesized, and overexpressed in the same species as the former, namely *P. pastoris*. The sequence comparison of the two revealed only a 5.5% identity in amino acid sequences and a 2.1% identity in the DNA sequence. The nucleotide sequence encoding EglA from *A. niger* VTCC-F021 has been deposited in the GenBank with an accession number of GU445334.

Ten amino acids, in *A. niger* EglA, of N36, W38, G68, E131, M133, W135, P144, G146, F194, and E219, were absolutely conserved among the family 12 enzymes (Fig. 1). Two residues, of E132 and E220, might have been the active site nucleophile and proton donor, respectively, when based on comparisons between family 12 endoglucanases and family 11 xylanase by hydrophobic cluster analysis [1]. The 128-GDY**E**L**M**I**W**LA-137 and 215-QFGTEAFTGG-214 were conserved among the family 12 endoglucanases from *Aspergillus* sp. using MegAlign (DNAStar). This information based on sequence similarity (data not shown) clearly indicates that the cloned endoglucanase A is a member of the cellulase system; glycosyl hydrolase family 12. Endoglucanases family 12, to which our EglA thus belongs, has shown significant activity toward xyloglucan, in addition to arabinoxylans side-activity [33], whereas endoglucanases family 5 (EgI from *A. niger* IFO31125) has exhibited significant activity toward mannan and galactomannan.

Expression and Purification of Recombinant EglA

P. pastoris GS115 was transformed with pPEglA, and the resulting transformants were grown in a YP medium for β-

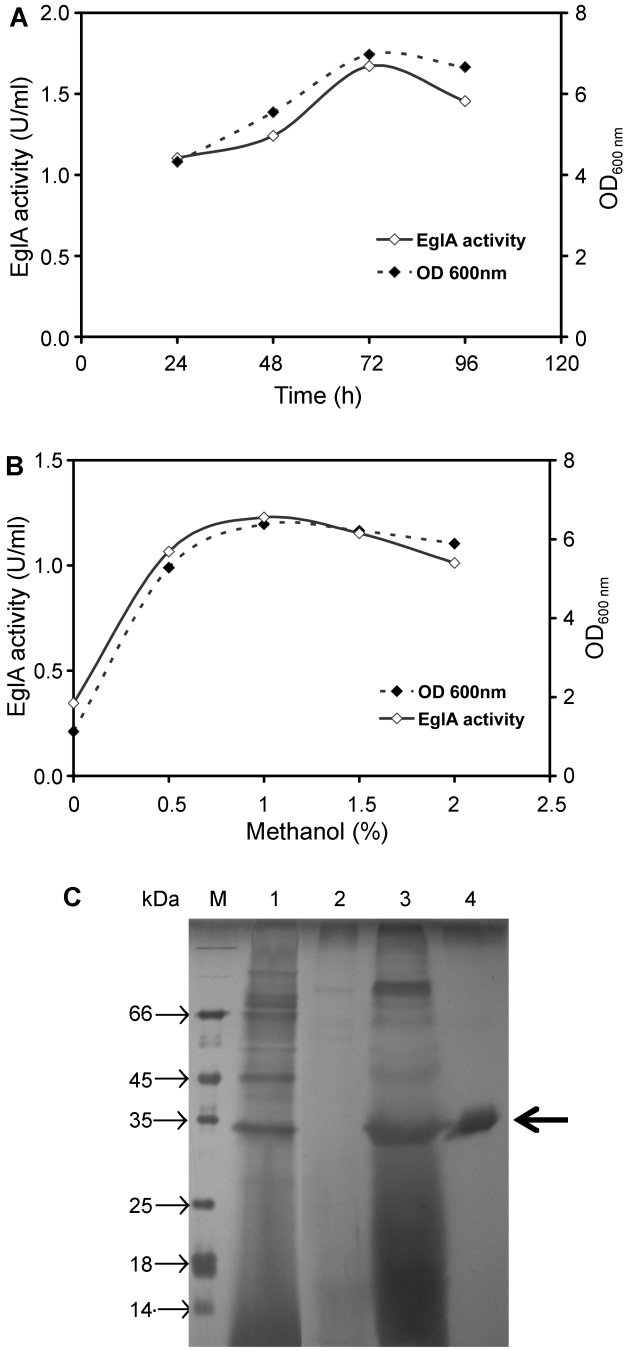

Fig. 2. EglA production curve (**A**), methanol induction on EglA production (**B**), and SDS–PAGE of EglA samples (**C**).
A. The cultivation was performed in 25 ml of YP medium at 30°C with agitation at 220 rpm with 1% (v/v) of methanol added every 24 h to maintain induction. **B**. The cultivation was performed in 25 ml of YP medium at 30°C with agitation at 220 rpm for 72 h with 0–2% (v/v) of methanol added every 24 h to maintain induction. EglA activity was determined at 55°C, pH 5 with 1% (w/v) CMC. **C**. A 20 µl aliquot of different samples of EglA was loaded on SDS–PAGE together with 4 µl of dyes. Lane **1**: culture supernatant of *A. niger* VTCC-F021; Lane **2**: culture supernatant of *P. pastoris* GS115; Lane **3**: culture supernatant of the recombinant *P. pastoris* GS115/pPEglA; Lane **4**: the recombinant purified EglA; Lane **M**: molecular mass of standard proteins (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA).

glucanase production. After 72 h of methanol induction, the culture supernatants were collected and used for enzyme activity assay. The *P. pastoris* GS115/pPEglA transformant, exhibiting the highest production level of β-glucanase (1.59 U/ml, Fig. 2A), was used for enzyme production, purification, and characterization. Conditions were first optimized to offer the highest production yield of the recombinant protein. A series of methanol induction concentrations (0.5–2%) were tested. The induction of 1% (v/v) of methanol produced the highest production level (1.229 U/ml) of the recombinant β-glucanase (Fig. 2B).

The recombinant β-glucanase was purified from the culture supernatant, by affinity chromatography with ProBond resin, and thus revealed only one protein band of approximately 33 kDa on SDS–PAGE (Fig. 2C, lane 4) with a specific activity of 16.24 U/mg protein, a little higher than that of wild-type EglA from *A. niger* VTCC-F021 (14.1 U/mg protein) (data not shown), whereas the vector control transformants had no detectable activity. The higher molecular mass of the recombinant β-glucanase, compared with the expected size (26 kDa) calculated from its deduced amino acid sequence, was possibly due to the glycosylation of rEglA. Moreover, as seen in Fig. 2 (lane 1), the wild-type protein also ran at a higher than expected position in the gel. This suggests that the wild-type protein was posttranslationally modified. The glycosylation prediction program NetOGlyc-3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/) revealed that the threonine, in position 234 (Fig. 1), was the predicted *O*-glycosylation site in the EglA sequence, with a G-score of 0.575. If the G-score is >0.5, the residue is predicted as glycosylated; the higher the score, the more confident the prediction [14].

Endoglucanase CelA, from *A. oryzae* KBN616 overexpressed under the control of a promoter of the *A. oryzae* taka-amylase A gene, has a molecular mass of 31 kDa [16]. The full-length cDNA, encoding a xyloglucan-specific endo-beta-1,4-glucanase (XEG) isolated from the filamentous fungus *A. aculeatus*, overexpressed in *A. oryzae*, has a molecular mass of 23.6 kDa [27]. These data suggest that *P. pastoris* is a suitable host for the overexpression of functional EglA.

Kinetic Parameters

The kinetic parameters of K_m, V_{max}, k_{cat}, and k_{cat}/K_m ratio, obtained for rEglA from *A. niger* VTCC-F021 with CMC substrate, were 10.17 mg CMC/ml, 28.99 U/mg protein, 579.71 min^{-1}, and 57.01, respectively; and with β-glucan substrate were 4.04 mg β-glucan/ml, 102.04 U/mg, 2,040.82 min^{-1}, and 505.05, respectively (Table 1). Interestingly, the K_m and V_{max} values obtained for wild-type EglA from *A. niger* VTCC-F021 were 8.5815 mg CMC/ml and 20.121 U/mg protein, respectively (data not shown), lower than those obtained for rEglA. There are several possible explanations as to why the K_m and V_{max} of rEglA were higher than that of the wild-type protein; one of the possible reasons is that the recombinant protein was in a glysosylated form, leading to a change in the three-dimensional structure of the catalytic site. This might have caused the increase in the activity of recombinant EglA when compared with the wild-type protein.

Table 1. Kinetic parameters of rEglA.

Kinetic parameters	β-Glucan	CMC
K_m (mg/ml)	4.04	10.17
V_{max} (U/mg)	102.04	28.99
k_{cat} (min^{-1})	2,040.82	579.71
k_{cat}/K_m	505.05	57.01

EglA activity was determined at 55°C, pH 5 with 1–10 mg/ml CMC or 2–12 mg/ml β-glucan.

The K_m value for rEglA from *A. niger* VTCC-F021, expressed in *P. pastoris*, was estimated to be 10.17 mg/ml CMC, lower than that (52–80 mg/ml) obtained for the enzyme from *A. niger* [12], but higher than that (8.7 mg/ml CMC) obtained for Egl from *S. sclerotorium* [34] and from the wild-type *A. niger* VTCC-F021 (8.5815 mg/ml CMC). The V_{max} (28.99 U/mg protein) obtained for rEglA from *A. niger* VTCC-F021 was much lower than that obtained for purified Egl from *A. terreus* AN_1 (200 U/mg protein) [22] but higher than that of *A. terreus* DSM 826 (4.35 U/mg protein) [8] when CMC was used as substrate.

The turnover number (k_{cat}) of rEglA from *A. niger* VTCC-F021 at 55°C, pH 5, 50 μg/ml rEglA, was found to be 579.71 min^{-1} with CMC substrate and 2,040.82 min^{-1} with β-glucan. The k_{cat} of rEglA from *A. niger* VTCC-F021, with CMC substrate (579.71 min^{-1}), was very similar to the value obtained for cellotriose of Egl from *T. reesei* QM 9414 (k_{cat}: 567 min^{-1}) [5]. The k_{cat}/K_m (catalytic efficiency), often referred to as the "specificity constant," is a useful index for comparing the relative rates of an enzyme acting on alternative, competing substrates. The higher the k_{cat}/K_m values, the higher the enzyme specificity with the substrate. In this study, the k_{cat}/K_m value of rEglA toward CMC was 57.01 min^{-1}, while it was 505.05 min^{-1} toward β-glucan (Table 1). Thus, rEglA was of a high specificity with β-glucan.

Temperature and pH Optima

The optimum temperature and pH for rEglA activity were 55°C (22.39 U/mg, Fig. 3A) and pH 6.5 (20.71 U/mg, Fig. 3B), respectively. This optimal temperature and pH are similar to those of the wild-type EglA from *A. niger* VTCC-F021 (data not shown).

Our results are somewhat different from those of previous studies, which showed that the optimum temperature and pH for β-glucanase from *A. niger* expressed in *P. pastoris* GS115 were 70°C and pH 5, respectively [35], whereas they were 70°C and pH 6 when the β-glucanase from *A.*

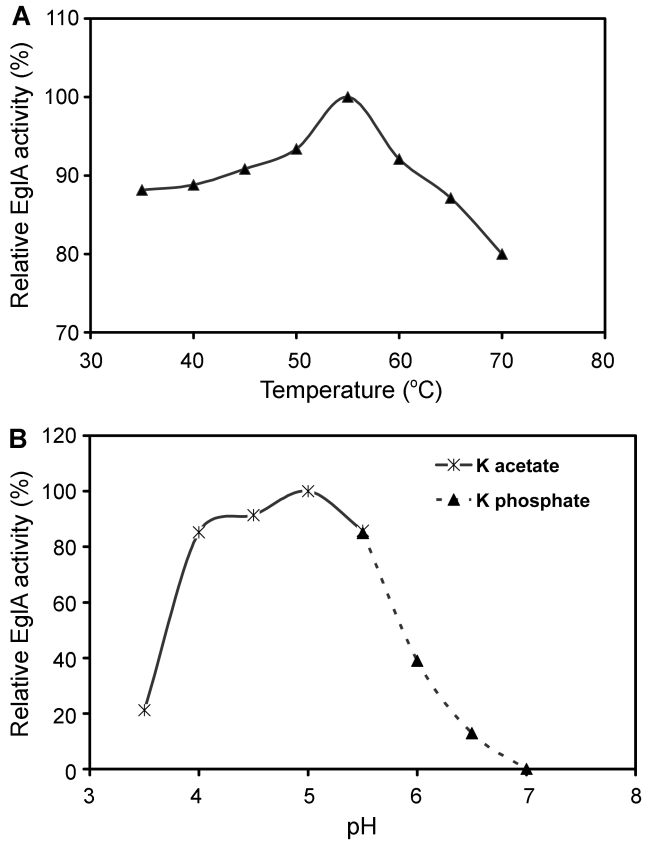

Fig. 3. Temperature (**A**) and pH (**B**) optima.
EglA activity (5 µg per reaction) was determined (**A**) at 35–70°C, pH 5 and (**B**) at 55°C, pH 3.5–7.0, with 1% (w/v) CMC.

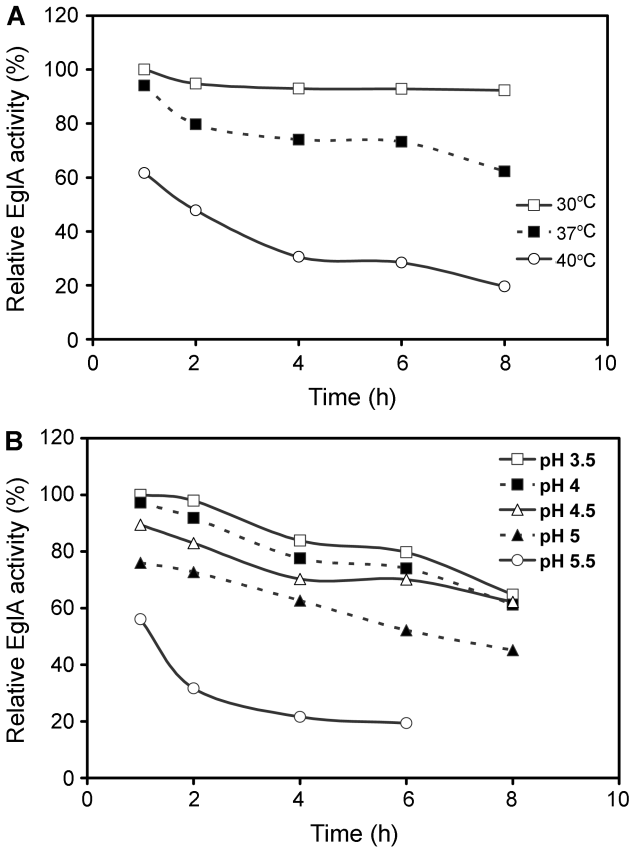

Fig. 4. Temperature (**A**) and pH stability (**B**).
Five µg EglA for each reaction was incubated (**A**) at 30, 37, and 40°C, pH 5 and (**B**) at 30°C, pH 3.5–5.5, for 1–8 h. The residual activity was then determined at 55°C, pH 5 with 1% (w/v) CMC.

niger was expressed in *S. cerevisiae* [10], and that from *A. aculeatus* expressed in *A. oryzae* were 50°C and pH 4.5 [29]. These differences may perhaps be due to amino acid sequence variations amongst these proteins, the different endoglucanase families from which they are derived, and because of the different protein processing mechanism(s) between the expression hosts.

Temperature and pH Stability

rEglA was stable at temperatures below 40°C and in a pH range of 3.5–4.5. The residual activity was over 60% after 8 h of treatment at 37°C (Fig. 4A) and in a pH range of 3.5–4.5 (Fig. 4B), whereas the activity of wild-type EglA from *A. niger* VTCC-F021 remained at 80% after 8 h treatment at 37°C and at pH 4 (data not shown), thus indicating that rEglA is less stable than wild-type EglA.

Zhao *et al*. [35] reported, in 2010, that the recombinant EgI (glycosyl hydrolase family 5) from *A. niger* IFO31125 produced in *P. pastoris* could withstand heating up to 70°C for 30 min without apparent loss of activity, and that more than 90% of the total activity was retained after incubation in a pH range of 3–9 at 40°C for 30 min. However, only 20% of the activity was maintained after it was heated to 80°C for 30 min. The recombinant Egl enzyme from *A. niger* expressed in *S. cerevisiae* retained 56% of the initial activity after 1 h of incubation at 80°C and was stable in the pH range of 3–10 [10]. The temperature and pH stability of F1-CMCase from *A. aculeatus* in *A. oryzae* were below 45°C and 2–9, respectively [29].

Since apparent inferior thermostabilities are probably related to lower levels of glycosylation, it is possible that because of the presence of less glycosylation, our expressed EglA may have a slightly narrower temperature optimum (55°C) and be less thermostable (< 40°C) than the wild type (data not shown), or than those expressed in other hosts. It has been demonstrated that N-glycosylation of cycloinulo-oligosaccharide fructanotransferase improved the thermostability of the enzyme expressed in *S. cerevisiae* [15]. Our hypothesis is further supported by other studies that have demonstrated a similar role for glycosylation in protein thermostability [17, 32].

Effects of Metal Ions on the rEglA Activity

The addition of various metal ions to the reaction mixture in various concentrations affected enzyme activity (Table 2).

Table 2. Effects of metal ions and EDTA on rEglA activity.

Metal ions (mM)	Activity remaining (%)		
	5	10	15
Ag^{+}	54	30	25
Ca^{2+}	77	68	59
Co^{2+}	96	89	68
Cu^{2+}	95	102	94
EDTA	106	98	78
Fe^{2+}	70	59	42
K^{+}	75	71	64
Mn^{2+}	56	57	67
Ni^{+}	64	78	82
Zn^{2+}	78	72	54

Five μg EglA for each reaction was incubated with 5–15 mM metal ions and EDTA at 37°C for 1 h. The residual activity was then determined at 55°C, pH 5 with 1% (w/v) CMC.

These metal ions and EDTA had different effects on rEglA activity. Co^{2+}, Cu^{2+}, and EDTA had only a slight effect on endoglucanase activity, whereas other metal ions, including Ag^{+}, Fe^{2+}, and Zn^{2+}, had an obvious inhibitory effect on endoglucanase activity. Despite this, enzyme activity retained one to three fourths of the original activity when it was treated with the various concentrations of metal ions (Table 2). These results are similar to those for wild-type EglA from *A. niger* VTCC-F021 (data not shown).

Effects of Organic Solvents and Detergents

All additives of organic solvents and detergents to the enzyme resulted in obvious inhibitory effect on rEglA (Fig. 5). The addition of 10–30% of ethanol, acetone, and methanol decreased enzyme activity to 40–90% of the original activity, and isopropanol and *n*-butanol decreased the activity to 23–53% (Fig. 5A). The addition of 0.5–2.0% (w/v) Tween 20 and Tween 80 decreased rEglA activity to 31%, whereas rEglA activity retained only 47% upon the addition of 0.5–2.0% Triton X-100 and 0.5–1.0% SDS, and activity was completely stopped by the addition of 1.5–2.0% SDS (Fig. 5B).

Wild-type endoglucanase from *A. niger* VTCC-F021 exhibited a similar profile for residual activity when it was treated with these additives (data not shown).

In conclusion, several *Aspergillus* genes encoding endo-1,4-β-glucanase have been isolated from *A. aculeatus* (cDNA: 711 bp, 237 aa, [25]), *A. kawachii* IFO 4308 (FI-CMCase, 717 bp, 239 aa, [31]), *A. oryzae* KBN616 (*celA*, 717 bp, 239 aa, [16]), *A. aculeatus* (XEG, 714 bp, 238 aa, [27]), and *A. niger* IFO31125 (*eng1,* 996 bp, 332 aa, [10]). Although the endo-β-1,4-glucanase has been expressed as an active enzyme in several systems, such as those of *S. cerevisiae* [10, 31] and *A. niger* [30], the recombinant proteins produced in these systems still have some limitations such as production yield as well as protein

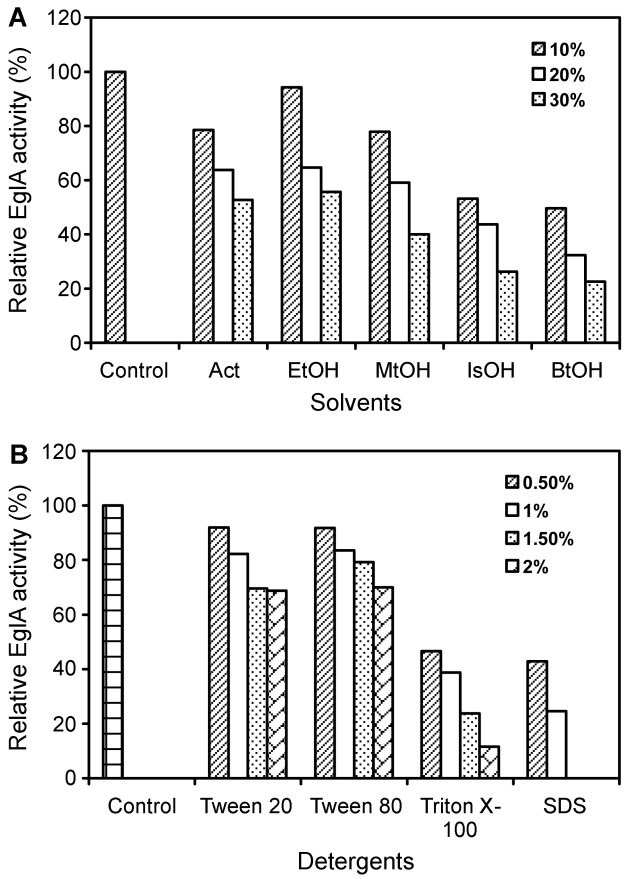

Fig. 5. Effects of organic solvents (**A**) and detergents (**B**) on the rEglA activity.
Five μg EglA for each reaction was incubated with (**A**) 10–30% (v/v) of organic solvents and (**B**) 0.5–2% (w/v) of detergents, at 37°C for 1 h. The residual activity was then determined at 55°C, pH 5 with 1% (w/v) CMC.

activity and stability. For practical applications, a system that can express active EglA at high expression levels is needed. In the present study, we have successfully cloned, expressed, and documented the gene coding for EglA isolated from *Aspergillus niger* VTCC-F021 in *P. pastoris*, and have demonstrated that *P. pastoris* would provide a good system for the expression of the *eglA* gene on an industrial scale. rEglA has been shown to be relatively stable, in addition to being highly specific toward β-glucan, and is expected to have high potential for use in downstream biotechnological applications.

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