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Molecular Cloning and Characterization of a Glutathione S-transferase Gene Repressed by Phenylacetic Acid From *Penicillium chrysogenum**

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Abstract Glutathione S-transferase (GST) gene, PcgstA was cloned from the penicillin producing strain *Penicillium chrysogenum*, which is important for understanding the industrial fermentation process. PcgstA gene has an open-reading-frame of 840 bp in length, which is interrupted by two introns. The deduced amino acid sequence shows about 50% identity to several characterized filamentous fungi GSTs. The recombinant PcGSTA in *Escherichia coli* were overexpressed and purified. Enzymatic assays showed that the recombinant PcGSTA had a specific activity with 1-chloro-2, 4-dinitrobenzene of (0.159±0.031) μmol/(min·mg). It was found that the expression level of PcgstA in the penicillin producing medium supplemented with phenylacetic acid, the side chain precursor of penicillin G, was significant down regulated than that in medium without phenylacetic acid. This result suggested that PcGST may be related to phenylacetic acid metabolism in the penicillin producing strain.

Key words *Penicillium chrysogenum*, glutathione S-transferase, phenylacetic acid

The filamentous fungus *Penicillium chrysogenum* has been used for the industrial production of penicillin for over 50 years. The biosynthetic pathways of penicillin in *P. chrysogenum* and other filamentous fungi have been well elucidated. Three precursor amino acids, α-aminoadipate, L-cysteine, and L-valine were condensed to form δ- (L-α-aminoadipyl) -L-cysteinyl-D-valine (ACV) tripeptide by the action of the enzyme ACV synthetase. The ACV is then cyclized into isopenicillin N (IPN) by the enzyme IPN synthase. In the final step, the aminoadipyl side-chain of IPN is exchanged with phenylacetic or phenoxyacetic acids to yield penicillin G or V. This reaction is catalyzed by acyl coenzyme A (CoA): isopenicillin N acyltransferase (IAT). The three respective genes: pcbAB, pcbC, and penDE have already been cloned and characterized [1]. As a secondary metabolite, the biosynthesis of penicillin involves a complex regulatory system. Identification of factors that limit the yield and rate of penicillin production has made it possible to improve strains by directed genetic modifications.

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) is structurally analogous to ACV tripeptide [2]. Many studies have shown that penicillin biosynthesis is interconnected with GSH metabolism. First, GSH may suppress penicillin production by inhibiting isopenicillin N synthase (IPNS) activities [3]. Second, GSH may be involved in the activation or detoxification of the penicillin side chain precursors, phenylacetic and phenoxyacetic acids[4~7]. Finally, GSH may also serve as an S-source in the biosynthetic process[8]. However, GSH related genes have not been characterized from *P. chrysogenum* to this date.

Glutathione S-transferases (GSTs, EC 2.5.1.18) are important enzymes that involving in GSH metabolism. They catalyze the conjugation of GSH to

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a wide variety of endogenous and exogenous electrophilic compounds[9]. They are a super-family of phase Ⅱ detoxification enzymes, which present in all aerobic organisms. GSTs also play multifunctional roles, such as transporting hydrophobic compounds and regulating cell signaling pathways. Many GSTs genes have been cloned and characterized from broad ranges of organisms. Based on primary and tertiary structure similarity, immunological cross-reactivity, and substrate specificity, GSTs are categorized into several classes. Mammalian GSTs have been classified into α, μ, π, θ, σ, κ, ξ and ω classes. Other classes include φ, τ classes in plant, β class in bacteria, and δ, ε classes in insects[10].

Up to date, relatively little is known about GSTs from fungi, especially filamentous fungi. And most characterized fungal GSTs do not fit into any of the previously characterized classes. Study of fungal GSTs will help us to gain better understanding of functions and evolutionary history of GSTs. In the present study, we report a GST gene that we cloned from *P. chrysogenum*, and our comparative analysis and functional expression in penicillin producing medium.

1 Materials and methods

1.1 Strains and culture conditions

Penicillium chrysogenum Wis 54-1255 (ATCC28089), a low-penicillin-producing strain, was used in this study. Spore suspensions of Wis 54-1255 were inoculated in 40 ml of seed medium (20 g/L corn steep liquor, 20 g/L sucrose, 5 g/L yeast extract, 5 g/L $CaCO_3$, pH 5.8) in 250 ml flasks and incubated in a rotary shaker (250 r/min) at 26℃ for 24 h. 2 ml of the seed culture were transferred to 40 ml of fermentation medium contained 35 g/L lactose, 30 g/L corn steep liquor, 5g/L $(NH_4)_2SO_4$, 1g/L KH_2PO_4, 1g/L K_2SO_4, 10 g/L $CaCO_3$, 2 g/L phenylacetic acid, 6 ml/L corn oil, pH 6.0 and grown at 26℃ with 250 r/min shaking. Production medium without phenylacetic acid was used as control when necessary.

Escherichia coli DH5α (Invitrogen) and XL1-Blue (Stratagene) were used for nucleic acids manipulations and bacteriophage propagations, respectively. *Escherichia coli* BL21 (DE3)-RP (Stratagene) was used as the host for expression vector pET11a (Stratagene). All bacterial strains were cultured according to standard procedures[11].

1.2 Manipulation of *P. chrysogenum* nucleic acids

P. chrysogenum Wis 54-1255 mycelium grown in production medium was harvested by filtration, washed three times with 0.9% NaCl, frozen in liquid nitrogen, and ground to a powder with mortar and pestle. Genomic DNA was isolated by CTAB method[12] and partially digested with Sau3A Ⅰ (Promega). Fragments about 20 kb were selected, ligated to vector λZAP Ⅱ and packaged with Giga pack Ⅱ Gold kit (Stratgene).

Total RNA was isolated with Trizol™ (Invitrogen) and treated with RQ1 RNase-free DNase (Promega) to remove genomic DNA contamination. The first strand cDNA was constructed by using the SuperScript™ Ⅱ RNase H^- (Inritrogen) and Random Hexamers (Promega).

1.3 Cloning of *P. chrysogenum* gstA gene

To isolate the *P. chrysogenum* gst gene, the conserved regions of *Aspergillus nidulans* GSTA (GenBank Accession No. AAM48104) [13] protein -NPNGRIPA- and -GDHITIADI- were used to design the degenerate PCR primers: GSTF, 5′ AAY CCX AAY GGX MGX ATH CCX GC 3′ and GSTR, 5′ DAT RTC XGC DAT XGT DAT RTG RTC XCC 3′. PCR amplification was performed with EX Taq (Takara) at 94℃ 5 min (1 cycle); 94℃ 15 s, 56℃ 30 s, and 72℃ 30 s (40 cycles), using genomic DNA of Wis 54-1255 as template. A 404 bp fragment was obtained. Sequence analysis demonstrated that the cloned fragment corresponds to a GST gene. To isolate the entire *P. chrysogenum* gst gene, a PCR-amplified probe was used to screen the *P. chrysogenum* DNA library in λZAP Ⅱ by plaque hybridization. Positive phagemids were isolated and fragments containing the hybridizing region were subcloned and sequenced.

1.4 Heterologous expression of PcgstA in *E. coli*

The ORF of PcgstA gene was amplified by PCR with cDNA as a template. The primers EGSTF: 5′ ATATTCTACCATATGTCTTCAAACATTACCCTG 3′ and EGSTR: 5′ TCAACGCGGATCCTCAATGC-TGATCGGAAGTAGTC 3′ were used and *Nde* Ⅰ /*Bam*H Ⅰ (Promega) sites were introduced to the 5′ and 3′ ends, respectively. The PCR product was subcloned into the pGEM-T vector (Promega) and confirmed by DNA sequencing. The insert was excised with *Nde* Ⅰ /*Bam*H Ⅰ and subcloned into the expression vector pET11a, which previously cut with the same restriction enzymes, to generate pET11a/PcGSTA. *E. coli* BL21 (DE3)-RP transformed with pET11a/PcGSTA was used to overexpress the recombinant PcGSTA according to the standard manual[11]. An overnight culture was diluted 1∶100 with Luria-Bertani (LB) broth containing ampicillin (100 mg/L) and grown at 37℃ until A_{600} reached about 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.5 mmol/L. After further grown at 37℃ for 3.5 h, bacteria were harvested by centrifugation at 10 000 *g* for 5 min at 4℃.

1.5 Purification of recombinant PcGSTA

Cells expressing the PcGSTA were resuspended in 10 volumes of buffer A (50 mmol/L Tris-HCl, 0.5 mmol/L EDTA, 50 mmol/L NaCl, 5% glycerol, pH 7.5) [14] and sonicated on ice-water bath. The inclusion bodies of recombinant PcGSTA were collected by centrifugation at 10 000 *g* for 10 min at 4℃ and washed twice with buffer A plus 2% sodium deoxycholate. The inclusion bodies were then dissolved in buffer A with 0.3% sodium lauryl sarcosinate, and protein refolding was obtained by dialyzing the solution against 100 volumes buffer A overnight at 4℃. The refolded protein was concentrated by ultrafiltration and purified by Superdex-200 gel filtration chromatography (column 1.6 cm × 58 cm). The purity of the recombinant PcGSTA was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

1.6 Enzyme assay

GST activity was measured with 1 mmol/L 1-chloro-2, 4-dinitrobenzene (CDNB, Sigma) and 1 mmol/L reduced GSH in 100 μl 0.1 mol/L PBS buffer (pH 6.5). The reaction was detected by measuring the increase in A_{340} as described by Habig *et al* [15]. Specific activity of GST was defined as micromoles CDNB utilized per minute and per mg of protein. The K_m and V_{max} values for CDNB were determined using a CDNB range from 0.2 to 1.5 mmol/L and a fixed GSH concentration of 1 mmol/L. The kinetic parameters were calculated using non-linear regression analysis in the Hyper32 program. Protein concentration was determined by the method of Bradford[16].

1.7 Phylogenetic analysis

Representative GSTs were selected from previously described GST classes. The amino acid sequences were obtained from GenBank and aligned with the deduced protein sequence of PcGSTA using ClustalW [17]. Based on the multiple alignments, phylogenetic trees were constructed by using the neighbor-joining (NJ) method and MEGA version 2.1 [18]. The data were analyzed by using p-distance model with 1 000 bootstrap tests.

1.8 Quantitative analysis of PcgstA expression

TaqMan probe method was used to detect the expression level of PcgstA in *P. chrysogenum*. γ-Actin gene was used as an internal control. TaqMan primers and probes were selected using the Primer Express software (Applied Biosystems). Oligonucleotide sequences were as follows: PcgstA forward primer, 5′ GGCTGGTCTCGGGCCTAT 3′, reverse primer, 5′ CGCGTGTTGGCGAAGAG 3′, and TaqMan probe, 5′ CAAGGACAAGCGAACCATTTCCG 3′. γ-Actin forward primer, 5′ CTCGCTGAGCGTGGTTACAC 3′, reverse primer, 5′ TTGATGTCACGGACGATTTCA 3′, and TaqMan probe, 5′ TTCTCCACCACCGCCGAGC 3′. Phenylacyl-CoA ligase (phl) forward primer, 5′ AGACATGCACGGTCGTAAGCT 3′, reverse primer, 5′ GCAAAGCGCCGGATGA 3′, and TaqMan probe , 5′ ACCCACCCGCACGATATCTGGCT 3′. IPN synthase (pcbC) forward primer, 5′ CCACCTGCCGCCATTAAG 3′, reverse primer, 5′ CCTCATGCCATTCGAAACTCA 3′ and TaqMan probe, 5′ CGGCGGAGGACGGCACCA 3′. The probes were labeled at the 5′ end with the reporter dye FAM (6-carboxyfluorescein), at the 3′ end with the quencher dye TAMRA (6-carboxytetramethylrhodamine).

cDNA templates were obtained from total RNA extracted from Wis 54-1255 mycelia grown for 6 h, 12 h and 24 h in fermentation medium with and without phenylacetic acid. PCR amplifications were performed in 50 μl volume with TaqMan Universal PCR Master Mix kit (Applied Biosystems) in the ABI-prism 7700 sequence detector under a condition of 2 min at 50℃ and 2 min at 94℃, followed by 15 s at 94℃ and 1 min at 60℃, 40 cycles. The relative expression level of PcgstA was measured in medium supplemented with PAA and without PAA by using $2^{-\Delta\Delta CT}$ method[19].

2 Results and discussion

2.1 Cloning the *P. chrysogenum* PcgstA gene

In order to isolated GST gene from *P. chrysogenum*, we designed a pair of degenerate PCR primers according to the conserved N-domain and C-domain of *A. nidulans* GSTA and indeed obtained a 404 bp DNA fragment from the *P. chrysogenum* Wis 54-1255 genomic DNA. Sequence analysis confirmed that it corresponds to a GST gene. To obtain the full length of the gene, we then screened a genomic library constructed with Wis 54-1255 in λZAP Ⅱ with the PCR product as a probe. After three consecutive rounds of hybridization-based screening, one of the positive phagemids was isolated and a 1.61 kb *Sma* Ⅰ fragment was sequenced. The sequence has been deposited in GenBank under accession number DQ462327.

Using FGENESH gene finding software (http://www.softberry.com/berry.phtml), we predicted an 840 bp open-reading frame (ORF). The ORF is interrupted by two putative introns of 64 bp and 59 bp in length at positions 138 and 408 of the genomic DNA sequence (numbered from ATG), respectively. The ORF encodes a peptide of 238 residues, which has

a predicted molecular mass of 26.9 ku and an isoelectric point of 5.75. The deduced amino acid sequence shows high identities with characterized GSTs from filamentous fungi (Figure 1), 54% identity with the plant pathogenic fungus *Botryotinia fuckeliana* GST1 (BfGST1, GenBank Accession No. AAG43132)[20], 52% identity with *A. nidulans* GSTA (AnGSTA), and 52% identity with the human pathogen *Aspergillus fumigutus* GSTA (AfGSTA, GenBank Accession No. AAX07321)[21]. We named this gene as PcgstA.

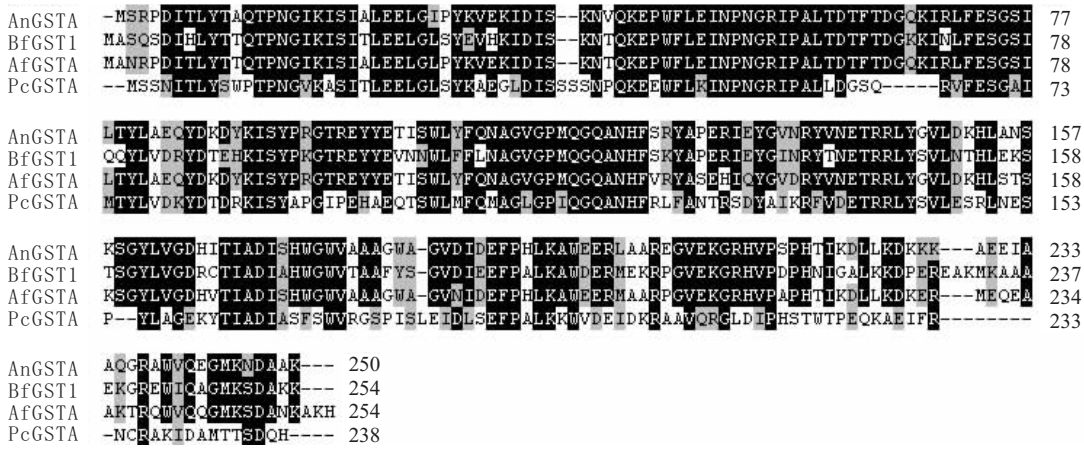

Fig. 1 Amino acid sequence alignment of four GSTs from filamentous fungi

The deduced protein sequence of PcGSTA was aligned with AnGSTA (*Aspergillus nidulans*. GenBank Accession No. AAM48104), BfGST1 (*Botryotinia fuckeliana.* GenBank Accession No. AAG43132), and AfGSTA (*Aspergillus fumigutus*. GenBank Accession No. AAX07321). Identical and conserved residues are shaded in different degrees of grayness.

We confirmed the length and position of the two introns by RT-PCR with Wis 54-1255 cDNA as template and EGSTF/EGSTR primers. Figure 2 shows the comparison of the four GST genes from filamentous fungi. All the genes are interrupted by two introns. The introns of the two *Aspergillus* GST genes, AngstA and AfgstA, are proximal to the 5′ end. In Bfgst1, the two introns are in the middle. The introns in PcgstA are also closed to 5′ end, but the length of the first and second exons are longer. The other two reported GST genes from *A. fumigutus*, gstB (GenBank Accession No. AAX070318) and gstC (GenBank Accession No. AAX070319) have no introns[21]. Their amino acid sequences identity with PcGSTA, are 45% and 37%, respectively.

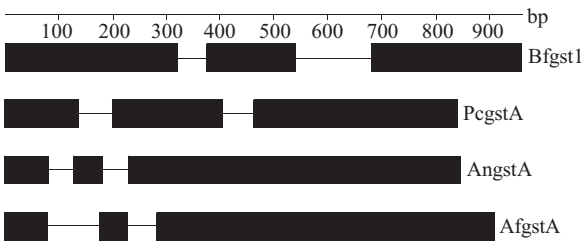

Fig. 2 Comparison of introns positions and lengths of GST genes from filamentous fungi

The thick and thin lines represent protein-coding and introns, respectively. The genes in addition to PcgstA are: AngstA (*Aspergillus nidulans*, GenBank Accession No. AF425746), Bfgst1 (*Botryotinia fuckeliana*, GenBank Accession No. AF061253), and AfgstA (*Aspergillus fumigutus*, GenBank Accession No. AY770045).

Conservation of the intron positions is shown as an important feature of corresponding genes of filamentous fungi, suggesting a common ancestor[22]. Since GSTs have many isoenzymes, more genes need to be characterized to reveal their evolutionary relationship among fungal GSTs. We constructed a phylogenetic tree to characterize GSTs (Figure 3) and showed that those from the five filamentous fungal GSTs, PcGSTA, BfGSTA, AnGSTA, and AfGSTA, are cluster together and significantly related to AfGSTB, AfGSTC, and GST Ⅰ and Ⅱ of *Schizosaccharomyces pombe*. They are all closely related to the beta class GSTs in bacteria. GST Ⅲ from *S. pombe* and other fungi GSTs from *Saccharomyces cerevisiae* (GST1, 2), *Issatchenkia orientalis* (GSTY1, Y2), and *Cunninghamella elegans* (GST1, 2) are more distantly related.

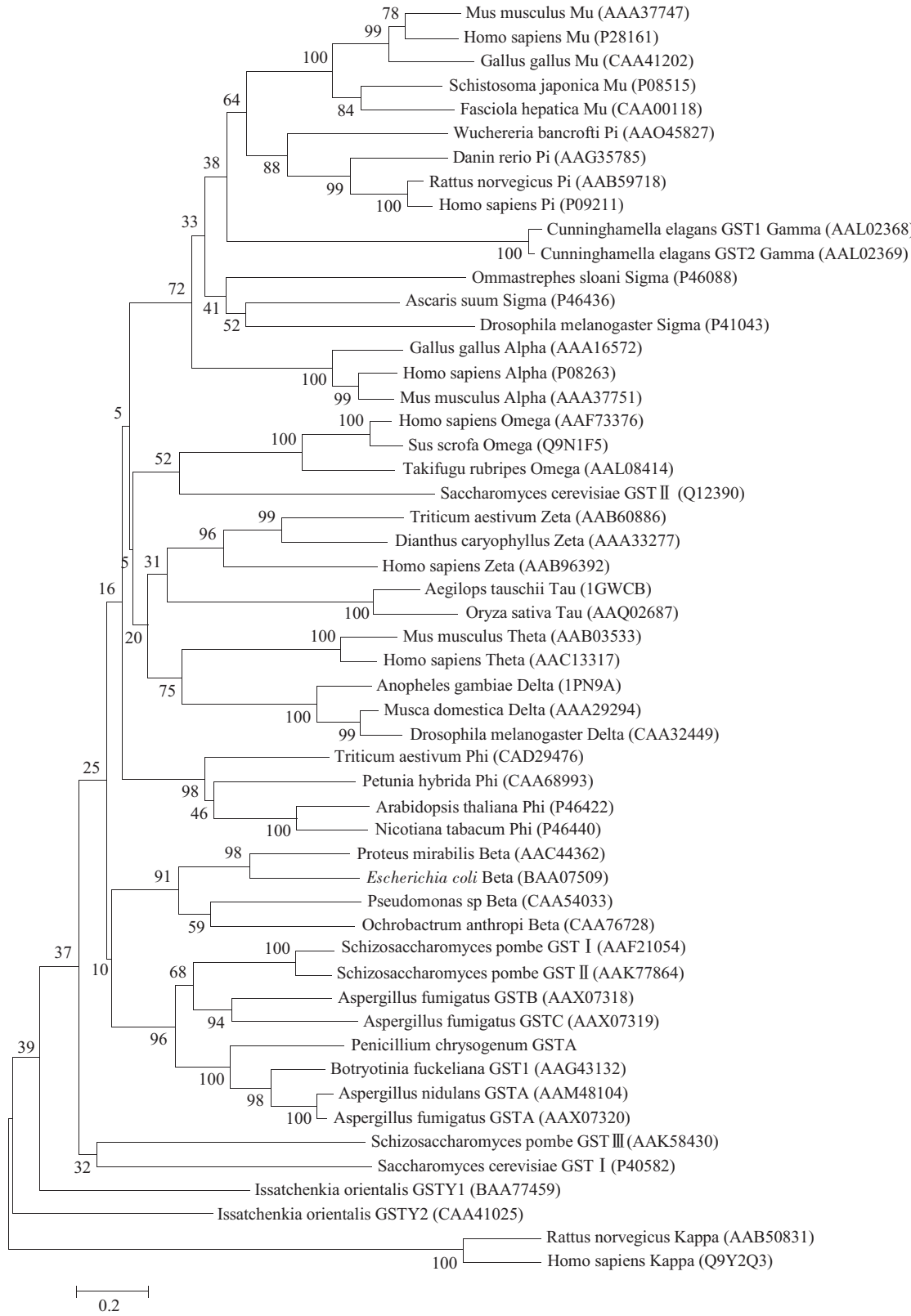

Fig. 3 Phylogenetic analysis of PcGSTA compared to other characterized GSTs

The amino acid sequences were obtained from GenBank and aligned with PcGSTA by using ClustalW. Neighbor-joining tree was generated by MEGA version 2.1 with bootstrap 1000. Percentage bootstrapping values are shown at branch points.

2.2 Expression, purification and activity analysis of recombinant PcGSTA protein

To determine if PcgstA gene is encoding functional GST, we constructed an expression vector pET11a/PcGSTA containing the entire ORF of PcgstA and transformed into *E.coli* BL21 (DE3)-RP. Upon induction with IPTG, recombinant PcGSTA protein was expressed both in cell lysate supernatants and precipitates, but most of the proteins were found as insoluble form (Figure 4). The cell lysate harbors pET11a/PcGSTA showed significant high CDNB-conjunction activity when a common substrate of GSTs, CDNB, was used to assess the enzymatic activity of PcGSTA. The result indicated that PcgstA indeed encodes a functional GST.

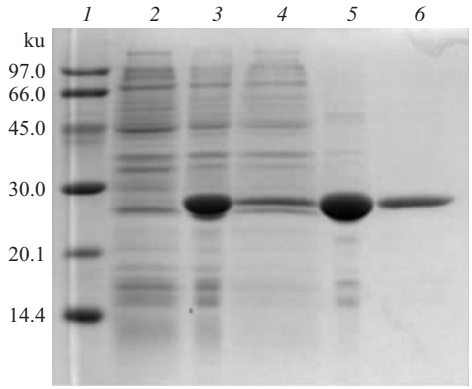

Fig. 4 SDS-PAGE analysis of the purified recombinant PcGSTA

1: Molecular mass markers with the size in ku; *2*: Total cellular extract from uninduced *E. coli* BL21 (DE3)-RP containing pET11a/PcGSTA; *3*: Total cellular extract from induced *E. coli* BL21 (DE3)-RP containing pET11a/PcGSTA; *4*: Supernatant after sonication and centrifugation of cells expressing recombinant PcGSTA; *5*: Pellet after sonication and centrifugation of cells expressing recombinant PcGSTA; *6*: The purified recombinant PcGSTA.

We further purified the recombinant PcGSTA from the inclusion bodies and determined its molecular mass (27 ku) on SDS-PAGE (Figure 4). The specific activity of the purified recombinant enzyme for CDNB was (0.159 ± 0.031) μmol/(min·mg). The specific activity is much higher than that of some characterized fungal GSTs, such as GSTA, GSTB and GSTC from *A. fumigutus* [21], but is lower than most of the mammalian GSTs [23]. At fixed GSH concentrations (1 mmol/L), the K_m and V_{max} values for CDNB were (0.153 ± 0.003) μmol/L and (4.57 ± 0.13) μmol/(min·mg), respectively.

2.3 Repression of PcgstA expression by phenylacetic acid

We used phenylacetic acid (PAA) as a side chain precursor for industrial production of penicillin G (benzylpenicillin) in *P. chrysogenum*. It has to be activated to phenylacyl-CoA prior to the incorporation. A gene encoding a phenylacyl-CoA ligase (phl) has been demonstrated to involve in penicillin production in *P. chrysogenum* [24]. However, disruption of phl indicated that other enzyme also contribute to PAA activation. Ferreo *et al*[7]. proposed an alternative PAA and phenoxyacetic acids (POA) activating route involving GSH S-derivation. But there is little evidence provided for this route *in vivo*. On the other hand, GST was suggested to participate in the detoxification of the PAA and POA in *P. chrysogenum* [4~6].

Using real-time quantitative RT-PCR, γ-actin gene[25] as internal control, we compared the expression level of PcgstA in penicillin producing medium with and without PAA. To our surprise, the expression level of PcgstA was significant down-regulated by PAA (Figure 5). Twelve hours after inoculation, the relative expression level of PcgstA in medium containing PAA was just about one third of that in medium without PAA. On the contrary, the expression level of phl was induced by PAA. The expression level of phl in medium supplement with PAA at 6, 12, 24 h after inoculation were (2.99 ± 0.21), (2.11 ± 0.09), and (1.77 ± 0.20) times higher than that in medium without PAA. We also compared the expression level of pcbC, the gene encoded isopenicillin N synthase [26]. In the three time points, the expression levels of pcbC in both media were almost equal (Figure 5).

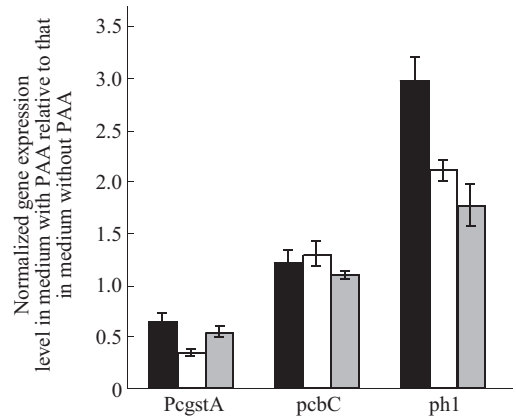

Fig. 5 Relative expression level of PcgstA in penicillin producing medium

TaqMan quantitative RT-PCR was performed using γ-actin gene as an internal control. All the gene expression levels in medium without PAA were normalized to 1 by $2^{-\Delta\Delta CT}$ method. Data are shown as $\bar{x} \pm s$ ($n = 4$). ■: 6 h; □: 12 h; ▩: 24 h.

In this study, we first cloned a *P. chrysogenum* GST gene and expressed it to prove its activity. We then address its activity in a system mimicking the

production process. We learnt that PcGSTA may be involved in phenylacetic acid metabolism since it is down regulated by PAA. Since increasing the utilization efficiency of PAA may reduce the cost of the penicillin fermentation process, investigations into this metabolic pathway is a major objective in the improvement of industrial strains. Although the role of PcgstA in the metabolism of PAA is still unclear, the cloning of PcgstA gives us a chance to study the relationship of GSH and penicillin biosynthesis using genetic methods.

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产黄青霉谷胱甘肽 S-转移酶基因 PcgstA 的克隆与鉴定 *

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摘要 从青霉素工业生产菌产黄青霉 (*Penicillium chrysogenum*) 中首次克隆了一个谷胱甘肽 S- 转移酶（GST）基因，定名为 PcgstA. 该基因的开放阅读框长 840 bp，含有两个内含子,编码一个 238 氨基酸残基的蛋白质. 其推断的氨基酸序列与一些已经鉴定的丝状真菌 GST 具有 50%左右的序列一致性. PcgstA 的完整编码区经 RT-PCR 扩增、验证，插入原核表达载体 pET11a，转化大肠杆菌 BL21(DE3)-RP 菌株，表达得到重组 PcGSTA 蛋白. 酶活测定证实，重组 PcGSTA 具有 GST 活性，其对底物 CDNB(1-chloro-2, 4-dinitrobenzene)的比活为(0.159 ± 0.031) μmol/(min·mg). 利用 TaqMan 探针法，对 PcgstA 的表达情况进行了比较. 结果表明，在添加了侧链前体苯乙酸的青霉素生产培养基中，PcgstA 的表达水平和在不含苯乙酸培养基中的表达相比明显下调，显示了该基因与苯乙酸代谢的关系.

关键词 产黄青霉，谷胱甘肽 S- 转移酶，苯乙酸
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