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New food for an old mouth: New enzyme for an ancient archaea

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ABSTRACT

As a multifunctional group of enzymes, glutathione *S*-transferases (GSTs) are capable of inactivation, degradation or excretion of wide range of compounds catalytically or non-catalytically. However, to date, no study has been addresses the presence of GSTs in archaea based on their enzymatic functions. In this study, beside glutathione (GSH) amount measurement, the determination of GST activity in halophilic archaeon called *Haloarcula hispanica* ATCC 33960 were aimed. According to the results, specific activity was determined as 19.68 nmol min^{-1} mg^{-1} protein and GSH content were found to be as 194 μg g^{-1} K_m and V_{max} values for CDNB and GSH calculated from Lineweaver-Burk plot were 0.46 mM and 27.93 nmol min^{-1} mg^{-1}, 0.13 mM and 22.03 nmol min^{-1} mg^{-1}, respectively. Hanes-Woolf and Eadie-Hofstee plots for CDNB and GSH were also found to be in co-relation with the results obtained from Lineweaver-Burk plot. To the best of our knowledge, GST enzymes have not been identified in archaea yet, at least based on their catalytic activities. Therefore, it is the first report on this area.

1. Introduction

In the late 70s, the archaea were first classified as a separate domain in the three-domain system by Woese and co-worker [1]. The word *archaea* comes from the Ancient Greek, meaning 'ancient things' [2]. Recently, scientists reveal that archaeans are not restricted to harsh environments like hot springs, cold habitats and extremely saline, alkaline or acid water, but are also present in soils, oceans and marshlands [3]. As a major part of global ecosystems, they may contribute up to 20% of earth's biomass [4]. According to the recent publication of Allocati et al. [5], archaea domain comprises three main phyla, namely *Euryarchaeota*, *Crenarchaeota* and *Thaumarchaeota*. However, there are other groups which have been tentatively hypothesized, such as Nanoarchaeota [6] and Korarchaeota [7]. Today, the phylogeny of archaeans is based on a single ribosomal RNA (rRNA) sequences for the hypothesized groups, which is indicative for the ambiguity of diversity in archaeans and also for its classification [8].

Haloarchaea (halophilic archaea) belong to the halophile community and live in extremely saline environments. Their requirement for salt concentration range about 10–37% of salinities for optimal growth in hypersaline lakes and solar salterns, where they are mainly found [9]. They are classified within the family *Halobacteriaceae* of the phylum *Euryarchaeota*. The family *Halobacteriaceae* currently possesses about 36 recognized genera [10], including genus *Haloarcula* which are found in salt lakes, marine salterns and saline soils. Similar to other *Halobacteriaceae* members, most of the *Haloarcula* strains grow best at 2.5–3.5 M NaCl [11]. In the *Haloarcula* genus, nine species were recognized (as of August 2011) those are namely *H. amylolytica*, *H. argentinensis*, *Haloarcula hispanica*, *H. japonica*, *H. marismortui*, *H. quadrata*, *H. salaria*, *H. tradensis* and *H. vallismortis* [12].

H. hispanica is first introduced in 1986 by Juez and co-workers [13]. However, the complete genome sequence of *H. hispanica* strain ATCC33960 was reported very recently by Liu et al. [14], as a representative of this species. The complete genome sequence was found to be 3890,005 bp, consisting of two chromosomes and one megaplasmid: a main chromosome I (2,995,271 bp), a minichromosome II (488,918 bp) and megaplasmid pHH400 (405,816 bp) (GenBank accession nos. CP002921, CP002922, and CP002923, respectively).

Similar to halophilic bacteria, halophilic archaea can have potential biotechnologic applications. Such as, increasing salt production in salt-crystallization lakes [15], usage of *Haloferax* and *Haloarcula* sp. for viscosity stabilization [16], producing poly-β-hydroxyalkanoate (PHA) for recyclable plastics [17] and the usage of ether-binding lipids of halophilic archaea to make liposomes for medicine and cosmetics. Although, the list can be extended by usage of a protein in *H. salinarum* as an antigen to detect certain cancer types [18], bioremediation of the petrol contaminants by destruction of hydrocarbons [19] and usage of non-toxic gas vesicles of *H. salinarum* NRC-1 strain to develop a new system for the epitop re-arrangement [20]. In addition, controlling the bacterial decomposition in salted foods and tanned leather by halocins

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[15] and usage of a new class of antibiotics, called archaeocins with novel modes of action [21] could also be counted for the different applications of halophilic archaea. On the other hand, some of the applications are dependent on enyzmatic activities, as enzymes from these archaea tend to be very stable in organic solvents that allow their use in green chemistry [22]. However, the exoenzymes (amylase, amyloglucosidase, protease and lipase) produced by archaea can be used in biological processes during the destruction of macromolecules in high salt concentrations [16].

As a multifunctional group of enzymes, glutathione *S*-transferases (GSTs; EC 2.5.1.18) are also capable of inactivation, degradation or excretion of both endogenous and exogenous electrophilic compounds through their ability to catalyze the addition of glutathione (GSH) [23]. GSTs are widely distributed from prokaryotes to eukaryotes and classified mainly based on their sequence analysis [24]. However, beside their catalytic actions, these group of enzymes are also non-catalytically acting as ligandins for their substrates [25]. In the literature, very limited information is available about the classification and occurrence of GSTs in archaea. However, currently, there are few genome profiling and phylogenetic relationships studies available. In which, the advantage of the availability of fully-sequenced genomes of archaea were recruited to assign the putative GSTs into the various classes that belong to the GST superfamily or identification of newly hypothesized genes coding for GSTs. Allocati et al. [5] have shown that several putative GSTs are present in archaea belonging to the *Euryarchaeota* and the *Crenarchaeota* phyla very recently. According to their results, majority of the putative archaeal GSTs (91% of all sequences found in the archaea) can be ascribed to the new Xi class, which is proposed by Meux and co-workers [26]. On the other hand, to date, no study has addressed the presence of GSTs in the domain of archaea based on their enzymatic functions [27,28]. Because, it is believed that the absence of these enzymes in archaea is consistent with the lack of GSH [5,29], which is known as a nonessential molecule of life. This thiol tripeptide (γ-L-glutamyl-L-cysteinyl-glycine) is synthesized in two ATP-consuming steps from the constituent amino acids by γ-glutamyl-cysteine synthetase (γ-GCS, EC 6.3.2.2) and GSH synthetase (GS, EC 6.3.2.3) [30].

This study was designed for the determination of GSH amount and optimization of GST activity in halophilic archaeon called *H. hispanica* ATCC 33960. In the presence of the universal GST substrate 1-chloro-2,4-dinitrobenzene (CDNB), optimized activity conditions were detected for several parameters. Based on their catalytic activities, GST enzymes have not been identified in archaea yet. Therefore, to the best of our knowledge, it is the first report on this area.

2. Materials and methods

2.1. Materials

1-Chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), sodium carbonate, copper sulfate, sodium hydroxide, potassium-sodium tartrate tetrahydrate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, hydrochloric acid, ethanol, bovine serum albumin (BSA), disodium hydrogen phosphate, sodium hydrogen phosphate, sodium acetate, acetic acid, Tris base, Ciocalteu's Folin phenol reagent, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and trichloroacetic acid (TCA) were purchased from Sigma (St. Louis, USA). Potassium hydroxide, ammonium sulfate, magnesium sulfate heptahydrate, sodium hydroxide, yeast extract, agar agar, magnesium chloride hexahydrate, sodium bromide, sodium bicarbonate, calcium chloride and sodium chloride were bought from Merck (Darmstadt, Germany). All other chemicals were analytical grade and were obtained from commercial sources at the highest purity available.

2.2. Archaea material and growth conditions

In this study a microorganism which belongs to archaea domain was used as test organism. Halophilic archaeon called *H. hispanica* strain ATCC 33960 were acquired from M. Dyall-Smith (Charles Sturt University, Australia) and they are already deposited into the departmental culture collection.

The cells of *H. hispanica* ATCC 33960 were inoculated in sea water (SW) agar medium and incubated for one week at 37 °C. Four loopful of archaeal growth from the culture was transferred into 100 ml of sea water liquid medium and then incubated for one week at 37 °C by 150 rpm shaking to multiply them to use as protein, glutathione and enzyme sources. The stock cultures maintained on sea water agar plates were stored at 4 °C for further studies.

2.3. Methods

2.3.1. Morphological studies

2.3.1.1. Staining of microorganisms by modified gram staining. Gram staining was performed according to Dussault method [31]. The cells grown in liquid medium for 7 days were used for staining. They were fixed for 5 min with 2% (v/v) acetic acid, then stained for 3 min with 0.25% (v/v) crystal violet and treated for 1 min with lugol's iodine. After decolorization for 10–15 s with 95% ethanol, they were stained for 30 s with safranin. Dried preparates were scanned by using immersion objective under light microscope. Intermediate washings were conducted with 25% NaCl solution.

2.3.2. Biochemical studies

2.3.2.1. Glutathione (GSH) determination. The cells grown in liquid medium for 7 days were homogenized in a ratio of 1:4 (w/v), with 5% (w/v) TCA by using UltraTurrax at 13,500 rpm for 90 s at 4 °C. The homogenate was centrifuged at 4 °C, 17,400 × *g* for 15 min and the pH of the supernatant was adjusted to 4.0–5.0 with 1 M NaOH. The content of GSH in crude extract was determined using the Ellmann (DTNB) procedure [32], in which DTNB is reduced by SH groups to form 1 mol of 2-nitro-5-mercaptobenzoic acid per mol of SH. The nitromercaptobenzoic acid anion has an intense yellow color that can be used to quantify SH groups by measurement at its maximum absorbance at 412 nm.

The reaction mixture comprised the sample (0.1 ml), 100 mM pH 8.4 Tris–HCl buffer (2 ml) and Ellmann reagent (0.1 ml; 60 mg/100 ml Tris–HCl buffer 0.1 M, pH 7.0). The absorbance of the reaction mixture was read at 412 nm. The GSH concentration in the samples was calculated from the standard curve calibrated by using reduced GSH. Data are expressed as $\mu g\, g^{-1}$ fresh weight of tissues.

2.3.2.2. Preparation of cytosolic extract. The freeze–thaw method of Koval and Sprott [33] was used for enzyme extraction. After a week of growth period (on 7th day), the liquid medium cultures were centrifuged at 12,100 × *g* for 15 min at 4 °C. The supernatant was discarded and the pellet was suspended with cold ultrapure water in a ratio of 1:2 (w/v) and suspended cells were frozen for 1 day at −80 °C. At the end of the time, the cells were first thawed at 4 °C, then centrifuged at 12,100 × *g* for 15 min at 4 °C. The supernatant fraction (referred to as crude extract) was immediately subjected to enzyme activity measurement without further storage; otherwise, it was stored at −80 °C as aliquots (1 ml) for later protein assays.

2.3.2.3. Soluble protein determination. The soluble protein content of the crude extracts was determined by the method of Lowry et al. [34]. In this method, BSA was used as a protein standard. The protein