

Molecular cloning and Characterization of a membrane-intrinsic (S)-2,3-di-O-digeranylgeranyl glyceryl phosphate synthase involved in the biosynthesis of archaeal ether-linked membrane lipids.

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Abstract

The second step in the biosynthesis of the core membrane diether lipids in archaea is synthesis of digeranylgeranyl glyceryl phosphate from geranylgeranyl glyceryl phosphate and geranylgeranyl pyrophosphate. The reaction is catalyzed by (S)-2,3-di-O-geranylgeranyl glyceryl phosphate synthase (DGGGP synthase). The gene encoding the DGGGP synthase was cloned from *Methanocaldococcus jannaschii* (MJ 0279) and expressed in the cells of *Escherichia coli* C41 (DE3). The membrane protein was then solubilized by 2% n-Octyl- β -D-glucopyranoside and purified to homogeneity by a combination of heat treatment, DEAE-Sepharose, Resource Q and Hydroxyapatite column chromatography. The native polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis of purified DGGGPS gave a single band at 30 kDa. The optimum temperature and pH of the purified enzyme was 70°C and pH 6.0, respectively. The enzyme requires Mg²⁺ for optimal activity, while EDTA inhibits the activity. Other characteristics, including substrate specificity, salt effects and detergents effects were determined.

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1. Introduction

The core structures of membrane lipids of archaea have some unique properties that permit archaea to be distinguished from the others, i.e., bacteria and eukaryotes. The structures of archaeal membrane lipids were analyzed and determined in methanogens, halophiles, and thermophiles (Gambacorta et al, 1995). Investigation of the unique archaeal membrane-lipid biosynthesis may provide a clue to the early evolution of life. Koga et al, (1998) have reported that sn-glycerol-1-phosphate dehydrogenase, which determines the enantiomeric specificity of ether lipids, does not show similarity to the eubacterial enzyme, sn-glycerol-3-phosphate dehydrogenase, which is responsible for enantiomeric ester lipid synthesis. They suggested that G-1-P dehydrogenase and G-3-P dehydrogenase originated from different ancestral enzymes and this difference is responsible for the

divergent evolution of archaea and bacteria (Koga et al, 1998). The structures of membrane lipids have some interesting and remarkable properties and the archaeal diether membrane lipids are homologues of glycerolipids in other organisms but they differ. The hydrocarbon moieties of the archaeal lipids are fully reduced C₂₀ or C₂₅ prenyl groups, whereas the ordinary glycerolipids contain linear acyl groups. The alkyl groups are attached to glycerol via an ether bond in archaeal lipids, while glycerol and the acyl chains are ester-bonded in the bacterial and eukaryotic glycerolipids. The complete polar lipid composition of *Thermoplasma acidophilum* HO-62 was determined by Shimada et al, (2001). Kon et al, (2002) also described the ether biosynthesis pathway in the thermoacidophilic archaeon. Recently the biosynthetic pathway of ether lipids in archaea has been analyzed and postulated by several researchers (Koga and Morii, 2006; Koga and Morii, 2005; Nishihara and Koga 1995). Ether bond formation proceeds in two steps (Fig. 1): geranylgeranyl glyceryl phosphate (GGGP) synthase (GGGPS) catalyzes the reaction between glycerol-1-phosphate (G-1-P) and geranylgeranyl pyrophosphate (GGPP) forming GGGP (Chen et al, 1993;

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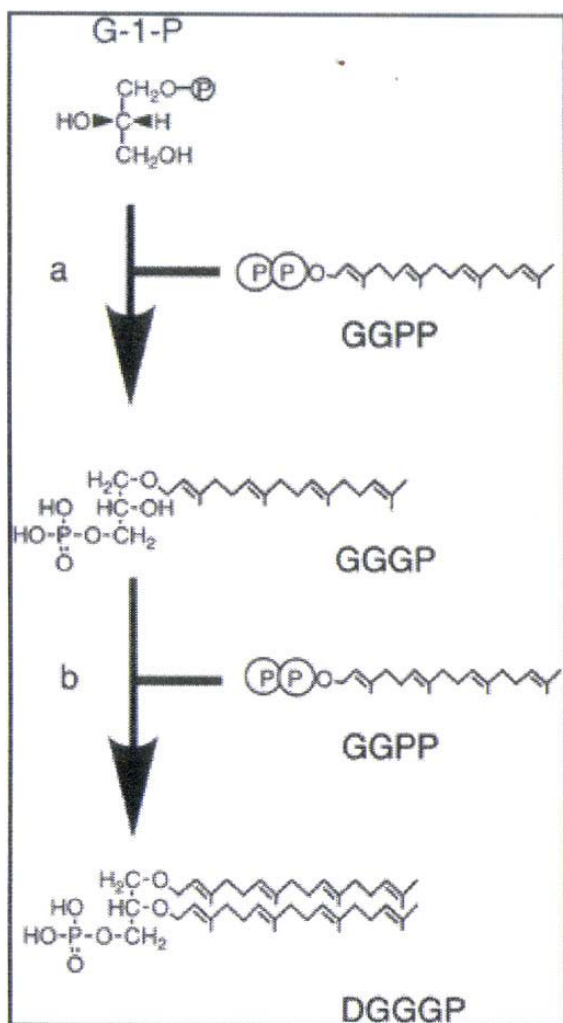


Fig. 1. Ether bond formation reaction between sn-glycerol-1-phosphate and geranylgeranyl pyrophosphate in Archaea. The reactions are catalyzed by (a) GGGP synthase and (b) DGGGP synthase.

Ohnuma et al, 1994), and then, (S)-2,3-di-O-geranylgeranylglycerol phosphate (DGGGP) synthase (DGGGP synthase) catalyzes the reaction between GGGP and GGPP forming DGGGP (Nemoto et al, 2003). In addition the cytosolic fraction contains the GGGP synthase activity, while the membrane fraction contains the DGGGP synthase activity (Ohnuma et al, 1994). Ether bond formation between geranylgeranyl diphosphate and sn-glycerol-1-phosphate in cell-free preparations from *Methanobacterium thermoautotrophicum* and *Halobacterium halobium* has been reported (Chen et al, 1993; Poulter and Zhang, 1993). The genes of geranylgeranyl diphosphate (GGPP) synthase (GGPS), which catalyzes the production of the precursor of the alkyl moieties of archaeal lipids GGPP, have been cloned from the thermophilic archaea *Sulfolobus acidocaldarius* (Wang et al, 1999) and *Archaeoglobus fulgidus* (Ohnuma et al, 1994; Nemoto et al, 2003) and homologues have been identified in the genomes of various archaea. The 1st step yielding GGGP, GGGPS was characterized in detail by Chen et al, (1993) and the GGGPS gene was cloned, and expressed in our laboratory from *Thermoplasma acidophilum* and reported (Yamagishi et al, 2003). The second step that involves the production of (DGGGP) from

GGGP and GGPP is catalyzed by an enzyme, DGGGP synthase, although further information concerning this prenyltransferase has not been obtained at this time. DGGGP, which has been shown to be the intermediate of archaeal membrane lipids as evidenced by an incorporation experiment (Eguchi et al, 2003), would be expected to subsequently undergo saturation of the alkyl group, modification of the polar head group and the formation of the cyclic tetraether structure (Kon et al, 2002). However, comprehensive information concerning these biosynthetic reactions is not available: the genes and proteins involved and even the order of the reactions are unknown at this time, except for some enzymes that catalyze the modification of polar head groups (Morii and Koga, 2003; Morii et al, 2000). Recently, the archaeal membrane lipid biosynthesis DGGGP synthase has been cloned and purified from *Sulfolobus solfataricus* (Hemmi et al, 2004). In this study, we cloned the gene encoding DGGGP synthase from genomic library of *Methanocaldococcus jannaschii*. The cloned genes were expressed in the cell of *Escherichia coli* C41 (DE3). The recombinantly expressed DGGGP synthase was purified and characterized and shown to specifically catalyze the formation of DGGGP from GGGP and GGPP.

2. Materials and Methods

2.1. Materials

Radio labeled GGPP triammonium salt was purchased from NEN Life Science Products (NEN Life science Products, Boston, Mass). Unlabeled Geranylgeranyl pyrophosphate (GGPP) ammonium salt, minimum 95% (TLC) was purchased from Sigma (Sigma, St. Louis, USA). Over Express TM host strains *Escherichia coli* C41(DE3) was purchased from Avidis (Avidis SA, Saint-Beuzire, France) Company Ltd. Sn-G-1, 3-P disodium salt hexahydrate and sn-G-3-P di (Monocyclohexylammonium) salt (approximately 95% purity) were purchased from Sigma (Sigma, St. Louis, USA). Alkaline phosphate (*Escherichia coli*) was purchased from Takara (Takara Bio Inc., Otsu, Shiga, Japan) and precoated reversed-phase TLC plates LKC-18F was purchased from Whatman Chemical Separation, Inc., (Whatman Inc., Clifton, New Jersey, USA). DEAE-sepharose was obtained from Pharmacia Biotech (Pharmacia Biotech, Uppsala, Sweden). All other chemicals were of analytical grade.

2.2. Microorganism and culture conditions

The genomic DNAs of *Methanocaldococcus jannaschii* were obtained from Mr. S. Yokobori in our laboratory. *Escherichia coli* C41 (DE3) cells were grown at 37°C in Luria-Bertani medium.

2.3. Isolation of MJ 0279 gene

The MJ 0279 gene was amplified by means of a PCR by using primers, 5-ACGTCATATGGGGGTTTTTATGGAGAAGTTA-3 and 3-TAGATAGGATCCTTATAGT--TTTATGGCTCCAACAACAATAAAT-5. The genomic DNAs of *M. jannaschii* was used as templates for PCR amplification. The restriction sites were introduced by the primers: *Nde*I and *Bam*HI sites are underlined. The amplified fragment was extracted from 0.7% agarose gel

after electrophoresis, digested with *Nde*I and *Bam* HI and then ligated into the *Nde*I-*Bam*HI sites of the pET-21c.

2.4. Expression and Purification of recombinant enzyme

Escherichia coli C41 (DE3) was transformed with plasmid and cultivated in 1 liter of M9YG broth supplemented with ampicillin. When the A_{660} of the culture reached 0.6, 1 mM IPTG was added to the culture media for gene expression. After an additional 5h cultivation, the cells were harvested and disrupted by sonication. The homogenate was centrifuged at 20,000Xg for 15 min and precipitate was collected. n-Octyl- β -D-glucopyranoside at a final concentration of 2% was added to the precipitate to solubilize the recombinant protein. The homogenate was centrifuged again at 20,000Xg for 15 min, and the supernatant was recovered as a crude extract. The supernatant after heat treatment at 70°C for 20 min was applied to a DEAE-sepharose column (diameter 1.5, height 5cm) which was equilibrated with 20 mM Hepes buffer pH 7.0 containing 1% n-Octyl- β -D-glucopyranoside. The enzyme was eluted with stepwise gradient 300 mM NaCl concentration. The active fraction, eluted at 0.3 M NaCl, was dialyzed against 20 mM Hepes buffer pH 7.0 and loaded onto a Resource Q column (Amersham-Pharmacia Biotech) equilibrated with same buffer. The active fractions were eluted with a 0-0.3M NaCl gradient. The purification process was repeated with a Resource column at pH 8.0. The active fractions were eluted with a 0-0.2M NaCl gradient. Finally the active fraction from Resource Q column was dialyzed against 5mM sodium phosphate buffer (pH 6.8) and loaded onto a hydroxyapatite column (BIO-RAD). The adsorbed proteins were eluted with a 5-500 mM linear gradient of sodium phosphate buffer (pH 6.8) at a flow rate of 1 ml/min. The DGGGP protein, eluted with 200 mM sodium phosphate buffer was collected and stored at 4°C. Protein concentration was determined with a BCA protein assay reagent (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. Protein fractions were analyzed by SDS-PAGE. Proteins bands were stained with Coomassie Brilliant Blue R-250.

2.5. Enzyme assay and product analysis.

The reaction mixture 100 μ l containing 100 mM Hepes buffer, pH 7.0, 5 mM $MgCl_2$, 0.2 mM G-1, 3-P, 0.14 μ M radio labeled GGPP and 1 μ g DGGGP synthase was incubated at 55°C for 20 min to produce radio labeled GGPP, which is the substrate for the next reaction. Then 1mM unlabeled GGPP and 1 μ g DGGGP synthase was added and incubated at 55°C for 10 min. The DGGGP formed was incubated with alkaline phosphatase (500 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$) at 37°C for 1h. The labeled products were extracted with 300 μ l methanol, 150 μ l chloroform, and 150 μ l pentane. The lower phase was collected and dried under a stream of nitrogen gas. The residue was dissolved in a small amount of chloroform-methanol (2:1, v/v) and analyzed by reversed-phase TLC using a percolated plate, LKC-18F, developed with acetone/H₂O (9/1). Radioactive spots of the products of the TLC plate were detected by autoradiography by X-ray film with Enhance (NEN life Science Products, Boston, Mass.). Each spot was scraped off the plate, the contents were extracted and the activity was estimated on a liquid

scintillation counter LSC-1000 (Aloka, Tokyo, Japan) with scintillation cocktail Scintisol AL-1 (Dojindo, Kumamoto, Japan).

2.6. Effect of temperature and pH.

The effect of temperature on DGGGP synthase activity of the purified enzyme was measured at different temperature 40, 50, 60, 70, 80, 90 and 100°C under optimal reaction conditions in 100 mM hepes buffer pH 7.0, 5 mM $MgCl_2$, 2 mM G 1, 3-P, 1 mM unlabeled GGPP and 0.14 μ M level GGPP at 55°C. DGGGP synthase of the purified enzyme was measured at different pH 4.0-9.0 under the same optimal reaction condition.

2.7. Effect of metal ions and detergents

The effects of various metal ions and detergents were tested on the activity of purified enzyme at reaction conditions in 100 mM Hepes buffer pH 7.0, 5 mM $MgCl_2$, 2 mM G 1, 3-P, 1 mM unlabeled GGPP and 0.14 μ M level GGPP at 55°C. The enzyme was incubated with 100 mM of metal ions, such as Na^+ , K^+ and NH_4^+ and 5 mM metal ions Mg^{++} , Ca^{++} , Mn^{++} and Zn^{++} , for 20 min under standard conditions. Activity was measured as described above. The relative enzyme activity was calculated.

3. Results and Discussion

3.1. Cloning, Expression and purification of DGGGP synthase

DDGGP synthase gene from *S. solfataries* (Morii et al, 2000) was used as a key sequence and DNA data bases were searched. We found candidate gene *MJ* 0276 as DGGGP Synthase in *Methanocaldococcus jannaschii* gene sequence. Primers were designed for ORF *MJ* 0276. PCR was done using these primers and the *Methanocaldococcus jannaschii* genomic DNA as the template. A DNA fragment with the expected length about 900 bp was amplified and cloned in a pCR T7/CT-TOPO vector. The cloned gene was subcloned into high-expression vector pET-21c. Sequence analysis of the subcloned fragment confirmed the full ORF *MJ* 0276 in the vector and the plasmid was named p*MJ* 0276. The recombinant DGGGP synthase was expressed in the cells of *E. coli* C41 (DE 3) harboring the p*MJ* 0276 plasmid. The *E. coli* cells harboring the plasmid were grown until mid-log phase and gene expression was induced by IPTG at 37°C. Fig. 2 shows the product of the DGGGOH by DGGGP synthase in the TLC plate after induction of *Escherichia coli* extract. Poulter and Zhang (1993) showed that the activity of DGGGPS is in membrane portion. For solubilization and purification, the membrane protein requires detergents for extraction from the membrane. By adding 2% n-Octyl- β -D-glucopyranoside or N,N-Dimethyldodecyl-amine N-oxide (LDAO) to the precipitate, we succeeded in solubilizing the enzyme, which enabled us to purify the enzyme. After solubilization of the protein, the extract was heated at 70°C for 30 min to remove *Escherichia coli* proteins. We then used DEAE-Sepharose, Resource Q ion-exchange column chromatography for further purification of the enzyme. Finally hydroxyapatite column was used to purify the enzyme described details in Materials and methods section. The SDS-PAGE showed the single band of protein of molecular mass 30 kDa (Fig 3), was similar

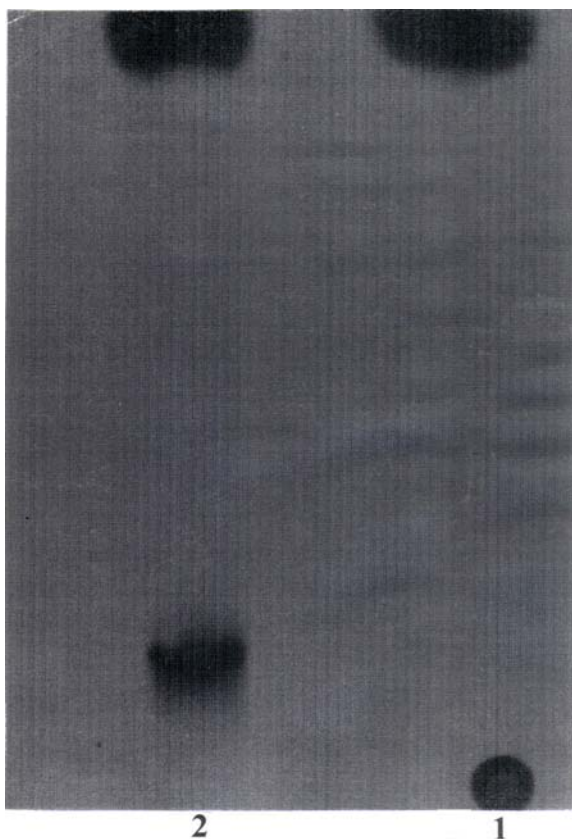


Fig. 2. TLC plate showing the product of the DGGGP synthase reaction of *E. coli* cell extract. Lane 1 represents reaction without DGGGP synthase and Lane 2 represents with DGGGP synthase.

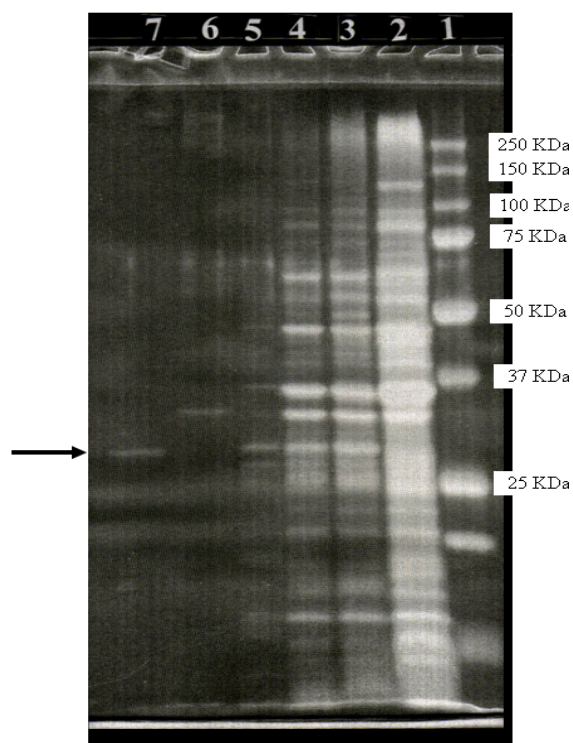


Fig. 3. SDS-PAGE analysis of samples from the purification steps of DGGGP synthase from *Methanocaldococcus jananschii*. Lane 1, protein standard marker; 2, After sonication; 3, After heat treatment; 4, After DEAE-sepharose column; 5, After Resource Q pH 7.0; 6, After Resource Q pH 8.0; 7, After Hydroxyapatite. Arrow indicates the DGGGP synthase.

to that calculated from its amino acid sequence (31.7 kDa). The purification process is summarized in Table-1. The specific activity at the final step of purification was 75 nmol/min/mg and purification fold is 750. Recently, the DGGGP synthase has been cloned and purified from archaea *Sulfolobus solftricus* and reported (Hemmi et al, 2004) but they did not mention the specific activities and purification yield due to instability of the activities of the enzyme.

3.2. General properties of DGGGP synthase

3.2.1. Optimum temperature and pH:

Maximal activity of DGGGP synthase was seen around 70°C (Fig. 4a) at temperatures in the normal range of *Methanocaldococcus jannaschii* growth temperature. At 90°C the DGGGP synthase lost about 80% activity and at 100°C almost no activity was detected. Purified DGGGP synthase was active over a wide range of pH between pH 5.0 to pH 7.0 and the optimum pH for the enzyme reaction was shown to be around 6.0 (Fig. 4b). The optimum pH value is similar to the optimum pH value of purified DGGGP synthase from *Sulfolobus solftricus* reported recently (Hemmi et al, 2004).

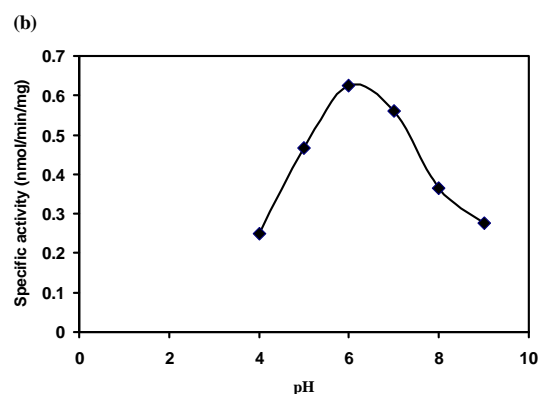


Fig. 4. (a) Temperature and (b) pH dependence of DGGGP synthase.

3.2.2. Effect of salts and metal ions:

The enzyme activity of DGGGP was significantly decreased when 5 mM Mg^{2+} was replaced with an equivalent concentration of EDTA, which indicates the requirement of a divalent metal ion for activity. The optimum concentration of Mg^{2+} was 2.5-5 mM and the enzymatic activity was slightly inhibited by higher concentrations of Mg^{2+} (Fig. 5). The metal ion could be replaced by 5 mM Ca^{2+} , although the enzyme activity fell by about 30% by Ca^{2+} , and NH_4^+ , whereas enzyme activity fell by 91.8% by 5mM Mn^{2+} and 91% by 5 mM Zn^{2+} but 100 mM Na^+ and K^+ did not affect the enzymatic activity of DGGGP synthase. The effects of different metal ions on purified DGGGP synthase were shown in Table 2. The enzyme activity of the purified DGGGP synthase was measured under optimal reaction conditions in 100 mM HEPES buffer pH 7.0, 5 mM $MgCl_2$, 2mM G 1, 3-P, 1 mM unlabel GGPP and 0.14 μ M level GGPP at 55°C. We also used sodium phosphate buffer instead of HEPES buffer and we did not see any change of the activity.

Table 1: Purification table of DGGGP synthase from *Methanocaldococcus jannaschii*

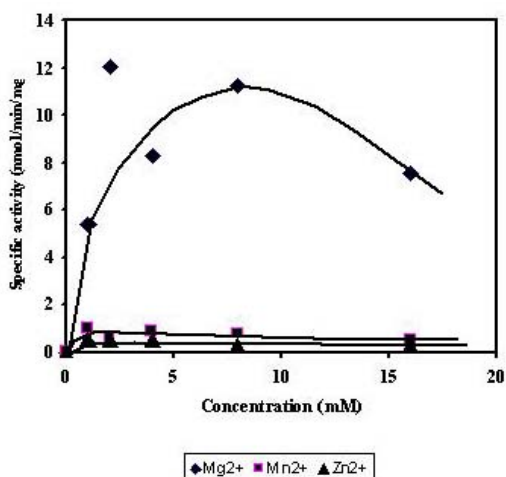
Steps	Volume (ml)	Protein (mg)	Activity (nmol./min)	Recovery (%)	Specific activity (nmol./min/mg)	Purification (fold)
Sonication	24	1000	100	100	0.100	1
Detergent	8	185	80	80	0.43	4.3
Heat treatment	7	79.4	75	75	0.94	9.4
DEAE-sepharose	7	50	64	64	1.28	12.8
Resource Q (pH 7.0)	5	2.25	47	47	20.9	20.9
Resource Q (pH 8.0)	5	0.6	40	40	66.7	667
Hydroxy-apatite	2	0.4	30	30	75	750

Table 2: Effects of metal ions on purified DGGGP synthase activity

Metal ion	Concentration (mM)	Relative activity (%)
MgCl ₂	5	100
CaCl ₂	5	71.2
MnCl ₂	5	8.2
ZnCl ₂	5	9.0
EDTA	5	7.3
NaCl	100	90.2
KCl	100	109.7
NH ₄ Cl	100	70.7

Table 3: Effects of detergents and substrate on purified DGGGP synthase activity.

Detergents or substrate	Relative activity (%)
n-Octyl-β-D-glucopyranoside 1%	100
N,N-dimethyldodecyl-amino N-oxide (LDAO) 1%	101.5
Triton X-100 1%	91.4
Chaps 1%	69.5
Nonidet P-40 1%	39.0
Deoxycholate 1%	36.7
G-1, 3-P	100
G-3-P	28.7

Fig. 5. Mg²⁺ dependence of DGGGP synthase. Reactions were carried out at 45°C

3.2.3. Effect of detergent:

To check the effect of detergents on DGGGP synthase, we used the detergents 1% Chaps, 1% Triton X-100, 1% Nonidet P-40, 1% Deoxycholate, 1% n-Octyl-β-D-glucopyranoside and 1% N, N-dimethyldodecyl-amino N-oxide (LDAO) described detailed in Material and Methods section. The DGGGP synthase activity was not changed when n-Octyl-β-D-glucopyranoside and LDAO was used. But the activity was decreased about 10% by Triton X-100, 30% by Chaps, and 61% by Nonidet P-40 was used (Table 3).

3.2.4. Substrate specificity of the enzyme

We used the substrate G-1, 3-P and GGPP to the reaction mixture for the enzymatic activity of DGGGP synthase. When the substrate G-1, 3-P was replaced with G-3-P, the enzymatic activity was decreased 71.3% (Fig. 6). The residual activity 28.7% can be attributed to the impurity of G-3-P (95% purity) and mixture of G-1, 2-P. Also when we did not use GGPP synthase enzyme in the 1st step reaction, the DGGGP synthase activity was almost completely lost. Similar result was obtained when we did not use GGPP in the reaction mixture. Thus DGGGP synthase is specific for G-1-P and GGPP. Specificity for G-1-P as a substrate has also been reported or GGPP synthases from our laboratory study (Yamagishi et al, 2003) and *M. thermotrophicum* (Chen et al, 1993) and *Halobacterium halkoobium* (Poulter and Zhang, 1993).

3.2.5. Molecular mass

The purified DGGGP synthase gave single peaks of absorbance at 280 nm corresponding to 29.6 kDa, on the gel filtration using a Superdex 200 HR column (GE Healthcare Bio-sciences Corp, Piscataway, NJ, USA; Fig. 7). This result indicated that the DGGGP synthase was a monomeric protein.

4. Conclusion

In summary, we have successfully purified DGGGP synthase from a cell free extract of the archaeon *Methanocaldococcus jannaschii*. The MJ 0996 gene of the

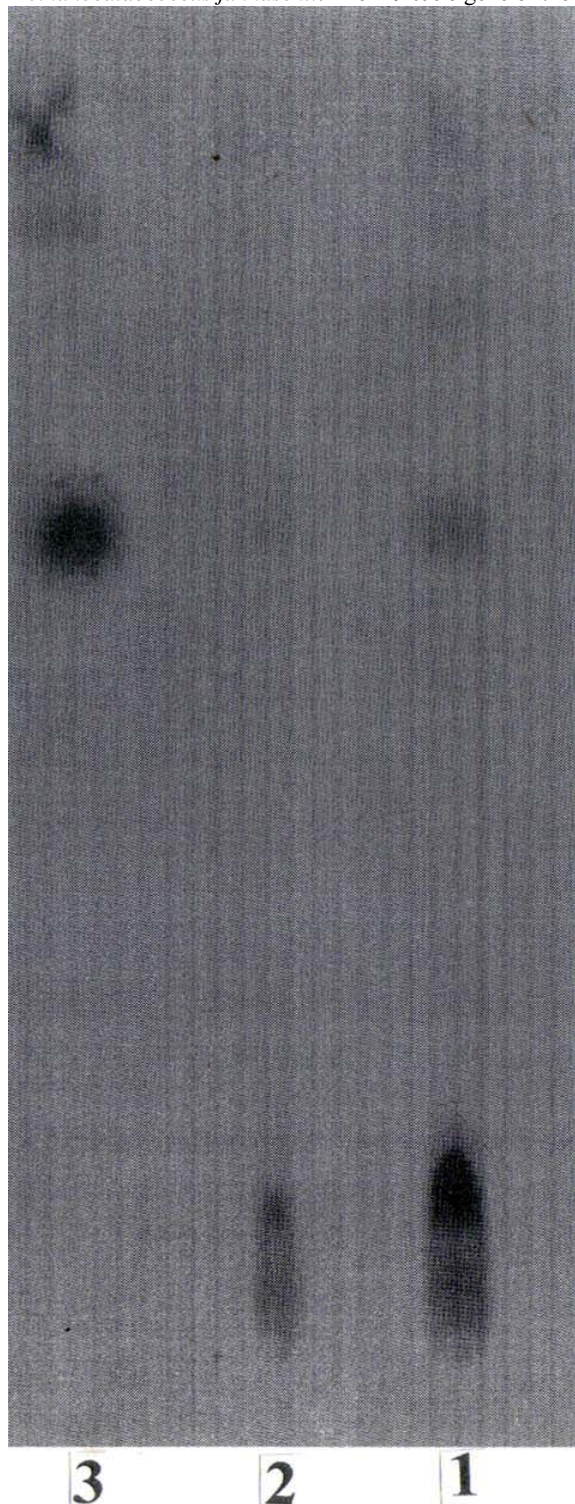


Fig. 6. TLC plate showing the product of the DGGGP synthase reaction of G-1, 3-P and G-3-P. Lane 1— G-1, 3-P; Lane 2— G-3-P; Lane 3— no glycerol phosphate.

Methanocaldococcus jannaschii genome can be functionally assigned as DGGGP synthase based on the N-terminal amino acid sequence of the purified enzyme.

Furthermore in this study, we cloned the gene encodes DGGGP synthase from genomic DNAs of *Methanocaldococcus jannaschii* and expressed in the cell of *Escherichia coli* C41 (DE3). The membrane protein DGGGP synthase after solubilized was purified by a combination of heat treatment and four chromatographic steps. The yield of the membrane protein is about 30%. So, it is possible to get enough protein by large culture using jar fermentor. It should be interesting to study the crystallize structure of the membrane protein and structure of the membrane protein DGGGP synthase and this study is underway in our laboratory.

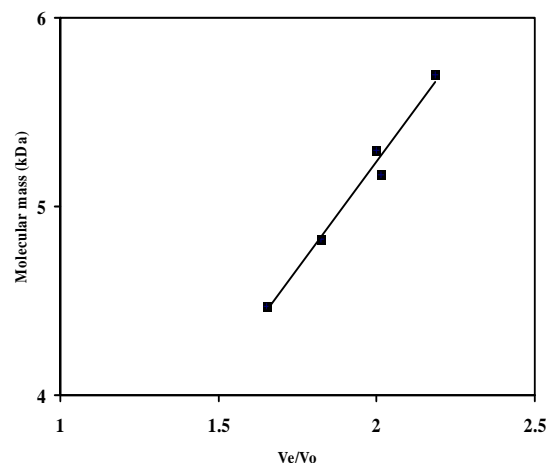


Fig. 7. Determination of the molecular mass of DGGGP synthase by gel filtration. Marker proteins were apoferritin (443 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 Kda), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). The relative retention volume of DGGGP synthase is indicated by an arrow.

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