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An Efficient System for the Expression and Purification of Yeast Geranylgeranyl Protein Transferase Type I

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To purify the geranylgeranyl protein transferase type I (GGPT-I) efficiently, a gene expression system using the pGEX-4T-1 vector was constructed. The *cal*1 gene, encoding the β subunit of GGPT-I, was subcloned into the pGEX-4T-1 vector and co-transformed into *E. coli* cells harboring the *ram*2 gene, the α subunit gene of GGPT-I. GGPT-I was highly expressed as a fusion protein with glutathione S-transferase (GST) in *E. coli*, purified to homogeneity by glutathione-agarose affinity chromatography, and the GST moiety was excised by thrombin treatment. The purified yeast GGPT-I showed a dose-dependent increase in the transferase activity, and its apparent K_m value for an undecapeptide fused with GST (GST-PEP) was 0.66 μM and the apparent K_m value for geranylgeranyl pyrophosphate (GGPP) was 0.071 μM.

Keywords: Geranylgeranyl protein transferase type I, Glutathione S-transferase, Purification, Thrombin cleavage

Introduction

Prenylation is a class of lipid modification involving covalent addition of either farnesyl (15-carbon) or geranylgeranyl (20-carbon) isoprenoids to conserved cysteine residues at or near the C-terminus of proteins. Known prenylated proteins include Ras and Ras-related GTP-binding proteins (G proteins), the subunits of trimeric G proteins, protein kinases, and at least one viral protein. This prenylation process is now recognized as a prerequisite for the membrane anchoring and biological activities of a variety of cellular proteins. Ras proteins are farnesylated with a modification that is required for oncogenic forms of Ras to transform cells (Casey *et al.*, 1989; Hancock *et al.*, 1989; Schafer *et al.*, 1989). These studies dramatically stimulated interest in the field of protein prenylation (Gibbs *et al.*, 1991).

Three known enzymes catalyze isoprenoid addition to proteins: farnesyl protein transferase (FPT), geranylgeranyl protein transferase type I (GGPT-I), and geranylgeranyl protein transferase type II (GGPT-II). FPT and GGPT-I are closely related to each other and transfer a farnesyl group or a geranylgeranyl group from farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), respectively, to the cysteine residue of CaaX-containing proteins (Reiss *et al.*, 1990; Casey *et al.*, 1991; Moores *et al.*, 1991; Seabra *et al.*, 1991; Yokoyama *et al.*, 1991; Reiss *et al.*, 1992; Omer *et al.*, 1993, Ying *et al.*, 1994; Zhang *et al.*, 1994). When "X" is either serine, methionine, or glutamine, proteins are recognized by FPT, whereas a leucine at this position results in modification by GGPT-I (Casey *et al.*, 1991; Yokoyama *et al.*, 1991). GGPT-II transfers geranylgeranyl groups from GGPP to both cysteine residues of CC- or CXC-containing proteins in a process mechanistically distinct from that of the CaaX-containing proteins (Horiuchi *et al.*, 1991, Seabra *et al.*, 1992).

GGPT-I, like FPT, is a zinc metalloenzyme (Moomaw *et al.*, 1992). However, the activity of metal-depleted GGPT-I can be restored with Zn^{2+} alone unlike FPT which requires both Zn^{2+} and Mg^{2+} (Zhang *et al.*, 1995). As noted earlier, Mg^{2+} is absolutely required for FPT activity. The lack of requirement for Mg^{2+} exhibited by GGPT-I is surprising since other properties of FPT and GGPT-I are so similar, and highlights the importance of determining the precise role(s) for the metal ions in the function of both enzymes. The role of zinc is likely to be the same in both FPT and GGPT-I. As with FPT, the zinc of GGPT-I is required for the binding of the peptide substrate but for not the isoprenoid binding (Trueblood *et al.*, 1993; Bukhtiyarov *et al.*, 1995; Mitsuzawa *et al.*, 1995; Yokoyama *et al.*, 1995; Yokoyama *et al.*, 1995).

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GGPT-I binds GGPP and peptide substrates independently. The GGPT-I/GGPP complex can be isolated by gel filtration, and the isoprenoid can be released intact upon denaturation of the enzyme (Yokoyama *et al.*, 1993). A photoactivatable GGPP analog has been shown to crosslink to the β subunit of GGPT-I (Bukhtiyarov *et al.*, 1995). Thus, as with FPT, it is reasonable to assume that both substrate binding sites are localized predominantly in the β subunit of GGPT-I. Steady-state kinetic analysis of GGPT-I is consistent with a random-sequential mechanism but, as with FPT, the preferred kinetic pathway might be that the isoprenoid substrate first binds to the free enzyme. FPT and GGPT-I are generally quite selective for their substrates. However, cross-specificity has been observed, and such ability to modify alternate substrates may be of biological significance. If the purified GGPT-I can be shown definitively to contain the same α-subunit as FPT, it will then be important to study the mechanism to determine whether the requirements for GGPT-I activity are the same.

Previously, FPT was purified and characterized from bovine (Ryu *et al.*, 1995) in our lab. Also the pFlag vector was used to subclone the *ram*2 and *cal*1 genes (Kim *et al.*, 1996) for its convenience in purifying pFlag-label fused protein by anti-Flag M1 affinity gel chromatography. But, far less expression level of β subunit relative to α subunit is the problem of using this affinity column. Purification with anti-Flag M1 affinity column was tried and elution fractions were analyzed by Western blotting assay but only the α subunit band was detected.

In this paper, we resolve some problems of the expression and purification of yeast GGPT-I by using the recombinant pFlag vectors (Kim *et al.*, 1996), *cal*1 gene encoding the β subunit of GGPT-I was subcloned into a new pGEX-4T-1 vector and GGPT-I was expressed using this recombinant vector, and purified from glutathione-agarose affinity column.

Materials and Methods

Materials [^{3}H]geranylgeranyl pyrophosphate ([^{3}H]GGPP, 20 Ci/mmol) was purchased from Dupont-New England Nuclear research products (USA). GST-PEP, undecapeptide (GCVKIKKCAIL) fused with GST, was expressed in *E. coli* and purified using a glutathione-agarose column (Kim *et al.*, 1996). The pGEX-4T-1 vector was purchased from Pharmacia LKB Biotechnology (Sweden), and glutathione insolubilized on cross-linked 4% bead agarose, and molecular size markers were from Sigma (USA). DNA primers were synthesized by Bioneer, Inc (Korea). Isopropyl-β-D-thiogalactoside (IPTG), *Taq* polymerase, and Wizard PCR preps were purchased from Promega (USA). Restriction enzymes were purchased from Promega, NEB, and Kosco. Chemicals used were obtained from Sigma, Aldrich (USA), and Merck (Germany).

Amplification of *cal*1 gene by PCR The α and β subunits of yeast GGPT-I are encoded by *ram*2 and *cal*1 genes, respectively (Mayer *et al.*, 1992). Oligonucleotide primers (5′CGGGATCC-ATGTGTCAAGCTACCAAT-3′, 5′-GCGTCGACTCAAAAACA GCACCTTTT-3′) were designed to insert the *cal*1 gene between *Bam*HI and *Sal*I sites of the pGEX-4T-1 vector. The reaction mixture of PCR contained 1 μg of yeast chromosomal DNA, 2.5 units of *Taq* polymerase, 0.5 μM of each PCR primer, 0.4 mM of 4 dNTPs, and 3 mM of $MgCl_2$ in 100 μl of water. After 35 cycles of reaction, PCR products were analyzed by 0.8% agarose gel electrophoresis.

Subcloning of the *cal*1 gene into an expression vector pGEX-4T-1 The pGEX-4T-1 vector contains a *tac* promoter, an open reading frame encoding GST, a thrombin cleavage site and unique restriction endonuclease sites for *Bam*HI, and *Sal*I. After obtaining a 1.13 kb DNA fragment, this PCR product was digested with *Sal*I and *Bam*HI. pGEX-4T-1 was digested with *Sal*I and *Bam*HI. After purification of digested DNA samples by phenol/chloroform extraction and ethanol precipitation, the fragments were ligated by T4 DNA ligase, and transformed into the *E. coli* NM522 strain. Plasmids were isolated from *E. coli* transformants, digested with *Eco*RI and *Sal*I, and identified by 0.8% agarose gel electrophoresis. This recombinant plasmid was named pGCAL1.

Expression of yeast GGPT-I in *E. coli* The pGCAL1 plasmid was co-transformed into the *E. coli* NM522 strain with the pFCRAM2 plasmid (Kim *et al.*, 1996) and plated on an LB agar plate containing both ampicillin and chloramphenicol. *E. coli* transformants harboring both recombinant plasmids were cultured overnight at 37°C to OD_{600} 0.5. This culture was diluted 10-fold to 2 l fresh LB medium with ampicillin and chloramphenicol and was grown for 2 h at 37°C. After 0.5 mM IPTG was added to the culture, the culture was grown for 6 h at 25°C. After that, cells were harvested using Centrikon 6.14 rotor (5000 $\times$ g_{max}, 10 min) at 4°C and washed in PBS buffer (8 g NaCl, 0.2 g KCl, 1.15 g $Na_2HPO_4 \cdot 7H_2O$, 0.2 g KH_2PO_4) and resuspended in 4 ml PBS buffer. Cells were disrupted by sonication. After centrifugation for 10 min at 10,000 $\times$ g_{max} and 4°C, the supernatants were collected for further purification and GGPT-I activity assay.

Assay for GGPT-I activity GGPT-I activity was determined by the measurement of the amount of [^{3}H]geranylgeranyl transferred from [^{3}H]GGPP to GST-PEP. The standard reaction mixture contained the following components in a final volume of 50 μl: 25 μM $ZnCl_2$, 1 mM DTT, 1.78 μM GST-PEP, and 0.26 μM [^{3}H]GGPP (43,000 dpm/pmol). After 30 min incubation at 37°C, the reaction was quenched by adding 1 ml of 1 M HCl in ethanol to the reaction mixture. The quenched reaction mixtures were allowed to stand at room temperature for 15 min. After adding 2 ml of 100% ethanol, the mixtures were vacuum-filtered through Whatman 3M filters using a Millipore 1225 sampling manifold. Filters were washed four times with 2 ml aliquots of 100% ethanol, mixed with 5 ml of Triton X-100 and toluene cocktail, and counted in a scintillation counter.

Purification of yeast GGPT-I Two ml of 50% G-agarose was added to supernatant and mixed gently for 2 min at room temperature for binding. After a brief centrifugation at 1000 $\times$ g_{max}, beads were washed by adding 5 ml ice-cold PBS, mixing, and centrifuging for 10 s at 1000 $\times$ g_{max}. The wash was

repeated two more times. The beads were resuspended in 1 ml of ice-cold PBS and transferred to a 1.5 ml microtube, centrifuged for 10 s at 1000 × g_{max} to collect beads and the supernatant was discarded. The fusion protein was eluted by adding 500 μl of 50 mM Tris-Cl (pH 8.0) and 15 mM reduced glutathione, mixing gently for 2 min, centrifuging 10 s at 1000 × g_{max} and collecting the supernatant. Elution was repeated two more times with 400 μl and 300 μl eluting solutions each. Each fraction was assayed for GGPT-I activity and analyzed by 10% SDS-PAGE.

Removal of GST carrier by thrombin cleavage The G-agarose beads (bearing fusion protein) were washed twice with 20 volume of 1% Triton X-100 in PBS. The beads were equilibrated by washing once with wash buffer (50 mM Tris·Cl (pH 7.5), 150 mM NaCl), then washed once with thrombin cleavage buffer (2.5 mM $CaCl_2$ in wash buffer). The beads were resuspended with one ml of cleavage buffer, 1% thrombin was added and incubated for 1 h at 25°C. Released protein was recovered by washing beads with 1 bed volume of wash buffer. Eluted proteins were assayed for GGPT-I activity and analyzed by 10% SDS-PAGE.

Characterization of yeast GGPT-I To determine the optimum pH and substrate dependency of yeast GGPT-I, an activity assay of this enzyme was conducted with the same reaction conditions as described above except the pH of sodium, HEPES, and concentration of [^{3}H]GGPP, or of GST-PEP.

Results and Discussion

Two different expression vectors were used for expressing yeast GGPT-I, which is a heterodimeric protein (Mayer *et al.*, 1992). Subcloning into a new vector system (pGEX-4T-1) was designed to surmount the problems of expressing and purifying GGPT-I from the pFlag vector system (Kim *et al.*, 1996). In the pFlag vector system, a few amount of fusion proteins were expressed, and purification was not efficient. The pGEX-4T-1 vector was selected as an expression vector for the *cal*1 gene because of its advantages of efficiency and rapidity of purification by G-agarose affinity gel and easy removal of the carrier protein by thrombin or factor Xa. The *cal*1 gene was subcloned into pGEX-4T-1 vector, this recombinant plasmid, pGCAL1 was digested with several restriction enzymes and identified with 0.8% agarose gel electrophoresis (Fig. 1). The two recombinant plasmids, pFCRAM2 (Kim *et al.*, 1996) and pGCAL1, were co-transformed into *E. coli* NM522. The *E. coli* cells containing both recombinant plasmids were selected by two antibiotics — ampicillin and chloramphenicol. The selected cells containing both recombinant plasmids were induced with 0.5 mM of IPTG. Cell lysates in various conditions were compared by SDS-PAGE (Fig. 2(a)). On induction with 0.5 mM IPTG at 25°C for 6 h, an induced protein bands were observed (Ram2 α-subunit: 45 kDa, GST-CalI β-subunit: 76 kDa) (Fig. 2(a), lane 2,3). To decrease the level of inclusion body formation, induction was carried out at a lower temperature (25°C) for a longer time. It was reported that the higher temperature (37°C) promotes inclusion body formation and the lower temperature (25°C) inhibits the formation of inclusion bodies (Lee *et al.*, 1993). The fusion proteins were contained in the supernatant after sonication and purified by the glutathione-agarose affinity chromatography (Fig. 2(a), lane 4–8). After the treatment of the fused proteins with thrombin, the sample only gave the two bands expected for Ram2 α-subunit (45 kDa) and the CalI (48 kDa) β-subunit, respectively (Fig. 2(b), lane 3), and had a high level of GGPT-I activity (data not shown).

The calculation of specific activity relative to the total protein amounts by Bradford assay (Bollag *et al.*, 1996) shows a definitely increased value during purification. Table 1 summarizes the quantitative results of this purification system. Purified GGPT-I had a specific activity of 2.95 U/mg protein with a 32-fold purification of the enzyme by one chromatography step. The purified enzyme was isolated in 73% yield and stored in elution buffer containing 20% glycerol at −80°C until needed.

To determine the optimum pH of yeast GGPT-I, sodium HEPES buffer was used. The enzyme showed an optimum pH range between 6.9 and 7.1 (data not shown), almost the same as reported earlier (Kim *et al.*, 1996). Michaelis constants for yeast GGPT-I were calculated by fitting hyperbolic plots of initial velocity versus GST-PEP concentration, in the presence of a fixed concentration of [^{3}H]GGPP as shown in Fig. 3(a). A Lineweaver–Burk plot of the initial velocity against GST-PEP is also presented (Fig. 3(b)). The apparent K_m value was 0.68 μM. The reaction to determine the [^{3}H]GGPP dependency of yeast GGPT-I was carried out for 30 min, and GGPP limiting conditions were avoided by reducing enzyme amounts (Fig. 4(a)). A Lineweaver–Burk plot of the initial velocity

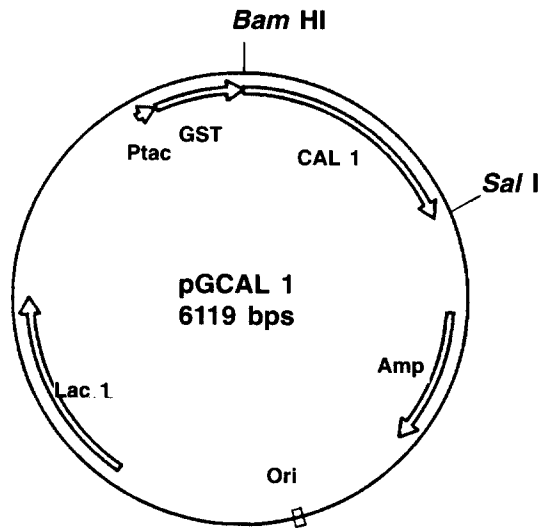

Fig. 1. Restriction enzyme map of pGCAL1.

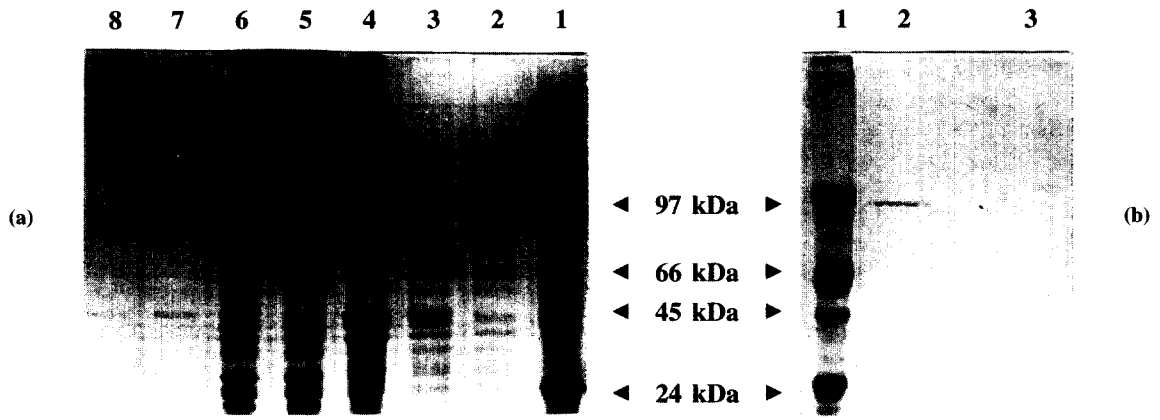

Fig. 2. Analysis of recombinant GGPT-1 by SDS-PAGE (10%). (a) Lane 1: Protein size marker (97, 66, 45, 24 kDa). Lane 2: *E. coli* lysates before IPTG induction. Lane 3: *E. coli* lysates after IPTG induction. Lane 4: Soluble fraction after ultrasonic disruption of cells. Lane 5: Cell pellet after ultrasonic disruption of cells. Lane 6: Unbound protein. Lane 7: 1st elution fraction released by reduced glutathione. Lane 8: 2nd elution fraction released by reduced glutathione, (b) Lane 1: Protein size marker (97, 66, 45, 24 kDa). Lane 2: Purified GST fused GGPT-I. Lane 3: Thrombin treated purified GGPT-1. One μg of each protein from *E. coli* ((a) lane 2–5), GST-GGPT-I after glutathione agarose bead purification ((a) lane 6–8), and purified GGPT-I after thrombin treatment was applied to an SDS-PAGE and Coomassie blue staining.

Table 1. Purification of *yeast* GGPT-I from recombinant *E. coli*.

Fraction	protein (mg)	Specific Activity (U/mg)	Total Activity (U)[a]	Purification (fold)	Recovery (%)
Cytosol	901.2	0.092	1.50	1	100
GST-GGPT-I	40.47	1.601	0.74	17.40	80.5
GGPT	20.52	2.95	0.51	32.06	73.1

[a] One unit (U) of enzyme activity is the amount of enzyme that transfers 1 pmol of [^{3}H]geranylgeranyl from [^{3}H]GGPP to acid-precipitable GST-PEP per h under standard conditions. Protein concentration of the various fractions was determined as described in Matherials and Methods.

Table 2. Steady-state kinetic parameters for the recombinant GGPT-I.

Substrate	K_m (μM)	V_{max} (pmol/min)	K_{cat} (min^{-1})	K_{cat}/K_m ($M^{-1} min^{-1}$)
GST-PEP	0.68	0.95	1.112	1.635×10^6
GGPP	0.071	0.34	0.394	5.549×10^6

of yeast GGPT-I against [^{3}H]GGPP was also presented (Fig. 4(b)). The apparent K_m value was 0.071 μM. The value of the steady-state kinetic parameters for GST-PEP and GGPP are shown in Table 2. The value we measured for V_{max} for yeast GGPT-I was approximately an order of magnitude larger than that determined previously for the mammalian enzyme (Zhang *et al.*, 1994). The K_m value for GGPP is much lower than that for GST-PEP. The second-order rate constant, k_{cat}/K_m, and not K_m alone, is the true measure of the specificity of a substrate for an enzyme. As we obtained the purified enzyme, we could measure the k_{cat}/K_m value. The specificity constant, k_{cat}/K_m, of geranylgeranyl protein transferase is much lower than other enzyme-substrate pairs. Compared to other enzymes, GGPT-I is a very slow enzyme. Thus, there is a possibility that allosteric activators may affect GGPT-I activity *in vivo*. It was reported that substrate binding to the enzyme occurs in random order (Mitsuzawa *et al.*, 1995). But a recent study has shown that the mechanism is random in principle but ordered in practice, since the preferred catalytic pathway is through the enzyme/GGPP binary complex (Mitsuzawa *et al.*, 1995). The yeast system provides a powerful tool for investigating the structure and function of GGPT-I. Given the sequence and biochemical similarities between yeast and mammalian GGPT-I, results from yeast GGPT-I would be useful for the understanding of human GGPT-I.

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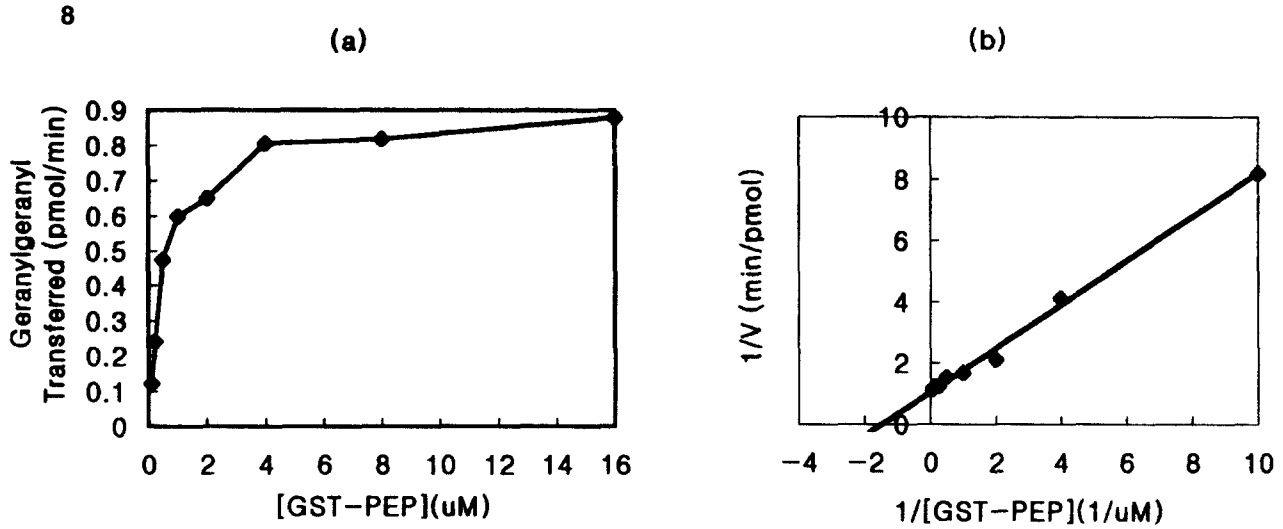

Fig. 3. The effect of GSP-PEP concentration on GGPT-I activity. (a) Michaelis–Menten plot, (b) Lineweaver–Burk plot of GST-PEP. Each reaction mixture contained 0.8 μg of GGPT-I. The reactions were carried out for 30 min under the standard reaction condition except for variations in GST-PEP concentration.

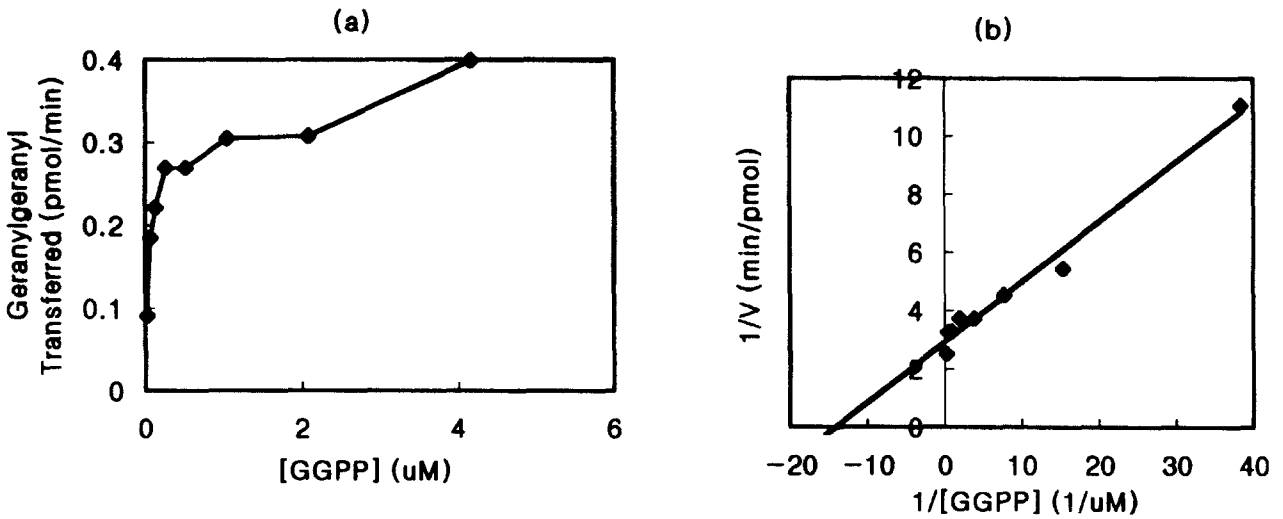

Fig. 4. The effect of [^{3}H]GGPP concentration on GGPT-I activity. (a) Michaelis–Menten plot, (b) Lineweaver–Burk plot of [^{3}H]GGPP. Each reaction mixture contained 0.8 μg of GGPT-I. Reactions were carried out with the indicated amount of GGPP under the standard condition described in Matherials and Methods.

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