J. Microbiol. Biotechnol. (2004), 14(3), 620–627

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF)-Based Cloning of Enolase, ENO1, from *Cryphonectria parasitica*

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Received: February 16, 2004
Accepted: April 28, 2004

Abstract On the foundation of a database of genome sequences and protein analyses, the ability to clone a gene based on a peptide analysis is becoming more feasible and effective for identifying a specific gene and its protein product of interest. As such, the current study conducted a protein analysis using 2-D PAGE followed by MALDI-TOF and ESI-MS to identify a highly expressed gene product of *C. parasitica*. A distinctive and highly expressed protein spot with a molecular size of 47.2 kDa was randomly selected and MALDI-TOF MS analysis was conducted. A homology search indicated that the protein appeared to be a fungal enolase (*eno*1). Meanwhile, multiple alignments of fungal enolases revealed a conserved amino acid sequence, from which degenerated primers were designed. A screening of the genomic λ library of *C. parasitica*, using the PCR amplicon as a probe, was conducted to obtain the full-length gene, while RT-PCR was performed for the cDNA. The *E. coli*-expressed *eno*1 exhibited enolase enzymatic activity, indicating that the cloned gene encoded the *C. parasitica* enolase. Moreover, ESI-MS of two of the separated peptides resolved from the protein spot on 2-D PAGE revealed sequences identical to the deduced sequences, suggesting that the cloned gene indeed encoded the resolved protein spot. Northern blot analysis indicated a consistent accumulation of an *eno*1 transcript during the cultivation.

Key words: *C. parasitica*, enolase, 2-D PAGE, MALDI-TOF, ESI-MS

The ascomycetous filamentous fungus *Cryphonectria parasitica* (Murr.) Barr, the causal agent of chestnut blight, was responsible for the virtual disappearance of chestnut orchards in North America at the beginning of the 20th century. However, its hypovirus infection is known to cause a reduced virulence, hypovirulence, which is a good example of biological control [1, 39]. Strains containing the double-stranded (ds) RNA viruses *Cryphonectria* hypovirus (CHV) exhibit the characteristic symptoms of hypovirulence, and display hypovirulence-associated changes, such as reduced sporulation, laccase production, oxalate accumulation, and pigmentation [12, 15, 32]. Interestingly, the symptoms caused by a hypoviral infection appear to be the result of the aberrant expression of specific sets of fungal genes in the hypovirulent strain, which have been suggested to be genes encoding cutinase, laccase, cryparin, and mating pheromones [6, 33, 41, 43, 44]. Thus, the chestnut blight fungus, *C. parasitica*, and its hypovirus are a useful model system for studying the mechanisms of fungal gene regulation by mycoviruses.

Besides its roles as a phytopathogenic fungus and biological control agent, *C. parasitica* is known to secrete an aspartic protease, endothiapepsin (EAP, EC 3.4.23.6), which is used as a milk-clotting enzyme in cheese making and has been produced on a commercial scale for more than 20 years. The gene encoding endothiapepsin has already been cloned and characterized [8, 31], and several studies have attempted to genetically modify the fungus to secrete a higher level of endothiapepsin [8, 17]. In addition, *C. parasitica*, as a member of hardwood pathogens, has also been suggested as a good degrader of polycyclic aromatic hydrocarbons (PAHs), which are a large group of xenobiotic pollutants [13].

A number of important *C. parasitica* genes have been identified based on their expression patterns or homologies to known genes in other organisms [5, 11, 18, 19, 21, 29, 30, 33, 41, 43, 44]. Nonetheless, more remains to be identified

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to explain the phenotypic changes in the fungal host and improve the industrial processes of *C. parasitica*.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) combined with mass spectrometry (MS) has emerged as a highly accurate and sensitive means of analyzing peptides and proteins. Moreover, with the help of the high-throughput capacity of DNA sequencing, which facilitates the rapid accumulation of abundant sequence data and corresponding deduced protein data from many organisms, it has become more accurate and efficient to infer the nature of proteins when they are resolved in 2-D PAGE followed by mass analysis. Accordingly, since this should be more feasible with *C. parasitica* than other pathogenic filamentous fungi, as there are at least some specific DNA data available [11], the current study reports on the cloning and characterization of a gene encoding a highly expressed protein using the combination of 2-D PAGE followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), following tryptic digestion, electrospray ionization mass spectrometry (ESI-MS), and degenerated PCR. As a result, the feasibility of cloning a gene and encoding a protein of interest from *C. parasitica* resolved on a protein gel is demonstrated.

MATERIALS AND METHODS

Fungal Strains and Growth Conditions

CHV1-713 containing the hypovirulent *C. parasitica* strain UEP1 and its isogenic virus-free strain EP155/2 (ATCC 38755) were maintained on PDAmb plates under a constant low light at 25°C [20]. The culture conditions and methods used to prepare the primary inoculum for liquid cultures have been previously described [20]. The mycelium was collected and lyophilized, as previously described, until used [30].

2-D PAGE Followed by MALDI-TOF MS

For 2-D PAGE, the total soluble proteins were extracted by homogenization of 2.0 g of ground mycelium in 20 ml of lysis buffer (40 mM Tris, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 0.1 mM PMSF). The samples were centrifuged twice at 15,000 ×*g* for 30 min. The supernatant was then precipitated using 80% ammonium sulfate, followed by dialysis against lysis buffer. The resolved proteins were further cleaned using a G-25 column and Ready Prep™ 2-D cleaning kit (Bio-Rad, Hercules, CA, U.S.A.) just before loading onto a slab gel. First, dimension tubes for isoelectric focusing were prepared using broad (pH 3- 10) ampholytes and 300 μg of protein was loaded in the first dimension. A 10% acrylamide/N,N'-methylene-bis-acrylamide (29:1) gel (140 mm×110 mm×0.5 mm) was used for the second dimension of electrophoresis. The 2-D gels were run following the procedure of the ReadyStrip™IPG Strips (Bio-Rad) instruction manual. The gels were stained with Coomassie blue R-250.

Electrospray ionization mass spectrometry (ESI-MS) was performed in the negative mode using a Mariner Biospectrometry Workstation (Applied Biosystems, Tokyo, Japan), as described previously [35]. Methanol 50% was used as the solvent at a flow rate of 5 μl/min. The oligosaccharide fraction derived from the above was dissolved in 40% acetonitrile containing 0.1% acetic acid and the spectra were recorded from *m/z* 40 to 4,000.

Cloning and Characterization of Enolase Gene, *eno*1

The degenerate primers specific to the consensus nucleotide sequences corresponding to the most conserved amino acids of fungal enolases are shown in Fig. 2. The primers used were eno-F (forward) 5'-GGWAACCCBACYGTY-GAGGT-3' and eno-R (reverse) 5'-AGGDGCACCRGCT-TGATCTG-3'. PCR was conducted with 50 ng of DNA from *C. parasitica* EP155/2 using the following parameters: preheating at 94°C (4 min); 30 cycles of denaturation at 94°C (30 s), annealing at 55°C (30 s), and an extension at 72°C (1 min); followed by one cycle of 94°C (30 s), 55°C (30 s), and 72°C (10 min). The 1,250-bp PCR amplicon was then cloned into a pGEM-T vector (Promega, Madison, WI, U.S.A.). The positive bacterial clone inserts were sequenced using the dideoxynucleotide method, and compared before being used as a hybridization probe for the screening of the genomic λ library of the EP155/2 strain according to the procedure described in standard protocols [34].

To obtain the cDNA clone of *eno*1, PCR using reverse transcriptase (RT-PCR) was performed with primers eno1-mF1 (forward) 5'-CTTGAATCTTCCCCAGTTA-3' and eno1-mR1 (reverse) 5'-TTCACTAACTACCGGCCAA-3'. The cDNA was sequenced using the dideoxynucleotide method with synthetic oligonucleotide primers.

To identify the transcriptional initiation site, a primer extension experiment was performed using the reverse primer 5'-CGATGGCACGGTGGAGGCCG-3' according to a standard protocol [34].

Southern Blot and Northern Blot Analysis

The genomic DNA from *C. parasitica* was extracted using the method described by Churchill *et al.* [9]. The DNA (10 μg) was then digested with restriction enzymes, including *Apa*LI, *Bam*HI, and *Hin*dIII, blotted onto a nylon membrane, and hybridized with the radioactive labeled *eno*1.

The RNA was extracted from cultures 1-, 3-, and 5-days after inoculation, then equal amounts (15 mg) of RNA were subjected to Northern blot analysis, as previously described [22]. The *eno*1 transcript was compared using the glyceraldehyde-3-phosphate dehydrogenase (*Gpd*) from

C. parasitica as an internal control for gene expression in *C. parasitica* [7].

Heterologous Expression of *eno*1 in *E. coli*

The full-length *eno*1 protein product ENO1 was expressed in *E. coli* as hexahistidine fusion proteins and purified by nickel affinity chromatography, according to the manufacturer's directions (Novagen, Madison, WI, U.S.A.). The cDNA encoding the full-length ENO1 was amplified by PCR using the primers 5'-CCATATGCCTATCTCCAAG-3' (forward) and 5'-GGAATTCTTACAGGGTAACAGA-3' (reverse). The primers were modified to incorporate the restriction sites (underlined) for *Nde*I and *Eco*RI, respectively. The full-length *eno*1 (1,423 bp) was inserted into the *Nde*I/*Eco*RI sites in the expression vector pET28a(+) (Novagen), then the resulting recombinant plasmids were transformed into *E. coli* strain BL21. The induction, purification, and confirmation of the recombinant ENO1 using the anti-hexahistidine antibody were conducted according to the manufacturer's directions (Novagen). The *E. coli*-derived inclusion body was solubilized, then refolded by the step-wise dilution dialysis of the denaturants [10].

The anti-ENO1 antibody was obtained by injecting 100 μg of purified full-length ENO1 into an 8-week-old BALB/c mouse, which was boosted with the same amount of the ENO1 emulsified in incomplete Freund's adjuvant 10 days after the initial injection. Polysera were obtained 5 days after the booster injection, then Western blot analysis was conducted according to the standard procedure [34].

Enolase Activity of ENO1

The enzymatic activity of the recombinant enolase was determined by the modified coupled assay described by Machida *et al.* [25]. The enzyme reaction using the recombinant ENO1 was monitored by UV spectrophotometry (Beckman, Fullerton, CA, U.S.A.) at room temperature and compared with that using rabbit muscle enolase (EC 4.2.1.11) (Boehringer Manheim, Manheim, Germany) as a standard control. One unit of enolase activity was defined as the conversion of 1 mmole NADH to NAD per min, based on an extinction coefficient for NADH at 340 nm, this being 6.22 $mM^{-1}cm^{-1}$.

RESULTS

Two-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE)

The Coomassie blue stained profiles of the gels are shown in Fig. 1. The proteins were distinctly separated and about 400 reproducible protein spots ranging from 113 kDa to 21.5 kDa were resolved in the gel. There were many protein spots, which represented the most easily detectable and consistent spots. Among these predominant protein spots, one at 48 kDa was randomly selected and the nature of this protein examined further. MALDI-TOF MS analysis of the trypsin-digested protein spot revealed 30 fragments with a molecular mass of 47,246 Da. A homology search using Mascot (www.matrixscience.com) indicated that the selected protein was most similar to the known fungal enolase from *Penicillium citrinum*. For consistency, three independent protein samples were resolved by 2-D PAGE and all the MS analyses of a corresponding digested protein spot followed by a homology search resulted in the same results for a fungal enolase.

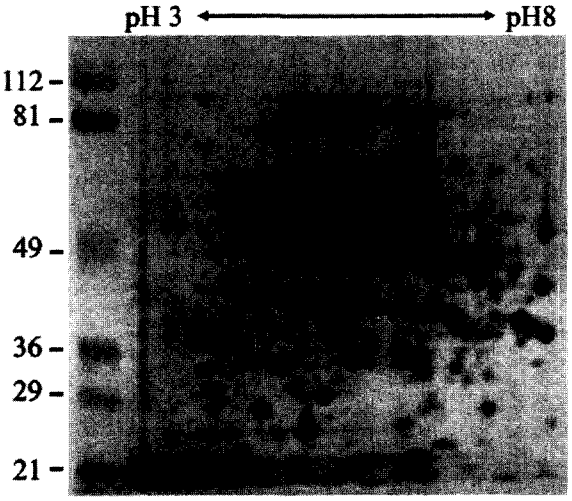

Fig. 1. Coomassie blue stained 2-D gel of *C. parasitica* EP155/2 cell-free extract. The first dimension was focused using ampholytes (pI range 3- 10).
The second dimension was performed by 10% SDS-PAGE. The spot marked with an arrow (MW 40 kDa) was analyzed by MALDI-TOF MS and ESI. The numbers on the left refer to the protein sizes in kDa.

Characteristics of *eno*1 Gene

Multiple alignments of fungal enolases were conducted to obtain the conserved region (Fig. 2). Using degenerated primers derived from the conserved domains of fungal enolases (Fig. 2), a DNA fragment with a size of 1,250 bp was amplified and sequenced. A sequence analysis of the cloned PCR fragment revealed the highest homology to the enolase gene from *Neurospora crassa*, which shared an 80% identity with both the nucleotide and amino acid sequences.

Southern blot analysis was used to determine the copy number of the *eno*1 gene. The genomic DNA was digested with various restriction enzymes, which were selected based on their ability to either not cut the *eno*1 (*Hin*dIII and *Bam*HI) or cut the *eno*1 only once, but not in the region of the probe (*Apa*LI). Each restriction digestion showed only one hybridizing band, and no additional bands were detected when the hybridization was conducted

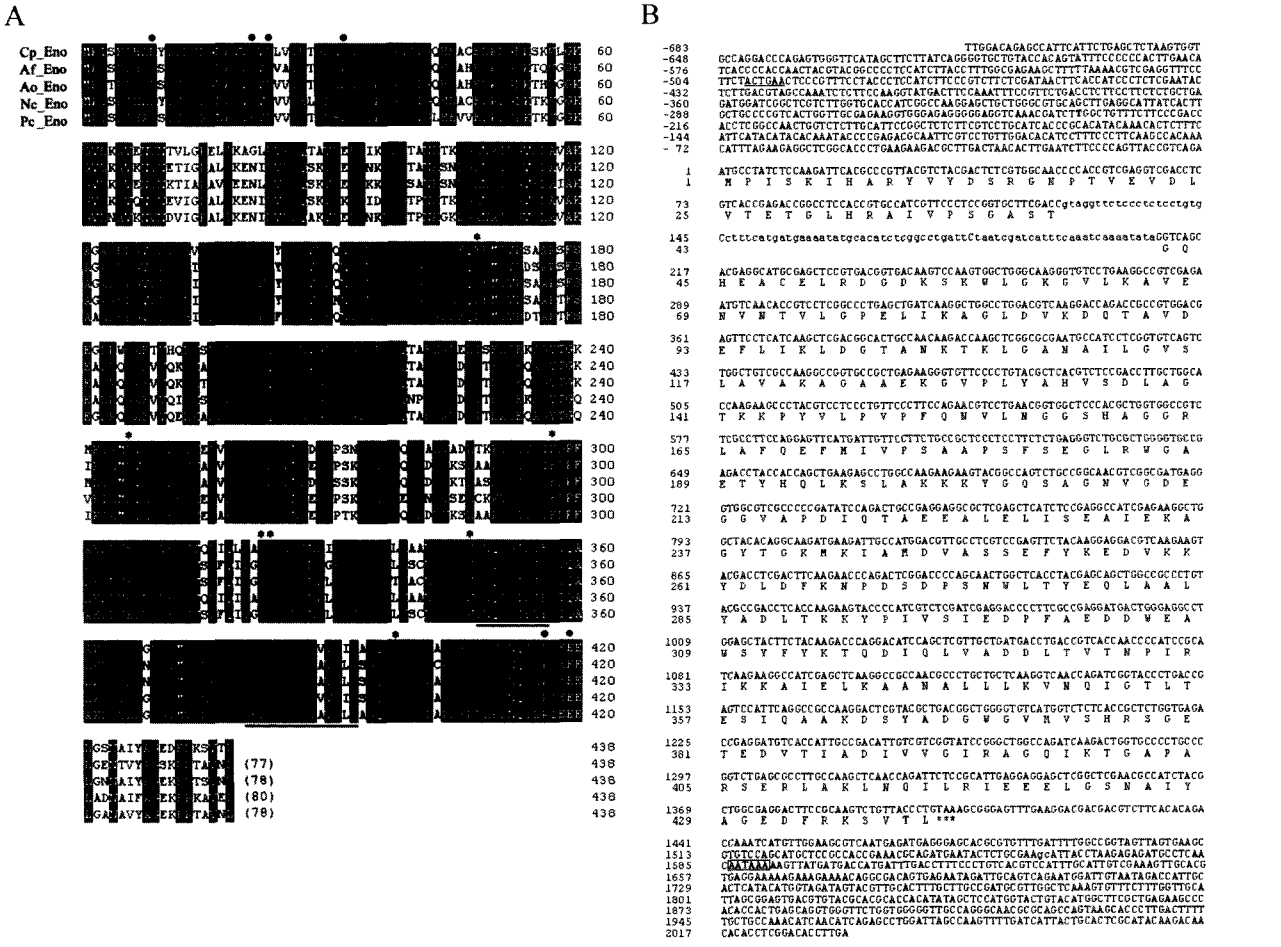

Fig. 2. **(A)** Amino acid sequence alignment of the predicted *eno*1 gene product with other enolases from filamentous fungi.
Identical amino acids are highlighted in white on the black background. The asterisks and closed circles indicate the residues important for the catalytic activity and subunit interaction, respectively [36]. Sequences identical to those by ESI-MS are indicated by the underlining. The homology (percent identity) of the *C. parasitica eno*1 in relation to other enolases is given in parenthesis at the end of each sequence. The numbering of the residues from the N-terminus of the whole protein is shown on the right. Cp_Eno, Af_Eno, Ao_Eno, Nc_Eno, and Pc_Eno are the enolases from *C. parasitica*, *A. fumigatus*, *A. oryzae*, *N. crassa*, and *P. citrinum*, respectively. **(B)** Nucleotide and deduced amino acid sequence of *eno*1 from *C. parasitica*. The intron and 5'- and 3'-flanking regions are shown in small letters. The 7-bp conserved element is underlined and the transcription initiation site shown by a bold letter. The putative poly(A) signal is indicated in the box. The GenBank accession number for the *eno*1 sequence is AY499570.

under low stringency at 52°C. As such, these results indicate that the *eno*1 gene is present in the *C. parasitica* genome as a single copy gene (data not shown).

The screening of the genomic λ library yielded 2 positive clones out of 40,000, and a 6.0 kb *Eco*RI-digested λ clone containing the full-length *C. parasitica* enolase gene, *eno*1, was selected for further analysis. RT-PCR using the primer pair eno1-mF1 and eno1-mR1 was conducted to amplify the cDNA fragment of the *eno*1, and the 1.5 kb amplicon was cloned into a pGEM-T vector. A sequence analysis of the cDNA clone obtained using RT-PCR indicated one long open reading frame (ORF) of 1,317 bp, starting at the first methionine codon (ATG, 28 nt position) and ending at a stop codon (TAA, 1,342 nt position). A sequence comparison with the corresponding genomic sequence indicated that the *eno*1 gene consisted of two exons with one intervening sequence of 89 bp in size. The sequence around the start codon of the *C. parasitica* enolase (5'-CAGAAUGCCT-3') closely resembles the optimal sequence environment for the eukaryotic initiation codon 5'-G/ANNAUGN-3' [23] and consensus sequence of yeast glycolytic mRNAs (5'-ANAAUGNNT-3') [3]. The 5'-end (GTAGGT) and 3'-end (TAG) of the intron were closely matched to the known consensus sequences, 5'-GTRRGT-3' and 5'-YAG-3', of *C. parasitica*, respectively. Although no internal consensus sequence was observed, the putative consensus sequence of 5'-TCTAAT-3' instead of 5'-NCTRAC-3' occurred 30 bp upstream from the 3' end of the intron [6, 14, 44]. In addition, the poly(A) signal, AATAAA, was located 183 bp downstream of the translational stop codon. The deduced *eno*1 protein product ENO1 consisted of 438 codons, with an estimated

molecular mass of 47 kDa and pI of 5.25 (The GenBank accession number for the *eno*1 sequence is AY499570). Alignment of the deduced amino acid sequence showed the cloned ENO1 to be highly related to the fungal enolases from *Aspergillus fumigatus* (77% identity), *A. oryzae* (78%), *N. crassa* (80%), and *P. citrinum* (78%). All the amino acid residues essential for catalytic activity and those involved in subunit interactions were conserved in the *C. parasitica* enolase [36].

The codon bias was distinctive in the *C. parasitica* enolase. The preference of GGPy for Gly (37 GGPy in 37 Gly codons) and rare usage of AGN (1 AGT and 3 AGT for Ser in 438 codons) observed in the highly expressed genes of *N. crassa* and *A. oryzae enoA* gene were also noted in the *C. parasitica* enolase. In addition, rare usage of codons ending with A (only 3 out of 438 codons), possibly attributed to the unique feature in the highly expressed genes, was also shown in the *C. parasitica* enolase. However, when compared with other filamentous genes, codons CAG, CCC, UGC, and UUC instead of CAA, CCA, UGU, and UUG encoding Gln, Pro, Cys, and Leu, respectively, were preferred in the *C. parasitica* enolase.

A primer extension experiment revealed that the transcription start site of the *eno*1 was located 235 bp upstream of the putative start codon. Neither a canonical TATA nor a CAAT box was observed in the promoter region.

Microsequencing of Separated Fragment of MALDI-TOF

Microsequencing of the separated protein fragments showed the sequences of VNQIGTLTE and TEDVTIADI, corresponding to the regions of 348 to 356 and 380 to 388 from the putative start codon, respectively. The occurrence of sequenced amino acid residues in the deduced sequence strongly indicated that the gene encoded the corresponding protein spot in the 2-D PAGE.

Enzymatic Activity of *E. coli*-Expressed ENO1

The expression of the full-length ENO1 was examined by SDS-PAGE. Following gentle purification using sonification and nickel affinity chromatography, a single protein band was observed at 49 kDa for the full-length ENO1 (Fig. 3). The observed sizes of the recombinant proteins were slightly larger than the calculated ones due to the presence of hexa-histidine. The enzymatic activity of the renatured *E. coli*-expressed ENO1 was examined as previously described [24]. The specific enolase activities of the purified fusion protein and standard rat muscle enolase were 2.52 and 40 U/mg, respectively, which was comparable to previous results from *E. coli*-expressed *A. oryzae* enolase and suggests that the cloned gene encodes a protein with enolase activity.

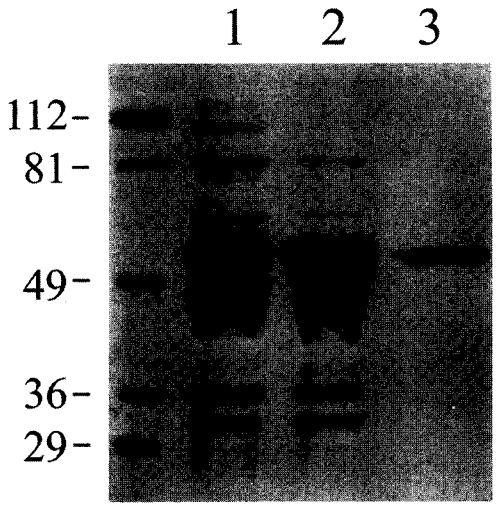

Fig. 3. Coomassie blue stained SDS-PAGE of *E. coli*-expressed ENO1.
Lanes 1 and 2 contain protein preparations from noninduced and induced *E. coli*, respectively. Lane 3 contains affinity-purified recombinant ENO1 from induced *E. coli*. The numbers on the left refer to the protein sizes in kDa.

Expression of *eno*1

Northern blot analysis was conducted to examine the expression pattern of the *eno*1 gene (Fig. 4). The total RNA was prepared from 1-, 3-, and 5-day cultures after inoculation and the accumulation of the *eno*1 transcript was examined using the cloned *eno*1 probe. In the 1-day culture, the *eno*1 transcript was detected at 1.7 kb, consistent with the cDNA clone size plus a poly(A) tract, which then gradually decreased until the 5-day culture. No difference was observed in the transcript accumulation between the wild-type and its virus-containing isogenic

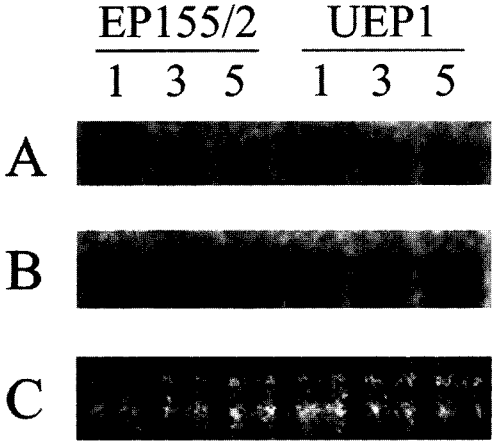

Fig. 4. Northern blot analysis of *eno*1 (A) and *Gpd* (B).
Equal loading of RNA was confirmed by a parallel blot hybridized with a *Gpd* probe as an internal control and an ethidium bromide stained-gel (C). Strains are indicated above the line, with the numbers on top of the lanes referring to the days after inoculation.

hypovirulent strain, indicating that the *eno*1 expression was unaffected by the presence of the hypovirus.

DISCUSSION

The high-throughput capacity of DNA sequencing has allowed the rapid accumulation of abundant sequence data from many organisms. In *C. parasitica*, the mRNA differential display has been used to profile the total host genes affected by a CHV1 infection [4]. Also, a *C. parasitica* EST library/database was recently constructed based on compiling 4,200 sequence files and cataloging 2,200 unique genes [11]. Thus, although never tried, systematic analyses of the protein of *C. parasitica* need to be conducted for a better understanding of fungal gene expression and its specific modulation by a mycovirus as a model system. Therefore, a new approach to gene cloning that can encode a protein spot of interest is needed for the efficient identification of a specific gene and its protein product of interest.

Enolase (EC 4.2.1.11) is a ubiquitous enzyme that catalyzes the interconversion of 2-phosphoglycerate to phosphoenolpyruvate, the only dehydration step in the glycolytic pathway. Enolases have already been identified from various living organisms, including bacteria, fungi, drosophila, amphibians, birds, plants, and humans, and their DNA sequences have been found to be highly conserved [40, 42]. A comparison of the amino acid sequence from the *C. parasitica* enolase with those from other filamentous fungal enolases revealed a high degree of conservation, as shown in Fig. 2. The *C. parasitica* enolase showed the highest similarity to that from *N. crassa* with an 80% identity. The predicted protein sizes of the five sequences were all 438 amino acid residues in length.

A comparison of the cDNA with the genomic DNA of the *C. parasitica eno*1 gene confirmed the presence of one intron in the protein-coding region, as predicted from the genomic sequence. The splicing sites in the intron follow the GT-AG rule for class-II introns and the internal conserved sequence was well preserved: [5'-GTAGGT-53nt-TCTAAT-21nt-TAG-3']. The intron size was comparable to the genes from other filamentous fungi, including *C. parasitica* [6, 28]. The primer extension experiment revealed the transcription initiation site 235 bp upstream from the putative start codon. A putative TATA box, observed in closely related fungal enolases, was not observed in the promoter region of the *C. parasitica eno*1 gene.

In yeast, enolase is encoded by two genes, such as *ENO1* and *ENO2*, whose transcriptions are governed by multifunctional transcriptional modulators, which bind to a short common sequence in the promoter region [2, 38]. The RAP1-binding sequence (5'-RMACCCANNCAYY-3', R=A/G, M=A/C, Y=T/C), one of the *cis*-acting elements common in the promoter region of both the ENO1 and ENO2 genes, was not observed in the promoter region of the *C. parasitica eno*1 gene. As such, this suggests that the recognition sequence of the regulatory factor may be different from that of *S. cerevisiae* or that the general transcription factor, such as Rap1p, may not be present in *C. parasitica*. Recently, a deletion analysis of the enolase gene promoter from *A. oryzae* revealed the transcription regulatory region, which was located -224 nt to -121 nt upstream of the start codon and included the 15-bp sequence essential for the transcription regulation of the *A. oryzae* enolase gene [37]. Within this 15-bp element (5'-GTGACTGAACCATCC-3') showing a close, yet not identical, similarity to the RAP1-binding sequence, the 7-bp nucleotide sequence (5'-ACTGAAC-3') was highly homologous to that found in the *A. oryzae* glycolytic genes, such as *gpmA* (5'-ACTGTAC-3') and *tpiA* (5'-ACTGAAC-3'), which showed a similar pattern of expression among all the glycolytic genes. Interestingly, the 7-bp nucleotide sequence, although located further (-500 nt) upstream of the start codon, was also found in the promoter region of the *C. parasitica eno*1 gene, possibly suggesting that the 7-bp element may be a common regulatory element with a similar regulatory mechanism for the glycolytic pathway existing in different filamentous fungi.

Enolase is known to be one of the most highly expressed proteins in certain organisms, including yeasts and filamentous fungi [16, 24]. Based on reproducible distinctive spots on 2-D PAGE, it can be further extended that the *C. parasitica eno*1 gene also appears to be highly expressed to the same extent as the enolases from other organisms. The regulated expression of yeast glycolytic genes has been extensively studied, and several regulatory factors and elements essential for the transcriptional regulation of the genes have already been identified [26, 38]. Similarly, all the glycolytic genes from *A. oryzae* have been cloned and most of those induced by glucose found to be identical to those seen in *S. cerevisiae* [27]. Owing to the analogy between *A. oryzae* and *C. parasitica*, similar regulatory mechanisms are expected to govern the strong promoter activity, which will provide a better understanding of the regulation of fungal genes, resulting in more extensive industrial uses of *C. parasitica*.

Acknowledgments

This work was supported by a grant (MG-02-0303-001-2-2-0) from the Microbial Genomics & Applications Center, the 21st Century Frontier Program funded by the Korean Ministry of Science and Technology. We also thank the Research Center for Industrial Development of BioFood Materials at Chonbuk National University for kindly providing the facilities for this research.

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