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Original Article

Expression analyses of endoglucanase gene in *Penicillium oxalicum* and *Trichoderma viride*

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Abstract: The expression of endoglucanase gene and protein profile belonging to two fungal species, *Penicillium oxalicum* 1SMS and *Trichoderma viride* 156MS with high cellulase enzyme activity, was investigated. Fungal isolates were cultured on inducer CMC medium and then the amount of released sugar and protein were assayed every three days for a month, using arsenate molybdate reagent and Bradford method, respectively. Detection of endoglucanase gene was performed by using specific primers. Expressed cDNA fragments in two isolates revealed a size of 1380 bp to 1434 bp length. The cDNAs were sequenced and queried in BLASTN and TBLASTX search against *eg1* genes from other fungi. These sequences showed 98% homology to *eg1* gene deposited in databases. SDS-PAGE pattern of protein separation revealed one protein band for each isolate having a molecular weight of 49.786 and 48.339 kDa with 487 and 460 amino acids. Bioinformatics analysis revealed glycosyl hydrolase domains, a cellulase banding domain and 6 PROSITEs for both proteins. One ORF in each gene region was determined for each species. The results indicated that carboxy methyl cellulose is able to induce expression of *eg1*, following endogluconase enzyme production.

Key words: β-1,4-endoglucanase, *eg1* gene, sequence homology, bioinformatics analysis, Prosite.

INTRODUCTION

Cellulose is a water insoluble polysaccharide and the most abundant degradable hydrocarbons in nature, which is composed of D-glucopyranosyl units joined by 1,4-glycosidic bonds. Cellulose plays an important role in changing stabilized carbon to CO_2, as well as producing sustainable biological and bio-energy products to replace fossil fuels (Chojoghi et al. 2005). Biological degradation of cellulose in soil could be performed by cellulase enzyme produced endogenously by many microorganisms such as fungi, bacteria and Protista (Li et al. 2009). A number of enzymes with cellulose activity classified in family 9 from various organisms from which cellulases have been reported (Arai et al. 2003).

Cellulolytic enzymes should be either secreted in the environment or connected to outer surface of cellulolytic microorganisms. These enzymes usually act together synergistically. They are able to hydrolyze β-1, 4 glycosidic bands in cellulose and change it to CO_2 and H_2O (in aerobic condition), and CO_2, H_2O, and CH_4 (in anaerobic condition). These enzymes are; 1-4-β-D- glucanase or endoglucanases (EG, EC 3.2.1.4), which hydrolyze internal glycosidic bands and 1-4-β-D-glucancellobiosehyrolase or exoglucanase (CBH, EC 3.2.1.9.1), which releases cellobiose units from the fungi can grow on cellulose as the sole source of carbon. Cellulase enzyme is one of the most important ends of the cellulose chain; 1-4-β-D-glucosidases (βGLC, 3.2.1.21) which separates glucose units from linear cellulose (cello-oligosaccharides) (Coughlan, 1990). Some microorganisms such as fungi are able to degrade cellulose. Fungi play an important role in degrading the cellulosic compounds due to their strong enzymatic systems (Jorgensen & Olsson 2006).

Detection and manipulation of some fungal species as cellulose producers has been interested many research centers, because of their application in starch processing, grain alcohol fermentation, extraction of fruit juices, pulp and paper industry and textile industry (Gao et al. 2008). Among hundreds of fungal species which are capable of developing on materials containing cellulose as the source of carbon, some of them could produce cellulose enzyme to degrade cellulose (Suurnakki et al. 2000). Previously, we have reported that some fungal species of different genera (*Fusarium solani*, *Trichoderma viride*, *T. reesei*, *T. koningii* and *Aspergillus terreus*) can produce cellulolytic enzymes with high activity rate (Leylai et al. 2011a). Saprophytic fungi could be regarded as one of the most beneficial sources of cellulolytic enzymes. More than one hundred

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industrial enzymes have been introduced in agricultural, food and paper industries (Bhat 2000).

With regard to the important role of these enzymes in industry, in this study, we attempted to determine the profile of endogluconase gene expression and its related proteins in two selected isolates of fungi with high cellulase activity.

MATERIALS AND METHODS

Fungal culture and media

Sampling was performed from soil of agricultural areas in north of Iran during 2009-2010. Soil suspension was prepared and cultured on water-agar medium. After growing fungi for five days, a six-mm disc of fungal colony was transferred to Potato Dextrose Agar (PDA, Merk, Germany) medium. A total of 300 isolates were used for celluase activity assay. Cellulose degradation *in vitro* was evaluated by providing minimal medium containing nitrogen sources, micro-elements, and carboxymethyl cellulose as the only carbon source (Leylai et al. 2011b). Induction of isolates was performed by carboxymethyl cellulose (CMC) to release cellulase in culture medium inside 100-ml conical flask containing fresh hypha of fungi. Experiments were done at 25 ºC in three independent replications.

Sugar and protein assay

Sugar assay was done four days after induction and continued for one month. Released glucose in culture media was detected by using ammonium molybdate reagent. Absorption was read by spectrophotometer at 575 nm wave length (Kossem & Nannipieri 1995). Endoglucanase activity was determined in a duplicate reaction mixture containing 200 µl 1% CMC-Na (w/v, dissolved in 100 mM sodium acetate buffer, pH 6) and 100 mM NaCl. The reactions were allowed to proceed for 10 min at 50 °C and terminated by adding 0.5 ml dinitrosalicylic acid (DNS) and then heating in a boiling water bath for 10 min. The absorbance was measured at 540 nm, using Novospect II spectrophotometer (Pharmacia). One unit of enzyme is defined as the amount of enzyme that catalyzes the formation of 1 µmol glucose/min under defined assay conditions (Li et al. 2009). Protein purification procedures were carried out at 4°C with chromatographic monitored at 280 nm wave length. Protein concentrations were determined by the method of Bradford (Bradford 1976), using serum albumin as standard treatment. SDS–PAGE was performed in 10% SDS–PAGE and proteins were visualized by silver staining (Morrissey 1981). A band of about 66 kDa was cut out and kept in CMC medium for 24 h at 25°C. Protein extraction and purification from gel was performed using AllPrep Mini kit (Qiagen) according to manufacturer's protocol. The sequence of N-terminal amino acid of the intact enzyme was determined by using an automated sequencer (Applied Biosystem, ABI, USA).

RNA isolation and reverse transcription

Total RNAs from induced hypha in culture medium was performed using a standard phenol-chloroform procedure and purified using RNA purification Kit (Qiagene). Total RNA was quantified using a Scandrop spectrophotometer (Analytika, Germany) and RNA quality was assessed by 1% agarose gel electrophoresis stained by Ethidium bromide. The integrity of RNA was evaluated with an Agilent 2100 bioanalyzer (Agilent Technologies). First-strand cDNA was synthesized from total RNA using Roch extraction solution (Roch, Germany). 50-100 ng of the total RNA from each CMC stimulated cell was used to prepare double-strand cDNA using Ominiscrip kit (Qiagene, Germany) according to manufacturer's protocol.

Primer design and polymerase chain reaction

Primers used in this study were designed by using NTI software and Primer 3 site (http://frodo.wi.mit.edu). The primers were checked for secondary structures with oligoanalyser software (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx). The primers used in this study are summarized in Table 1.

Table 1. The primers used in this study.

Primer name*	Tm	5'-3' sequence	Length	Tm
PO1	60	ATGTCTTTCACAAGGAGACAG	21	60
PO11	60	TCACAGGCACTGAGAGTAGTAG	22	60
TV1	65	TTGTCCCAATATGGCGCCCTC	21	65
TV11	66	GCAATGCCTTTAGAGCGTTGAC	22	66

*PO: *Penicillium oxalicum* 1SMS and TV: *Trichoderma viride* 156M

PCR assay

To identify β-1,4-endoclocanase sequence, the PCR assay was performed by using related specific primers (Table 1). The reaction mixtures were prepared in a total volume of 25 µl with a final concentration of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM of each dNTP and 1.5 mM $MgCl_2$. For each reaction, 1.5 U of *Taq* DNA polymerase (Fermentas, France), 15 pmol of each primer and 25 ng of fungal template DNA were added. The PCR reaction was performed in a thermocycler (Eppendorf Mastercycler Gradient, Hamburg, Germany) using the following program: an initial denaturation step at 94° for 2 min, 40 cycles of 94 °C (30 s), 60 °C - 66 °C (30 s), 72 °C (60 s), and a final extension step at 72 °C for 10 min. A negative control, deleting DNA template, was used in every set of reactions. PCR products were separated by electrophoresis on 1.5% agarose gels stained with Ethidium bromide (0.5 µg/ml) in 40 mM Tris–acetate and 1.0 mM EDTA and then photographed under UV light. Extraction of purified PCR fragment was done using a Silica Bead DNA Gel Extraction kit (Fermentase, USA), according to the manufacturer's protocol.

DNA Sequencing

The 5'-end DNA sequencing was conducted with an ABI 3700 sequencer at Macrogen Company in South Korea. The cDNA sequences were screened for quality and then queried using the National Center for Biotechnology Information (NCBI) standalone BlastAll program against the BLASTN (nucleic acid), TBLASTX (translated protein query search), Swissprot, version 6.0 UniProt and UniRef100. PROSITEs were specified using NPSA server (http://npsapbil.ibcp.fr/cgibin/npsa_automat.pl?page=/NPSA/npsa_server.html). Sequence similarities above 98% and E values less than $1E^{-10}$ were considered as statistically significant positive matches (data not presented). Sequences that matched to hypothetical proteins or had low similarity to a known protein were considered as unknown genes.

RESULTS

Sugar and protein assay

Sugar and protein released in inducer medium were measured at different time intervals. There was fluctuation in the amount of released glucose during sugar measurement in studied isolates.

Presence of glucose in fungal culture medium showed cellulose decomposition. According to these results, variation in amount of glucose are significantly observed during the measurement which is due to fungal activity to provide required conditions for growing and producing the maximum amount of enzyme. Regarding the amount of the released sugar and protein, isolates were graded; two isolates with high activity were selected for future experiments (Table 2). The maximum amount of the enzyme was obtained 13 days after inoculation for different isolates in culture media. Results showed that the amount of enzyme was decreased. This might be due to either aging of fungi and presence of secondary metabolites or reduction of materials in culture media. The resulted data indicated that two isolates, *Penicillium oxalicum* 1SMS and *Trichoderma viride* 156MS possessed the maximum enzyme production rates which were 0.078 mg/L and 0.075 mg/L, respectively.

Endoglucanase enzyme evaluation showed that the two isolates could produce 0.06578 and 0.05989 mg/L protein, respectively. SDS-PAGE analysis also indicated a protein with molecular weight of 45-66.2 kDa, which is able to release glucose according to enzyme verification test (Fig 1).

Table 2. Bioinformatics analysis of N-glycosylation site related to endoglucanase gene in two studied fungal species

N-glycosylation site in *Penicillium oxalicum* access number: PS00001	N-glycosylation site in *Trichoderma viride* access number: PS00001
Randomized probability: 5.138e-03	Randomized probability: 5.138e-03
Site: 97 to 100 NYTS. Identity	Site: 78 to 81 NTTL. Identity
Site: 202 to 205 NGTL. Identity	Site: 164 to 167 NGSL. Identity
	Site: 204 to 207 NGTL. Identity
	Site: 208 to 211 NTSH. Identity
	Site: 394 to 397 NSTA. Identity

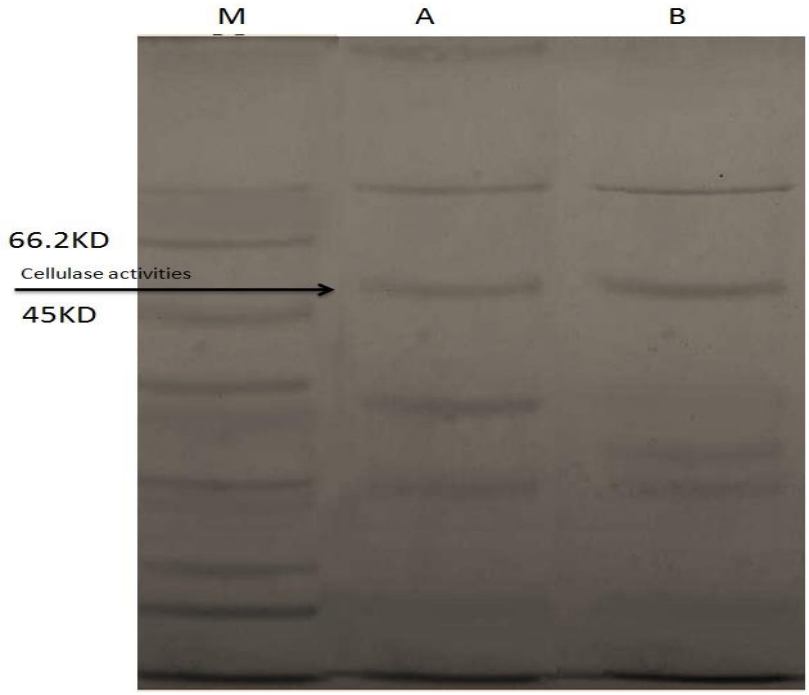

Fig. 1. SDS–PAGE analysis of purified enzyme stained with coomassie R-250 brilliant blue. (**A**): protein profile produced by *Trichoderma viride* (lan 1) and (**B**):.*Peninicilium oxalicum* at inducer CMC medium (lane 2). **M:** protein size marker.

Trichoderma viride 156MS

Amplified product of endogluconase gene in *Trichoderma viride* 156MS isolates, which was provided by using specific primer, showed a yielded fragment ranging from 1400 to 1600 bp (Fig 2A). This sequence showed 98% similarity with the same gene in *Trichoderma* isolates having a fragment of 1700 bp in database. The sequence of *T. viride*156MS which was characterized in this part has been recorded in GenBank under accession number of JN247430. Bioinformatic analyses of queried sequence showed an open reading frame (Fig 2B).

Penicillium oxalicum 1SMS

Amplified fragments produced in PCR reaction by usingspecific primer showed a ranging in size from 1400 to 1700 bp on electrophoresisgel (Fig 3A). Nucleotide sequence related to coding region of *eg1* in *Penicillium oxalicum* cited in NCBI database, has the size of 1437 bp. Comparison of amplified sequences with those of 5-P flanking regions of cellulase genes in other fungi revealed the large areas of homology about 98%. The sequence related to coding region of β-1,4 endoglucanase (CDs) showed a high degree of nucleotide sequence similarity to genes assigned in the genomes of *Penicillium* isolates. Protein sequence also showed a strong sequence similarity to endoglucanase genes in *Penicillium* isolates. The sequence determinationof this isolate has been deposited in GenBank under accession number of JF309107. Bioinformatics analysis of queried sequence showed an open reading frame (Fig 3B).

Fig. 2. (**A**) Related fragments of endoglucanase gene (Line 3) and cDNA of expressed *eg*1 gene (Lines 1-2) amplified by using specific primers for *Trichoderma viride* 156MS. (**B**) ORF of coding sequence region (cDNA) related to Endoglucanase gene in *T. viride* 156MS.

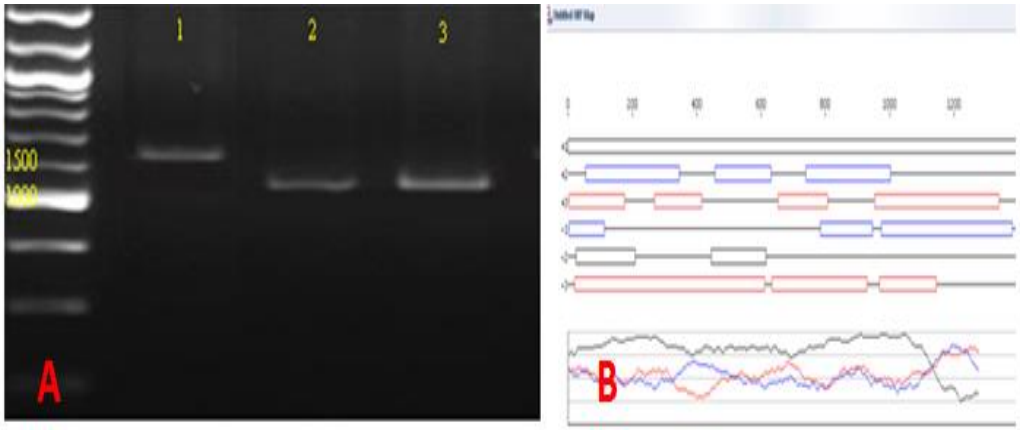

Fig. 3. (**A**) Related fragments of endoglucanase gene (Line 1) and cDNA of expressed *eg*1 gene (Lines 2-3) amplified by using specific primers for *Penicillium oxalicum* 1SMS. (**B**) ORF of coding sequence region related to endoglucanase gene in *P. oxalicum* 1SMS.

Bioinformatics assays

Two queried sequences were analyzed in CDD (Conserve Domain Database) at NCBI. Results from bioinformatics analysis indicated that our queried sequence has two glycosylhydrolase and Cellulase Binding Domain (CBD). As with CBDs, catalytic domains have been delineated in some cellulases by proteolysis (Ghangas & Wison 1988, Mo & Hayashida 1988, Tomme et al. 1988, Gilkes et al. 1989). These results revealed that duplicated sequences are related to endoglucanase I gene, which change crystalline cellulose to linear chains. Prosites analysis revealed that the specified proteins have six prosites which are quite similar to that of all six prosite active sites. This domain has glycosation sites which hydrolyze glycosidic band between two or more carbohydrates or between carbohydrate and non-carbohydrate (Table 2). Their difference is in passive sites. Molecular weight of the two proteins was evaluated using ClC bio software. Molecular weight was determined as 49.786 kDa (IEPs = 5/21) for *P. oxalicum* 1SMS, and 48.339 kDa (IEPs = 5.45) for *T. viride* 156MS.

DISCUSSION

The ability of different fungi such as *Trichoderma* and *Aspergillus* to produce cellulolytic enzymes on different sources of carbon in submerged culture has already been investigated (Ilm`en et al. 1997; Morikawa et al. 1995). These enzymes consist endo-L-1,4-glucanases (EGs), cellobiohydrolases (CBHs) and L-glucosidases (BGLs). In this research, cellulase activity of two soil fungi was investigated. CMC was used as enzyme production inducer in culture medium. Biomass growth of induced fungi and presence of free sugar in culture medium showed the ability of studied fungal isolates to produce cellulose enzyme. Macris (1983) surveyed some genera like *Trichoderma*, *Fusarium*, *Aspergillus*, *Phanerocheate*, *Chrysosporium* and *Sclerotium* showed some differences in their cellulase activity. A study about surveys on cellulase activity of different genera reported that *T. harzianum* and *A. niger* showed highest and *Trichothecium roseum, Trichoderma reesei*, *A. ochraceus* and *Penicillium italicum* exhibited lower activity against CMC (Sazci et al. 1986). Measurement of cellulase activity among 115 isolates of fungi showed that *Trichoderma*, *Aspergillus* and *Fusarium*s trains had the highest activity (Jahangeer et al. 2005). In this study, biochemical analysis of sugar and protein assays showed different ranges of enzyme production in tested isolates. Screening of isolates' enzyme activity determined two isolates with high activity. Study of structure and expression properties of the endo-L-1,4-glucanase, a gene from the filamentous fungus *A. nidulans*, revealed that *eglA* promoter nucleotide sequence has no major areas of homology with 5 P-flanking regions of other fungal cellulose genes (Chikamatsu et al. 1999). The analysis of related sequences of studied gene showed a high sequence homology with those sequences related to enzyme production in other fungi. These results indicated the high potential of these species for industrial application. The comparison of deduced amino acid sequences showed a slight variation between isolates with high and low cellulose activity. This variation may be caused by mutation in nucleic acid sequence. This fact has been demonstrated using mutated strain in *engl* gene but in this study we tested our wild strains in natural condition. DNA sequencing has allowed rapid determination of amino acid sequences of cellulases. Sequences analysis and comparison with similar sequences in database revealed that the conserved stretches are common to both cellulases and xylanases. The conserved sequences occur in discrete domains connected by linkers which allow the domains to function independently. Some studies have demonstrated strong correlations between *N*-glycosylation and cellulose activities (Knowles et al. 1987, Stals et al. 2004, Wei et al. 2005). Glycosylation has also an important role in stability and activity of endogluconase. DNA sequencing of

eng1 in studied species revealed different Prosite of *N*-glycosylation with known endoglucanase genes. Our results indicated that formation of endoglucanase enzymes is inducible in both *P. oxalicum* and *T. viride*. This is in congruence with several other fungi that have been grown on different media as carbon sources (Ilm`en et al. 1997, Morikawa et al. 1995). This finding also confirms the high level of enzyme activity of studied species. At this time, the mechanism of cellulase induction is obscure and non-explicit knowledge is available. So, further molecular studies such as DNA manipulation and signaling pathways involved in regulatory mechanisms will be necessary for clarification. These results highlight the industrial potentials of studied isolates as possible raw materials for cellulase enzyme production and landscape of application of these enzymes to produce chemical fuels. These results illustrate the industrial potentials of studied species as possible microorganism for cellulase enzyme production

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تجزیه و تحلیل بیان ژن اندونوکلئاز در *Pecicillium oxalicum* و *Tricchoderma viride*

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چکیده: در این تحقیق مشخصات پروتئین و ژن اندوگلوکاناز در دو جدایه دارای فعالیت بالای سلولازی *Penicillium oxalicum* 1SMS و *Trichoderma viride* 156MS، ردیابی و مورد بررسی قرار گرفت. جدایه های قارچی در محیط القاء کننده CMC (کربوکسی متیل سلولز) کشت داده شدند و سپس میزان قند و پروتئین آزاد شده در محیط هر سه روز تا یک ماه به ترتیب با استفاده از معرف آرسنات مولیبدات و محلول برادفورد اندازه گیری شد. ردیابی ژن اندوگلوکاناز توسط آغازگرهای اختصاصی انجام شد. قطعات cDNA بیان شده مربوط به دو جدایه اندازه ۱۴۳۴- ۱۳۸۰جفت باز را نشان دادند. توالی های cDNA مربوط در هر جدایه توالی یابی و سپس در BLASTN و TBLASTX همردیف گردیدند که ۹۸٪ شباهت با ژن*eng1* در بانک های اطلاعاتی نشان داد. الگوی SDS-PAGE پروتئین های جدا شده در ژل، یک پروتئین به ترتیب با وزن ملکولی۴۹/۷۸۶ کیلودالتون و ۴۸/۳۳۹ کیلودالتن و با ۴۸۷ و ۴۶۰ اسید آمینه را برای جدایه های 1SMS و 156MS نشان داد. داده های بیوانفورماتیک برای دو پروتئین دو دامنه به نام های گیلوکوزیل هیدرولاز و دامنه متصل شونده سلولاز (Cellolase binding domain) و ۶ پروسایت را مشخص کرد. همچنین یک منطقه Opean Reading Fram)ORF) در هر ناحیه ژنی برای هر جدایه تشخیص داده شد. نتایج نشان داد که کربوکسیمتیل سلولزقادر به القاءبیان ژن*eng1* و به دنبال آن تولیدآنزیم اندوگلوکاناز می باشد.

واژه های کلیدی:۱-۴ اندوگلوکوناز، ژن *eg1* , تشابه توالی، آنالیز بیوانفورماتیک، پروسایت

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