Bacterial Phytoene Synthase: Molecular Cloning, Expression, and Characterization of *Erwinia herbicola* Phytoene Synthase†

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ABSTRACT: Phytoene synthase (PSase) catalyzes the condensation of two molecules of geranylgeranyl diphosphate (GGPP) to give prephytoene diphosphate (PPPP) and the subsequent rearrangement of the cyclopropylcarbinyl intermediate to phytoene. These reactions constitute the first pathway specific step in carotenoid biosynthesis. The *crtB* gene encoding phytoene synthase was isolated from a plasmid containing the carotenoid gene cluster in *Erwinia herbicola* and cloned into an *Escherichia coli* expression system. Upon induction, recombinant phytoene synthase constituted 5−10% of total soluble protein. To facilitate purification of the recombinant enzyme, the structural gene for PSase was modified by site-directed mutagenesis to incorporate a C-terminal Glu−Glu−Phe (EEF) tripepetide to allow purification by immunoaffinity chromatography on an immobilized monoclonal anti-α-tubulin antibody YL1/2 column. Purified recombinant PSase-EEF gave a band at 34.5 kDa upon SDS−PAGE. Recombinant PSase-EEF was then purified to >90% homogeneity in two steps by ion-exchange and immunoaffinity chromatography. The enzyme required Mn^{2+} for activity, had a pH optimum of 8.2, and was strongly stimulated by detergent. The concentration of GGPP needed for half-maximal activity was ∼35 μM, and a significant inhibition of activity was seen at GGPP concentrations above 100 μM. The sole product of the reaction was 15,15′-*Z*-phytoene.

Carotenoids are a major class of isoprenoid metabolites synthesized de novo in bacteria, fungi, algae, and higher plants (*1*). Although they are found primarily in the photosynthetic members of these groups, carotenoids are synthesized in a limited number of nonphotosynthetic organisms and accumulate exogenously in many organisms. In those organisms that synthesize carotenoids de novo, the function of the metabolites is tied to photosynthesis, their ability to absorb and transfer energy, and as scavengers of singlet oxygen (*2*).

Carotenoids are an essential part of the human diet as precursors for a number of nutritionally important metabolites. Most notable are the retinoids, including retinal, which in conjunction with the protein opsin forms the visual pigment rhodopsin in mammalian retinas (*3*), retinoic acid, which is essential for normal epithelial cell differentiation, vertebrate limb development, embryogenesis (*4*), and vitamin A (*5*). Carotenoids, in particular β-carotene, have also found important clinical uses in the treatment of photosensitivity associated with diseases such as erythropoietic protoporphyria (*5*). In addition, there is a growing body of epidemiological data that suggest an important role for β-carotene, and possibly other carotenoids, in the prevention of certain cancers (*5*−*7*). Carotenoids are also being studied for their potential role in modulating immunological responses (*5*), for their possible chemopreventive value in the development of cataracts (*8*), and as dietary anti-oxidants (*9*).

The early stages of carotenoid biosynthesis share a common pathway with other major classes of isoprenoids. These reactions include initial formation of isopentenyl diphosphate (IPP)[1], isomerization of IPP to dimethylallyl diphosphate (DMAPP), and chain elongation of DMAPP to geranylgeranyl diphosphate (GGPP) by sequential addition of IPP (*1*, *10*). The coupling of two molecules of GGPP to yield phytoene (see Scheme 1) is mediated by phytoene synthase (PSase) and represents the first committed step in carotenoid biosynthesis (*1*, *10*). Like squalene synthase, which catalyzes a similar coupling reaction in the sterol biosynthetic pathway (*11*), PSase has attracted considerable interest for the mechanistic complexities inherent in the transformation of GGPP to phytoene and for its potential role in regulation.

Although PSase has been purified from natural sources (*12*), preparations of the enzyme are not readily available for mechanistic and structural studies. The assignment of a number of structural genes in both prokaryotic and eukaryotic species for PSase (*13*) opens the possibility of using molecular cloning techniques to obtain recombinant enzyme for structural and mechanistic studies. Recently, the cloning of the *crtB* gene from *Erwinia uredovora* and characteriza-

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[1] Abbreviations: BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidoproyl)-dimethylammonio]-1-propanesulfonate; DMAPP, dimethylallyl diphosphate; D-MEM, Dulbecco's modified eagle media; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; PMSF, phenylmethylsulfonyl fluoride; PSase, phytoene synthase; PPPP, prephytoene diphosphate.

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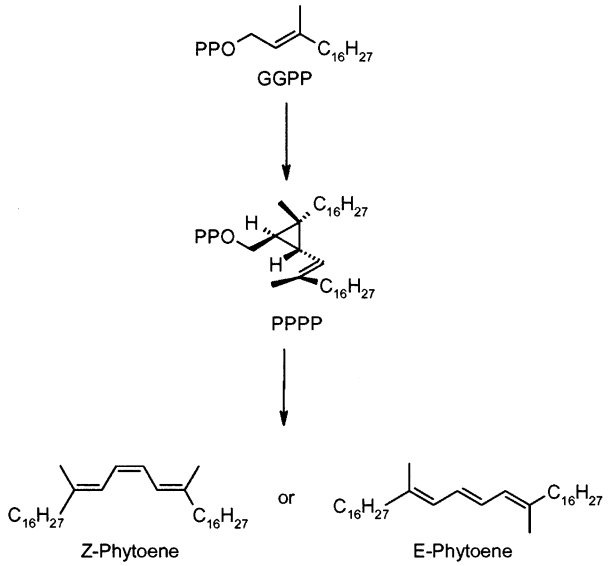
Scheme 1 : Conversion of Geranylgeranyl Diphosphate to Phytoene



tion of partially purified encoded bacterial PSase was reported (*14*). We have constructed an *Escherichia coli* clone containing the *crtB* gene from the nonphotosynthetic bacterium *Erwinia herbicola* that directs synthesis of PSase. Several of the properties of our recombinant enzyme, including specific activity, cofactor requirements, and the structure of the product of the reaction, differ from those reported for *E. uredovora* PSase.

EXPERIMENTAL PROCEDURES

General Methods and Materials. GGPP used as substrate for phytoene synthase activity assays was synthesized by the procedure of Davisson et. al. (*15*). [1-^{3}H]Geranylgeranyl diphosphate was purchased from NEN. Radioactivity was quantitated by liquid scintillation counting using a Packard Tri-Carb 4530 Liquid Scintillation Counter with Opti-Fluor (Packard) or CytoScint (ICN) scintillation cocktail. HPLC analysis was carried out with a Waters 625 multisolvet delivery system equipped with a 991 photodiode array detector. Cells were disrupted with a Branson Sonifer 350 cell disrupter. Gel electrophoresis was performed with a BRL Model S2 gel apparatus for DNA sequencing, a Hoefer Scientific Instruments model HE 33 Minnie Submarine unit for agarose electrophoresis, and a BioRad Mini PROTEAN II unit for protein analysis. Protein chromatography was performed by FPLC (Pharmacia) with DE-52 (Whatman) and Gamma Bind Plus Sepharose (Pharmacia). Protein concentrations were determined with coomassie protein reagent (Pierce or BioRad) according to the method of Bradford (*16*), using bovine serum albumin (BSA) as a standard. SDS−PAGE analysis of proteins was performed using 12% gels and visualization with coomassie brilliant blue (*17*).

DNA fragments generated from restriction digests were purified with either the GeneClean (Bio 101) or the Spin Bind (FMC) kits according to the manufacturer's instructions. Plasmid midi-preparations (∼100 μg) were carried out with the Tip-100 kit from Qiagen according to the manufacturer's instructions. Restriction digests and ligations were conducted with enzymes from New England Biolabs (NEB), Boeringer Mannheim (BM), United States Biochemical (USB), and Stratagene. Analysis of restriction digests was carried out in 0.8−1.4% agarose gels (SeaKem, FMC). All DNA sequencing was performed on double-stranded plasmid DNA utilizing the dideoxy chain termination method of Sanger (*18*) with the Sequenase kit (USB) according to manufacturer's instructions. Site-directed mutagenesis of the *crtB* gene followed the method of Kunkel (*19*, *20*) using the MutaGene kit from BioRad according to the manufacturer's instructions. Plasmid mini-preparations (∼15 μg), transformations, ligations, and other standard molecular biology techniques were carried out as described (*17*). Plasmid pAPU211 was a gift from Dr. John Hearst of the University of California at Berkeley. Dr. Robert Schackman at the University of Utah Regional Cancer Protein/DNA Core Facility synthesized DNA primers for sequencing and mutagenesis.

Bacterial Strains, Media, and Growth Conditions. Cloning was performed in *E. coli* strains **DH5α** (F$^-$, ϕ80d*lac*ZΔM15, *end*A1, *rec*A1, *hsd*R17 (r^-_K,m^-_K), *sup*E44, *thi*-1, λ^-, *gyr*A1, *rel*A1; Bethesda Research Laboratory), **JM101** (*sup*E, *thi*, Δ(*lac-pro*AB), F′[*tra*D36, *pro*AB$^+$, *lac*I^q, *lac*ZΔM15]), **CJ236** (*dut*-1, *ung*-1, *thi*-1, *rcl*A1; pCJ105(Cm^r); BioRad), **MV1190** (Δ(*lac*-proAB), *thi*, *sup*E, Δ(*srl-rec*A)306::Tn10-(tet^r)[F′:*tra*D36, *pro*AB, *lac* I^q ZΔM15]; BioRad), and **BL21-(DE3)** (*hsdS*, *gal* (λ*c*I*ts*857, *ind* 1, *S*am7, *nin*5, *lac*UV5-T7gene*1*)). Cells were grown at 37 °C in LB, 2 × YT (*15*), or minimal glucose, or at 30 °C in M9 + CAGM media, supplemented with chloramphenicol (15 μg/mL) and/or ampicillin (100 μg/mL) as required. Media components were from Difco. Hybridoma cells producing the YL 1/2 antibody were obtained from the European Collection of Animal Cell Cultures (ECACC #92092402). D-MEM and protein-free hybridoma media were from Gibco/BRL; fetal calf serum was from Hyclone.

Construction of an E. coli Expression System for crtB. The *E. herbicola crtB* gene was excised from pAPU211 (*21*) as a 3.44 kb *Bgl*II/*Eco*RI fragment and ligated into the *Bam*HI/*Eco*RI sites of pBluescript SK(+) to give pSM-III92. A 1.9 kb fragment of DNA downstream from *crtB* was removed by digestion of pSM-III92 with *Eco*RI followed by a partial digestion with *Bam*HI. Fill-in with Klenow and ligation with T_4 ligase gave pSM-III104. The *crtB* gene, minus the first 36 base-pairs, was then excised from pSM-III104 as a 1.1 kb *Nco*I/*Hin*dIII fragment and ligated into the *Nco*I/*Hin*dIII sites of pSM-III108, a pKEN2 derivative (*22*) containing a synthetic DNA fragment consisting of the first 36-bp of *crtB* with an *NdeI* site as part of the translation initiation codon, giving pSM-III110. DNA sequencing confirmed the structure of the gene. The intact *crtB* gene was then subcloned as a 1.1-kb *Nde*I/*Hin*dIII fragment into the *Nde*I/*Hin*dIII sites of the *E. coli* expression vectors pDR200, a derivative of pLM1 (*23*), and pARC306N (*24*), to give pDR-I201 and pSM-III145, respectively.

Engineering of a C-Terminal EEF Motif into crtB. The *crtB* gene was excised from pSM-III110 as a 1.1-kb *Xho*I/*Hin*dIII fragment and ligated into the *Sal*I/*Hin*dIII sites of pTZ18U (BioRad), to give pDR-I38. Uracil-containing ssDNA was obtained by infection of *E. coli* strain CJ236/pDR-I138 with helper phage M13K07 and isolation of the ssDNA from the resulting phagemids by phenol/$CHCl_3$ extraction. The uracil-containing ssDNA was then used as template for mutagenesis with a 39-mer oligonucleotide

containing codons for Glu−Glu−Phe (underlined) immediately preceding the stop codon (italics) and a unique *Nae*I restriction site (bold) downstream from the stop codon [5′-(ATGGCC**GCCGGC***CTA*GAACTCCTCAACGGGACGCTGCCA)-3′]. The reaction mixture was transformed into competent *E. coli* MV1190 cells, and transformants were selected for ampicillin resistance. Positive transformants were screened for modified *crtB* by restriction analysis of plasmid DNA from mini-preps, and the mutation was confirmed by DNA sequencing. EEF-modified *crtB* was subcloned as an *Nde*I/*Hin*dIII fragment into expression vectors pDR200 and pARC306N to give pDR-II194 and pCLP-II49, respectively. The plasmids were transformed into *E. coli* strains BL21-(DE3) and JM101 for over-production of recombinant EEF-modified phytoene synthase (PSase-EEF).

Expression of Wild-Type and EEF-Modified crtB in E. coli. A single colony of *E. coli* JM101/pSM-III145 or JM101/pCLP-II49 was used to inoculate 3 mL of M9 + CAGM containing 100 μg/mL ampicillin in a culture tube. Colonies of *E. coli* BL21(DE3)/pDR-I201 and BL21(DE3)/pDR-II194 were inoculated into LB containing 100 μg/mL ampicillin. The cultures were incubated at 30 °C and 250 rpm for approximately 12 h. A 1 mL sample was then used to inoculate 50 mL of M9 + CAGM, or LB, containing 100 μg/mL ampicillin in 250 mL Erlenmeyer flasks, and the cultures were allowed to grow overnight. A 6 mL portion of the overnight culture was used to inoculate 300 mL of M9 + CAGM or LB containing 100 μg/mL ampicillin in 2.8 L Fernbach flasks. The cultures were incubated at 30 °C and 250 rpm until they reached an OD_{600} of 0.7−0.9, at which time nalidixic acid (JM101) or IPTG (BL21(DE3)) was added to a final concentration of 50 μM or 500 μM, respectively. The cultures were grown an additional 4 h, harvested by centrifugation, and frozen in liquid N_2.

Phytoene Synthase Assays. Assays for PSase-EEF were conducted in 13 × 100 mm glass test tubes in a final volume of 200 μL. The standard assay buffer contained 50 mM MOPS (pH 7.2), 10 mM KF, 2.0 mM DTT, 2.0 mM $MnCl_2$, 2% Tween 80, and 100 μM [1-^{3}H]GGPP (1.0 μCi/μmol). The tubes were equilibrated at 30 °C for 10 min. The reaction was initiated with the addition of enzyme and terminated after 5−15 min by the addition of 300 μL of 1:1 40% (w/v) aqueous KOH/methanol. Saturated brine (0.5 mL) and solid sodium chloride were added, followed by 2 mL of 10% (v/v) diethyl ether in ligroin. The tubes were vortexed for 30 s, and 1 mL of the organic extract was loaded onto a column of activated alumina (2 mL) in a Pasteur pipet preequilibrated in 10% (v/v) diethyl ether in ligroin. The column was eluted with 4 × 1 mL portions of the same solvent, and the eluent was collected directly into scintillation vials. Scintillation cocktail (10 mL) was added, and the radioactivity was measured.

Production of YL 1/2 Antibodies and Construction of an Immunoaffinity Chromatography Column. Hybridoma cells were initially grown from frozen stocks in D-MEM + 10% fetal calf serum in T-flasks at 37 °C with 5% CO_2. After recovery, the cells were transferred gradually to protein-free hybridoma medium, then seeded at 2 × 10^5 cells/mL into 5 × 200 mL of fresh protein-free hybridoma medium in roller bottles (1 L). The cultures were grown at 37 °C with 5% CO_2 for ∼1 week, then centrifuged (1000*g*, 15 min) to remove the cells, and the supernatant was recovered. The typical concentration of YL 1/2 in the supernatant was 100 mg/L. To generate an immunoaffinity column, a portion of the supernatant was loaded directly on a column of GammaBind Plus Sepharose equilibrated in PBS buffer. After washing thoroughly with PBS buffer, the column was ready for use.

Purification of Recombinant EFF-Modified Phytoene Synthase. Frozen cell paste (1.5 g) of *E. coli* strain JM101/pCLP-II49 was suspended to a density of 150 mg/mL in buffer containing 50 mM Tris (pH 7.5); 2.0 mM DTT; 1 μg/mL each of leupeptin, chymostatin, and antipain; 1.0 mM PMSF; and 20% glycerol. The cells were disrupted by sonication (micro probe, power setting of 3, 6 × 30 s) while being cooled in an ice bath. The extract was clarified by centrifugation (12 000*g*, 15 min), and the supernatant was loaded onto a DE-52 column (1.5 × 15 cm, 0.75 mL/min) equilibrated in 50 mM Tris (pH 7.5), 2.0 mM DTT, 1 μg/mL leupeptin, 0.2 mM PMSF, and 20% glycerol. The column was eluted with 60 mL of starting buffer, followed by a linear gradient to 500 mM NaCl in starting buffer (120 mL). Fractions containing PSase-EEF were combined and applied (0.2 mL/min) in three batches to a 0.5 × 9.0 cm immunoaffinity column containing YL 1/2 antibodies (*25*) noncovalently bound to GammaBind Plus Sepharose (Pharmacia) preequilibrated with 50 mM Tris (pH 7.5), 2.0 mM DTT, 75 mM KCl, and 20% glycerol. The column was eluted (flow 0.6 mL/min) with equilibration buffer until the absorbance of the eluent at 280 nm had returned to the baseline. PSase-EEF was eluted with equilibration buffer containing Asp−Phe (5 mM). The column was washed with 50 mM Tris (pH 7.5), 2.0 mM DTT, 500 mM NaCl, and 20% glycerol after each run, and then reequilibrated with loading buffer.

Metal Ion, pH, and Detergent Dependence of Phytoene Synthase. All assays contained 100 μM [1-^{3}H]GGPP (1−170 μCi/μmol), 2 mM DTT, 10 mM KF, and 6.1 μg of PSase-EEF purified through the affinity step. The samples were incubated at 30 °C for 15 min in a final volume of 200 μL. The reactions were stopped and worked-up as described for the standard assay. The pH studies were conducted in 50 mM buffer containing 0.03% Tween 80 and 0.25 mM $MnCl_2$. The pH of the assay solutions was determined at 30 °C with nonradioactive samples containing all of the reaction components. For studies on the metal dependence of PSase-EEF, the assays contained 50 mM HEPES (pH 8.2) and 0.08% Tween 80, with the concentration of $MgCl_2$ varied from 0.2 to 20 mM and the concentration of $MnCl_2$ varied from 0.1 to 5.0 mM. For studies on the detergent dependence of PSase-EEF, the assays contained 50 mM HEPES (pH 8.2) and 0.25 mM $MnCl_2$. Studies on GGPP dependence contained 50 mM HEPES (pH 8.2), 0.25 mM $MnCl_2$, and 0.08% Tween 80, with [1-^{3}H]-GGPP concentrations varied from 1.0 to 200 μM. Two to six independent determinations were conducted at each concentration or pH, and these values were averaged for each data point plotted.

Product Analysis of the Phytoene Synthase Reaction. HPLC analysis with diode array detection was carried out on a Vydac 218TP104 reversed phase column with elution at 0.7 mL/min beginning with isocratic elution for 10 min with 85:2.5:2.5:10 acetonitrile/dichloromethane/hexane/methanol, followed by a linear gradient to 45:22.5:22.5:10 acetonitrile/dichloromethane/hexane/methanol over 45 min on a 10 μL sample of the product isolated from a reaction

Table 1: Purification of Recombinant PSase-EEF from *E. coli* Jm101/PCLP-II49[a]

sample	total protein (mg)	total units (nmol/min)	specific act. (U/mg)	yield (%)	purification (*n*-fold)
cell-free	118	69	0.58	100	1
DE-52	37	72	1.95	106	3.4
affinity	3	23	7.8	34	13

[a] Based on 1.5 g of cell paste.

using cold GGPP that was conducted and worked-up in the dark. Two other samples were exposed to a fluorescent lamp for 30 and 60 min, respectively, before HPLC analysis.

RESULTS AND DISCUSSION

Synthesis and Purification of Recombinant Phytoene Synthase-EEF. The *crtB* gene was cloned into a number of bacterial expression vectors, including derivatives of pKEN2 (*22*), pARC306N (*24*), and pLM1 (*23*), with different configurations of promoters, leader sequences, and ribosomal binding sites. Of these, pSMIII-145, a derivative of pARC306N, and pDR-I201, a derivative of pLM1, gave PSase as approximately 30% of the total cellular protein based on SDS−PAGE of crude homogenates. Unfortunately, in both cases essentially all of the recombinant protein segregated into inclusion bodies as inactive enzyme. Attempts to refold the denatured enzyme under a variety of conditions were unsuccessful. We were able to obtain a significant portion of the recombinant protein in soluble, active form at levels of 5−10% of total cytosolic protein by changing to a minimal growth medium (M9 + CAGM) and lowering the incubation temperature to 30 °C.

To facilitate purification of PSase by immunoaffinity chromatography, we appended a C-terminal Glu−Glu−Phe (EEF) α-tubulin epitope to the protein (*25*). This approach was recently used to purify recombinant protein farnesyltransferase (*26*), farnesyl diphosphate synthase (*27*), HIV-1 reverse transcriptase (*28*), and *ras* GTPase-activating proteins (*29*). EEF-modified *crtB* was subcloned as an *Nde*I/*Hin*dIII fragment into expression vectors pDR200 and pARC306N to give pDR-II194 and pCLP-II49, respectively. Colonies of *E. coli* BL21(DE3)/pDR-II194 and JM101/pCLP-II49 were screened for PSase activity, and the most active transformants were selected for synthesis of PSase-EEF. Addition of the EEF sequence to the C-terminal of PSase did not noticably alter the level of protein synthesis or the activity of the enzyme. The intensity of bands on SDS gels and the PSase activity of cell-free preparations were indistinguishable for wild type and EEF-modified proteins.

In a typical purification, the cell-free extract was chromatographed on DE-52 or fractionated with ammonium sulfate prior to the affinity step. A purification of recombinant PSase-EEF is summarized in Table 1. In this case, the cell-free extract was applied to a DE-52 column and eluted with a gradient of 0−0.5 M NaCl in loading buffer. PSase-EEF eluted as a broad peak at ∼0.2 M NaCl (Figure 1). This step usually provided a 3.0−3.5-fold purification. The protein was then applied in portions directly to the immunoaffinity column (*28*). After washing the column thoroughly with loading buffer, PSase-EEF eluted as a sharp peak with loading buffer containing 5 mM Asp−Phe.

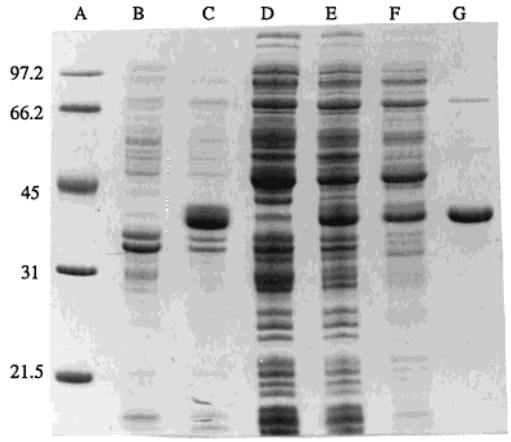


FIGURE 1: SDS−PAGE of samples from the purification of recombinant PSase-EEF. Lane A—molecular weight markers: phosphorylase B, 97.2 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; and trypsin inhibitor, 21.5 kDa. Lane B—pellet from JM101/pARC306N. Lane C—pellet from JM101/pLP111. Lane D—cell-free extract from JM101/pARC306N. Lane E—cell-free extract from JM101/pCLP−II49. Lane F—after DE52 chromatography. Lane G—after immunoaffinity chromatography.

The concentration of PSase-EEF recovered after affinity chromatography was typically 0.3−0.5 mg/mL, and the protein could be used without further treatment. The presence of Asp−Phe had no effect on the enzymatic activity (data not shown). For long-term storage, the enzyme was flash frozen in liquid N_2 and stored at −80 °C. PSase-EEF was stable for at least 6 months when stored in this manner. For more concentrated protein solutions, PSase-EEF was precipitated by the addition of ammonium sulfate to 60% saturation and resuspended in 50 mM Tris (pH 7.5), 1.0 mM DTT, and 20% glycerol. The SDS gel shown in Figure 1 for purified recombinant PSase-EEF gave an intense band at 34.5 kDa consistent with the predicted molecular mass for the protein. The sample was judged to be ≥90% pure.

Characterization of Recombinant Phytoene Synthase. Like squalene synthase, PSase binds a water-soluble substrate and releases a water-insoluble product. Both enzymes require a detergent (Tween 80 in these studies) for maximal activity. However, the activity of squalene synthase increased in a hyperbolic manner with increasing concentrations of Tween 80, with maximal activity occurring at ∼2.0 mM (*30*), while PSase activity increased sharply to maximal activity at ∼0.08% Tween 80 and then dropped off gradually with increasing Tween 80 concentration (Figure 2). Similar behavior was observed with CHAPS and *n*-dodecyl-*β*-D-maltoside (data not shown). Interestingly, when squalene synthase was assayed in the absence of NADH, such that presqualene diphosphate was produced instead of squalene, a pattern similar to that observed for PSase-EEF was observed, with maximal activity occurring at ∼0.2% Tween 80. Those results were rationalized on the basis of the differential water solubility of presqualene diphosphate and squalene, with squalene requiring high detergent concentrations to provide a means of removing the hydrocarbon from the enzyme. Given that phytoene is less soluble than squalene in aqueous solvent systems, and thus even more dependent on detergent for efficient removal from the enzyme surface, the lower activity observed here for phytoene synthase at higher detergent concentrations may reflect decreased stabil-

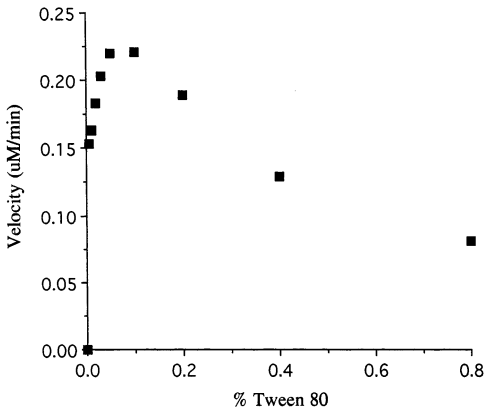


FIGURE 2: Initial velocity plot for recombinant PSase-EEF at different concentrations of Tween-80.

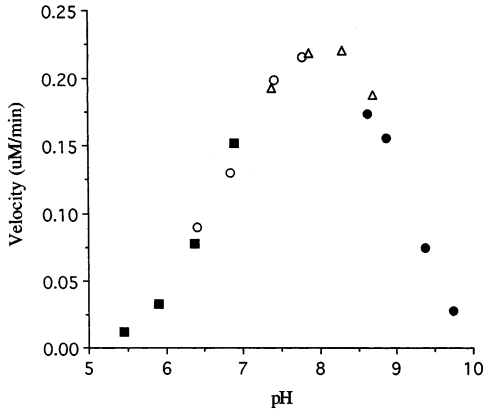


FIGURE 3: pH-rate profile for recombinant PSase-EEF from pH 5.5−9.7. (■) Mes, pH 5.46−6.90. (○) MOPS, pH 6.41−7.77. (△) HEPES, pH 7.38−8.70. (●) CHES, pH 8.63−9.74.

ity of the enzyme under those conditions or a greater tendency for the more lipophilic GGPP (as compared with FPP) to become sequestered within detergent micelles where it may not be as readily available to the enzyme.

E. herbicola PSase-EEF shows a well-defined bell-shaped response to pH (Figure 3), with inflection points at ∼pH 6.6 and 9.0 and optimal activity at pH 8.2, somewhat higher than reported for the eukaryotic enzyme (pH 7.6) (*12*) or squalene synthase (pH 7.2) (*30*).

The enzymes of isoprenoid biosynthesis have an almost universal requirement for Mg^{2+}. Exceptions include the Mn^{2+} dependent enzymes dehydrosqualene synthase (*31*), which catalyzes the synthesis of the C_{30} homologue of phytoene as the precursor to the C_{30} carotenoids in some bacteria, and eukaryotic PSases, including enzymes from *Lycopersicon esculentum* chloroplasts (*32*) and *Capsicum annum* (*12*). This trend extends to bacterial *E. herbicola* PSase-EEF. The enzyme has a strict dependence on Mn^{2+}, with a maximal activity observed at ∼0.25 mM $MnCl_2$. Importantly, no activity was observed when the enzyme was assayed in buffer containing a range of different Mg^{2+} concentrations (activity <2% of activity with 0.25 mM Mn^{2+}). These results differ from those described recently by Neudert et. al. (*14*) who reported activity for the recombinant *E. uredovora* PSase in the presence of Mg^{2+} (∼40% of the activity in the presence of Mn^{2+}). Given the extensive similarity between the bacterial enzymes (65% amino acid identity), it is unusual that they differ so significantly in their metal ion dependence. The discrepancy might be due to an artifact of the assay

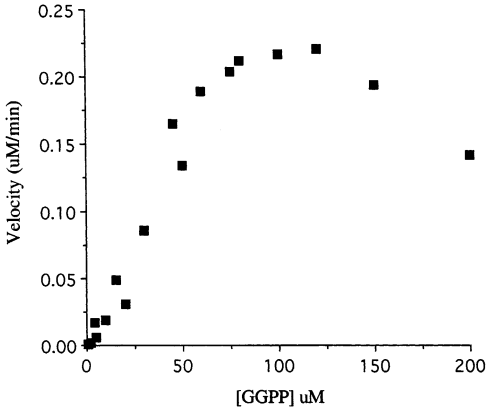


FIGURE 4: Initial velocity plot for recombinant PSase-EEF at different concentrations of GGPP.

employed in the analysis of the *E. uredovora* PSase. In those studies, detergent was not added to the assay buffer, and turnover was extremely inefficient. Assays required 15 h at 37 °C with stoichiometric levels of enzyme to obtain measurable product formation, resulting in a reported specific activity almost 20 000-fold lower than we observed for the *E. herbicola* protein. As discussed above, we believe that the detergent provides a hydrophobic reservoir to facilitate product release from the surface of the enzyme.

A plot of velocity versus the concentration of GGPP for *E. herbicola* PSase-EEF did not show the hyperbolic Michealis−Menten profile reported for the eukaryotic and *E. uredovora* enzymes (*12*). Instead, the rate of phytoene synthesis reached a maximum at ∼100 μM GGPP and then decreased at higher concentrations (Figure 4). S_{05} (the concentration required for half-maximal activity) was ∼35 μM. Similar behavior was reported for yeast squalene synthase (*30*).

In contrast, Neudert et.al. (*14*) reported Michaelis−Menten behavior for *E. uredovra* PSase with $K_M = 41$ μM, based on rates measured at GGPP concentrations between 2 and 10 μM. At these low substrate concentrations, the *E. herbicola* enzyme also gave linear double reciprocal plots of rate versus [GGPP] typical of Michaelis−Menten kinetics. However, distinctly nonlinear behavior was seen when the range of GGPP concentrations was extended beyond what was required for half-maximal velocity.

Early reports using crude preparations of PSase suggested that the enzyme requires ATP for activity (*33*, *34*). Similar claims were made recently for the *E. uredovora* protein (*14*). In contrast, we found no effect by ATP on the enzymatic activity of recombinant *E. herbicola* PSase-EEF. There is no mechanistic basis for an ATP requirement, and ATP is not required for the purified PSase from *C. annum* (*12*). In addition, yeast squalene synthase, which catalyzes a similar coupling of isoprene units in the sterol biosynthetic pathway, shows no dependence on ATP for activity, and furthermore, is capable of synthesizing the C_{30} analogue of *Z*-phytoene when incubated in the absence of its normal cosubstrate NADPH (*35*).

The Z- and E-central double-bond isomers of phytoene are both found naturally. *Z*-Phytoene is prevalent in higher plants, fungi, algae, and photosynthetic bacteria, while *E*-phytoene often predominates in nonphotosynthetic bacteria (*31*). Interestingly, in many organisms that produce *Z*-

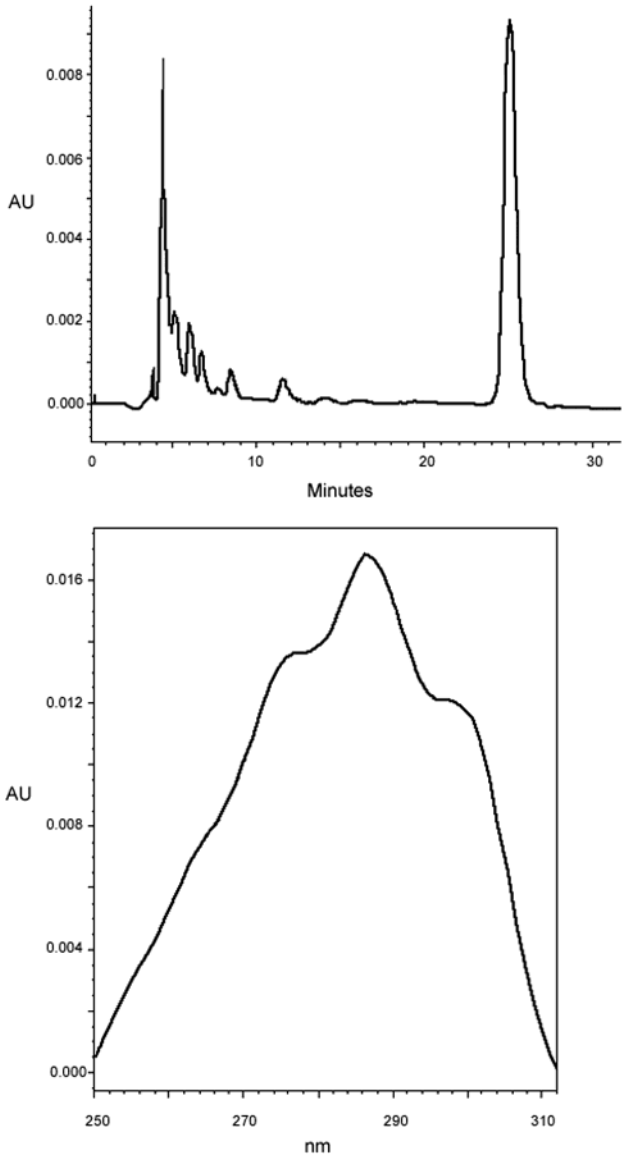


FIGURE 5: HPLC trace and UV spectrum of *Z*-phytoene produced from recombinant *E. herbicola* PSase-EEF.

phytoene, the carotenoid intermediates directly downstream from phytoene have an E−15−15′ double bond. It has generally been assumed that the E isomer of phytoene is required for phytoene desaturase, the next enzyme in the pathway. Speculation about formation of both double-bond isomers has included the existence of discreet *Z*- and *E*-phytoene synthases, the presence of a single enzyme with low stereochemical fidelity capable of producing both isomers, or a single enzyme specific for the Z isomer. In the latter case, interconversion of the two isomers has been proposed to occur through the action of a specific isomerase or by nonenzymatic photoisomerization.

Incubation of recombinant PSase with GGPP in the dark gave a single product that eluted sharply at ∼25 min upon reversed-phase HPLC (*36*). UV spectra measured throughout the elution of the peak were identical and had a maximum at 286 nm with inflection points at 276 and 297 nm (Figure 5), identical to that reported for the Z-central double-bond isomer of phytoene (15,15′-*Z*-phytoene) (*37*). When the product was exposed to white light (fluorescent lamp), the width of the HPLC peak at ∼25 min broadened considerably. UV spectra obtained at the beginning of the peak were similar to those for *Z*-phytoene before photoisomerization. Spectra collected from the latter part of the peak also had a maximum at 286 nm but also had clearly resolved bands at 276 and 298 nm characteristic of the E isomer (*14*, *37*). It is also possible that other double bond isomers of the central triene chromophore were present. In contrast to the report that both isomers are formed by recombinant *E. uredovora* Psase (*14*), we were unable to detect *E*-phytoene as a primary product from Psase-EEF. Given the similarity of the amino acid sequences for the *E. uredovora* and *E. herbicola* proteins and the widespread occurrence of *Z*-phytoene in nature, it is likely that the reported formation of the E isomer is an artifact. *Z*-phytoene is prone to photoisomerization (*34*), and inadvertent exposure to light during the unusually long incubation times required for the marginally active *E. uredovora* enzyme may be responsible for its formation.

The results presented here, together with evidence that PSase from tomato (*38*) and *Phycomyces blakesleeanus* (*39*) produce only the *Z*-phytoene, while the enzyme from *Mycobacterium* sp. produces only the E isomer (*40*), suggest that phytoene synthases have inherently high stereochemical fidelity. The structural genes for PSase in prokaryotic carotenoid biosynthetic gene clusters similar to *Erwinia* that have been sequenced (*40*−*43*) appear to encode proteins with similar structures. However, the stereochemistry of the central double bond in phytoene does not ultimately appear to be crucial. Synthesis of carotenoids in recombinant strains of *E. coli* with plasmids containing the carotenoid biosynthetic genes from *Erwinia* species (*41*, *42*) and *Agrobacterium aurantiacum* (*43*) proceeds to give downstream products with E−15,15′ double bonds. Since the host strains lack a dedicated carotenoid double bond isomerase, that activity is clearly not necessary for the Z/E isomerization. Recombinant phytoene desaturase from *E. uredovora* has been shown to efficiently utilize *Z*-phytoene as the substrate in the production of *E*-lycopene (*44*), demonstrating that the Z/E isomerization is catalyzed by phytoene desaturase. Related genes in *Arabidopsis thaliana* apparently do not have isomerase activity and require photoisomerization of intermediates for the pathway to produce the final carotenoid products (*45*).

Thus, it is likely that *Z*-phytoene is the sole product of PSase in a phylogenetically diverse group of organisms, while a smaller subset of organisms have an analogous enzyme that appears to be specific for synthesis of the E isomer. Interestingly, the stereoisomer of phytoene produced from PSase appears ultimately to be unimportant with respect to the stereochemistry of the 15−15′ double bond. In some organisms the Z → E isomerization can be efficiently carried out by phytoene desaturase, and in others the double bond is isomerized photochemically. It will be of particular interest from the context of both the evolution and the structural biology of PSase to obtain structures for the Z- and E-specific enzymes and to determine the differences in active-site architecture responsible for the observed stereochemical specificity.

Phytoene synthase catalyzes the first pathway specific reaction in the carotenoid biosynthetic pathway and controls flux into the pathway (*46*, *47*). In plants, the gene is under photochrome control as suggested by regulation of mRNA levels by light. (*48*−*50*). In *Mucor circinelloides* and the related carotenoid-producing fungus *Phycomyces blakesleea-*

nus, the genes for phytoene synthase, which catalyzes the first reaction in carotenoid biosynthesis, and lycopene cyclase, which catalyzes the third reaction, are fused to give an unusual bifunctional protein that catalyzes nonconsecutive steps in the pathway (*51*, *52*). The intervening step is catalyzed by phytoene dehydrogenase. The two closely linked genes, which encode the three enzymatic activities, are oriented in opposite directions, thereby providing an opportunity for joint regulation.

In summary, we have cloned the *crt*B gene of *E. herbicola* from a plasmid containing the carotenoid gene cluster and expressed it in *E. coli*. The encoded recombinant enzyme was purified to >90% homogeneity in two steps by ion exchange and immunoaffinity chromatography. Recombinant PSase-EEF requires Mn^{2+}, is stimulated by detergent, which presumably provides a hydrophobic reservoir for the water insoluble product, and stereoselectivly synthesizes 15,15′-*Z*-phytoene.

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