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# Overexpression, purification, and characterization of selenomethionyl farnesyl diphosphate synthase of *Bacillus stearothermophilus*

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**Abstract**

To facilitate X-ray crystal structure solution of farnesyl diphosphate (FPP) synthase of *Bacillus stearothermophilus*, selenomethionyl recombinant enzyme was overproduced in a methionine (Met) auxotrophic strain of *Escherichia coli*, and purified to homogeneity by two chromatographic steps. About 50 mg of the pure selenomethionyl enzyme was obtained from 2 g of *E. coli* cells. Inductively coupled plasma (ICP) emission spectrometric analysis for selenium content showed that all of the Met residues in the FPP synthase were substituted by selenomethionine (SeMet). The selenomethionyl recombinant enzyme showed similar chromatographic behavior, heat stability, immunochemical property, product specificity, and kinetic parameters to those of the wild-type enzyme, indicating that SeMet substitution has little effect on the prenyltransferase with respect to substrate binding, enzymatic activity, and structure. 

*Keywords:* Isoprenoid; Prenyltransferase; Farnesyl diphosphate synthase; Selenomethionyl recombinant enzyme

## 1. Introduction

Farnesyl diphosphate (FPP) synthase (EC 2.5.1.10) occupies a central branch point in isoprenoid biosynthetic pathway. It catalyzes the consecutive condensation of isopentenyl diphosphate (IPP) with dimethylallyl diphosphate (DMAPP) and then with the resulting product, geranyl diphosphate (GPP), to produce FPP as the final product. FPP is a key precursor for most of isoprenoid compounds of physiological significance [1]. So far, cDNAs or genomic clones encoding FPP synthase have been isolated from various sources, including human [2], yeast [3], *Escherichia coli* [4], *Bacillus stearothermophilus* [5], white lupin [6], *Arabidopsis thaliana* [7], and rubber tree [8]. The structure–activity relationship of this enzyme have been extensively studied by site-directed mutagenesis to define the roles of specific amino acids in catalysis and the binding of substrates [9–15]. The three dimensional structure of avian recombinant FPP synthase has been determined by Tarshis et al. [16].

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FPP synthase from *B. stearothermophilus* is a homodimeric enzyme with subunit size of 297 amino acids. This enzyme has been overproduced in *E. coli* cells and purified [5]. Overproducing strains are able to produce the FPP synthase in an amount equivalent to ~20% of the total soluble proteins. This recombinant enzyme is so stable that it retains most of the original activity even after heating at 65°C for 1 h. The catalytic turnover for the synthesis of FPP from GPP and IPP is 660/min at 55°C. Because of its stability and abundance, the enzyme is extremely interesting from both mechanistic and synthetic viewpoints, in that it catalyzes the formation of new carbon–carbon bonds with latent stereospecificity [5]. To develop a novel method of the synthesis of chiral compounds using this stable enzyme, Nagaki et al. [17,18] and Maki et al. [19,20] investigated the substrate specificity of the bacterial FPP synthase using various artificial substrate analogues and compared it with that of animal FPP synthases. The bacterial FPP synthase was shown to have some differences in substrate recognition relative to pig and avian FPP synthases, especially with respect to the acceptability of the prenyl chain of allylic substrates. Comparison of the deduced amino acid sequences of FPP synthases obtained from *B. stearothermophilus* and avian liver indicates the presence of conserved regions typical of (*E*)-prenyltransferases although these FPP synthases shared only 25% identity. In addition, *B. stearothermophilus* FPP synthase was crystallized and a preliminary X-ray diffraction analysis was carried out to about 3 Å resolution [21]. These studies prompted us to determine the tertiary structure of the bacterial FPP synthase so that we are able to exploit the potential of this enzyme for chiral synthesis and to analyze its thermostability.

The incorporation of the unnatural amino acids, selenomethionine (SeMet) and telluromethionine (TeMet), for L-methionine (Met) into proteins have been used to facilitate X-ray crystal structure solution [22,23] and nuclear magnetic resonance (NMR) spectroscopy [24]. These experiments are conducted under the assumption that incorporation of SeMet or TeMet into proteins will have a very limited effect on protein structure and function. In order to obtain an insight into selenomethionyl FPP synthase, an expression plasmid, pET22EXF, carrying *B. stearothermophilus* FPP synthase gene, was used for transformation of a Met auxotrophic strain of *E. coli* cells, and the cells were cultured in LeMaster's medium containing SeMet as the sole source for Met. After purification, the SeMet FPP synthase was characterized and compared with the wild-type enzyme. This paper describes the results demonstrating that the SeMet substitution has little effect on substrate binding, enzymatic activity, and immunochemical property of *B. stearothermophilus* FPP synthase.

## 2. Experimental

### *2.1. Materials*

DL-SeMet, the common L-amino acids, adenosine, guanosine, thymine, and uracil were purchased from Sigma. [1-$^{14}$C]IPP (1.95 TBq/mol) was obtained from Amersham. Nonlabeled IPP, DMAPP, and GPP were synthesized according to the procedure of Davisson et al. [25]. Precoated reversed phase thin layer chromatography (TLC) plates, LKC-18, were purchased from Whatman. Isopropyl β-D-thiogalactopyranoside (IPTG) was a product of Wako. The antiserum against FPP synthase of *B. stearothermophilus* was the same preparation described previously [5]. *E. coli* B834 (DE3) competent cells were obtained from Novagen. All other chemicals were of analytical grade.

### *2.2. Construction of an expression system for B. stearothermophilus FPP synthase*

The *B. stearothermophilus* FPP synthase gene [5] was amplified by polymerase chain reaction

(PCR) using the appropriate primers. The sense and antisense primers, 5′-GGAGGAGGA-GTAACATATGGCGCAGCTTTC-3′ and 5′-CAAGGGCGGGCGTTAAGCTTTTAATGGT-CG-3′ (newly created restriction sites are underlined), were designed to create an *Nde*I site overlapping the starting codon, ATG, and an *Hin*dIII site downstream of the stop codon, TAA, respectively. The PCR product, which was digested with *Nde*I and *Hin*dIII, was separated on a 0.8% agarose gel. The desired band was eluted and ligated into the same sites of pET22b vector (Novagen), yielding an expression plasmid for *B. stearothermophilus* FPP synthase, pET22EXF. The recombinant plasmid constructed was then used for transformation of *E. coli* B834 (DE3).

### 2.3. Culture conditions

Two ml of an overnight culture of *E. coli* cells harboring pET22EXF plasmid in LB medium containing 50 μg/ml ampicillin was inoculated into 2 l of M9YG [5] or LeMaster's (defined) medium [26] containing 50 μg/ml ampicillin and 50 μg/ml L-Met or DL-SeMet replacing L-Met. The cells were grown at 37°C to an approximate $A_{600}$ value of 0.7, and IPTG was added to a final concentration of 1 mM, and then the incubation was continued for an additional 3 h at 30°C. Overproduction of the protein was confirmed by profile analyses after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### 2.4. Purification of the SeMet FPP synthase

The SeMet FPP synthase overproduced in *E. coli* cells was purified essentially according to the procedure as described in our previous work [5]. The pET22EXF/B834 (DE3) cells were suspended in 50 ml of 25 mM Tris–HCl buffer, pH 8.5, containing 1 mM EDTA and 10 mM 2-mercaptoethanol and disrupted by sonication with a sonifier (Branson). The resulting lysate was heated at 55°C for 1 h and then centrifuged at $100{,}000 \times g$ for 1 h. The protein fraction precipitated from the supernatant by 35–60% saturation of ammonium sulfate was chromatographed on a Butyl-Toyopearl column ($2.5 \times 10$ cm) with a decreasing linear gradient from 35% to 0% saturation of ammonium sulfate. FPP synthase fractions were collected and then applied to a Mono Q HR16/10 column (Pharmacia Biotech) with a linear gradient of 0–0.5 M NaCl. The fractions of FPP synthase were analyzed for purity by SDS-PAGE with Coomassie Brilliant Blue staining, and the fractions that showed more than 99% purity were used for further characterization. Protein concentrations were measured by the method of Bradford [27] with bovine serum albumin (BSA) as a standard.

### 2.5. Selenium analysis

Determination of selenium content of 0.22 mg/ml SeMet FPP synthase samples was made by ICP emission spectrometric analyses with a sequential plasma spectrometer (Shimadzu) at an excitation wavelength of 196.09 nm. Selenium standard solution (Kanto) was used as standards for the elemental selenium analysis.

### 2.6. FPP synthase assay and product analysis

The enzyme activity was measured by determination of the amount of [1-$^{14}$C]IPP incorporated into butanol-extractable prenyl diphosphates. In a standard experiment, the incubation mixture contained in a final volume of 0.2 ml, 50 mM Tris–HCl buffer, pH 8.5, 50 mM $MgCl_2$, 50 mM $NH_4Cl$, 50 mM 2-mercaptoethanol, 25 μM DMAPP or GPP, 25 μM [1-$^{14}$C]IPP (37 MBq/mol), and a suitable amount of the enzyme. After incubation at 55°C for 20 min, the reaction products were immediately extracted with 1-butanol saturated with water, and the radioactivity in the butanol extracts was measured with an Aloka LSC-1000 liquid scintillation counter. For kinetic studies with the wild-type or SeMet enzyme, the concentrations of

allylic substrate DMAPP/GPP or [1-$^{14}$C]IPP were 2.5, 5.0, 7.5, 10, 20, 30, 40, 50, or 100 μM, while the other substrate, [1-$^{14}$C]IPP or DMAPP/GPP was kept constant at 100 or 200 μM, respectively, and the incubation period was 15 min.

The radioactive products in the reaction mixture were hydrolyzed to the corresponding alcohols with potato acid phosphatase according to the method reported previously [28], and analyzed by reversed phase TLC plates with a solvent system of acetone/water (19:1). The position of authentic standards were visualized with iodine vapor, and distribution of radioactivity was detected. The TLC plates were exposed on a Fuji imaging plate at room temperature for 1 day, and then the plate was analyzed with a Fuji BAS 1000 Mac bioimage analyzer.

### 2.7. Immunochemical analysis

The inhibition of the enzymatic activity of SeMet FPP synthase by antiserum against *B. stearothermophilus* FPP synthase was examined by incubating various volumes of the antiserum solution with SeMet FPP synthase at the concentration of 0.75 ng/μl at 37°C for 1 h, then at 4°C overnight. After centrifugation at 10,000 rpm for 20 min, the supernatants were assayed for FPP synthase activity as described above.

## 3. Results

### 3.1. Overproduction of SeMet FPP synthase

The expression plasmid pET22EXF for *B. stearothermophilus* FPP synthase was constructed by inserting an open reading frame cassette into an expression vector, pET22b, which carries a T7*lac* promoter effective for suppressing basal expression in λDE3 lysogens before addition of IPTG. To obtain the SeMet FPP synthase, the plasmid was used for transformation of *E. coli* B834 (DE3), which is a Met auxotroph. The growth of pET22EXF/B834 (DE3) in LeMaster's (defined) medium containing DL-SeMet differed markedly from that of cells grown L-Met-containing or M9YG medium, by exhibiting an extended lag phase (7 h) as shown in Fig. 1. The induction with IPTG was effective for overproduction of the SeMet FPP synthase, with levels more than 20% of the total proteins similar to the same culture in L-Met-containing or M9YG medium as estimated by SDS-PAGE analysis (Fig. 2).

### 3.2. Purification of SeMet FPP synthase

The SeMet FPP synthase was purified essentially according to the purification procedure of the wild-type enzyme, including heat treatment followed by two chromatographic steps. The heat treatment of cell extracts was effective because *E. coli* proteins could be denatured and removed without loss of the enzymatic activity (data not shown). After hydrophobic chromatography followed by ion-exchange chromatography with a Mono Q column and separation on SDS-PAGE gels, the proteins were stained by Coomassie Brilliant Blue. The purified enzyme was found to be more than 99% pure (Fig. 2). Starting with 2 g of wet cells grown in DL-SeMet medium, we obtained approximately 50 mg of pure SeMet FPP synthase.

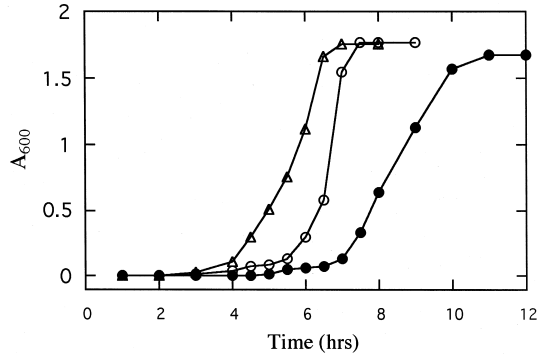


Fig. 1. Growth curves for *E. coli* B834 (DE3) cells harboring pET22EXF in M9YG medium (△), defined L-Met-containing medium (○), or defined DL-SeMet-containing medium (●).

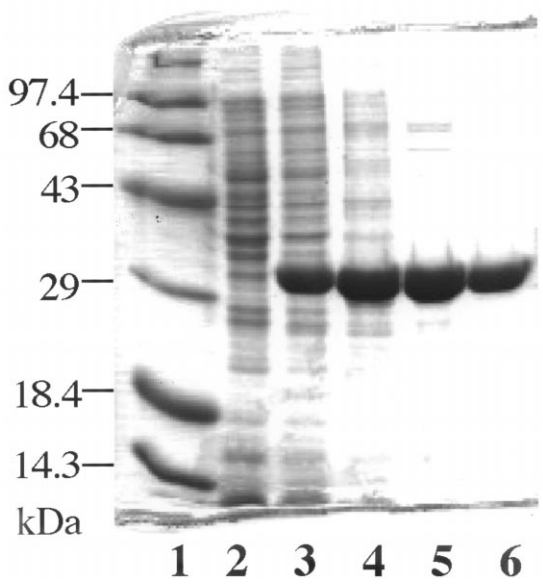


Fig. 2. SDS-PAGE analysis of overproduction (lanes 2 and 3) and purification (lanes 4–6) of the SeMet FPP synthase from *E. coli* cells harboring pET22EXF. Lane 1, molecular weight markers; lane 2, total protein extracts from *E. coli* B834 (DE3) with pET22EXF without IPTG; lane 3, total protein extracts from *E. coli* (DE3) with pET22EXF after induction with IPTG; lane 4, 100,000× *g* supernatant of the cell homogenate heated at 55°C for 60 min; lane 5, after Butyl-Toyopearl chromatography; lane 6, after Mono Q chromatography.

During the purification of the wild-type FPP synthase of *B. stearothermophilus*, the enzymatic activity was found in two fractions obtained after elution from a Mono Q chromatography column (Fig. 3a), which is similar to that reported earlier [12]. Similar observations have been reported for pig liver FPP synthase [29–31] and geranylgeranyl diphosphate synthase of *Methanobacterium thermoautotrophicum* [32]. These authors have attributed this phenomena to the oxidoreductive conversion of sulfhydryl and disulfide groups of the enzymes. We measured the enzyme activity in each fraction after Mono Q chromatography of the SeMet FPP synthase. The SeMet enzyme was also resolved into two fractions, one being eluted with 100 mM NaCl and the other with 150 mM NaCl similar to those of the wild-type enzyme (Fig. 3b). These results indicate that SeMet substitution for Met in *B. stearothermophilus* FPP synthase has little effect and Met is not involved in the oxidation–reduction reaction of this enzyme. The proteins eluted with 100 mM NaCl from Mono Q chromatography were used for further characterization of the enzyme.

### 3.3. Elemental selenium analysis

*B. stearothermophilus* FPP synthase is a homodimeric enzyme with an approximate molecular mass of 32.3 kDa. Each subunit is comprised of 297 amino acid residues, eight of which are Met at positions 1, 38, 77, 91, 111, 156, 164, and 187 [5]. Selenium content of the SeMet FPP synthase solution was measured by ICP emission spectrometric analysis. Triplicate determinations with 0.22 mg/ml of the SeMet FPP synthase sample yielded 4.22 μg/ml value of selenium, which is consistent with the pres-

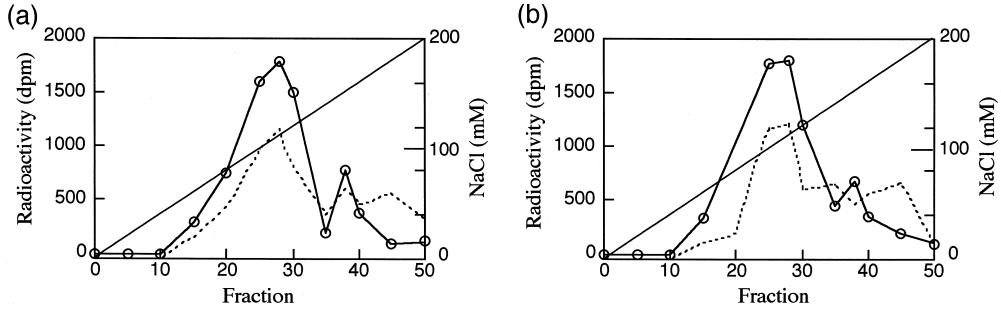


Fig. 3. Mono Q chromatography of the wild-type FPP synthase (a) and SeMet FPP synthase (b). (---), Protein absorbance at 280 nm; (-○-), FPP synthase activity; (—), NaCl gradient.

Table 1
Kinetic parameters of the wild-type and the SeMet FPP synthases

| | $K_m$ (DMAPP) (μM) | $K_m$ (GPP) (μM) | $K_m$ (IPP)[a] (μM) | $V_{max}^b$ (nmol/min/mg) |
|---|---|---|---|---|
| Wild-type | 16.4 ± 1.7 | 11.9 ± 1.0 | 9.6 ± 0.7 | 4394 ± 52 |
| SeMet mutant | 15.9 ± 1.7 | 11.5 ± 0.9 | 10.1 ± 0.8 | 4426 ± 70 |

[a] For the reaction with GPP.
[b] For the reaction with [1-$^{14}$C]IPP and GPP. Nanomoles of [1-$^{14}$C]IPP converted to FPP per minute by 1 mg of enzyme.

ence of eight SeMet per subunit of FPP synthase. This result indicates that all of Met residues in the enzyme were substituted with SeMet.

### 3.4. Kinetic and product analyses

Kinetic data were determined for both the wild-type and SeMet FPP synthases, and the results are summarized in Table 1. The SeMet enzyme exhibited similar kinetic parameters with the wild-type enzyme, showing that the replacement of sulfur by selenium in the Met residues has little or no effect on these kinetic properties of the enzyme. After enzymatic reaction with DMAPP/GPP and [1-$^{14}$C]IPP as substrates, the radioactive prenyl diphosphate products were hydrolyzed to the corresponding alcohols. TLC analyses of the alcohols indicated that both the wild-type and SeMet enzymes catalyzed the formation of FPP as the ultimate product (Fig. 4).

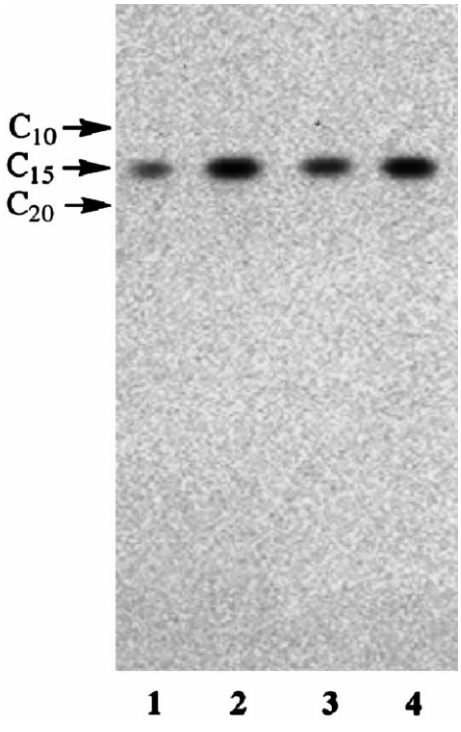


Fig. 4. TLC autoradiogram of the alcohols obtained by enzymatic hydrolysis of the products formed by the wild-type FPP synthase (lanes 1 and 2) or the SeMet FPP synthase (lanes 3 and 4) using [1-$^{14}$C]IPP and DMAPP (lanes 1 and 3) or GPP (lanes 2 and 4) as substrates. Arrows indicate the positions of authentic alcohols: $C_{10}$, geraniol; $C_{15}$, (all-*E*)-farnesol; $C_{20}$, (all-*E*)-geranylgeraniol.

### 3.5. Inhibition of the enzymatic activity of SeMet FPP synthase by antiserum against the wild-type enzyme

The antiserum against the wild-type FPP synthase of *B. stearothermophilus* was used for

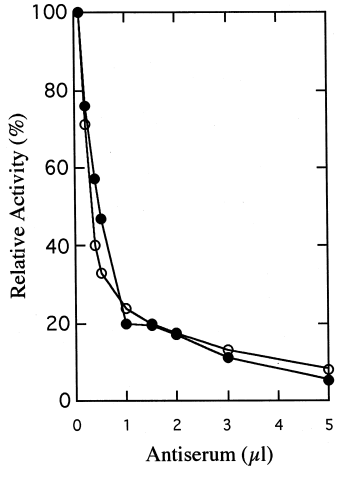


Fig. 5. Inhibition of the enzymatic activity of the SeMet FPP synthase by the antiserum against the wild-type enzyme. The indicated amount of antiserum was incubated with the wild-type FPP synthase (○), or with the SeMet FPP synthase (●) and the residual enzymatic activity was assayed using GPP and [1-$^{14}$C]IPP as substrates. Activities relative to that of the enzyme without treatment by antiserum are indicated.

examination of the inhibitory effect on SeMet FPP synthase activity. After immunoprecipitation by the antiserum at various volumes, the remaining FPP synthase activities in the supernatants of both wild-type and SeMet enzymes were determined. As shown in Fig. 5, the enzymatic activities of both enzyme preparations were similarly inhibited, indicating that both enzymes have identical immunochemical properties.

### *3.6. Heat stability*

Heat stabilities of both wild-type and SeMet enzymes were examined by analyzing their remaining activity after heat treatment at various temperatures for 1 h. The SeMet FPP synthase exhibited similar thermostability to that of the wild-type, without losing activity even after heating at 65°C for 1 h (data not shown).

## 4. Conclusions

An overexpression system for SeMet FPP synthase of *B. stearothermophilus* in *E. coli* cells was constructed and the recombinant protein was purified to homogeneity by two chromatographic steps. The SeMet FPP synthase showed chromatographic behavior, enzyme kinetics, product specificity, immunochemical property, and heat stability closely similar to those of the wild-type enzyme, indicating that the SeMet substitution in this enzyme has little effect on its substrate binding, enzymatic activity, and structure. These achievements may facilitate the X-ray crystal structure solution of this interesting enzyme.

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