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A kinetic spectrophotometric method for the determination of pyridoxal-5′-phosphate based on coenzyme activation of apo-D-phenylglycine aminotransferase

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ABSTRACT

A new PLP assay method based on the coenzyme activation of apo-D-phenylglycine aminotransferase (apo-D-PhgAT) is reported. The assay process is comprised of two steps. First, PLP present in plasma samples is allowed to reconstitute apo-D-PhgAT, forming active holo-D-PhgAT. In the second step, the enzymatic activity of reconstituted D-PhgAT is determined using D-4-OH-phenylglycine as the amino donor substrate with 4-OH-benzoylformate (OH-BZF) as the reaction product. OH-BZF absorbs UV light strongly at 334 nm (molar absorption coefficient = 25.4×10^3 $M^{-1}cm^{-1}$) and its rate of formation is monitored spectrophotometrically. The rate of the transamination reaction catalyzed by the reconstituted D-PhgAT is directly proportional to the amount of PLP in the sample. The method is applicable for determining PLP in the concentration range from 5.2 to 250 nM and requires 50 μL of plasma sample. The mean within- and between-run coefficient of variations (CVs) were 8.1% and 12.4%, respectively. Analytical recoveries ranged from 98 to 108%. The assay was specific and showed good correlation with the established method (CDC, Method No: 4002.05). The assay requires one reaction catalyzed by a single enzyme, does not require a radioactive substrate, and a derivatization reagent is not needed. This PLP determination process is relatively simple to perform and can be completed using common laboratory equipment.

1. Introduction

Vitamin B_6 comprises a group of 6 interrelated vitamers which include pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM), and their phosphorylated derivatives, pyridoxine-5′-phosphate (PNP), pyridoxal-5′-phosphate (PLP), and pyridoxamine-5′-phosphate (PMP) [1–3]. Each of these vitamers can be converted to PLP [4,5], the biologically active form of vitamin B_6. PLP functions as a coenzyme for large numbers of essential enzymatic reactions in the human body including synthesis, interconversion, degradation of amino acids and biogenic amine, as well as several other metabolic pathways. PLP is not only required for all transamination but also for a wide variety of metabolic reactions catalyzed by PLP-dependent enzymes [6–9]. The lack of vitamin B_6 precursors is known to cause or be associated with several diseases. Although vitamin B_6 deficiency is a rare condition, it causes serious neurological symptoms including neonatal epileptic encephalopathy (NEE) [10]. According to reports from the US National Health and Nutrition Examination Survey (NHANES), vitamin B_6 deficiency was found to be closely associated with neurological abnormalities, such as depression and neurocognitive dysfunction [11]. In addition to neurological symptoms, anemia, steroid hormone function, immune function, atherosclerosis [12], as well as type I diabetes [13] were associated with vitamin B_6 deficiency. Decreased plasma PLP concentration has also been observed in several pathologic conditions including rheumatoid arthritis [14–16], cystic fibrosis [17] and various types of cancer [18–22]. Over the past 30 years, intensive clinical investigations have been performed to uncover the link between vitamin B_6 metabolism and a wide range of cancers and the possibility of using vitamin B_6 metabolic status as a prognostic factor in cancer patients [22].

Plasma PLP has been proposed to be an indicator of vitamin B_6 status [23,24] necessitating the need for precise methods for analysis of plasma PLP concentrations. Several methods have been developed for plasma PLP measurement. The fluorometric method is based on the reaction between semicarbazide and the PLP-protein Schiff base to form a fluorescent PLP semicarbazone [25]. In the radio-enzymatic assay, PLP present in the sample activates the apo-tyrosine decarboxylase which then converts [^{3}H]tyrosine to [^{3}H]tyramine. In another method,

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activated tyrosine decarboxylase releases $^{14}CO_2$ from the substrate [^{14}C]tyrosine. Radioactive products formed in these latter two methods are extracted and monitored to determine PLP levels [26,27]. In 2002, a non-radioactive enzymatic assay for plasma PLP was developed using apo-homocysteine-α,γ-lyase (apo-rHCYase). PLP in plasma samples reconstitutes rHCYase to an active form. In the presence of the substrate homocysteine (L-HCY) active rHCYase yields H_2S which can then combine with chromogenic reagents and be monitored spectrophotometrically [28]. PLP determination methods based on reconstitution of PLP-dependent enzymes, such as those utilizing apo-tyrosine decarboxylase, could be combined with high-performance liquid chromatographic (HPLC) to facilitate analysis. Products from the enzymatic reactions are separated by HPLC and detected electrochemically [29] or spectrofluorometrically [30]. Plasma PLP and other vitamin B_6 vitamers can be analyzed using HPLC with post-column derivatization and spectrofluorometric detection [31]. Highly sensitive methods for quantification of PLP in biological samples using advanced instrumentation, such as HPLC-tandem mass spectrometry (HPLC-MS/MS) [32], and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) [33], have also been developed and utilized.

The sensitive PLP assay methods currently available typically require complicated instrumentation and other sophisticate equipment that have high operational costs. Here, we report the development of an enzymatic method for the determination of PLP based on coenzyme activation of apo-D-phenylglycine amino transferase (apo-D-PhgAT) which is sensitive, specific, rapid, simple to perform, utilizes common laboratory instruments, non-radioactive, and requires no added chromogenic reagent.

2. Materials and methods

2.1. Chemicals

Pyridoxal-5′-phosphate (PLP) and pyridoxine hydrochloride (PN) were purchased from Sigma-Aldrich (St Louis, MO, USA); pyridoxamine-5′-phosphate (PMP) was from Fluka; pyridoxal hydrochloride (PL) and pyridoxamine dihydrochloride monohydrate (PM) were from Tokyo Chemical Industry Co., LTD.

2.2. Preparation of D-phenylglycine aminotransferase (D-PhgAT)

D-phenylglycine aminotransferase (D-PhgAT) used in this study was the D-PhgAT-(Q444E)-H_6C variant constructed in our laboratory [34], and for simplicity it is subsequently referred to as D-PhgAT. D-PhgAT was overexpressed in a recombinant host and the enzyme was purified according to the published procedures [34]. To obtain the purified D-PhgAT by using a single step purification, *E. coli* Tuner (DE3) pLysS harboring the plasmid carrying the gene construct for C-terminus hexahistidine tagged enzyme was used. The recombinant *E. coli* Tuner (DE3) pLysS was pre-cultured in 5 mL LB broth containing 50 μg/mL ampicillin and 34 μg/mL chloramphenicol at 37 °C with 200 rpm shaking to mid-log phase (OD600 ~0.8). Each of 250 μL of the pre-culture was transferred to 250 mL LB medium containing 50 μg/mL ampicillin and 34 μg/mL chloramphenicol in a 1000 mL baffled flask. The culture was incubated at 37 °C with 200 rpm until OD600 reached 0.6, then IPTG was added to a final concentration of 0.4 mM and the culture was further incubated for 4 h at 25 °C with slow shaking at 100 rpm. The induced cells were harvested by centrifugation at 4868 ***g***, 4 °C for 5 min (Sorvall RC 5C, SLA1000). To lyse the cells, the induced cell pellets were re-suspended in 9 volumes of lysis buffer (20 mM Tris–HCl, pH 7.6, 0.01% v/v β-mercaptoethanol and 50 μM PLP). The cells were subjected to disruption by ultrasonication (Vibra cell, Sonics & Materials Inc. USA). Sample tubes were placed in an ice-bath throughout the process and the disruption was carried out in the cycles of 10 s burst with 10 s intermittent cooling. The cell debris was removed by centrifugation at 11,227 ***g*** (Sorvall RC-5C, SS-34) for 45 min at 4 °C. The clarified cell lysate was purified in one step purification by immobilized metal ion chromatography (IMAC). The BD TALON™ (BD Biosciences) cobalt-based IMAC resin was packed in a chromatographic column (Tricorn 10/150, GE Healthcare) to a bed volume of 10 mL. The column was pre-equilibrated with 50 mM Tris-HCl buffer pH 7.6 containing 2.5 μM PLP and 100 mM NaCl. Sixty mL of the cell lysate was loaded onto the column through a static loop at a flow rate of 1 mL/min (ÄKTApurifier, GE Healthcare Bio-Sciences AB). Then, 3 bed volumes of buffer were applied to the column to wash out the unbound proteins at a flow rate of 1 mL/min. The D-PhgAT bound to the column was eluted with a linear gradient of imidazole from 0 to 60 mM in 15 bed volumes. The purity of the enzyme was monitored by SDS-PAGE. Activity of D-PhgAT was assayed using the method previously described [35].

2.3. Preparation of apo-D-PhgAT

Apo-D-PhgAT was prepared as previously reported [36] with modifications. In brief, the purified D-PhgAT was incubated with 10 mM L-glutamate in 50 mM Tris-HCl (pH 7.6) at 30 °C for 30 min to convert PLP to the PMP form of the coenzyme, which is no longer covalently-linked to the D-PhgAT protein. PMP released from the D-PhgAT was removed using an Amicon Ultra-15 10 K ultrafiltration device under 4000 rpm (3756 ***g***) centrifugation, at 10 °C for 15 min (Hettich Rotina 380R Centrifuge, UK). The filtrate containing free PMP was discarded and the retentate containing primarily apo-D-PhgAT was washed by gently mixing with 10 ml of 20 mM NaH_2PO_4 (pH 6.6) and ultrafiltration was repeated. The washing step was repeated 3 more times to ensure complete removal of PMP. One additional wash was performed with 50 mM Tris-HCl (pH 7.6) containing 1 mM EDTA. The concentrated apo-D-PhgAT solution was transferred to a fresh tube and kept in an ice box prior to use in the PLP assay.

2.4. Measurement of PLP in holo- and apo-D-PhgAT

During the preparation of apo-D-PhgAT, the PLP content was monitored using the chlorite post-column derivatization HPLC method of Rybak and Pfeiffer [37] with modifications as described below.

2.5. Ethics statement

All experimentation with human subjects were approved and certified by Mahidol University Central Institutional Review Board (MU-CIRB), Bangkok, Thailand (Protocol No. 2016/101.2106).

2.6. Preparation of plasma samples

Blood samples from 50 healthy donors were aseptically collected into Vacutainer Tubes containing EDTA (BD Vacutainer® K_2EDTA). The blood was immediately centrifuged (2600 ***g***, 10 min) to obtain plasma. Each plasma sample was aliquoted into three microcentrifuge tubes and stored at −80 °C until utilized for analysis. All plasma samples were protected from light during preparation and storage.

2.7. D-PhgAT reconstitution activation method for PLP determination

The method based on PLP activation of apo-D-PhgAT was applied for the determination of PLP concentration in plasma samples. The assay process is comprised of two steps. First, the reconstitution step, 50 μL of varying concentrations of standard PLP solution (5, 12.5, 25, 50, 125, and 250 nM) or plasma was mixed with 20 μL of 0.2 mg/mL of the apo-D-PhgAT and 430 μL of 10 mM citrate-phosphate buffer, pH 5.4. The mixtures were then incubated at 30 °C for 60 min allowing the PLP in the sample to combine with apo-D-PhgAT to form the holo-D-PhgAT. Second, the enzymatic reaction step, the activity of the reconstituted holo-D-PhgAT was determined using a kinetic spectrophotometric