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The first nonradioactive fluorescence assay for phosphatidylglycerol:prolipoprotein diacylglyceryl transferase that initiates bacterial lipoprotein biosynthesis

Srividhya Sundaram, Sanchari Banerjee, Krishnan Sankaran *

Centre for Biotechnology, Anna University, Chennai 600 025, India

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ABSTRACT

The unique and physiologically vital bacterial enzyme, prolipoprotein diacylglyceryl transferase (Lgt), which catalyzes the committed first step in the posttranslational transfer of diacylglyceryl group from phosphatidylglycerol to the prospective N-terminal cysteine of prolipoproteins, remains to be characterized for want of a simpler but equally sensitive nonradioactive assay. We, for the first time, report a coupled enzymatic fluorescence assay for Lgt using the de novo synthetic peptide substrate MKATK SAVGSTLAGCSSHHHHHH. The assay is based on the conversion of the by-product, glycerol-1-phosphate, to dihydroxyacetone using an alkaline phosphatase–glycerol dehydrogenase combination and estimating the fluorescence of the coupled reduction of resazurin to resorufin. The minimum amount of glycerol-1-phosphate, and hence the modified peptide, detected by this method is approximately 20 pmol, thereby making this assay a promising alternative to the radioactive assays. The assay is rapid, more convenient, less laborious, and suitable for purification and characterization of Lgt.

More than 15,000 posttranslationally modified lipoproteins have been either identified or predicted in both gram-positive and gram-negative bacteria. These lipoproteins perform essential functions in structural integrity, nutrient uptake, environmental signal transduction, cell division, sporulation, conjugation, protein secretion, adhesion, and antibiotic resistance [1]. They are synthesized as precursors with tripartite signal sequence, with a characteristic C-terminal "lipobox" (consensus sequence [LVI] [ASTVI] [GAS] [C]) [2]. During maturation, the invariant Cys sequentially undergoes diacylglyceryl modification catalyzed by phosphatidylglycerol:prolipoprotein diacylglyceryl transferase (Lgt),[1] signal sequence cleavage at the modification site catalyzed by lipoprotein-specific signal peptidase (Lsp II), and N-acylation of the partially lipid-modified Cys catalyzed by apolipoprotein *N*-acyl transferase (Lnt) [3]. The resultant *N*-acyl-*S*-diacylglyceryl-Cys anchors bacterial lipoproteins to the cell membrane at the N-terminal end so that bulk of the protein efficiently functions in the aqueous compartment. Recently, the role of Sec (secretory) and TAT (twin arginine translocase) pathways in differentially recognizing the slow- and fast-folding lipoproteins, respectively, for lipid modification has been identified [4]. This unique adaptation at the hydrophobic–aqueous interface by bacteria has potential in protein engineering applications, including enzyme-linked immunosorbent assay (ELISA), liposomal integration, targeted drug delivery, and biosensors [5].

Of the three enzymes, Lgt is most significant because it catalyzes the first committed step of the unique and ubiquitous pathway seen only in bacteria. This enzyme, associated on the cytosolic side of the inner membrane, catalyzes the characteristic transfer of diacylglyceryl moiety from phosphatidylglycerol (PG) to the thiol group of lipobox Cys. The 31.6-kDa [6] enzyme is essential for the growth and viability of gram-negative organisms [7]. Lgt mutants of gram-positive bacteria were viable but showed physiological deficiencies and attenuated virulence [8–11]. In-depth study of the unique activity of Lgt has been hindered due to its low abundance, difficulty to overexpress by recombinant methods, poor stability, and lack of simpler nonradioactive assays. The original radioactive assay developed for Lgt using a synthetic peptide corresponding to the N-terminal 24 amino acids of the prototype prolipoprotein, the precursor of the most abundant murein lipoprotein, led to the discovery of Lgt, its preference for negatively charged phospholipid substrate, particularly PG, and the actual pathway [3]. The fate of glycerol-1-phosphate released in the Lgt reaction is not yet known, but homologues of glycerol-1-phosphate dehydrogenase (G1PDH) genes in gram-positive *Bacillus subtilis* predict the possible use of glycerol phosphate in the synthesis of glycerophospholipid backbone [12]. Comparison of primary

* Corresponding author. Fax: +91 44 2235 0299.
E-mail address: ksankran@yahoo.com (K. Sankaran).

[1] *Abbreviations used*: Lgt, prolipoprotein diacylglyceryl transferase; Sec, secretory; TAT, twin arginine translocase; PG, phosphatidylglycerol; G1PDH, glycerol-1-phosphate dehydrogenase; IMV, inverted membrane vesicle; OG, β-octyl glucopyranoside; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; GDH, glycerol dehydrogenase; LB, Luria–Bertani; RFU, relative fluorescence units; WE, water extract; CPM, counts per minute; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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sequences of Lgt from phylogenetically distinct species of bacteria—*Escherichia coli, Salmonella typhimurium, Haemophilus influenza,* and *Staphylococcus aureus*—revealed a conserved 103-HGGLIG-108 motif, possibly involved in catalysis [6]. Site-directed mutagenesis of all the histidine residues and seven of the tyrosine residues and chemical modification of Lgt with diethyl pyrocarbonate (DEPC) implicated His103 and Tyr235 in Lgt activity [13]. The enzyme is very basic (deduced p*I* of 10.4), with an abundance of Arg residues in gram-negative organisms and an equal abundance of both Lys and Arg residues in gram-positive organisms.

The inability to precipitate hydrophilic peptide substrates and their diacylglyceryl-modified product by 90% acetone saturated with ammonium sulfate in the original peptide assay led to the development of a generic radioactive assay for Lgt employing a hydrophilic peptide synthesized de novo [14] and the differential paper-electrophoretic separation of PG (negatively charged and radioactive), unmodified substrate (positively charged and nonradioactive), and lipid-modified product (positively charged and radioactive) owing to their opposite charges. This assay led to the discovery that *E. coli* Lgt is superficially associated with the inner leaflet of inner membrane by hydrophobic interactions. Although low abundance of the enzyme and the inability to overexpress were major disadvantages in its isolation, water extraction of the inverted membrane vesicle (IMV) provided a convenient enriched source of the enzyme [14]. Very recently, it has been shown using engineered lipo-EGFP (enhanced green fluorescent protein) that Sec- or TAT-bound prolipoproteins could be the actual substrates of Lgt in vivo [4].

Both the radioactive assays indeed helped to unravel important characteristics of Lgt, but the serious disadvantages associated with radioactive assays have hindered the kinetic characterization and purification of this interesting enzyme. Therefore, we have developed a new, enzymatically coupled fluorescence assay for Lgt by estimating the by-product of the reaction, glycerol-1-phosphate. The newly developed nonradioactive assay is shown to be as sensitive and reliable as the radioactive assays.

Materials and methods

All fluorescence measurements were made with the BioTek Synergy HT multimode microplate reader at λ_{ex} = 550 ± 12.5 nm and λ_{em} = 590 ± 15 nm.

Reagents

The following chemicals were used in the study. The synthetic peptide MKATKSAVGSTLAGCSSHHHHHH was dissolved in 0.1 N HCl at 5 mg/ml concentration and stored in aliquots at −20 °C. PG, glycerol-1-phosphate, β-octyl glucopyranoside (OG), and resorufin were obtained from Sigma Chemical. NAD^+, NADH, glycerol, resazurin, diaphorase of *Clostridium* sp. (500 U), and alkaline phosphatase of calf intestinal mucosa (200 U) were purchased from Sisco Research Laboratories. [9,10] Palmitate (50 Ci/mmol) was purchased from GE Healthcare.

Buffers used in the study were as follows: TED buffer (20 mM Tris–HCl, pH 8.0, containing 5 mM ethylenediaminetetraacetic acid [EDTA] and 4 mM dithiothreitol [DTT]), TBS buffer (20 mM Tris–HCl, pH 8.0, containing 0.9% [w/v] NaCl), TED–NaCl buffer (20 mM Tris–HCl, pH 8.0, containing 5 mM EDTA, 4 mM DTT, and 0.5 M NaCl), 100 mM Tris–HCl buffer (pH 8.0), 100 mM sodium carbonate–bicarbonate buffer (pH 10.0), and 0.1 M sodium acetate buffer (pH 4.5).

Phosphatidylglycerol was solubilized in TED buffer containing 1% OG. Glycerol dehydrogenase (GDH) and diaphorase were dissolved in 100 mM Tris–HCl, and alkaline phosphatase was dissolved in 100 mM sodium carbonate–bicarbonate buffer. All of the enzyme reagents were stored at −20 °C. NAD^+, NADH, glycerol-1-phosphate, resazurin, and resorufin were dissolved in water and stored at −20 °C for approximately a month. PG was also extracted from *S. aureus.*

Design of fluorometric assay

The new fluorescence assay was designed based on the sensitive enzymatic estimation of glycerol-1-phosphate released in Lgt-catalyzed reaction. The sequential steps (Fig. 1) in the assay are as follows:

(i) Lgt catalyzes the transfer of diacylglyceryl moiety from PG to the thiol group of the invariable cysteine in the lipobox of the protein/peptide substrate, releasing the modified prolipoprotein/peptide and glycerol-1-phosphate;
(ii) glycerol-1-phosphate is cleaved in the presence of alkaline phosphatase into glycerol and inorganic phosphate (P_i);
(iii) the released glycerol undergoes dehydrogenation in the presence of NAD^+ and GDH, forming dihydroxyacetone and $NADH^+H^+$; and
(iv) the NADH is then coupled to diaphorase-catalyzed reduction of resazurin to resorufin (λ_{ex} = 550 nm and λ_{em} = 590 nm), a highly fluorescent product.

The resulting fluorescence will be directly proportional to the amount of glycerol-1-phosphate formed in the reaction catalyzed by Lgt and, hence, will reflect the Lgt activity.

Assay components

Partial purification of GDH from E. coli DH5α

The extraction and partial purification of GDH was carried out as described previously [15] but with alterations in the buffers used. *E. coli* DH5α was grown overnight in 1.5 L of Luria–Bertani (LB) medium, harvested by centrifugation, and washed twice with 0.9% NaCl. The pellet was resuspended in 0.1 M Tris–HCl (pH 7.5) and sonicated at 15,000 psi. The turbid supernatant was centrifuged at 12,000*g* for 45 min to obtain the cytosolic fraction, which was heated at 70 °C for 90 min to get enriched heat-stable protein preparation. After removing the denatured protein by centrifugation at 12,000*g* for 20 min, the enzyme present in the supernatant (because the enzyme is heat stable) was precipitated with 90% ammonium sulfate. The pellet was dissolved in 50 mM Tris–HCl (pH 7.5) to a final protein concentration of 10 mg/ml and dialyzed against the same buffer for 16 h at 4 °C to remove residual ammonium sulfate. The enzyme extract was then stored in aliquots at the final protein concentration of 5 mg/ml at −20 °C.

Kinetic characterization of GDH preparation

The activity of GDH was determined by measuring the absorbance of NADH at 340 nm during the enzymatic reaction (15 min at 37 °C) with 10 mM glycerol and 1 mM NAD^+ in 100 mM sodium carbonate–bicarbonate buffer (pH 10.0). The K_M of GDH for glycerol was measured by varying it from 25 to 500 mM with 1 mM NAD^+, and the K_M for NAD^+ was measured by varying it from 0.5 to 50 mM with 500 mM glycerol. Using a Lineweaver–Burk plot of the kinetic data, the K_M values of *E. coli* GDH for glycerol and NAD^+ were found to be 95 and 2.3 mM, respectively. The best preparations had a specific activity of 74 nmol/min/mg protein. The preparation was stable for approximately a week when stored at −20 °C.

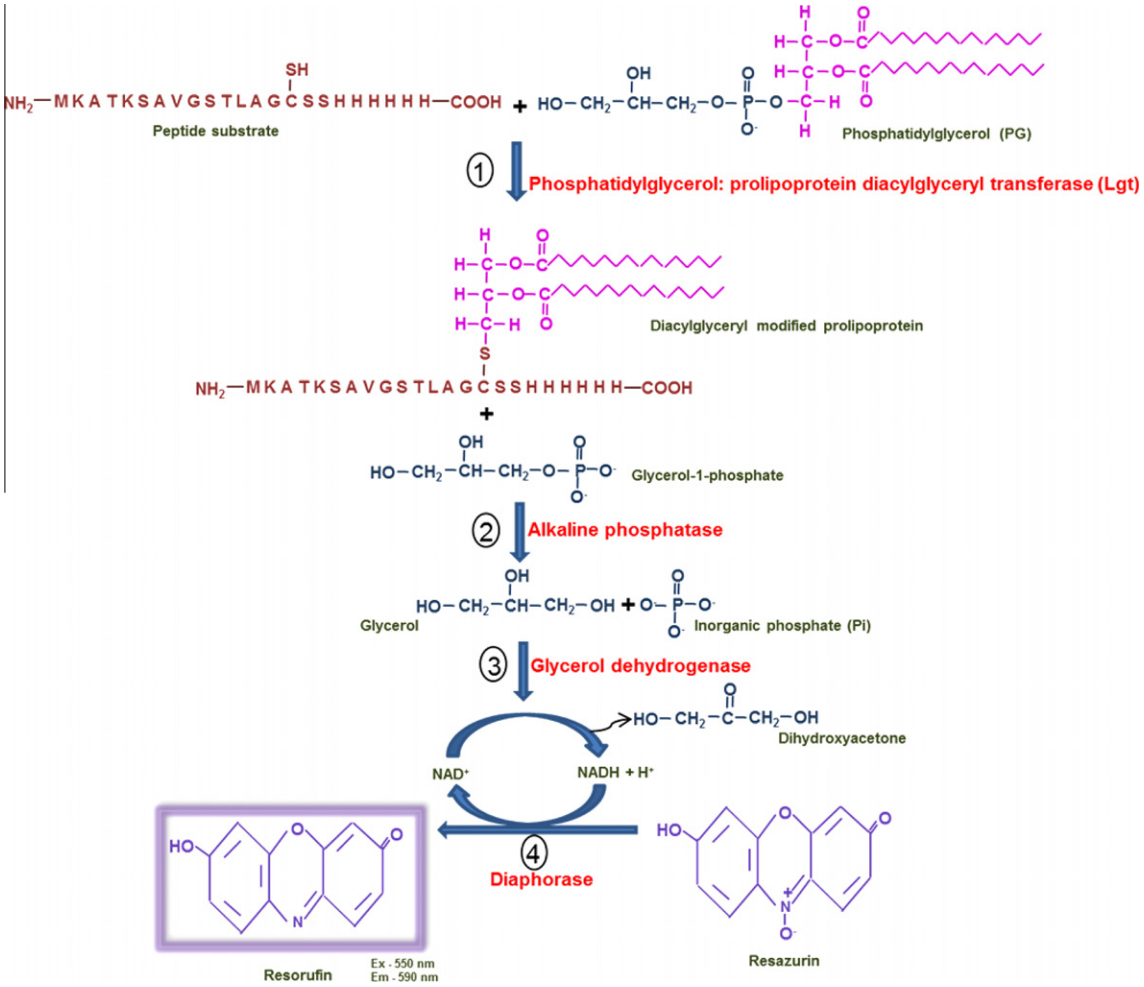


Fig.1. Schematic representation of steps involved in the new fluorometric assay for Lgt.

Estimation of glycerol-1-phosphate using GDH preparation: partial reaction in Lgt assay

The high K_M of GDH for glycerol demanded high enzyme concentration in the assay mix to estimate glycerol-1-phosphate in the range of 20 to 500 pmol. Here, 150 μg of partially purified GDH along with 0.1 U of alkaline phosphatase, 10 mM NAD^+, 0.2 U of diaphorase, and 1 mM resazurin in TED buffer was found to be optimal for the estimation of glycerol-1-phosphate. After 15 min of incubation at 37 °C in the dark, fluorescence was measured in a fluorescence microplate reader, where 200 relative fluorescence units (RFU) corresponded to 1 pmol of resorufin.

Preparation of IMV and its extraction with water as Lgt source

The preparation of IMV and water extract (WE) of Lgt was based on the method described by Selvan and Sankaran [14]. *E. coli* BL21(DE3) cells were used as host for hyperexpressing Lgt in the plasmid pT7GT under the T7 promoter system. Host cells containing T7GT and the vector control plasmids were grown in 1.5 L of LB medium overnight, with 100 μg/ml ampicillin selection. After harvesting the cells, the pellet was washed twice with 150 ml of TBS buffer, suspended in 20 ml of TE buffer (pH 8.0) containing 0.5 M NaCl, and passed twice through French press at 15,000 psi. After removing the debris by low-speed centrifugation at 3000*g* for 5 min, the turbid supernatant was centrifuged at 1,25,000*g* for 1 h. The IMV pellet obtained in this manner was suspended in TED buffer to a final protein concentration of 5 mg/ml and stored as 50-μl aliquots at −80 °C. The water-soluble fraction of Lgt (WE) was prepared by suspending the IMV pellet thoroughly in double distilled water at a protein concentration of 5 mg/ml and incubating the suspension on ice for 1 h, which was then centrifuged at 1,00,000*g* for 1 h. The supernatant was used as the water-soluble fraction of IMV, and the pellet, suspended in the same volume of TED buffer, served as the water-insoluble fraction of IMV.

Preparation of radioactive PG from S. aureus

S. aureus, which has approximately 70% PG in its total phospholipid content [16], was inoculated in nutrient broth and allowed to grow at 37 °C. At A_{600nm} = 0.3, 10 μCi/ml [9,10] palmitate (specific activity = 50 Ci/mmol) was added and grown for another 3 h at 37 °C. The radiolabeled cells were then harvested and suspended in 0.3 ml of TE buffer (pH 8.0) and extracted with 2:1 chloroform:methanol according to the Bligh and Dyer method [17]. The chloroform phase containing PG was aspirated into a fresh tube; radioactivity was counted in a liquid scintillation counter, and then the phase was dried in aliquots. Whenever required, the dried residue was suspended in TED buffer to the final concentration of 100,000 counts per minute (CPM)/μl [14].

Coupled fluorescence assay

Diacylglyceryl modification of peptide by Lgt

The assay for Lgt was performed in a total volume of 100 μl containing 20 μl of 5x TED buffer, 40 μM peptide, 200 μM PG, 0.1% (w/

v) OG, and IMV (50 μg of protein) or WE (10 μg of protein), GDH (150 μg of protein), 10 mM NAD^+, 0.2 U of diaphorase, and 1 mM resazurin. IMV or WE from the vector control served as negative control. After 20 min of incubation at 37 °C in the dark, fluorescence was measured in a fluorescence microplate reader. The activity of Lgt enzyme was converted from relative fluorescence units to reaction velocity (v) (i.e., picomoles of peptide converted per minute) using the resorufin standard graph.

Tricine SDS–PAGE of the modified and unmodified peptide

Tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [18] of the reaction mix demonstrates slower mobility of lipid-modified peptide (product) over unmodified peptide (substrate). The gel was prepared with the separating portion with 16.5% acrylamide with 6% crosslinking and the stacking portion with 5% acrylamide and 3% crosslinking. Lgt assay mix containing 200 μM PG, 40 μM peptide, and TED buffer with different dilutions of the WE–Lgt as enzyme source was mixed with sample solubilizing buffer after 20 min of the reaction time and 80 μl of the assay mix were loaded in the tricine gel. The electrophoresis was performed at a constant voltage of 100 V until the dye front touched the bottom of the gel. Gels were stained by the silver staining method [19].

Radioactive assay for diacylglyceryl modification of peptide

The radioactive assay and paper-electrophoretic separation of assay components were performed as described by Selvan and Sankaran [14]. The assay mix contained 40 μM peptide, PG corresponding to 200,000 counts, 0.1% OG, and IMV or WE of Lgt corresponding to 50 or 10 μg, respectively. After incubation at 37 °C for 20 min, 2.5 μl of this mix (corresponding to 20,000 counts) was applied at the center of 19 × 0.6-cm Whatman No. 2 paper strips wetted with 100 mM Tris borate buffer (pH 8.2) and electrophoresed at 500 V for 1 h. The strips were then dried at room temperature. From the air-dried strip, a 1-cm-long piece was cut at 1.5 cm away in the cathodic direction from the center (which corresponds to modified peptide) and counted in a liquid scintillation counter.

Functional characterization of Lgt

Determination of K_M and V_{max}

To determine the linearity between enzyme amount and activity, fluorescence change was measured at different enzyme concentrations varying from 10 to 50 μg for IMV in a 100-μl reaction volume and from 2 to 10 μg for WE in a 100-μl reaction volume. For both fluorescence and radioactive assays, to determine the K_M and V_{max} for the peptide, it was varied from 10 to 80 μM at a constant PG concentration of 200 μM. To determine the K_M for PG, it was varied from 25 to 250 μM at a constant peptide concentration of 40 μM.

Thermal and pH inactivation studies

The effect of temperature on Lgt activity was assessed by performing the assay with IMV and WE fractions preincubated at 50 and 80 °C for 15 min. In addition, to validate the effect of pH, the assay was performed with 0.1 M sodium acetate buffer (pH 4.5) and 0.1 M sodium carbonate–bicarbonate buffer (pH 10.0). Both fluorometric and radioactive assays were performed in parallel.

Results

The radioactive assays of Lgt based on the direct estimation of the amount of tritiated palmitate incorporated into the synthetic peptide substrate after reaction with the enzyme preparation and palmitoyl-labeled PG were not convenient for the kinetic characterization and purification of this unique enzyme and its interesting enzyme activity. Hence, an enzymatic fluorescence assay based on the estimation of the by-product, glycerol-1-phosphate, after converting it to glycerol and oxidizing it to dihydroxyacetone in the presence of NAD^+ with concomitant reduction of resazurin to highly fluorescent resorufin had been developed. It has been shown below that the assay was comparable to the radioactive assay in terms of sensitivity but better suited for higher throughput needed for kinetic characterization and purification.

The fluorescence measurement of resorufin could determine 1 pmol of the final product

Because resorufin is the ultimate product of the coupled enzymatic resazurin reduction assay, the linearity and limit of sensitivity of the assay were determined as a function of the amount of resorufin expected under the assay conditions. As shown in Fig. 2, the fluorescence of resorufin was linear in the broad range of 1 to 1000 pmol and permitted the measurement of a minimum of 1 pmol of the substance. Although this was the lowest practical

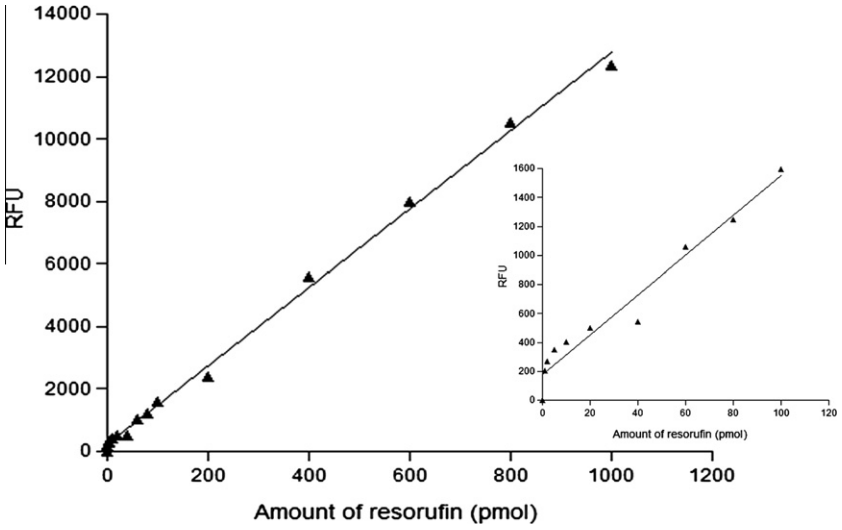


Fig.2. Standard graph of resorufin. The fluorescence response due to resorufin was linear from 1 to 1000 pmol, indicating its utility in developing a sensitive optical assay for Lgt. The inset shows the useful range of 1 to 100 pmol under the assay conditions for Lgt.

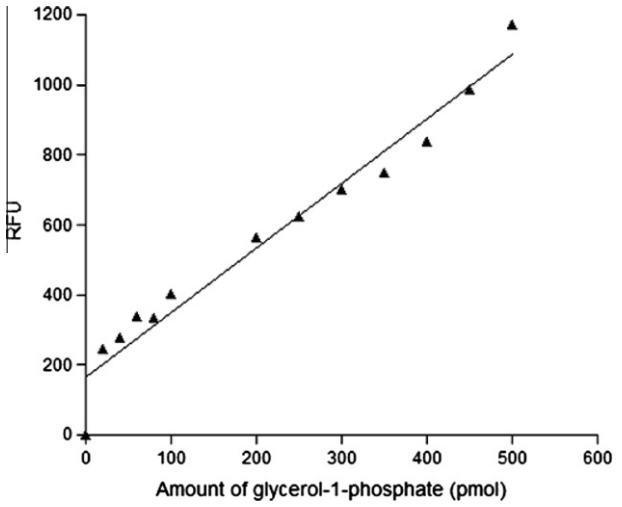


Fig.3. Standard graph of glycerol-1-phosphate using the new method. The minimum amount of glycerol-1-phosphate that could be estimated with the new assay is approximately 20 pmol, and the range covers the initial velocity conditions for Lgt.

limit, suitable for the measurement of the amount of glycerol-1-phosphate, expected from the Lgt reaction of 4000 pmol of the substrate, the actual sensitivity was actually dependent on the coupling of alkaline phosphatase, GDH, and diaphorase activities in measuring glycerol-1-phosphate released.

The detection level of glycerol-1-phosphate was as low as 20 pmol under the assay conditions used for measuring Lgt activity

The sensitivity of the new assay, therefore, was determined using glycerol-1-phosphate standard and measuring the fluorescence after the enzyme-coupled dye reduction under the conditions used in the Lgt assay. The increase in fluorescence was linear in the range of 20 to 500 pmol of glycerol-1-phosphate (Fig. 3). For 4000 pmol of peptide, this would correspond to the conversion of 0.5 to 12.5% of the substrate, the initial velocity conditions. In other words, the amount of Lgt required to convert 1% of the substrate in 15 min can be measured under the standardized assay conditions. Under the same assay conditions, 1 pmol of glycerol and 1 pmol of NADH could be estimated (data not shown), showing that these do not limit the overall sensitivity.

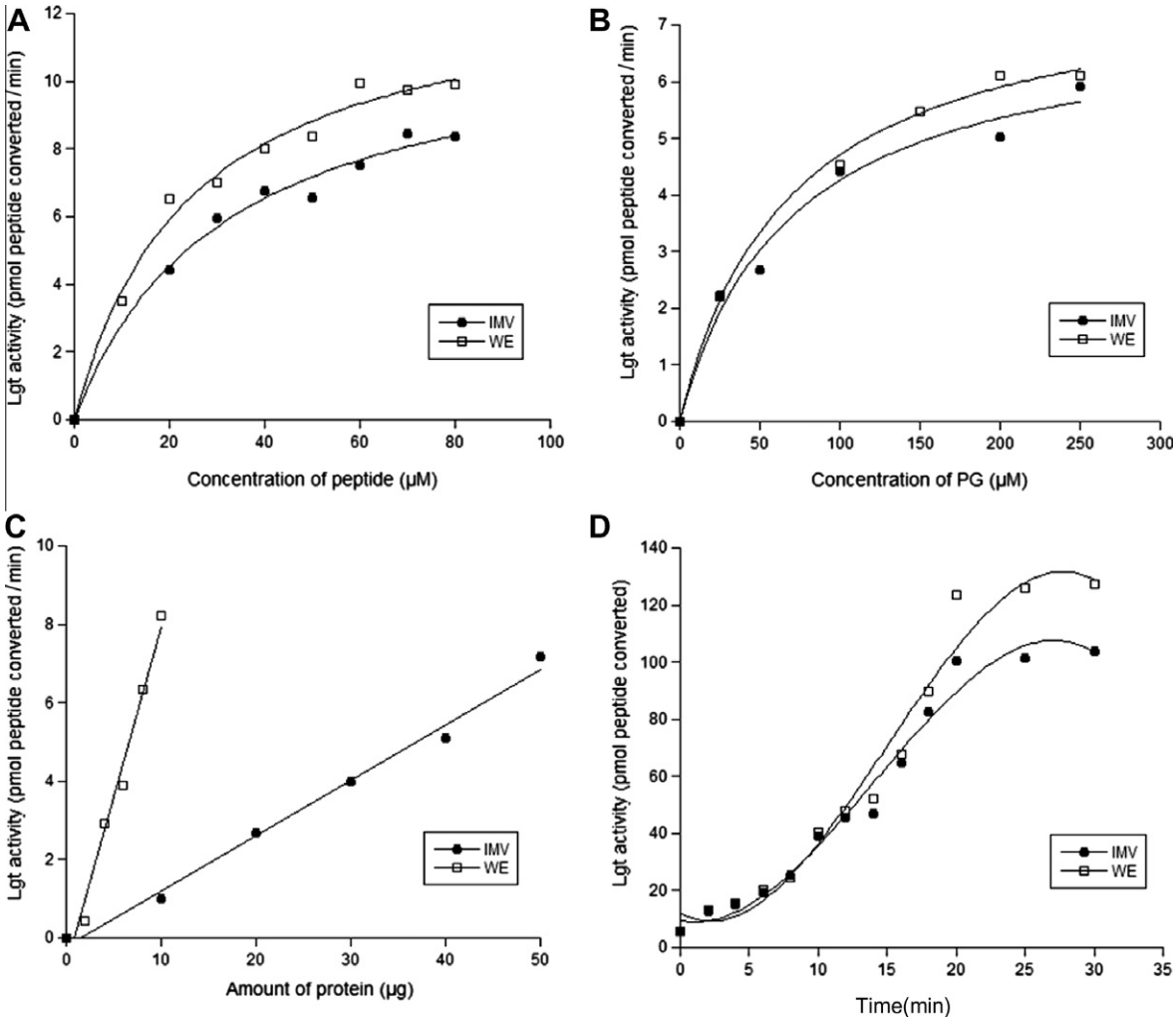


Fig.4. Effects of peptide substrate concentration (A), PG substrate concentration (B), amount of enzyme (C), and time (D) on the reaction rate of Lgt in both IMV and WE preparations. The assay conditions are as described in Materials and Methods. The Michaelis–Menten kinetics with respect to the two substrates is apparent and reproducible in terms of K_M already reported. The rate of the reaction increased linearly as a function of enzyme concentration, with the WE showing 5 times higher specific activity compared with IMV. The Lgt reaction with respect to time (panel D) was linear for 30 min, unlike the abrupt cessation of reaction at 20 min as reported in the radioactive assay. The rate of the reaction (panel D) was similar for both IMV (5 pmol/min) and WE (6 pmol/min).

Basic kinetic characteristics matched with what has been established by radioactive methods

The new assay was tested against the known kinetic behavior of the enzyme as established by the radioactive methods. The activity of Lgt with respect to both lipid and peptide substrate showed the typical Michaelis–Menten behavior, with the activity linear up to 40 μM peptide and 200 μM PG (Figs. 4A and B). The rates increased linearly in the range, where the primary reaction catalyzed by Lgt remained rate limiting for the coupled system. V_{max} of WE–Lgt was 823 pmol/min/mg protein, whereas V_{max} of IMV–Lgt was 143 pmol/min/mg protein, indicating that the specific activity of the WE–Lgt was approximately 5 times that of IMV–Lgt (Fig. 4C). The Lgt reaction was seen to progress linearly beyond 20 min, unlike what was reported in the radioactive method, but began to stop by 30 min (Fig. 4D).

The lipid-modified peptide showed a clear shift in the movement on a tricine gel

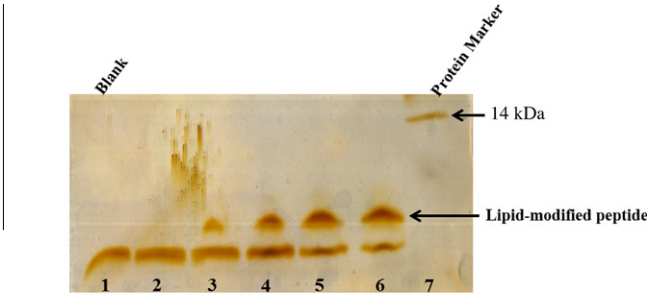
Because the fluorescence enzymatic assay is an indirect measure of Lgt, a direct demonstration of the correlation needed to be established. The tricine gel analysis of the modified and unmodified peptides provided the direct evidence. As can be seen from Fig. 5, the silver staining revealed the product, modified peptide, as a retarded band appearing as a result of Lgt activity. The intensity of the retarded band increased as the radioactive and fluorescence intensities increased with the product formation. The sensitivities of the fluorescence and radioactivity were comparable, with the latter being slightly more sensitive.

Comparable kinetic constants derived from both fluorescence and radioactive assays for Lgt

To establish the applicability of the newly developed nonradioactive assay for the kinetic characterization and purification of Lgt, both the fluorescence and radioactive assays were performed in parallel and the results compared. The kinetic constants derived from rate of reaction versus substrate concentration (peptide or PG), as shown in Table 1, revealed a good match between the two assays employing crude IMV and enriched or partially purified WE. As can be seen from Table 1, the values for basic kinetic parameters (K_M and V_{max}) of Lgt for the peptide as well as PG matched those obtained by the radioactive assay performed in parallel. In addition, both fractions of the enzyme, membrane bound and WE, showed identical kinetic behavior (Table 1), indicating nearly 100% recovery, a fact readily verifiable with the radioactive assay results. In our previous study using the radioactive assay, we had reported nearly identical affinity of Lgt for the peptide and PG substrates [14], unlike the results obtained in the current study. The K_M for PG was higher for both of the Lgt preparations, IMV and WE, as confirmed by both fluorescence and radioactive assays performed simultaneously.

Matching results on thermal and pH stabilities from radioactive and fluorescence assays

In another comparative test between the radioactive and fluorescence methods to establish the general applicability of the newly developed assay, heat and pH inactivation of the two enzyme preparations, IMV and WE, were performed; the results are shown in Table 2. By both radioactive and fluorescence assays, IMV and WE preparations of Lgt exhibited maximum activity at 37 °C and pH 8.0 [14]. As seen from the values in Table 2, the enzyme lost most of its activity when pretreated at 50 °C and pH 10.0. In addition, complete abolition of activity was observed at pH 4.5. The same behavior was observed for both membrane-bound and WE fractions in the radioactive assay reported.



	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
RFU	678	1547	2181	3014	3897
CPM	1556	3551	5006	6919	8946

Fig.5. Tricine SDS–PAGE analysis of the Lgt reaction. In this standard analysis, the lipid-modified peptide appeared as the retarded band, and its intensity after silver staining shows a progressive increase with a concomitant decrease in the intensity of the peptide substrate as a function of enzyme concentration. Evidently, fluorescence and radioactivity associated with lipid modification in respective assays are proportional to enzyme concentration (lanes 3–6). The sensitivities of radioactive and fluorescence assays are comparable, with the former being slightly more sensitive than the latter. Both radioactive and fluorescence assays are more sensitive than silver staining.

Discussion

The three major turnover events of PG in gram-negative bacteria are its conversion to cardiolipin, the transfer of glycerol-1-phosphate to the membrane-derived oligosaccharides, and the transfer of diacylglyceryl group to N-terminal cysteine of prolipoproteins [20] by Lgt that initiates the bacterial lipid modification pathway. None of the enzymes of PG turnover is essential for cell division and growth except Lgt [21]. Lgt is the sole member of transferases known to transfer diacylglyceryl moiety of PG, and the novelty of Lgt action, although intriguing, has remained only partially investigated for its substrate specificity, membrane localization, and basic kinetics using difficult radioactive assays [3,14]. It is to be noted that, due to the difficulty of working with this low-abundant enzyme and not-easy-to-assay conditions, the biochemical characterization of Lgt has not been reported elsewhere. The commercial potential in comprehensive lipid modification in a single enzymatic step led us to develop the first laboratory bioreactor (prototype) containing immobilized *E. coli* Lgt for in vitro lipid modification of proteins and peptides. For deeper understanding of this interesting enzyme and its exploitation for commercial applications, we needed a simpler optical assay, but one as sensitive as the radioactive assay, to save on the expensive peptide/PG substrates.

The fluorescence assay is comparable to radioactive assay in terms of sensitivity and reliability

A number of fluorescence assay methods with reagents such as iodoacetamido fluorescein, dansyl aziridine, monobromobimane, dansyl chloride, and 4-amino fluorescein have been attempted (unpublished results) for either the direct measurement (modified peptide) or indirect measurement (unmodified peptide) of Lgt activity. But all of these methods were not suitable owing to problems of low sensitivity or poor stability of the fluorogenic reagents or interference from by-products. Because coupled enzymatic

Table 1
Kinetic constants of Lgt for peptide and PG substrates: Comparison of fluorescence and radioactive assays.

	Peptide				PG			
	IMV		WE		IMV		WE	
	K_M (μM)	V_{max} (pmol/min)	K_M (μM)	V_{max} (pmol/min)	K_M (μM)	V_{max} (pmol/min)	K_M(μM)	V_{max}(pmol/min)
Fluorescence	32	12	24	13	69	7	68	8
Radioactive	13	12	10	13	59	15	52	16

Table 2
Fluorescence and radioactivity associated with heat-inactivated, pH-inactivated enzyme reactions and reaction blanks.

	Fluorescence (RFU)				Radioactive (CPM)			
	Clone		Vector control		Clone		Vector control	
	IMV	WE	IMV	WE	IMV	WE	IMV	WE
Control (37 °C, pH 8.0)	982	1302	87	102	1256	2989	58	96
Δ50 °C	112	125	21	42	42	56	32	35
Δ80 °C	26	3	36	25	78	34	28	55
pH 4.5	21	26	15	47	85	73	51	47
pH 10.0	165	142	62	93	112	142	67	47
Peptide blank	95	88	34	61	52	59	46	46
PG blank	118	124	101	87	64	87	69	34
Enzyme blank	48	65	26	48	52	51	49	44

methods have been reported and are commercially available for glycerol estimation, we attempted to extend such a method to the measurement of glycerol-1-phosphate, which is the other product of Lgt reaction. To match the sensitivity of the radioactive assay with minimal use of the peptide substrate, we needed to improve the detection limit of glycerol-1-phosphate by coupling the reaction to a fluorogenic dye with high molar extinction coefficient and emission peak in a region where there is minimal background signal or autofluorescence [22]. Resazurin is a redox-sensitive and stable water-soluble dye whose highly fluorescent reduced form, resorufin, has been used effectively to measure cell death [22] and viability and drug resistance [23] as well as to perform analysis of triglycerides in serum [24], diagnostic procedures of tuberculosis (TB) specimens [25], and high-throughput analysis of lipases and esterases [26]. By employing three sequential enzymatic reactions catalyzed by alkaline phosphatase, GDH, and diaphorase, subsequent to Lgt action, we were able to develop the reported fluorescence assay for Lgt. The K_M and V_{max} of each enzyme reagent was measured (results not shown) to determine the optimal coupling conditions in facilitating the detection of Lgt activity under initial velocity conditions. In terms of sensitivity, even 0.5% conversion of the 4 nmol of the peptide normally used, which is as low as 20 pmol of the modified peptide, could be detected.

The coupled enzymatic fluorescence assay points to product inhibition by glycerol-1-phosphate

The fluorescence assay exhibited two features not seen in radioactive assay when the Lgt reaction was measured as a function of time. The initial lag for 5 min and the extension of the linearity beyond 20 min with gradual cessation after 30 min appear to be the consequence of coupled enzymatic reactions. However, on performing the assay in two stages (i.e., by adding the coupling enzymes along with the dye at stipulated time intervals during Lgt reaction), the cessation could be observed at 20 min, indicating product inhibition by glycerol-1-phosphate. In other words, the use of glycerol-1-phosphate by alkaline phosphatase in the coupled single-step assay may be providing some relief, resulting in the reaction extending and gradually stopping by 30 min. When the assay was performed in two steps, Lgt reaction at 37 °C followed by enzymatic coupling reaction at 50 °C, the optimal temperature of commercial *Cellulomonas* GDH at which Lgt is inactivated, cessation was observed after 20 min, supporting the inference of product inhibition by glycerol-1-phosphate (data not shown). The initial lag until 5 min was found to be due to the accumulation of substrate required for the detectable activity of alkaline phosphatase given that the assay of alkaline phosphatase as a function of time using commercially available glycerol-1-phosphate showed the same lag during the initial 5 min (data not shown).

With respect to the kinetics of Lgt, comparable K_M values of IMV (32 μM) and WE (24 μM) fractions indicated similar affinity of both fractions for peptide substrate. These values did not differ much from the reported K_M values for the hydrophilic peptide (6.1 μM) [14] (radioactive assay data in this study) and the hydrophobic peptide (30 μM) [13] determined by radioactive assays. For PG substrate, however, higher K_M values of 69 and 68 μM were observed for IMV–Lgt and WE–Lgt, respectively. These values are approximately 5-fold higher than the previously reported values of 10 μM [13] and 9.6 μM [14]. However, the kinetic constants derived from radioactive assays performed in parallel closely match the current data. Direct evidence for the reliability of the fluorescence assay, however, was provided by comparing the results of the fluorescence and radioactive assays with the gel analysis of the reaction products for peptide modification as a function of enzyme concentration.

The easier and more convenient fluorescence assay will now aid the detailed study of Lgt, a unique enzyme of great interest to enzymologists and protein engineers

Developing a cost-effective yet sensitive assay was the major aim of this study, and thus the assay was optimized from that angle. One of the assay components, GDH, is commercially available from *Cellulomonas* sp. (specific activity = 100 U/mg protein), where 0.01 U/assay will suffice. However, the assay needed to be performed in two steps because the optimal temperature of *Cellulomonas* GDH is 50 °C, whereas for Lgt it is 37 °C. Hence, we resorted to using *E. coli* GDH. Owing to its high thermal stability, a simple enrichment step by heating the *E. coli* lysate for 90 min at 70 °C provided the second enzymatic reagent. Despite the simple preparation, the problems encountered with *E. coli* GDH are

its shorter shelf life (50% activity loss after 1 week in −20 °C) and the need to use a greater amount (150 μg of protein) of approximately 0.07 U for each reaction. In the final analysis, however, the easier extraction procedure of GDH from *E. coli* and the availability of cost-effective components such as NAD^+, diaphorase, alkaline phosphatase, and resazurin served as major advantages of this assay, especially in replacing the difficult and cumbersome radioactive methods.

In our recent study, we reported that, in vivo, the translocation of lipoproteins could be either Sec dependent (slow-folding lipoproteins) or TAT dependent (fast-folding lipoproteins), and it was postulated that Sec-bound (unfolded proteins) or Tat-bound (folded proteins) prolipoproteins are in fact the substrates for lipid modification by Lgt [4]. However, in vitro, Lgt is shown to modify free peptide/prolipoprotein substrates. The mechanistic reason for this behavior of Lgt has not yet been investigated. This property, however, may be attributable to the physiological necessity of the low-abundant Lgt to efficiently modify during translocation approximately 100 different lipoproteins per cell, with some of them such as murein lipoprotein being present in very high numbers and some others being present in very low abundance. The comparison of in vitro conversion of peptide substrates containing both Sec and TAT signals with lipobox would be an interesting study using this simple assay.

The availability of this generic and rapid assay will aid not only such mechanistic studies but also the purification of the enzyme, screening for its potent inhibitors as possible antibacterials, and immobilization for protein engineering applications. The method is readily adaptable for high-throughput screening of Lgt clones in a 96-well format and is now being used by our group for biochemical characterization and purification of Lgt.

Conclusion

We have developed a new fluorescence-based assay for Lgt based on the estimation of glycerol-1-phosphate in which direct measurement of resorufin, in a series of enzymatic reactions, would be related to its activity. The assay is an ideal method for further characterization of this significant enzyme and analysis of inhibitors.

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