Molecular cloning, expression and characterization of a novel class glutathione S-transferase from the fungus *Cunninghamella elegans*

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The structural gene for glutathione S-transferase (CeGST1-1) in the fungus *Cunninghamella elegans* was cloned by screening a cDNA library using a degenerate oligonucleotide probe based on the N-terminal sequence of the purified protein. Open reading frame analysis indicated that the *cegst1* gene encodes a protein of 210 amino acid residues. The deduced amino acid sequence showed 25 % sequence identity with the sequence of the Pi-class GST from *Danio rerio* (zebrafish). Similarity was also shown with the Alpha-class GST from *Fasciola hepatica* (liver fluke; 23 % identity), the Mu class from *Mus musculus* (22 %) and the Sigma class from *Ommastrephes sloani* (squid; 21 %). Further screening of a cDNA library with the *cegst1* gene probe revealed the presence of another GST isoenzyme (CeGST2-2) in this fungus, which shows 84 % sequence identity with CeGST1-1 at the amino acid level. Reverse transcription PCR revealed that *cegst2* was also expressed at the mRNA level in the fungus *C. elegans*. Both *cegst* genes were overexpressed in *Escherichia coli* using the expression vector pQE51, displaying specific activities with 1-chloro-2,4-dinitrobenzene of 2.04 and 0.75 μmol/min per mg of protein respectively. Both enzymes exhibited a similar substrate specificity and inhibition profile, indicating that CeGST1-1 and CeGST2-2 belong to the same GST class. Mutagenesis analysis revealed that Tyr^{10} in the N-terminal region is essential for catalysis of CeGST1-1. We propose from these results that the CeGSTs are novel Gamma-class GSTs and designated as GSTG1-1 and GSTG2-2 respectively.

Key words: fungal enzyme, overexpression, sequence analysis, substrate specificity.

INTRODUCTION

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a family of multi-functional enzymes involved in the cellular detoxification and excretion of a variety of xenobiotic substances, representing an integral part of phase II biotransformation enzymes [1,2]. These enzymes catalyse the S-conjugation between the thiol group of GSH and an electrophilic moiety in hydrophobic toxicants. GSTs are found in most aerobic eukaryotes and prokaryotes, and occur as multiple enzyme forms. Mammalian cytosolic GSTs have been well characterized and classified into Alpha, Mu, Pi and Theta classes on the basis of primary and tertiary structure, substrate/inhibitor specificity and immunological properties [1,3–5]. New classes of GSTs, such as Sigma [6], Kappa [7], Zeta [8] and Omega [9], have been identified from various organisms. Phi and Tau, and Beta classes are specific for plant and bacteria respectively [5,10]. Different classes of GSTs from insects have been found that are involved in antibiotic resistance [11–14]. In contrast, there is a paucity of information on the molecular phylogeny of fungal GSTs [5].

The filamentous fungus *Cunninghamella elegans* has been used as a microbial model of mammalian metabolism in many laboratories [15]. The zygomycete has the ability to metabolize a wide variety of xenobiotics, including polycyclic aromatic hydrocarbons and pharmaceutical drugs [16], and possesses phase I and phase II enzymes [17]. A GST from the fungus *C. elegans* has been purified and characterized in our laboratory [18], and evidence suggests that this fungal GST belongs to a novel class. The present study describes the cDNA cloning, expression and characterization of the GST genes of the fungus *C. elegans*.

MATERIALS AND METHODS

cDNA cloning and DNA sequence analysis

The N-teminal amino acid sequence of the purified *C. elegans* GST protein (CeGST1-1) [18] was used to design a degenerate oligonucleotide probe for the GST gene. The probe (5′-ACN-TTYGARAAYGTNACN-3′) was labelled with a digoxigenin (DIG) oligonucleotide 3′-end labelling kit, according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN, U.S.A.), and was used to screen a previously prepared cDNA library (λgt11) of *C. elegans* [19]. A plaque-lift hybridization procedure was used for the screening, as recommended by the manufacturer of the DIG labelling kit (Boehringer Mannheim), with prehybridizing for 2 h at 37 °C and hybridizing overnight at 37 °C.

The cDNA inserts of the positive phage clones were amplified by PCR using primers that hybridized to the λgt11 vector upstream (gt11-A1) and downstream (gt11-A2) of the insert [19]. After an initial denaturation step at 94 °C for 5 min, amplification was carried out for 33 cycles (94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min). PCR products were sequenced directly using the same primers after agarose-gel purification (Qiagen, Valencia, CA, U.S.A.). The ABI Prism BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (PerkinElmer, Foster City, CA, U.S.A.) and an automatic ABI Prism 310 Genetic Analyzer (PerkinElmer) were used for sequence determination. Homologous sequences were identified using the BLASTX program [20] from the National Center for Biotechnology Information.

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; *C. elegans*, the abbreviated form of the fungus *Cunninghamella elegans* and not the worm *Caenorhabditis elegans*; GST, glutathione S-transferase; CeGST, GST from the fungus *C. elegans*; RT-PCR, reverse transcription PCR; RACE, rapid amplification of cDNA ends.

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The nucleotide sequence data reported in this paper have been deposited in the GenBank®/EMBL/DDBJ Nucleotide Sequence Databases under the accession numbers AY053501 and AY053502.

DNA sequence analysis, translation and alignment with related genes and proteins were carried out using the Lasergene (DNASTAR, Madison, WI, U.S.A.) and BIOEDIT suite [21].

Construction of plasmids expressing *cegst* gene

The nucleotide sequence of the GST gene (*cegst1*) was used to design PCR primers to amplify the coding region. These primers, primer I (5′-CGGGATCCGACTTTCGAAAACGTTAC-3′, where the *Bam*HI site is underlined) and primer II (5′-GGGGTACCCCCTACTCCTTAAGGAAA-3′, where the *Kpn*I site is underlined), also added restriction sites to facilitate cloning. The PCR reaction was performed as described in the paragraph above. The resulting PCR product was digested with *Bam*HI and *Kpn*I and inserted into the plasmid expression vector pQE51, which contains the T5 promoter. The resultant plasmids were used to transform *Escherichia coli* M5 (Qiagen). Colonies containing the appropriate insert were identified by sequencing.

Screening for other *cegst* genes

The DNA of the cloned gene (*cegst1*) was also labelled with a DIG DNA random primed labelling kit (Boehringer Mannheim) and used as a probe to search other possible GST genes from the cDNA library. Positive clones were identified and sequenced as described in the subsection above.

5′-Rapid amplification of cDNA ends (RACE) and cloning of *cegst2* cDNA

The positive clone, which was identified as a second GST gene (*cegst2*), did not include the putative start codon of the GST gene. To isolate a cDNA containing the full *cegst2* coding region, 5′-RACE was carried out using the forward primer gt11-A1 and 3′-specific primer 5′-GGGGTACCCCTTATTTAAGAGAAG-TGA-3′ (primer IV, where the *Kpn*I site is underlined), which had been designed from the 3′-end region of partially identified *cegst2* gene. The resulting PCR products were sequenced as described in the subsection above. A second pair of PCR primers, primer III (5′-AAGGATCCAATGGCTCTCAATAACGCTA-CTC-3′, where the *Bam*HI site is underlined), which had been designed from the 5′ region of the full *cegst2* gene, and primer IV (see above) were used to amplify the complete *cegst2* gene. Other procedures for PCR amplification, cloning, sequencing and expression of *cegst2* were the same as those used for *cegst1*.

RNA isolation and reverse transcription (RT)-PCR

C. elegans strain ATCC 36112 was cultured in Sabouraud dextrose broth at 28 °C with shaking for 48 h. Mycelia were harvested by filtration, frozen in liquid nitrogen and ground into a fine powder. Total RNA was isolated from the powder using RNA-Bee (Tel-Test, Friendswood, TX, U.S.A.). The RNA was treated with RNase-free DNase I (Promega, Madison, WI, U.S.A.), and RT-PCR was performed using the Access RT-PCR System (Promega) with 1 μg of total RNA as a template and primers III and IV. Following RT at 48 °C for 45 min, 40 amplification cycles were performed, each consisting of 94 °C for 30 s, 60 °C for 1 min and 68 °C for 2 min with a final extension step at 68 °C for 7 min.

Mutagenesis of the *cegst1* gene

A primer containing a single nucleotide mismatch, designed to replace Tyr^{10} with a phenylalanine residue, was used to amplify and mutate the *cegst1* gene. The phage clone carrying the *cegst1* gene insert was used as a template. The PCR product was cloned and expressed, as described in the subsection above, and the mutant was confirmed by DNA sequencing.

Expression and purification of recombinant CeGSTs

E. coli M5 harbouring each of the *cegst* genes was cultured to $D_{600} = 0.7–0.9$ in 1 litre of Luria–Bertani broth containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml). Expression of the recombinant enzyme was induced by the addition of 0.1 mM isopropyl β-D-thiogalactoside and incubation was continued for a further 3 h at 37 °C. The cells were then harvested by centrifugation at 5000 ***g*** for 10 min at 4 °C, resuspended in 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol and disrupted by ultrasonication. The cell-free extract was obtained by centrifugation at 100000 ***g*** for 1 h at 4 °C. The enzyme was purified as described previously [18]. The crude extract was loaded on to a DEAE ion-exchange column, which had been equilibrated previously with 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol. The enzyme was eluted from the column with a linear gradient of 0–0.6 M NaCl. The active fractions were pooled and subjected to glutathione–agarose (Sigma, St. Louis, MO, U.S.A.) affinity chromatography. The sample was loaded on the column and washed with the same phosphate buffer containing 0.1 M NaCl. The enzyme was eluted with 50 mM Tris/HCl buffer (pH 9.5) containing 10 mM GSH.

Enzyme assay, protein assay and SDS/PAGE

GST activity was assayed spectrophotometrically with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 1 mM GSH in 1 ml of potassium phosphate buffer (pH 6.5) [22]. The reaction was monitored by measuring the increase in A_{340}. Activities on other substrates were assayed as described by Habig and Jakoby [22]. For inhibition studies, CeGST1-1 (20 μg/ml) or CeGST2-2 (50 μg/ml) was incubated for 10 min at 25 °C in the absence or presence of inhibitor. Protein concentration was determined as described by Bradford [23]. SDS/PAGE was performed as described by Laemmli [24] using a Mini-Protean A apparatus (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

RESULTS

Cloning and sequence analysis of cDNAs encoding GST

After screening the *C. elegans* cDNA library with an oligonucleotide probe based on the N-terminal sequence of purified GST from *C. elegans* [18], several positive clones were obtained and their DNA sequences were determined. Among them, one positive phage was found to have a DNA sequence coding exactly for the N-terminal amino acid sequence of *C. elegans* GST. The cDNA (*cegst1*) contained a 630 bp open reading frame (excluding the stop codon) (GenBank® accession number AY053501), which encoded a 210-amino-acid protein with a theoretical molecular mass of 24519 Da. At 42 bp downstream of the stop codon (TGA) in the 3′-untranslated region, a putative polyadenylation signal (AATAAA) was found.

A second GST gene (*cegst2*) was identified from the cDNA library using *cegst1* as a probe, which was initially found to lack a putative start codon. The full-length open reading frame of *cegst2* was determined by 5′-RACE to be 627 bp, encoding a protein of 209 amino acid residues (GenBank® accession number AY053502). The predicted molecular mass of the translated protein was 24551 Da. Two putative polyadenylation signals

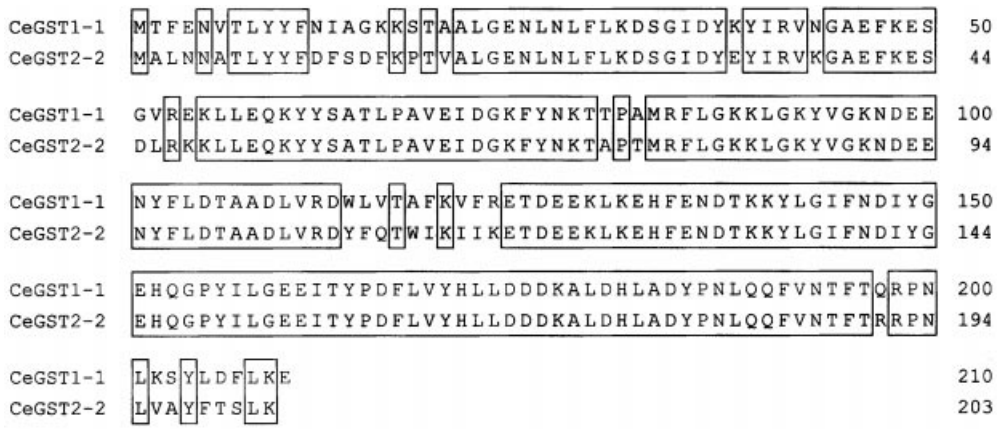

Figure 1 Alignment of deduced amino acid sequences of CeGST1-1 and CeGST2-2

Identical amino acid residues between the two proteins are boxed.

Table 1 Pairwise amino acid sequence identities among different GST classes

Sequence identities were determined using BIOEDIT Suite. The values represent the percentage identity, and the GST sequences that were selected for this Table showed the highest percentage identity of the given class. 1, *C. elegans* (GenBank® accession number AY053501); 2, *F. hepatica* (GenBank® accession number A00993); 3, *D. rerio* (GenBank® accession number AF285098); 4, *M. musculus* (GenBank® accession number J03953); 5, *Homo sapiens* (GenBank® accession number X79389); 6, *Ommastrephes sloani* (GenBank® accession number L02053); 7, *H. sapiens* (GenBank® accession number XM_007412); 8, *Rattus norvegicus* (GenBank® accession number S83436); 9, *R. rattus* (GenBank® accession number AB008807); 10, *E. coli* (Genbank AE000259); 11, *Anopheles gambiae* (GenBank® accession number Z71480); 12, *Aegilops tauschii* (GenBank® accession number AF004358); 13, *Arabidopsis thaliana* (GenBank® accession number D17672); 14, *Issatchenkia orientalis* (GenBank® accession number X57957); 15, *Saccharomyces cerevisiae* (GenBank® accession number Z38061); 16, *Schizosaccharomyces pombe* (GenBank® accession number AF213355); and 17, *Botrytis cinerea* (GenBank® accession number AF061253).

	GST class															
GST (class)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 CeGSTG1-1																
2 FhGST (Alpha)	23															
3 DrGST (Pi)	25	28														
4 MmGST (Mu)	22	46	25													
5 HsGST (Theta)	14	22	19	18												
6 OsGST (Sigma)	21	22	26	22	17											
7 HsGST (Zeta)	17	21	18	18	22	20										
8 RnGST (Kappa)	14	13	17	11	17	13	21									
9 RrGST (Omega)	17	15	20	19	19	19	19	15								
10 EcGST (Beta)	18	19	22	20	24	22	23	14	21							
11 AgGST1-1 (insect)	16	17	20	18	28	19	18	18	18	25						
12 AtauGST (Tau)	16	22	18	20	19	18	24	17	25	21	21					
13 AthaGST (Phi)	17	22	21	18	21	20	24	15	20	24	21	22				
14 IoGST Y-2 (yeast)	20	18	16	17	21	14	21	15	16	15	20	17	21			
15 ScGST (yeast)	14	15	17	19	16	17	16	15	16	13	16	19	21	17		
16 SpGST (yeast)	15	16	19	19	22	18	26	14	20	21	24	20	23	20	24	
17 BcGST (fungus)	11	20	18	18	18	17	19	13	21	19	20	23	16	19	21	32

(AATAAA) were also located at the downstream region of the translational stop codon, TAA, followed by a polyadenylated tail (results not shown).

Primary structure comparison

The BLAST program analysis showed that the nucleotide sequences of the *cegst1* and *cegst2* genes share homology with other classes of GSTs, indicating that both cloned genes encode GST enzymes. The predicted amino acid sequences from these two cDNA species were 84 % identical with each other (Figure 1). An alignment of the deduced amino acid sequences of the CeGST proteins with the sequences of various classes of GST proteins resulted in 22–25 % sequence identities at most with the major classes of GSTs, such as Alpha (*Fasciola hepatica*, liver fluke), Pi (*Danio rerio*, zebrafish) and Mu (*Mus musculus*), suggesting that the fungal GSTs do not belong to any known GST class. The sequence identities of CeGST1-1 with representative GST classes are summarized in Table 1.

Detection of mRNA transcript of *cegst2*

To determine whether *cegst2* is expressed in *C. elegans*, total RNA was isolated from a 48 h culture. The RNA was treated with DNase I and used as template in RT-PCR with primers specific to *cegst2*. The resulting product was approx. 600 bp in

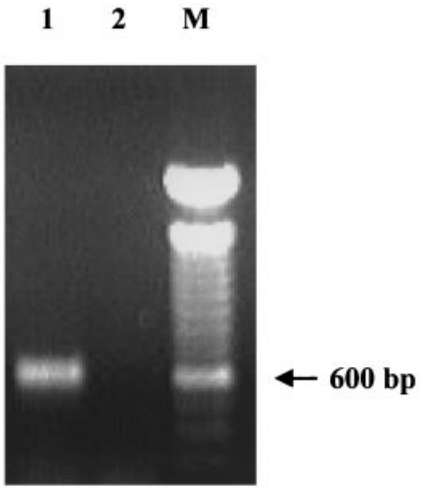

Figure 2 Expression of *cegst2* in *C. elegans*

Primers III and IV were used with 1 μg of total *C. elegans* RNA in RT-PCR to examine the expression of *cegst2*. An additional reaction omitting the reverse transcriptase was performed to ensure that no DNA contamination was present in the total RNA sample. Lane 1, RT-PCR of *cegst2*; lane 2, PCR without reverse transcriptase; lane M, DNA molecular-size standards.

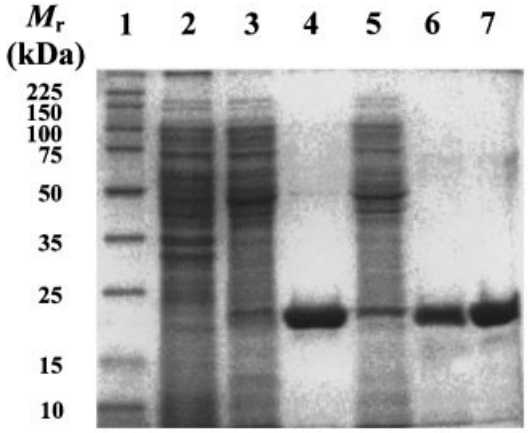

Figure 3 SDS/PAGE of the expression and purification of recombinant CeGST1-1 and CeGST2-2

Lane 1, molecular-mass markers (in kDa); lane 2, crude extract from uninduced bacteria containing pQE51; lane 3, crude extract from induced bacteria containing pNCK23 (CeGST1-1); lane 4, recombinant CeGST1-1 purified from *E. coli* M5; lane 5, crude extract from induced bacteria containing pNCK25 (CeGST2-2); lane 6, recombinant CeGST2-2 purified from *E. coli* M5; and lane 7, CeGST1-1 purified from *C. elegans*.

length (Figure 2), as expected, indicating that the *cegst2* gene was expressed at the mRNA level in the fungus under the experimental conditions used.

Expression, purification and characterization of recombinant CeGST

To obtain *E. coli* clones overexpressing recombinant *C. elegans* GST enzymes, the GST genes were amplified by PCR from cDNA clones using primers I and II for CeGST1-1 and primers III and IV for CeGST2-2. The PCR products were cloned after *Bam*HI and *Kpn*I digestion in the same enzyme sites of pQE51 vector under the T5 promoter, obtaining the plasmids pNCK23 (CeGST1-1) and pNCK25 (CeGST2-2). Both recombinant *E. coli* strains, carrying the *cegst1* and *cegst2* genes respectively, overexpressed the corresponding CeGST proteins with a molecular mass of 27600 Da when induced with isopropyl β-D-thiogalactoside (Figure 3). Both of the recombinant CeGST proteins bound to a glutathione–agarose affinity column and were purified to homogeneity as described previously [18]. Gel-filtration chromatography of the recombinant CeGST1-1 and CeGST2-2 showed the respective single peaks with molecular masses of 59400 and 59800 Da respectively, indicating the homodimeric structure as shown previously in the wild-type fungus [18]. The specific activity of CeGST1-1 towards CDNB (2.04 μmol/min per mg of protein) was approx. 3-fold greater than that of CeGST2-2 (0.75 μmol/min per mg of protein) (Table 2). The inhibition profile showed inhibitory effects of alizarin, Cibacron Blue, haematin and quecetin on both recombinant CeGSTs (Table 3). The Student's *t*-test indicated no significant difference between the inhibitory effects on both enzyme activities ($P > 0.05$), although CeGST2-2 appeared to be slightly more resistant to inhibitors than CeGST1-1.

Table 2 Substrate specificity of recombinant CeGSTs

Activities are presented as means of triplicate measurements and the S.E.M.s are less than 5%. n.d., not detectable (activities were not higher than non-enzymic reactions).

	Specific activity (μmol/min per mg of protein)	
Substrate	CeGST1-1	CeGST2-2
CDNB (1 mM)	2.04	0.75
1,2-Dichloro-4-nitrobenzene (1 mM)	n.d.	n.d.
Ethacrynic acid (0.2 mM)	0.13	0.11
Cumene hydroperoxide (0.1 nM)	0.12	0.005

Table 3 Inhibition profiles of recombinant CeGSTs in *E. coli*

Enzyme activity was measured with 1 mM CDNB after an incubation of 20 μg/ml of CeGST1-1 or 50 μg/ml of CeGST2-2 for 10 min in the absence or presence of the inhibitor. 100% activities of CeGST1-1 and CeGST2-2 are 2.04 and 0.75 μmol/min per mg of protein respectively.

		Activity (%)	
Inhibitors	Concentration (μM)	CeGST1-1	CeGST2-2
Alizarin	0	100	100
	1	91.2	94.7
	10	63.9	85.2
	100	35.7	73.4
Cibacron Blue	0	100	100
	1	4.7	64.2
	10	2.0	12.3
	100	0	10.4
Haematin	0	100	100
	1	28.8	63.6
	10	22.0	30.3
Quecetin	0	100	100
	1	85.9	88.7
	10	62.6	66.8
	100	17.1	43.0

Table 4 Enzyme activity of wild-type and mutant CeGST1-1 expressed in *E. coli*

Enzyme activity was measured from cell-free extracts of *E. coli* cells.

Enzyme	Specific activity (μmol/min per mg of protein)
Wild-type CeGST1-1	0.325
Mutant CeGST1-1	8.5×10^{-5}
Control (*E. coli* M5)	6.6×10^{-5}

Site-directed mutagenesis of the *cegst1* gene

To investigate whether the conserved Tyr^{10} residue is important for catalysis of CeGST, a mutated *cegst1* gene, in which Tyr^{10} was replaced with a phenylalanine residue, was constructed by site-directed mutagenesis. SDS/PAGE showed that *E. coli* M5 transformed with the mutated *cegst1* displayed a similar level of GST overexpression as the strain of M5 carrying the wild-type *cegst1* (results not shown). When the cell-free extract from a control culture of *E. coli* M5 was tested for GST activity against CDNB, only a low level of endogenous activity in *E. coli* [25] was detected (66 nmol/min per mg of protein) (Table 4). No significant GST activity was measured in a crude extract of the mutant strain towards CDNB. The mutant GST was able to bind to glutathione–agarose affinity matrices, but neither the eluent nor the unbound fraction showed any CDNB-conjugating activity.

DISCUSSION

Essentially all eukaryotic species appear to possess multiple isoenzymes of GSTs [1]. Although GSTs are known to be important in enzymic detoxification of xenobiotics in mammals, not much is known about the functional and physiological role of GSTs in micro-organisms. Bacterial GSTs have been studied extensively [26] and found to play roles in antibiotic resistance [27], dehalogenation of environmental pollutants [28] and degradation of polycyclic aromatic hydrocarbons [29]. However, relatively few studies on fungal GSTs have been reported. A number of fungal species were found to possess GSTs [30,31], and some of them were shown to be related to the Alpha and Mu classes of GSTs in rat [32], suggesting that fungal GSTs are more related to mammalian GSTs than to their bacterial counterparts. A GST from the hymenomycete *Phanerochaete chrysosporium* [33] cross-reacted with antisera to rat Theta-class GST5-5 and also showed N-terminal sequence similarity to the Theta class, including a conserved serine residue that has been specifically implicated in the catalytic site of this class [34]. Although fungal GSTs have been purified and characterized in several species [33,35–37], little is known about the molecular genetics of this group and the definitive class allocation has not been determined [5]. Full comparisons are difficult, because the only complete DNA sequences of fungal GSTs that have been reported are sequences from the plant pathogenic fungus *Botrytis cinerea* [38] and yeasts *Issatchenkia orientalis* [39], *Saccharomyces cerevisiae* [40] and *Schizosaccharomyces pombe* [41]. In the present study, we report the complete cDNA sequence of a fungal GST that has been purified and characterized previously [18]. As suggested in our previous study [18], the GST from *C. elegans* appears to be a novel class of the GST family on the basis of the biochemical and immunological properties and the N-terminal amino acid sequence. GSTs that share greater than 40 % identity are generally included in the same class, and those that possess less than 30 % identity are assigned to separate classes [1]. As shown in Table 1, CeGST1-1 exhibited only 14–25 % sequence identity with representatives of different GST classes. Therefore these results clearly indicate the novelty of this fungal enzyme. The fungal GST was relatively distantly related (14 % sequence identity) to the ancestral Theta-class GST. A phylogenetic and

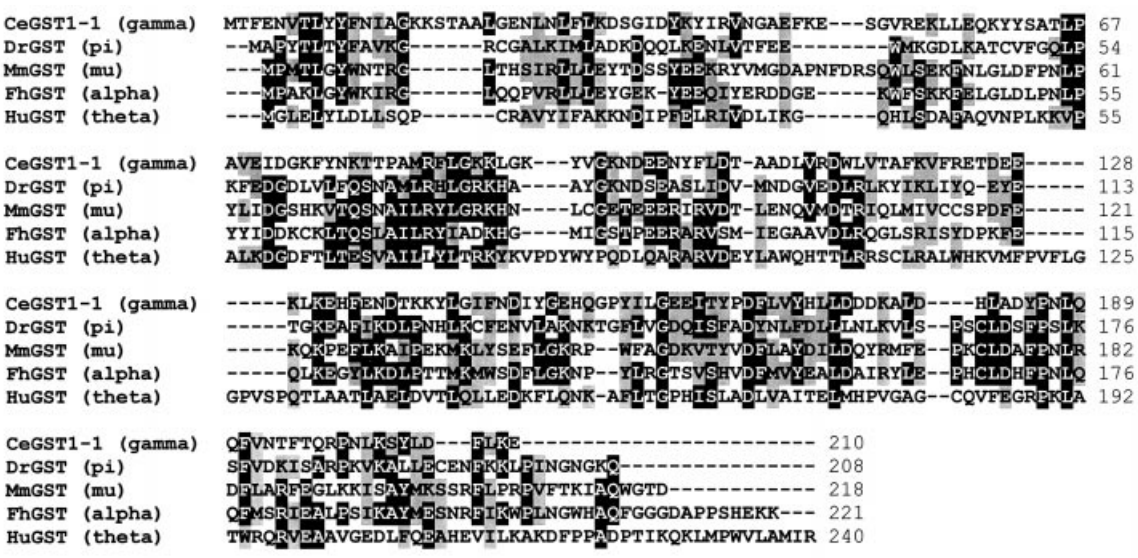

Figure 4 Alignment of CeGST1-1 sequence with representative eukaryotic GST enzymes of Alpha, Mu, Pi and Theta classes

Sequence alignment was based on the ClustalW algorithm implemented in the BIOEDIT program. Black shading indicates that more than two residues are identical among the five aligned residues. When less than three residues are identical and amino acid residues are related, letters are shaded in grey. The sources of GST sequences include: DrGST (Pi) from *D. rerio* (GenBank® accession number AF285098); MmGST (Mu) from *M. musculus* (GenBank® accession number J03953); FhGST (Alpha) from *F. hepatica* (GenBank® accession number A00993); and HuGST (Theta) from *Homo sapiens* (GenBank® accession number X79389).

evolutionary tree for GST classes has been provided by Pemble and Taylor [42], indicating that fungal GSTs arose from the mitochondrial Theta-class GST at an early stage of GST evolution. Our results also suggest that the CeGST might have evolved independently from Alpha-, Mu- and Pi-class GSTs after early divergence from the progenitor Theta class.

Although there is no specific region showing a high degree of similarity between the CeGSTs and other GSTs, Tyr^{10} in the N-terminal region, which is essential for catalysis in Alpha-, Mu-, Pi- and Sigma-class GSTs [43–45], is conserved. In contrast, site-directed mutagenesis experiments with the GSTs from *E. coli* and *Proteus mirabilis* have demonstrated that tyrosine is not essential for catalysis [46,47]. In the present study, we report almost complete loss of glutathione-conjugating activity with CDNB in the mutated CeGST1-1, suggesting that this tyrosine residue is essential for catalysis at the glutathione-binding site (G-site) of the fungal GST. However, the mutated protein was still able to bind to glutathione–agarose affinity matrices. Sequence alignment (Figure 4) indicated that amino acid residues that are conserved at the G-site and hydrophobic-binding site (H-site) of GSTs are present in CeGST1-1: Gly^{15}, Leu^{30}, Leu^{66}, Pro^{67}, Gly^{95}, Glu^{100}, Asp^{105}, Asp^{113}, Gly^{159}, Asp^{166}, Leu^{173}, Pro^{186} and Leu^{188} [48].

Another GST sequence (*cegst2*) with 84% sequence identity with CeGST1-1 was found in *C. elegans* by Southern hybridization using the *cegst1* gene as a probe. In our previous study [18], it was not clear whether *cegst2* was expressed in the fungus at the protein level, since the affinity-purified sample turned out to be CeGST1-1. RT-PCR using *cegst2*-specific primers, however, revealed that *cegst2* was also expressed at the mRNA level in *C. elegans* (Figure 2). Both recombinant enzymes expressed in *E. coli* were similar in substrate specificity (Table 2) and inhibition profile (Table 3), indicating that they belong to the same class of GST. Similar to *C. elegans*, *Anopheles gambiae* was also reported to possess two cDNA species encoding for GST with 80% sequence identity, which presumably represent the transcription products of closely related genes within a gene family [11]. Interestingly, three variable-sequence regions, which are different from the sequences of CeGST1-1, were found by pairwise sequence alignment of these two amino acid sequences (Figure 1): region I, Ala^{2}–Val^{22}; region II, Tyr^{114}–Lys^{123}; and region III, Val^{202}–Ser^{207}.

To find other members of GST, the cDNA library was screened with several oligonucleotide probes designed from *cegst1* and *cegst2*. However, no additional GST-like sequences were obtained, suggesting that CeGST1-1 and CeGST2-2 are major members of GSTs in this class. The presence of another GST enzyme has been suggested by biochemical studies [18,49], which appears to be another class of GST displaying a different substrate specificity compared with the CeGSTs. Further studies will be required to find out the presence of multiple GST classes in this fungus.

In summary, the present study reports the complete cDNA sequences of GST genes from a filamentous fungus. Sequence comparisons indicate that the CeGSTs are a novel class of GST proteins. Therefore we propose that the GSTs from *C. elegans* be Gamma-class GSTs and designated as GSTG1-1 and GSTG2-2 respectively. Further investigation on the structure and function of new microbial GSTs may provide new insights into GST evolution, possible detoxification pathways and ecological fates of toxic chemicals in the environment.

We thank Dr J. B. Sutherland, Dr B. D. Erickson and Dr B. F. Coles for critically reading the manuscript. This work was supported in part by an appointment to the Postgraduate Research Program at the National Center for Toxicological Research, administered by the Oak Ridge Institute for Science and Education, through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration. This work was also supported in part by the Postdoctoral Fellowship Program of Korea Science and Engineering Foundation (KOSEF).

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Received 11 March 2002/20 August 2002; accepted 27 August 2002
Published as BJ Immediate Publication 27 August 2002, DOI 10.1042/BJ20020400