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# Cloning, Expression, Purification and Characterization of Membrane Protein Di-geranylgeranylglyceryl Phosphate Synthase from Archaea

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**Abstract:** The core structure of membrane lipids of archaea have some unique properties that permit archaea to be distinguished from the others, i.e., bacteria and eukaryotes. Di-geranylgeranylglyceryl phosphate synthase (DGGGPS), which catalyzes the transfer of a geranylgeranyl group from geranylgeranyl diphosphate to geranylgeranylglyceryl phosphate, is involved in the biosynthesis of archaeal membrane lipids. Our laboratory already cloned, expressed and purified the geranylgeranylglyceryl phosphate synthase (GGGPS) from *Thermoplasma acidophilum* and reported. The second enzyme in the archaeal membrane lipid biosynthesis DGGGPS has not been cloned and purified from archaea yet. In this work, we cloned the gene encoding DGGGPS after PCR amplification of the gene from genomic library of *Thermoplasma acidophilum*. The cloned genes were subcloned into a high-expression vector and expressed in the cell of *E. coli* C41(*DE*3). The membrane protein was solubilized by 2% n-Octyl- β-glucopyranoside. Then the protein was then purified by heat treatment, DEAE-sepharose and Resource Q column chromatography. The protein gave single band on SDS-PAGE analysis. The molecular mass of 31 KDa was obtained by SDS-PAGE which is full agreement with the DNA sequence. The optimum temperature and pH of the purified protein was 65°C and pH 7.0, respectively.

**Key words:** Arechaea, Di-geranylgeranylglyceryl phosphate synthase, *Thermoplasma acidophilum*

## Introduction

The structures of membrane lipids have some interesting properties that enable us to distinguish archaea from other organisms, i.e., eukaryotes and bacteria. Although the archaeal dither membrane lipids are homologues of glycerolipids in other organisms, they differ with respect to the following features: 1) the hydrocarbon moieties of the archaeal lipids are fully reduced C20 or C25 prenyl groups, whereas the ordinary glycerolipids contain linear acyl groups. 2) The alkyl groups are attachéd to glycerol via an ether bond in archaeal lipids, while glycerol and the acyl chains are ester-bonded in the bacterial and eukaryotic glycerollipids. 3) The two groups of membrane lipids have opposite chiralities at their glycerol moieties. Morover, the existence of circular tetraether lipids, which are synthesized from two molecules of diether lipids in methanogenic and thermophilic archaea, emphasizes the uniqueness of the archaeal membrane lipids.

Ether bond formation proceeds in two steps: GGGP synthase catalyzes the reaction forming GGGP from G-1-P and geranylgeranyl pyrophosphate (GGPP)and then, digeranylgeranylglyceryl phosphate (DGGGP) synthase catalyzes the reaction forming DGGGP from GGGP and GGPP. The cytosolic fraction contains the GGGP synthase activity, while the membrane fraction contains the DGGGP synthase activity (Nemoto *et al*., 2003). The biosynthesis of the core structure of archaeal membrane lipids has been studied to date. The genes of geranylgeranyl diphosphate (GGPP) synthase (GGPS), which catalyzes the production of the general precursor of the alkyl moieties of ordinary

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archaeal lipids, have been cloned from the thermophilic archaea (Wang *et al*., 1999) and homologues have been identified in the genomes of various archaea. Nishihara and Koga (1997) reported that the formation of sn-glycerol-1-phosphate (G-1-P), the precursor of the glycerol moiety, from dihydroxy acetone phosphate, is catalyzed by G-1-P dehydrogenase in *M. thermoautotrophicum*. Zhang and Poulter (1993) and Chen *et al*. (1993) showed that the formation of ether linkages between G-1-P and two molecules of GGPP in *M. thermoautotrophicum* are catalyzed by distinct prenyltransferases. The 1st step, yielding GGGPS enzyme was characterized in detail by Zhang and Poulter (1993) and the GGGPS genes were cloned from *Thermoplasma* sp. The 2nd step that involves the production of DGGGP from GGGP and GGPP is catalyzed by a microsomal enzyme, DGGGPS, although further information concerning this prenyltransferase has not been obtained at this time. As far our knowledge, this is the first report of cloning, expression and purification of DGGGPS from *Thermoplasma acidophilum*. So, in this study, we cloned the gene encoding DGGGPS from genomic library of *Thermoplasma acidphilum*. The cloned genes were expressed in the cell of *E. coli* C41(*DE*3)and the recombinantly expressed enzymes were shown to specifically catalyze the formation of DGGGP from GGGP and GGPP. Furthermore we also purified and characterized the DGGGPS from the recombinant strain.

## Materials and Methods

Radioisotope GGPP triammonium salt was purchased from NEN Life Science Products. Unlabel GGPP was purchased from Sigma. Sn-G-1, 3-P disodium salt hexahydrate were purchased from Sigma. Alkaline phosphatase (*E. coli*) was purchased from Takara and precoated reversed-phase TLC plates, LKC-18F was purchased from Whatman Chemical Separation, Inc. DEAE-Sepharose was obtained from Pharmacia Biotech. All other chemicals were of analytical grade.

*Isolation of and TA Genes*

The *TA* gene was amplified by means of a PCR by using primers specific to the 5- and 3-ends; 5-ACGTCATATGGGGGTTTTATGGAGAAGTTA-3 and 3-TAGATAGGATCCTTATAGTTTTATGGCTCCAACAACAATAAAT-5, respectively. The genome of *Thermoplasm acdophilum*, as a templateand DNA polymerase were used in the reaction. The restriction sites introduced into the primers, the *Nde*I and *Bam*HI sites are indicated with underlines. The amplified fragment, extracted from 0.7% agarose gel after electrophoresis, was digested with *Nde*I and *Bam*HI and then ligated into the *Nde*I-*Bam*HI sites of the pET-21c to construct plasmid.

*Expression and Purification of the Recombinant Enzymes*

*E. coli* C41 (DE3) transformed with each of the plasmid was cultivated in 1 L of M9YG broth supplemented with ampicillin. When the A600 of the culture reached 0.7, the transformed bacteria were induced with 1 mM IPTG. After an additional 5 h cultivation, the cells were harvested and disrupted by sonication buffer. n-Octyl-β-glucopyranoside at a final concentration of 2% was added to the crude extract to solubilize the recombinant protein before the heat treatment. The supernatant after heat treatment was applied to DEAE-sepharose column which was equilibrated with 20 mM Hepes buffer pH 7.0 containing 1% n-Octyl-β-glucopyranoside. The DGGGPS protein was adsorbed in the column and eluted at 0.3 N NaCl gradient containing the same buffer. The active fraction from DEAE-sepharose column were dialyzed against the same buffer and applied to Resource column at pH 7.0 which was equilibrated with the same buffer. The active fraction was eluted at 0.3M NaCl gradient. The active fraction from resource Q pH 7.0 was applied to Resource Q column again at pH 8.0 with the same buffer. The DGGGPS protein was eluted at 0.1% NaCl concentration with 20 mM Hepes

buffer pH 8.0. and 1% n-octyl-β-glucopyranoside. The level of purification was determined by 12% SDS-PAGE. Proteins bands were stained with Coomassie Brilliant Blue R-250. Protein concentration was determined with a BCA protein assay reagent (Pierce) using bovine serum albumin as standard.

*Enzyme Assay and Product Analysis*

The reaction mixture contained G-1-P, radio labeled GGPP and GGGP synthase was incubated 55°C for 20 min to produce radio labeled GGGP, which is the substrate for the next reaction. Then unlabeled GGPP and DGGGP synthase was added and incubated 55°C for 10 min. The DGGGP product formed was incubated at 37°C for 1 h with alkaline phosphatase. Labeled products were extracted from the mixture according to the method of Bligh and Dyer (1959), that is 300 μL of methanol and 150 μL of chloroform were added and the reaction mixture was agited vigorously on a voltex mixer. The 150 μL of chloroform and 150 μL distilled water were added to the suspension and the suspension was centrifuged. The lower chloroform phase was collected and dissolved in a small amount of chloroform:methanol (2:1) and analyzed by TLC. Acetone-water (9:1) was used for TLC development. Radioactive spots of the products on the TLC plate were detected by autoradiography with X-ray film with ENHANCE (NEN Life Science).

## Results

*Expression of DGGGPS Enzyme*

Primers were designed from ORF *TA 0996*. PCR was done using these primers and the *Thermoplasma acidophilum* genome DNA as the template. A DNA fragment with the expected length (about 900 bp) was amplified in Fig. 1 and cloned in a pet 21c vector. Sequence analysis of the cloned fragment confirmed the full ORF *TA* (*0996*) in the vector. The recombinant DGGGP synthase was expressed in the C41 (*DE3*) cells harboring the plasmid. The *E. coli* cells harboring the plasmid were grown until mid-log phase and the gene expression was induced by IPTG.

*Purification of DGGGPS enzymes*

The purification of DGGGPS enzymes were described details in materials and methods section. The heat treatment experiment was done at 80°C for 25 min which removed many contaminant

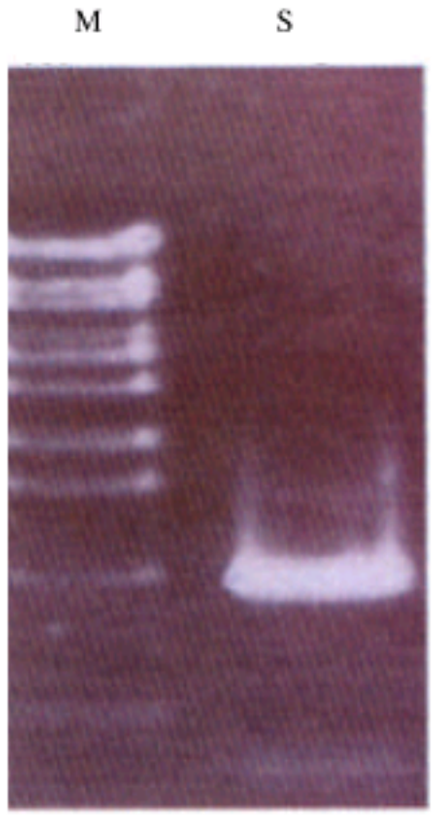


Fig. 1: The amplification of gene of *Thermoplasma acidophilum* by PCR. M: Marker, S: Sample

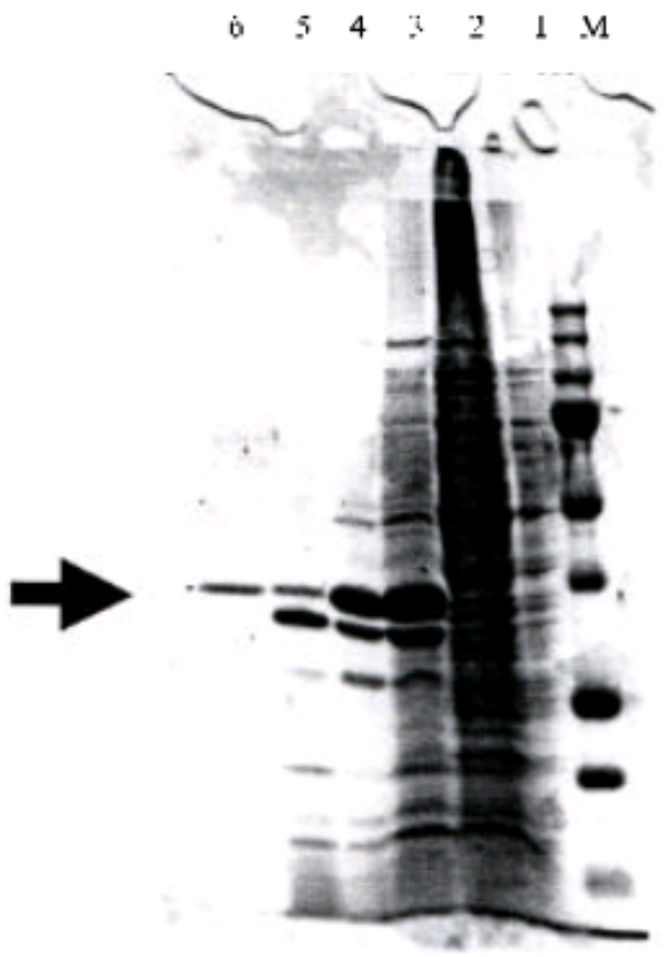


Fig. 2: SDS-PAGE analysis of purified DGGGPS from *Thermoplasm* sp. Lane M: Protein standard marker; 1: Crud extract; 2: Sonication sample; 3: Sample before heat treatment at 80°C; 4: Sample after heat treatment; 5: Sample after DEAE sepharose; 6: Sample after resource Q. Arrow indicates the target band

Table 1: The purification of DGGGPS from *Thermoplasma acidophilum*

| Steps | Volume (mL) | Protein (mg) | Activity (U) | Recovery (%) | Species activity | Puri. fold |
|---|---|---|---|---|---|---|
| Extracts | 30 | 1200.0 | 500 | 100.0 | 0.416 | 1.00 |
| Heat treatment | 15 | 218.0 | 300 | 60.0 | 1.37 | 3.29 |
| DEAE | 10 | 26.0 | 250 | 50.0 | 9.61 | 23.10 |
| Resource Q pH 7.0 | 4 | 14.0 | 72 | 14.4 | 5.14 | 12.30 |
| Resource Q pH 8.0 | 3 | 5.3 | 48 | 9.6 | 9.05 | 21.70 |

proteins. After heat treatment experiment, the supernant was applied to DEAE-sepharose column, resource column at different pH described above. Figure 2 shows the SDS-PAGE analysis of different steps of purification of DGGGPS from *Thermoplasma acidophilum*. The SDS-PAGE showed the single band of molecular mass of 31 kDa DGGGPS protein (Fig. 2), which means the homogeneity of the protein. The purification process is summarized in Table 1. Table 1 describes the purification process of DGGGPS protein in three steps; heat treatment, DEAE-Sepharose and Resource Q chromatographies. The amount of protein from 5 L culture was 1200 mg, activity 500 U and specific activity 0.416 U $mg^{-1}$. In the heat treatment experiment major contaminants protein was removed. After DEAE-Sepharose column chromatography, the specific activity was increased 9.61 and recovery is 50%. The specific activity of final step of purification was 9.05 and purification fold is 21.7.

## Discussion

In this study we cloned the gene encoding DGGGPS from genomic DNAs of *Thermoplasma* sp. and expressed the gene in the cells of *E. coli* C14 (DE3). For solubilization of membrane protein

requires detergents for extraction from the membrane. They solubilize proteins by binding to the hydrophobic parts of the proteins on one side and interacting with the aqueous phase on the other side. The expressed membrane proteins was solubilized by n-Octyl-β-glucopyranoside. Actually we tried with different detergents such as Chaps, Nonidet P-40, SDS, Deoxycholateand Triton X-100 but we succeeded to solubilize the protein only using detergents n-Octyl-β-glucopyranoside, which enabled us to purify the protein. The DGGGPS from *Thermoplasma* sp. was then purified by heat treatment, DEAE-Sepharose and Resource Q column chromatography. Recently, the DGGGPS has been cloned and purified from another archaea *Sulfolobus solfataricus* (Hemmi *et al*., 2004). Archaea and the other organisms can be uniquely distinguished not only by taxonomy based on rRNA sequences but also by the structures of their membrane lipids. This fact led us to the hypothesis that archaea were generated from the common ancestor of all living organisms at the same period in which they acquired a membrane formed by ether-linked lipids. The long evolutional history of DGGGPS might be supported by the early divergences observed among enzymes included in the DGGGPS cluster. Therefore, we can even imagine a scenario in which the archaeal enzyme played the role as the evolutional origin of all enzymes in the family, although the possibility of lateral transfer of prenyltransferase genes from other organisms to archaea cannot be ignored.

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