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Cloning and Heterologous Expression of a Novel Endoglucanase Gene *egVIII* from *Trichoderma viride* in *Saccharomyces cerevisiae*

Xiao-Mei Huang · Qian Yang · Zhi-Hua Liu · Jin-Xia Fan · Xiu-Ling Chen · Jin-Zhu Song · Yun Wang

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Abstract Endoglucanase is a major cellulolytic enzyme produced by the fungus *Trichoderma viride*. The 1,317 bp cDNA of endoglucanase gene *egVIII* was cloned from *T. viride* AS3.3711, encoding a 438 amino acid protein with a calculated molecular mass of 46.86 kDa and isoelectric point of 4.32. Sequence analysis suggested that EGVIII belonged to the glycosyl hydrolase family 5. The N-terminal region of EGVIII contains a signal peptide sequence of 19 amino acid residues, indicating that it is an extracellular enzyme. Transcription of the *egVIII* gene in *T. viride* AS3.3711 can be induced by carboxymethyl cellulose sodium (CMC-Na), sucrose, microcrystalline cellulose, and corn stalk, and inhibited by glucose and fructose. The α-mating factor signal can effectively enhance the secretion of the recombinant EGVIII in *Saccharomyces cerevisiae*, as demonstrated by the enzymatic activity of recombinant yeast IpYEMα-xegVIII in the supernatant, which was 0.86 times higher than that of the IpYES2-egVIII. Recombinant endoglucanase EGVIII showed optimal activity at a temperature of 60°C and pH of 6.0. It was stable when incubated from 35°C to 70°C for 1 h. The enzymatic activity of recombinant EGVIII was stable at a pH3.0 to 7.5 at 50°C and reached the highest level at 0.174U when activated by 75 mM of Zn^{2+}. The Michaelis–Menten constant (*Km*) and *Kcat* values for CMC-Na and cellotriose hydrolysis were 3.82 mg/ml, 9.56 s^{-1} and 1.75 mg/ml, 7.08 s^{-1}, respectively. Transgenic yeast strain IpYEMα-xegVIII might be useful for renewable fuels industries.

Keywords *Trichoderma viride* · Endoglucanase · Gene cloning · Yeast expression

Introduction

Lignocellulosic biomass, which is partly comprised of cellulose, is widely recognized as a promising source of raw material for production of renewable fuels and chemicals [1, 2].

X.-M. Huang · Q. Yang (✉) · Z.-H. Liu · J.-X. Fan · X.-L. Chen · J.-Z. Song · Y. Wang
Department of Life Science and Engineering, Harbin Institute of Technology, Harbin 150001, China
e-mail: YangQ@hit.edu.cn

Saccharomyces cerevisiae, one of the most popular microorganisms used for ethanol production, cannot use cellulose as the only source of carbon and energy due to the lack of endogenous cellulolytic enzymes. For the high-efficient use of cellulosic biomass, the cooperative action of three cellulolytic enzymes (endoglucanase, exoglucanase, and β-glucosidase) in hydrolysis to glucose is needed. Many genes of cellulolytic enzymes, cloned from different microorganisms such as *Trichoderma reesei*, *Trichoderma viride*, *Clostridium thermocellum*, and *Cellulomonas biazotea*, have been expressed in yeast [3-9]. However, yeast expression levels and recombinant enzyme activity are generally lower than that of the native enzyme. Penttilä et al. [9] reported that specific activity of recombinant *T. reesei* cellobiohydrolase (CBHI) was twofold lower compared to the native CBHI enzyme. In another study, the specific activity of *Aspergillus aculeatus* CBHI produced in *S. cerevisiae* was slightly lower than that of the native enzyme [10]. One reason for lower expression could be that the signal peptide of an exogenous gene may not be efficiently recognized by the host yeast cell. Therefore, a vector harboring a yeast signal peptide may improve the secretion of a heterologous protein from *S. cerevisiae*, as well as potentially enhancing the enzymatic activity and yield. Zhang et al. [11] constructed a vector with a signal peptide from *Brevibacillus brevis* to express a recombinant methyl parathion hydrolase; its activity was 8.1 times higher than that of enzyme with the original strain M6. Signal peptide from *B. brevis* directed post-translational extracellular transport of methyl parathion hydrolase.

In the present study, a novel endoglucanase gene *egVIII* was cloned from *T. viride* AS3.3711, and the response of *egVIII* in *T. viride* to different substrates was studied. Two recombinant endoglucanase yeast strains (IpYEMα-xegVIII and IpYES2-egVIII) were constructed. We determined that the yield, secretion, and activity of the recombinant EGVIII enzyme were enhanced when the native signal peptide was replaced with an α-mating factor (αMF) signal peptide from *S. cerevisiae*.

Materials and Methods

Strains and Cultivation Conditions, Plasmids

T. viride strain AS3.3711 was routinely maintained on potato dextrose agar (PDA; Difco, Detroit, MI, USA) at 28°C. One milliliter of a suspension containing 1×10^6 spores of *T. viride* AS.3711 was washed from the PDA plate with sterile water and used to inoculate 100 mL of potato dextrose broth (Difco Laboratories) in a 500-mL Erlenmeyer flask. The spores were then grown at 28°C on a shaker at 250 rpm for 48 h. *S. cerevisiae* H158 was used as the host and grown in yeast peptone dextrose broth medium (1% yeast extract, 2% peptone, and 2% glucose (*w*/*v*)). For expression studies, yeast cells were grown on synthetic complete lacking uracil (SC-U) medium (0.67% yeast nitrogen base, 0.01% (adenine, arginine, lysine, leucine, threonine, and tryptophan), and 0.005% (aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, valine, and tyrosine)) [12]. The pYES2 vector (Invitrogen, Madison, WI, USA) was used for the expression of endoglucanase in *S. cerevisiae*.

Cloning of the Endoglucanase Gene

In order to obtain the cDNA of the *egVIII* gene, total RNA was extracted from mycelia of *T. viride* AS3.3711 using Trizol reagent (Invitrogen, Madison, WI, USA) and digested with

DNaseI (Promega, Madison, WI, USA). Total RNA (2 μg) was reverse transcribed with oligo(dT) in a volume of 10 μl to produce the cDNA that was used as the polymerase chain reaction (PCR) template. The sense primer P1 (5′-ACGCTCCTCGTTTCTCC-3′) and the antisense primer P2 (5′-TCAAAACGCTACCAGC-3′) were designed specifically to amplify the endoglucanase gene *egVIII* from *T. viride*. The PCR reaction mixture consisted of 0.125 μl *Taq* (5 units μl^{-1}), 2.5 μl 10 × buffer, 2.5 μl deoxynucleotides (dNTP mixture, each at 2.5 mM), 0.5 μl primer P1 (20 μM), 0.5 μl primer R1 (20 μM), 1.25 μl template cDNA (1 ng μl^{-1}), and ddH_2O to a final volume of 25 μl. After 5 min denaturation at 94°C, 34 cycles of 30 s denaturation (94°C), 30 s annealing (55°C), and 1 min polymerization (72°C) were carried out. In the final cycle, the temperature of 72°C was held for an additional 7 min.

The PCR product was ligated into the pMD18-T vector (TaKaRa, Japan) to obtain pMD18-T/*egVIII*, which was transformed into *Escherichia coli* DH5α competent cells by the heat-shock method [13]. The transformants were spread onto Luria–Bertani plates containing ampicillin (100 μg ml^{-1}), X-Gal (0.2 mM), and isopropyl β-D-1-thiogalacto-pyranoside (40 μg ml^{-1}) and incubated at 37°C for 24 h. White colonies were selected for identification by PCR.

Bioinformatic Analysis of the *egVIII* Gene

The open reading frame (ORF) of the *egVIII* gene was searched using the ORF program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The theoretical molecular mass and isoelectric point of the EGVIII protein were calculated using the Protparam tool (http://us.expasy.org/tools/protparam.html), and the catalytic domain of the EGVIII protein was identified by InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/). The signal peptide was predicted using SignalP3.0 server (http://www.cbs.dtu.dk/services/SignalP/). Endoglucanase amino acid sequences from different fungi were aligned by the ClustalX program [14].

Response of *egVIII* in *T. viride* to Different Substrates

To study the transcriptional response of *T. viride* AS3.3711 *egVIII* to various substrates, 5 ml of a suspension of spores was inoculated into 50 ml MENDELS medium [15] (carbon source 10 g/L, $(NH_4)_2SO_4$ 1.4 g/L, urea 0.3 g/L, KH_2PO_4 2.0 g/L, $CaCl_2$ 0.3 g/L, $MgSO_4{\cdot}7H_2O$ 0.3 g/L, peptone 0.5–1.0 g/L, Tween 801.0–2.0 g/L, $FeSO_4{\cdot}7H_2O$ 5.0 mg/L, $ZnSO_4{\cdot}7H_2O$ 1.4 mg/L, $MnSO_4{\cdot}H_2O$ 1.6 mg/L, and $CoCl_2$ 2.0 mg/L, pH5–6) in a 150-ml Erlenmeyer flask. Six types of carbon sources (inducing substrates) included glucose, sucrose, microcrystalline cellulose, carboxymethyl cellulose sodium (CMC-Na), corn stalk, or fructose. Mycelia were harvested after induction for 1, 2, 3, 4, and 5 days.

Total RNA (10 μg) was extracted from mycelia of *T. viride* AS3.3711 using Trizol reagent (Invitrogen, Madison, WI, USA), separated on a 1.2% agarose gel containing 1.5% formaldehyde and blotted onto a Nylon membrane. Digoxigenin High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany) were used for the probe preparation and transcript detection of the *egVIII* gene. The *egVIII* gene was digested by *Xba*I and *Eco*RI from the plasmid (pYES2-egVIII) and labeled with digoxigenin as a probe. Hybridization probe was prepared by using the random primer extension method. The blotted membrane was prehybridized at 65°C in a digoxigenin Easy Hyb solution and then hybridized in the same solution supplemented with a digoxigenin-labeled *egVIII* probe overnight. The membrane was washed with 2 × saline-sodium citrate (SSC) and 0.1% sodium dodecylsulphate (SDS) at room temperature, followed by 0.1 × SSC and 0.1% SDS at

68°C. After washing the membrane with a solution containing 0.1 M maleic acid and 0.15 M NaCl, pH7.5, the hybridized signal was detected using a chemiluminescent substrate CDP-STAR in the detection kit (Roche, Germany) according to the manufacturer's instructions.

Construction of Recombinant Plasmids and Yeast Transformation

Initially, the coding region of the *egVIII* gene was amplified from *T. viride* AS3.3711 with primers e1 (5′-TGCGAATTCACGCTCCTCGTTTCTCC-3′, *Eco*RI site is underlined) and e2 (5′-CGCTCTAGATCAAAACGCTACCAGC-3′, *Xba* I site is underlined). The primers were designed according to the *Cel5b* (GenBank Acc. No. AY281373) from *T. reesei*. The *egVIII* fragment was digested with *Eco*RI and *Xba* I and inserted into vector pYES2, yielding pYES2-egVIII.

In addition, to improve the expression, an αMF signal sequence was amplified from *S. cerevisiae* with primers M1 (5′-CGGGATCCATGAGATTTCCTTC-3′, *Bam*HI site is underlined) and M2 (5′-CGGAATTCAGCTTCAGCCTCTC-3′, *Eco*RI is underlined). The αMF factor sequence was digested with *Eco*RI and *Bam*HI and inserted into pYES2, yielding pYES2-MFα. The *egVIII* fragment without signal peptide was amplified from *T. viride* AS3.3711 using primers eg3 (5′-TGCGAATTCGGCAAGATCAAATATCTG-3′, *Eco*RI site is underlined) and eg4 (5′-CGCTCTAGATCAAAACGCTACCAGC-3′, *Xba* I site is underlined). The non-signal peptide *egVIII* fragment was digested with *Eco*RI and *Xba*I and inserted into pYES2-Mα, yielding pYEMα-xegVIII.

The two recombinant plasmids (pYES2-egVIII and pYEMα-xegVIII) and control vector pYES2 were transformed into *S. cerevisiae* H158 by the use of lithium acetate method, respectively, as described by Krautwurst et al. [16].

Detection of Recombinant *egVIII* Expression in Yeast

To measure the expression of *egVIII* at the transcriptional level, transformants cultured in SC-U medium containing 2% galactose and yeast cells were collected after induction for 6, 12, 24, 36, and 48 h for RNA isolation. Total RNA was extracted from yeast cells using a Yeast RNA mini kit (Watson Biotechnologies, China). Northern blot hybridization was performed using the DIG-Labeling and Detection Kit (Roche, Germany; Kit no: 1585614).

The transgenic yeast IpYES2-egVIII, IpYEMα-xegVIII, and IpYES2 were grown on minimal medium SC-U and then each inoculated into 100 mL SC-U medium containing 2% galactose and induced for 96 h at 30°C. The supernatant was collected every 12 h by centrifugation to measure enzymatic activity.

Enzymatic Activity Assays of Recombinant Endoglucanase EGVIII

The CMCase activity was measured by using Meinke's procedure [17] with some modifications. The reaction mixture, consisting of 1 ml CMC-Na as the substrate and 0.5 ml of cultured supernatant after centrifugation, was incubated at 50°C for 30 min, supplemented with 0.5 ml of 3,5-dinitrosalicylic acid and boiled for 10 min. After cooling, the reduced sugars released in response to CMCase activity were measured at 540 nm. One unit of CMCase activity was defined as the amount of enzyme required for releasing total reduced sugar equivalent to 1 mmol glucose per minute. The analysis of variance and multiple comparisons were performed using SAS 9.0.

Stability Assays of EGVIII

The yeast transformants were induced by 2% galactose for 96 h at 30°C. The yeast cells were centrifuged, and solid ammonium sulfate was added to the supernatant up to 20% saturation. The precipitate was discarded by centrifugation at 2,500 rpm for 5 min. Further, solid ammonium sulfate was added up to 80% saturation then dialyzed at 4°C overnight. The dialyzed fraction was collected by centrifugation at 12,000 rpm and 4°C for 10 min. The dialyzed fraction was used in the following experiments.

The optimum pH was measured at 50°C between pH 3 and 9 at intervals of 0.5 pH units. pH stability was assayed by incubating the dialyzed fraction in different pH using buffers 50 mM sodium citrate (pH 3.0–6.0), sodium phosphate (pH 6.5–8.0), and Tris–HCl (pH 8.5–9.0) for 48 h at 4°C, and then the residual activity was measured under standard conditions (pH = 6.0).

The optimum temperature was measured at pH 5.2 between 30°C and 90°C for 30 min at 5°C intervals. Thermal stability was assayed by incubating the dialyzed fraction at different temperatures (ranging 30–90°C) in 50 mM citrate buffer, pH 5.2, for 1 h, and then the residual activity was measured by incubation at 60°C for 30 min.

To investigate the effect of ions on enzymatic activity, the dialyzed fraction was assayed in the reaction buffer supplemented with 75 mM of metal ion. Several different buffer solutions were prepared, each spiked with a different metal salt ($MgSO_4$, $CuSO_4$, $FeSO_4$, $ZnSO_4$, $MnSO_4$, $Al_2(SO_4)_3$, KCl, NaCl, $CaCl_2$, $BaCl_2$, $CoCl_2$, $FeCl_3$, and $AgNO_3$).

All of the above experiments were completed in triplicate, and average values were calculated based on results from three independent experiments.

The Kinetic Properties of Recombinant EGVIII

The effect of various substrate concentrations (from 0.5 to 2.5 mg/mL) on the enzyme activity was studied under the above standard assay conditions of pH (5.0) and temperature (50°C). The Michaelis–Menten kinetic constants *(Km)* and maximum velocity (*Vmax*) for CMC-Na, Avicel, cellobiose, and cellotriose were obtained using the reciprocal plot (Line-weaver-Burk plot). The protein concentration was determined according to Bradford' procedure [18], with bovine serum albumin as the standard.

Results

Cloning and Sequence Analysis of Endoglucanase Gene *egVIII*

The coding region of endoglucanase gene *egVIII* was 1,317 bp in length, encoding 438 amino acids with a calculated molecular mass of 46.86 kDa and a predicted isoelectric point of 4.32. The BlastP search indicated that the amino acid sequence of *egVIII* from *T. viride* shared the highest similarity (70%) with a known endoglucanase (AAR29981) from *Trichoderma* sp. C-4. The cDNA sequence of *egVIII* gene was deposited into the GenBank database with the accession number EU518928. SignalP prediction showed that there is a signal peptide (19 amino acids) in the N-terminal sequence of endoglucanase EGVIII. The signal peptidase cleavage site of EGVIII amino acid sequence is at A19 and G20 (ALA/GK), indicating that endoglucanase EGVIII is an extracellular enzyme. InterProScan analysis revealed that the EGVIII protein had a catalytic domain of glycoside hydrolase family 5

(InterPro Acc. No. IPR001547), indicating that it is an endoglucanase [19]. EGVIII has a C-terminal hydrophobic region and a consensus sequence, suggesting that it may be modified by a glycosylphosphatidylinositol anchor attached to serine 411 [20]. Therefore, EGVIII might represent a membrane bound endoglucanase.

Multiple sequence alignment of chitinases from 28 fungal species/strains was conducted using the ClustalX program, which revealed that the amino acid sequences of these endoglucanases were conserved, with identities ranging from 47% to 100%. Furthermore, the catalytic domains of these different endoglucanases were highly conserved. The consensus sequence of catalyzing domains is FG[L/I]MNEP (Fig. 1).

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ABP02069       WTLLATKYK-NDPNVIFGLMNEPHDLD-VPTWAGSVQAAVNAIRAAGA  190
Q04469         WTLLATKVTSNDPNVIFGLMNEPHDLD-VSTWAGSVQAAVNAIRAAGA  191
EED79362       WTQLADEYGSNDR-IIFGIMNEPHDLN-VTEWVASVQYVVNAVRSAGS  183
EED77769       WTQLADEYGSNDR-IIFGIMNEPHDLN-VTEWVASVQYVVNAVRSAGS  183
AAU12275       WTQLAQKYGSNQR-VIFGIMNEPHDIPSISTWVNSVQGAVNAIRAAGA  234
EED77763       WSQLSKFYGANDR-LIFGIMNEPHDLD-IAAWATSVQYVVNDIRAAGA  180
ACD36972       WVQIAKYYEDNDK-IIFGLMNEPHDLD-IEIWAQTCQKVVTAIRKAGA  189
AAP57754       WVQIAKYYEDNDK-IIFGLMNEPHDLD-IEIWAQTCQKVVTAIRKAGA  189
ACH92572       WSQLASKYASQSR-VWFGIMNEPHDVN-INTWAATVQEVVTAIRNAGA  264
ABQ95572       WSQLASKYASQSR-VWFGIMNEPHDVN-INTWAATVQEVVTAIRNAGA  243
ABA64553       WSQLASKYASQSR-VWFGIMNEPHDVN-INTWAATVQEVVTAIRNAGA  264
P07982         WSQLASKYASQSR-VWFGIMNEPHDVN-INTWAATVQEVVTAIRNAGA  264
AAQ21383       WSQLASKYASQSR-VWFGIMNEPHDVN-INTWAATVQEVVTAIRNAGA  264
BAA36216       WSQLASKYASQSR-VWFGIMNEPHDVN-INTWAATVQEVVTAIRNAGA  264
AAR29981       WSQLAQKYASQSK-VWFGIMNEPHDVN-INTWATTVQAVVTAIRNAGA  265
CAA61740       WTQLATHYKSNSR-VIFGIMNEPHDLN-IATWAATVQKTVTAIRNTGA  256
YP_449282      WRRLALEFKDDKA-VIFGLMNEPNGIS-APDWANAAQGAINAIRKTGA  198
AAW73535       WRRLALEFKDDKA-VIFGLMNEPNGIS-APDWANAAQGAINAIRKTGA  274
YP_001916047   WRRLALEFKDDKA-VIFGLMNEPNGIS-APDWANAAQGAINAIRKTGA  188
ZP_02241148    WRRLALEFKDDKA-VIFGLMNEPNGIS-APDWANAAQGAINAIRKTGA  188
NP_640         WRRLALEFKDDKA-VIFGLMNEPNGIS-APDWANAAQGTITAIRKTGA  198
YP_361764      WRRLALEFKDDKA-VIFGLMNEPNGIS-APDWANAAQGTINAIRKTGA  198
NP_635423      WRRLALEFKDDKQ-VIFGLMNEPNGIS-STDWAAAAQAAINTIRKTGA  195
YP_001901436   WRRLAIAFKSDNA-VIFGLMNEPYDIS-PEGWAAAAQASIDSIRATGA  191
NP_640384      WRRLALAFNSDNA-VMFGLMNEPNNIS-ASDWAGAAQAAIDAIRRTGA  190
YP_198922      WRRLALTFKSDNA-VMFGLMNEPNNIS-ASEWAGAAQAAIDAIRKTGA  190
YP_002283697   WARLAVEFANQDG-VLFGLMNEPHDIK-ATDWLEAANAAIRSIRAVGA  181
YP_002275184   WTRLGTAFRDRPD-VWFGLMNEPQQKS-AEAWRDIEQQAILGIRAAGA  183
               *  :.         : **:****        *    :  :  :* .*:
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Fig. 1 Multiple sequence alignment of catalyzed domains of endoglucanase from 28 fungal species/strains. All these endoglucanase are members of glycoside hydrolase family 5; their catalyzing domains are highly conserved. The *asterisks* refer to the conserved amino acid residues. ABP02069: *Cryptococcus* sp. S-2; Q04469: *Cryptococcus flavus*; EED79362: *Postia placenta* Mad-698-R; EED77769: *P. placenta* Mad-698-R; AAU12275: *Sporotrichum pruinosum*; EED77763: *P. placenta* Mad-698-R; ACD36972: *Trichoderma viride* AS3.3711; AAP57754: *Trichoderma reesei*; ACH92572: *Trichoderma* sp. SSL; ABQ95572: *T. viride*; ABA64553: *Hypocrea jecorina*; P07982: *T. reesei*; AAQ21383: *T. viride* AS3.3711; BAA36216: *T. viride*; AAR29981: *Trichoderma* sp. C-4; CAA61740: *Penicillium janthinellum*; YP_449282: *Xanthomonas oryzae* pv. *oryzae* MAFF 311018; AAW73535: *X. oryzae* pv. *oryzae* KACC10331; YP_001916047: *X. oryzae* pv. *oryzae* PXO99A; ZP_02241148: *Xanthomonas oryzae* pv. *oryzicola* BLS256; NP_640: *Xanthomonas axonopodis* pv. *citri* str. 306; YP_361764: *Xanthomonas campestris* pv. *vesicatoria* str. 85-10; NP_635423: *Xanthomonas campestris* pv. *campestris* str. ATCC 33913; YP_001901436: *X. campestris* pv. *campestris* str. B100; NP_640384: *X. axonopodis* pv. *citri* str. 306; YP_198922: *X. oryzae* pv. *oryzae* KACC10331; YP_002283697: *Rhizobium leguminosarum* bv. *trifolii* WSM2304; YP_002275184: *Gluconacetobacter diazotrophicus* PAl5

Expression Pattern of *egVIII* in *T. viride* Cultured with Different Carbon Sources

The expression patterns of *egVIII* in *T. viride* AS3.3711 were analyzed in the presence of different carbon sources. No *egVIII* transcripts were detected in mycelium cultivated with glucose (2%) or fructose (2%) as carbon sources, at any time point (Fig. 2a, f). When sucrose or corn stalk was the carbon source, *egVIII* mRNA reached the highest levels at 4 days, while no signal was detected at 1 day (Fig. 2b, e). Similar results were observed when microcrystalline cellulose was the carbon source (Fig. 2c). However, the strongest signal was observed with CMC-Na as only carbon source (Fig. 2d): the expression was induced by CMC-Na at 1 day, peaked at 4 days, and decreased beyond 4 days (Fig. 2d).

Heterologous Expression of *egVIII* Gene in *S. cerevisiae*

The presence of the RNA transcript from the expressed *egVIII* gene in transformants IpYEMα-xegVIII and IpYES2-egVIII induced by galactose were analyzed by Northern blotting (Fig. 3). No hybridization signals were observed from transformant IpYES2 using *egVIII* gene as a probe (Fig. 3a), but a single band was observed from transgenic yeast IpYES2-egVIII (Fig. 3b) and IpYEMα-xegVIII (Fig. 3c). The transcript level of endoglucanase gene *egVIII* could be detected at 6 h after inoculation, increased rapidly at 12 h, reached the highest levels at 24 h, and decreased thereafter. The transcriptional patterns of the *egVIII* gene in transgenic yeasts IpYES2-egVIII and IpYEMα-xegVIII were similar (Fig. 3b, c).

The recombinant EGVIII activity of the transgenic yeast IpYEMα-xegVIII and IpYES2-egVIII showed a peak value at 36 h after galactose induction, while CMCase activity of the transformants IpYEMα-xegVIII was higher than that of IpYES2-egVIII. The highest activity was about 0.110U (Fig. 4). Additionally, CMCase activity was not detected in the

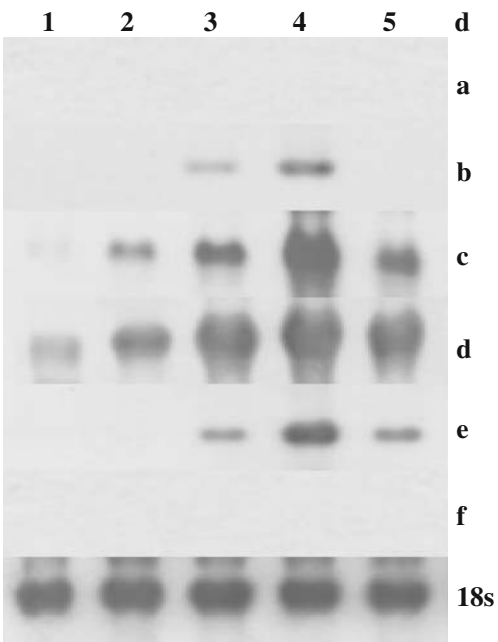

Fig. 2 Expression patterns of *egVIII* gene in *Trichoderma viride*. Total RNA (10 μg) was extracted from mycelia of *T. viride* AS3.3711 cultured in MM with different carbon sources. *a*: 1% glucose; *b*: 1% sucrose; *c*: 1% microcrystalline cellulose; *d*: 1% CMC-Na; *e*: 1% corn stalk; *f*: 1% fructose. Mycelia were harvested after induction for 1, 2, 3, 4, and 5 days. The *egVIII* and *18S rDNA* sequences were used as hybridization probes

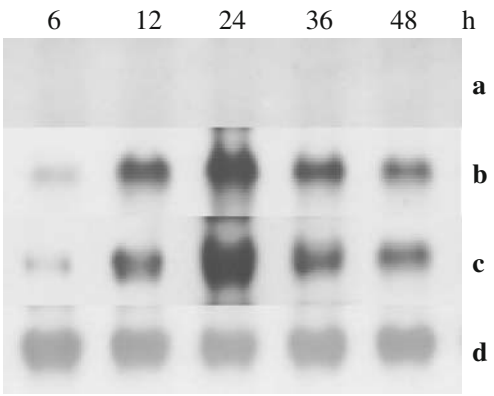

Fig. 3 Northern blot analysis of *egVIII* transcripts in the yeast transformants. Total RNA was isolated from yeast cells induced in SC-U medium containing 2% galactose. Listed at the top are hours after inoculators of the galactose media. *a*, *b*, and *c*: The *egVIII* expression in transformants IpYES2, IpYES2-egVIII, and IpYEMα-xegVIII at different induced times, respectively. *d*: 18S rRNA expression in *Saccharomyces cerevisiae* H158 as control

culture supernatant of *S. cerevisiae* IpYES2 when galactose as the only carbon source, indicating that CMCase activity displayed in transgenic yeast cells was due to expression of the exogenous *egVIII* gene. Furthermore, the αMF-factor signal played an important role in the secretion of the recombinant EGVIII whose activity in the IpYEMα-xegVIII supernatant was 0.86 times than that of the IpYES2-egVIII.

Properties of Recombinant Endoglucanase EGVIII

To characterize the enzyme properties, the supernatant of fermentation broth was dialyzed, and the dialyzed fraction was used for measuring the CMCase activity. EGVIII activity increased slowly from 30°C to 40°C, reached its peak at 60°C, and reduced beyond 60°C; therefore, the optimal reaction temperature of recombinant EGVIII was 60°C (Fig. 5a). Enzymatic activity of recombinant EGVIII was stable when incubated from 35°C to 70°C for 1 h and decreased rapidly when temperature reached 75°C (Fig. 5b). At 50°C, the enzyme activity of recombinant EGVIII was stable at a pH3.0 to 7.5, with a peak of activity at pH6.0. CMCase activity was reduced dramatically at pH8.0 (Fig. 5c). The highest activity of CMCase was at pH6.0 (Fig. 5d).

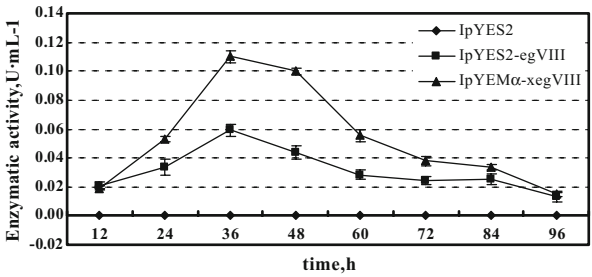

Fig. 4 Activity of endoglucanase EGVIII from different transgenic yeasts. The culture supernatant of recombinant yeast with the *egVIII* gene was used for measuring endoglucanase activity. The supernatant was boiled at 100°C for 30 min and used as control. The transformants were induced by galactose at 30°C for 12 to 96 h, at 12 h intervals. The enzyme activity at different induced times was measured. *Square*: EGIII activity of yeast IpYES2-egVIII, *triangle*: EGIII activity of yeast IpYEMa-xegVIII, *diamond*: EGIII activity in yeast IpYES2

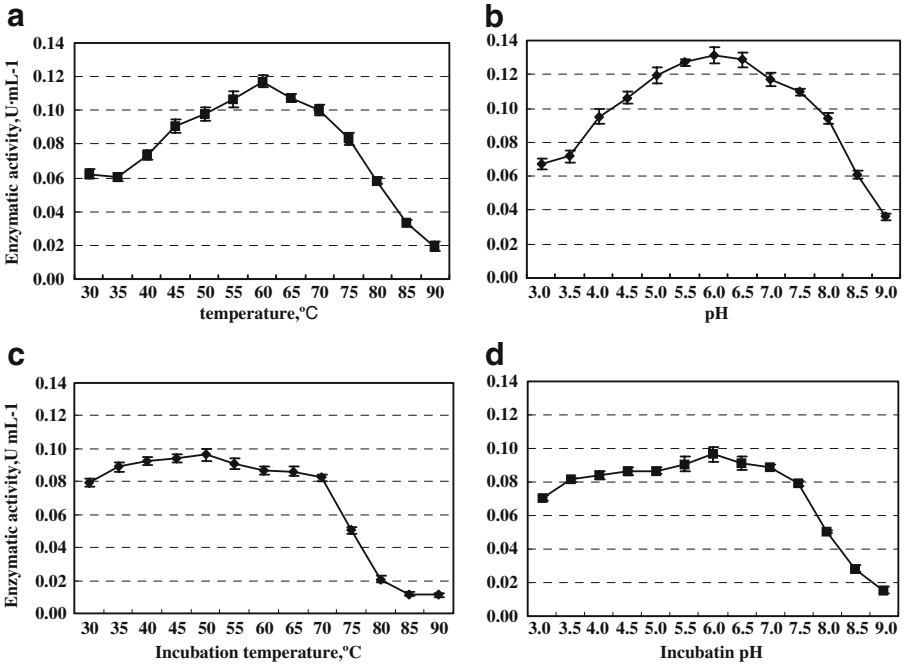

Fig. 5 Activity and properties of recombinant endoglucanase EGVIII. **a** Effects of temperature on endoglucanase EGVIII activity. The enzyme activity was measured at 30°C to 90°C for 30 min at 5°C intervals (pH5.2). **b** Effects of temperature on endoglucanase EGVIII stability. The dialyzed fraction (pH5.2) was incubated at 30°C to 90°C for 1 h at 5°C intervals, and then the residual activity was measured by incubation at 60°C for 30 min. **c** Effects of pH on endoglucanase EGVIII activity. The enzyme activity was measured in the reaction buffer at different pH values between 3 and 9 at intervals of 0.5 pH units. **d** Effects of pH on endoglucanase EGVIII stability. The dialyzed fraction was incubated in different pH buffers (pH3.0–9.0) for 48 h at 4°C, and then the residual activity was measured under standard conditions (pH= 6.0). All the experiments were performed three times

To investigate the effect of metal ions on enzymatic activity, the dialyzed fraction was dissolved in the reaction buffer that was supplemented with a metal ion at a concentration of 75 mM. Several different buffer solutions were prepared, each spiked with a different metal.

The variance analysis showed that CMCase activity was significantly different ($F = 156$, $df = 13$, $P < 0.01$) in the presence of different metal ions. The activity of recombinant EGVIII was strongly stimulated by Mg^{2+}, Zn^{2+}, K^{+}, and Fe^{3+} but was inhibited strongly by Al^{3+} and Ag^{+}. In particular, the activity of EGVIII reached 0.172 and 0.174 U/ml when activated by Mg^{2+} and Zn^{2+}, respectively (Fig. 6).

Kinetic constants for CMC-Na, Avicel, cellobiose, and cellotriose hydrolysis were obtained from Lineweaver-Burk plots, and the results were shown in Table 1. The *Km* values of recombinant EGVIII were 3.82 and 1.75 mg/ml for CMC-Na and cellotriose hydrolysis, respectively. The recombinant EGVIII showed the highest affinity towards cellotriose hydrolysis and the lower affinity towards Avicel (12.4 mg/ml) and cellobiose (6.9 mg/ml). The values of *Kcat* for cellotriose and CMC-Na were similar, while the values of *Kcat* for Avicel and cellobiose hydrolysis were substantially lower. The value of catalytic efficiency factor (*Kcat/Km*) for CMC-Na (2.502 s^{-1} ml mg^{-1}) was slightly higher than that of the cellotriose (2.038 s^{-1} ml mg^{-1}). The catalytic efficiency for cellobiose was the lowest (0.112 s^{-1} ml mg^{-1}).

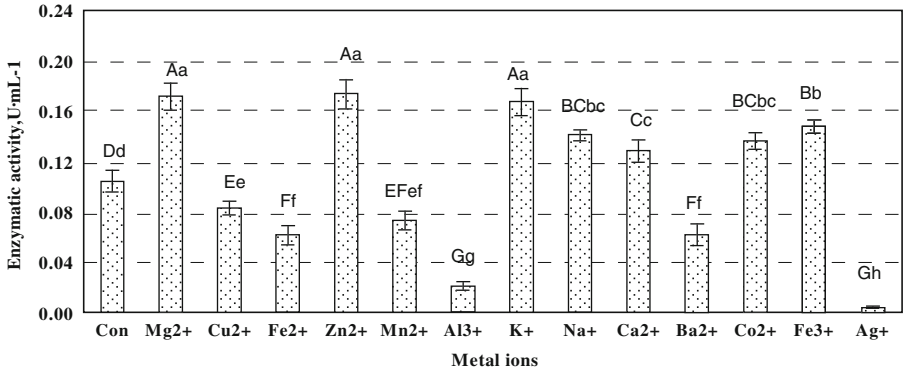

Fig. 6 Effects of exposure to different metal ions on the activity of endoglucanase EGVIII. Several different reaction buffers were prepared, each spiked with 75 mM of a metal ion. One unit of CMCase activity was defined as the amount of enzyme required for releasing total reduced sugar equivalent to 1 mmol glucose per minute. *Con*: control, the activity of EGVIII was measured under the normal reaction condition without any additional ions. The experiments were performed three times. *Different letters above the columns* indicate a significant difference determined by Duncan's multiple comparisons test ($P<0.01$)

Discussion

T. viride can produce a series of extracellular enzymes which are important factors for degrading cellulose. The *T. viride* secretes at least five different endoglucanases, EGIV (Cel7B), Cel5A, Cel12A, Cel61A, and Cel45A, that have been purified from a mutant strain of *T. viride* T 100-14 [21]. The *egVIII* (*cel5B*) genes from *Paenibacillus polymyxa* GS01 [22], *Piromyces rhizinflata* [23], *Thermobifida fusca* [24], and *T. reesei* [23] had been studied, while the *egVIII* gene (*cel5B*) from *T. viride* was not previously reported. The *egVIII* cDNA isolated in this present study was a novel gene coding a 46.86 kDa endoglucanase from *T. viride* AS3.3711. BlastP analysis against the GenBank non-redundant protein database revealed that EGVIII has a relatively high degree of similarity to endoglucanase (EC.3.2.1.4) belonging to the glycoside hydrolase, cataloged as family 5 (http://www.ebi.ac.uk). The highest identity in sequence was found with *cel5B* from the fungus *T. reesei*, demonstrating that EGVIII from different fungi was highly conserved. However, the sequence identity was very low with *cel5B* from bacterium, perhaps due to phylogenetic evolution.

The expression analysis of *egVIII* in *T. viride* confirmed that the expression of the endoglucanase gene *egVIII* can be induced by different carbon sources, and CMC-Na was the best inducing substrate (Fig. 2d). Not only was expression of *egVIII* inhibited by glucose or fructose, no *egVIII* transcripts were detected at any time in the presence of either

Table 1 Kinetic properties of recombinant EGVIII.

Substrates	*Km* (mg/ml)	*Kcat* (s^{-1})	*Kcat/Km* (s^{-1}ml mg^{-1})
CMC-Na	3.82 ± 0.19	9.56 ± 0.13	2.502 ± 0.09
Avicel	12.4 ± 0.26	1.80 ± 0.09	0.144 ± 0.01
Cellobiose	6.90 ± 0.26	0.77 ± 0.05	0.112 ± 0.01
Cellotriose	1.75 ± 0.04	7.08 ± 0.10	2.038 ± 0.05

Results are shown as means ± SD

carbon sources (Fig. 2a, f). Transcription of the endoglucanase gene *egIV* from *T. reesei* was reported to be also depressed by glucose [25], although it was induced when glucose was the only carbon source [26]. Cel5B of *T. fusca* could be induced by microcrystalline cellulose powder [24], and *egVIII* of *T. viride* could also be induced by the same substrate (Fig. 2c). Transcriptional regulation of *egVIII* gene in *T. viride* should be further studied.

Although the expression regulation of EGVIII (*Cel5B*) from *T. reesei* has been studied [21], the enzymatic characterization of endoglucanase EGVIII (*Cel5B*) was not detected. This is the first report that the *egVIII* from *T. viride* was functionally expressed in *S. cerevisiae* and the recombinant enzyme characterized. The endoglucanase EGVIII expressed in the *S. cerevisiae* was stable over a wide pH range (3.0–7.5; Fig. 5d) and at temperatures up to 70°C with an optimal temperature of 60°C (Fig. 5b), thus making the enzyme suitable for use in cellulose saccharification at moderate temperatures and pH levels. In comparison, the optimal temperature of recombinant EGVIII was higher and had a wider pH range than that of the *Mucor circinelloides* (55°C, pH4.0–7.0) [27]. Ding et al. [28] reported that a recombinant endoglucanase from *Volvariella volvacea* was inhibited by Zn^{2+}, Mg^{2+}, and Ca^{2+}. In the current study, we found that the activity of EGVIII was stimulated to a high degree by Mg^{2+}, Zn^{2+}, K^{+}, and Fe^{3+}, while being inhibited by Al^{3+} and Ag^{+} (Fig. 6). These results showed that EGVIII can be activated or inhibited by certain metal ions, although endoglucanases from different species may be differentially regulated by different ions.

A hyperbolic curve was obtained from the activities of the cellulase enzyme. The Line Weaver-Burk plot was used to obtain the maximum velocity (*Vmax*) and the Michaelis–Menten constant (*Km*) of the recombinant EGVIII. With the *Km* value of 3.82 mg/mL for CMC-Na, the recombinant EGVIII has higher affinity than that of endoglucanase from *Chaetomium thermophilum* CT2 [29], *Trichoderma longibrachiatum* [30], and *Gymnoascella citrina* [31] but lower affinity than that of endoglucanase from *Arachniotus citrinus* [32]. The EGVIII affinity ability for cellotriose was lower than that of EGIII from *T. viride* [33] and higher than that of EGIV from *T. viride* [34]. The catalytic efficiency of EGVIII enzyme for cellotriose and cellobiose was higher than that of EGIII from *T. reesei* [35].

The signal peptide ensures effective transport of the expressed product across the cell membrane and has been used to enhance secretion and activity of recombinant proteins. Kang et al. [36] improved the secretion of α1-antitrypsin by introducing the inulinase signal sequence, resulting in 70% biologically active α1-AT secreted into the medium. Ding et al. [28] constructed *Pichia pastoris* expression plasmids pPICZA-EG1 and pPICZAαB-EG1, which contained the native *S. cerevisiae* α-factor secretion signal, and showed that the enzymatic activity of the recombinant transformant harboring the signal peptide was enhanced by 6%. This result demonstrated that the α-factor secretion signal sequence adopted from the yeast host could enhance the secretion for recombinant protein. Furthermore, although the signal sequence of *Syncephalastrum racemosum* (BCC18080) endoglucanase was recognized and properly processed by *P. pastoris*, the endoglucanase was truncated; a recombinant vector carrying the α-factor secretion signal sequence could secrete the full-length endoglucanase protein [37]. In our study, we constructed two vectors for improving recombinant endoglucanase secretion and expression. The native signal peptide of *T. viride* was recognized and secreted by *S. cerevisiae*, but the use of the secretion signal factor from *S. cerevisiae* effectively improved the secretion of enzyme EGVIII and increased the activity by 0.86-fold (Fig. 4). Enhancement of secretion of the recombinant EGVIII was also advantageous for the protein purification process and reduced the deleterious (e.g., toxic) effects for the yeast host cells.

In summary, we have successfully cloned the *egVIII* gene (GenBank Acc. No. EU518928) from *T. viride* AS3.3711 and obtained recombinants yeast strains IpYES2-egVIII and IpYEMα-xegVIII using either its native signal sequence or the *S. cerevisiae* αMF secretion signal sequence. Recombinant endoglucanase EGVIII from yeast strain IpYEMα-xegVIII showed the highest activity (0.174U/ml) at 60°C, pH6.0, and 75 mM of Zn^{2+}. The enzymatic activity of recombinant EGVIII was stable at pH3.0 to 7.5 at 50°C or when incubated from 35°C to 70°C for 1 h. This strategy may be useful for industrial production of EGVIII and other cellulolytic enzymes.

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