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Functional classification and biochemical characterization of a novel rho class glutathione *S*-transferase in *Synechocystis* PCC 6803

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ABSTRACT

We report a novel class of glutathione *S*-transferase (GST) from the model cyanobacterium *Synechocystis* PCC 6803 (sll1545) which catalyzes the detoxification of the water pollutant dichloroacetate and also shows strong glutathione-dependent peroxidase activity representing the classical activities of zeta and theta/alpha class respectively. Interestingly, sll1545 has very low sequence and structural similarity with these classes. This is the first report of dichloroacetate degradation activity by any bacterial GST. Based on these results we classify sll1545 to a novel GST class, rho. The present data also indicate potential biotechnological and industrial applications of cyanobacterial GST in dichloroacetate-polluted areas.

1. Introduction

Dichloroacetate (DCA), a by-product of water chlorination and a metabolite of drug and industrial chemicals is known to cause nerve and liver damage [1–3]. It is a xenobiotic of interest to both environmental toxicologists and clinicians [3–5]. Its accumulation in groundwater and at certain Superfund sites is considered a potential health hazard. It has been shown that the chronic administration of DCA in mice induces hepatotoxicity and neoplasia [6]. In contrast, DCA stimulates the activity of pyruvate dehydrogenase enzyme complex of mitochondria, resulting in increased oxidation of blood glucose and lactate and thus can help in treatment of lactic acidosis [7–10]. A low dose of DCA is highly effective for treatment of congenital lactic acidosis [7]. Recent researches suggest that it may also enhance cellular energy metabolism and can help in cancer treatment [11,12]. Though human clinical studies showed that the elimination half-life of DCA increases with repeated doses of DCA. At higher concentrations in water bodies it can cause liver damage and tumors [6]. Hence detoxification of DCA containing wastewater is essential before its release [6].

Glutathione *S*-transferases (GSTs E.C. 2.5.1.18) are isozymes having central roles in the cellular detoxication of a diverse group of exogenous and endogenous harmful compounds [13]. GSTs catalyze the initial step in the formation of mercapturic acid derivatives of a wide range of foreign compounds [14]. They conjugate reduced glutathione (GSH) with compounds that contain an electrophilic center through the formation of a thioether bond between the sulphur atom of GSH and the substrate .In addition, these enzymes also carryout a range of other functions like nucleophilic aromatic substitution reactions, reversible Michael additions to α, β-unsaturated aldehydes and ketones, isomerizations, epoxide ring openings, and peroxidase reactions [13,15–17]. Despite low amino acid sequence identity, the global fold of GSTs is remarkably similar. Most cytosolic GSTs are dimeric and consists of two structurally distinct domains – the N-terminal thioredoxin like domain and a larger C-terminal all α-helical domain. The N-terminal domain contains the GSH binding site while the C-terminal domain is involved in substrate recognition. In some eukaryotes monomeric GSTs are also reported. GSTs have been divided into a number of classes – alpha, mu, pi, theta, sigma, beta, chi, omega, zeta,

Abbreviations: GST, glutathione *S*-transferase; GSH, reduced glutathione; DCA, dichloroacetate; CDNB, 1-chloro-2,4-dinitrobenzene; GSTZ, glutathione *S*-transferase zeta

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etc. – that is based on amino acid sequence similarity, immunological, kinetic, and tertiary/quaternary structural properties. Cytosolic GSTs within a class share >40% identity whereas there is only approximately 25% identity between classes [18].

It has been reported that zeta class GSTs are dimeric and can degrade DCA [19,20]. GSTZ are widely distributed in organisms including plants, fungi and eukaryotes [21–23]. Few structures of zeta class GSTs have been resolved by using X-ray crystallography [24,25]. Although these structures have the GST canonical fold, a number of structurally important differences exist. Presence of high concentrations of reduced glutathione suggests the occurrence as well as importance of GSH utilizing enzymes in cyanobacteria [26–29]. GSTs of Cyanobacteria have not been characterized in detail. We studied DCA degradation ability of a GST of the cyanobacterium *Synechocystis* PCC 6803. Database suggests that *Synechocystis* PCC 6803 encodes at least 3 GSTs. sll1545 is a *Synechocystis* GST that cannot be assigned to any class on the basis of sequence similarity. It shows only 21% sequence identity with zeta class and with alpha/theta classes of GSTs. Also most zeta class GSTs are ~25 kDa protein, while sll1545 is encoded by 816 nts and is of about 30 kDa. In contrast to all previously reported dimeric bacterial GSTs, the recombinant sll1545 was found to be a monomeric protein of 30 kDa. Molecular modeling studies showed major differences between sll1545 and zeta class GSTs. Therefore in order to assign sll1545 its correct hierarchal position in GST superfamily we cloned sll1545 from *Synechocystis* PCC 6803, inserted into a His-tagged *Escherichia coli* prokaryotic expression system and studied its biochemical nature. The monomeric 30 kDa protein showed high specific activity and affinity for DCA as a substrate. Additionally, sll1545 shows peroxidase activity that is a signature of theta and alpha class of GSTs. Though, the structural and sequence similarity of sll1545 with these classes is very less. On the basis of these results we propose a novel rho class GST in *Synechocystis* PCC 6803 with potential for detoxification of DCA contaminated wastewater.

2. Materials

The molecular biology kits and Ni–NTA agarose were purchased from Qiagen, CA, USA. The dNTPs and enzymes were purchased from New England Biolabs, MA, USA. All other reagents and chemicals were purchased either from Sigma–Aldrich Chemical Company, St. Louis, MO, USA, or Sisco Research Laboratories, Mumbai, India and were of the highest purity available. Bacterial culture media was purchased from Himedia Laboratories, Mumbai, India.

2.1. Construction of a prokaryotic expression plasmid

The *Synechocystis* PCC 6803 was cultured in BG-11 medium. The genomic DNA was isolated using DNA isolation kit (Qiagen, USA). sll1545 gene was amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs, UK) using 5′-CGGGATCCATGCTTGAGCTT-3′ and 5′-AACTGCAGCTACTCAATGGTG-3′ as forward and reverse primers respectively. The restriction site consists of BamHI and PstI for the forward and reverse primers respectively. The PCR involved 30 cycles of denaturation at 98 °C for 20 s, annealing at 66 °C for 15 s followed by elongation at 72 °C for 15 s. This PCR product was digested with EcoRV and cloned into already EcoRV digested and purified pSK+ vector. The clone was verified by sequencing. After sequencing, the correctly cloned plasmid and pQE30 vector were both digested by BamHI and PstI restriction enzymes. The sll1545 gene fragment and the linear plasmid were recycled after agarose electrophoresis; connected by T4 DNA ligase to construct the recombinant expression plasmid pQE30-sll1545. The plasmid was transformed into *E. coli* DH5α competent cells and positive clones were screened. The correct pQE30-sll1545 clone was transformed into M15 competent cells for protein expression.

2.2. Induction of expression and purification of recombinant protein

Recombinant sll1545 was overexpressed in *E. coli* M15 cells and purified as follows. Single colony from transformed plates was inoculated in 5 mL of LB broth containing 100 μg/mL ampicillin and 50 μg/mL kanamycin. Cells were grown for 4–5 h at 37 °C with continuous shaking at 160 rpm. Next day a 400 mL LB broth flask containing above–mentioned antibiotics was inoculated with 1% (v/v) of overnight grown culture and incubated at 37 °C with shaking. Culture was grown until the OD_{600} reached 0.5–0.6. At this stage culture was induced with 1 mM IPTG. The culture was grown overnight at 23 °C. Next day culture was harvested and pelleted by centrifugation at 7000 rpm for 10 min at 4 °C. The pellet was then suspended in 1/50th culture volume of lysis buffer. The dissolved cells were lysed by sonication with pulse–rest cycle (60 cycles; 20 s pulse at 40% amplitude with 10 s interval after each pulse). The lysate was centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatant was collected. All further steps were performed at 4 °C temperature. The supernatant was poured on Ni–NTA agarose matrix (3 mL) pre-treated with equilibration buffer (50 mM phosphate buffer pH 8.0 containing 300 mM NaCl) and was allowed to bind slowly. Non-specifically bound and contaminating proteins were removed by washing with equilibration buffer containing 50 mM imidazole. Recombinant protein was eluted with 10 mL of elution buffer (equilibration buffer containing 400 mM imidazole). The protein was dialyzed against 20 mM phosphate buffer pH 8.0 containing 150 mM NaCl, Protein concentration was determined by Bradford method using BSA as a standard.

2.3. Size exclusion chromatography

The determination of the native molecular weight of sll1545 was performed by size exclusion chromatography on a Superdex™ S-200 column (GE Healthcare Biosciences, USA). The calibration curve was made using the logarithm of the molecular mass of standard proteins vs. the elution volumes for conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa) (Gel Filtration Calibration Kit, GE Healthcare Biosciences).

2.4. Enzymatic activities

GST activity using GSH and CDNB as substrates was determined spectrophotometrically at 340 nm on the basis of the extinction coefficient for the product S–(2,4–dinitrophenyl) glutathione (ε_{340} nm = 9.6 mM^{-1} cm^{-1}). The assay mixture (1 mL) comprisedsll1545 enzyme and 1 mM GSH in 100 mM phosphate buffer pH 8.0 containing 150 mM NaCl. The reaction was started by addition of 0.5 mM CDNB and reading was recorded for 60 s. During this period, the rate of reaction was linear with time. One unit of GST activity was defined as the conjugation of 1 μmol of CDNB with GSH per minute at 25 °C. The data was recorded with a Cary 50 Bio UV–Visible spectrophotometer at 25 °C.

The spectrometric method of Vogels and Van Der Drift was used to quantify glyoxylic acid formation using DCA as a substrate [30]. One mL of assay mixture contained 100 mM phosphate buffer (pH 7.4), 0.5 mM DCA and 1 mM GSH. Reaction was started by addition of DCA and 50 μL of trifluoroacetic acid was added to stop the reaction after 20 min of incubation at 37 °C. The reaction mixtures were placed on ice for 10 min and then centrifuged for 5 min to remove precipitated proteins. 850 μL of supernatant was transferred into a clean 5 mL microfuge tube and neutralized with 0.5 mL of 1 M