HHS Public Access

Author manuscript

Results Chem. Author manuscript; available in PMC 2023 December 21.

Published in final edited form as:

Results Chem. 2023 December ; 6: . doi:10.1016/j.rechem.2023.101044.

Facile immobilization of pyridoxal 5′-phosphate using p-diazobenzoyl-derivatized Sepharose 4B

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Abstract

Pyridoxal 5′-phosphate (PLP) is a ubiquitous and versatile cofactor utilized by numerous enzymes involved in amino acid biosynthetic pathways. Immobilized PLP is a valuable tool to isolate unknown PLP-dependent enzymes in nature or to perform *in vitro* selection or directed evolution on existing or *de novo* PLP-dependent enzymes. The C-6 position is preferred for covalent immobilization of PLP because it maintains all important functional groups in their native, unmodified form. Previously reported diazonium derivatization methods for C-6 immobilization utilized an azide linker compound that is hazardous and not readily available. Here we report a safer and more accessible method to synthesize p-diazobenzoyl-derivatized Sepharose 4B using the *N*-hydroxysuccinimide (NHS) ester chemistry. The derivative was used to immobilize PLP, and the resulting C-6 immobilized PLP had a loading of ~2.6 μmol PLP per mL of resin, comparable to commercially available products of other immobilized cofactors.

Keywords

Pyridoxal 5′-phosphate (PLP); Cofactor immobilization; Bioconjugation; NHS ester chemistry; p-Diazobenzoyl derivatized resin

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CRediT authorship contribution statement

Kun-Hwa Lee: Methodology, Investigation, Formal analysis, Visualization, Writing – original draft. **Mark D. Distefano:** Methodology, Validation, Writing – review & editing. **Burckhard Seelig:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rechem.2023.101044.

Introduction

Pyridoxal S′-phosphate (PLP), commonly known as vitamin B_6, is a versatile cofactor crucial for enzymatic reactions in essential metabolic pathways, including amino acid biosynthesis. Approximately 4% of all known enzymatic activities utilize PLP as a cofactor, and 1.5% of all genes encoded in prokaryotic genomes encode PLP-dependent enzymes [1]. Most PLP-dependent enzymes utilize PLP by forming a Schiff base (internal aldimine). Subsequently, the enzyme coordinates the substrate to facilitate external aldimine formation between PLP and the substrate for the catalysis [2]. Notable reactions catalyzed by PLP-dependent enzymes include transamination, α- and β-elimination, and decarboxylation. These reactions are crucial for amino acid biosynthetic pathways and *de novo* synthesis of other cofactors such as biotin, folate, and NAD^+ [3-5].

The immobilization of PLP on solid supports enables the selective purification of PLP-binding proteins by affinity chromatography. Putative PLP-dependent enzymes make up ~20% of the human genome and ~29% of the *Bacillus subtilis* genome, many of which have not been experimentally characterized [5,6]. Affinity chromatography with immobilized PLP facilitates the confirmation of putative PLP-binding proteins and enables the search for additional previously undiscovered PLP-binding proteins. Furthermore, immobilized PLP is also crucial for the directed laboratory evolution of proteins for their ability to bind to PLP. For example, directed evolution techniques such as mRNA display can be used to select and evolve protein binders, yet they require an immobilized target to enrich for target binders [7].

Several immobilization methods for PLP on solid supports have been described (Table 1). However, all previous approaches had one or more serious drawbacks. The simplest method for covalent PLP immobilization is to utilize the formyl group of PLP, as it can form a Schiff base with an amine group on the solid phase. Further reduction of the Schiff base results in a stable secondary amine linkage formation [8,9]. However, this method renders the formyl group no longer accessible for interaction with PLP-dependent enzymes.

Ikeda et al. reported C-6 immobilized PLP, hydroxy-3 immobilized PLP and N-1 immobilized PLP methods using bromo-acetamidohexyl-Sepharose 4B and p-aminobenzamidohexyl-Sepharose 4B described by Cuatrecasas (Table 1) [10,12]. These immobilization positions are less disruptive to the activity of PLP since the formyl group remains intact. The C-6 immobilization of PLP preserves all the functionally crucial moieties available for protein-cofactor interaction. It also yielded a higher PLP loading density and the highest non-enzymatic catalytic activity of PLP among the three types of immobilized species described. However, there is still a major drawback in implementing the C-6 immobilization method of Ikeda et al., which is sourcing the azide-containing reagent, p-nitrobenzoyl azide, that is needed to couple the p-nitrobenzoyl group onto the ω-aminohexyl-Sepharose 4B (**2**). Not only does it require expensive custom synthesis since it is not commercially available, but also it imposes potential safety problems due to the hazardous nature of the azide compound (C/N ratio: 2.5) [13,14]. There are possible synthetic routes for the in-house synthesis, but those options involve other hazardous azide or hydrazide compounds along with expensive catalysts [15,17].

To overcome the various drawbacks of the existing methods described above, we implemented *N*-hydroxysuccinimide (NHS) ester chemistry to derivatize ω-aminohexyl-Sepharose 4B resin (AHS, **2**) with a p-nitrobenzoyl group which was then activated for C-6 immobilization of PLP under aqueous conditions without the use of any azide-containing compound (Scheme 1).

The alternative reagent employed here to install the p-nitrobenzoyl group was synthesized by first reacting NHS and p-nitrobenzoyl chloride (pNB-Cl) in anhydrous pyridine. The resulting NHS-ester, p-nitrobenzoyl-oxy succinimide (pNBOS, **1**), was successfully synthesized in 40% yield. An extractive workup and washing steps yielded pure product, as confirmed by ^{1}H NMR analysis (Fig. S1) [18].

pNBOS was then reacted with AHS in sodium borate buffer (pH 8.5) to form p-nitrobenzamidohexyl-Sepharose 4B (pNBA-HS, **3**). A colorimetric assay using sodium 2,4,6-trinitrobenzenesulfonate (TNBS) was used to monitor the reaction converting the primary aliphatic amine group (yielding an orange color) to the amide (yielding a yellow color) (Table S1). The nitro group in the pNBA-HS resin (**3**) was reduced to the corresponding amine-containing material (pABA-HS, **4**) with $Na_2S_2O_4$ in $NaHCO_3$ buffer (pH 8.5). The pABA-HS resin (**4**) gave an orange color with the TNBS reagent due to the presence of the newly generated aromatic amine group. Next, diazotization with $NaNO_2$ in cold 0.5 M HCl yielded the desired p-diazobenzamidohexyl-Sepharose 4B (pDBA-HS, **5**), which was yellow in color.

The pDBA-HS resin (**5**) was reacted with PLP in sodium borate buffer (pH 8.0), which yielded the immobilized PLP resin (**6**) and resulted in a change in the resin color from yellow to bright orange (Fig. S2). The loading of immobilized PLP was determined by comparing the UV–Vis absorbance at 376 nm of the resin (**6**) suspended in 0.1 M HCl with 75 % glycerol and to that of a related reference compound, MRS 2159, measured under the same conditions (Fig. 1). MRS 2159 is structurally similar to the new reagent reported here, and it showed an absorbance maximum at 376 nm with an extinction coefficient of 11162 cm^{-1} M^{-1} (Fig. S3). The suspension of the resin in a high viscosity solution of 75% glycerol enabled the reliable UV–Vis absorbance measurement by slowing the resin particle settling, which had been described previously for determining PLP resin loading [12]. The PLP-coupled resin (**6**) showed a broadened absorbance towards longer wavelengths that overlapped with the main absorbance peak of reference compound MRS 2159. The PLP loading was determined to be 2.6 μmol per mL of resin, corresponding to a conjugation of 29% of the ω-aminohexyl groups on the initial resin (9 μmol/mL, according to the manufacturer).

The Schiff base formation of the formyl group is crucial for the function of PLP and PLP-dependent proteins. Therefore, we confirmed the presence of the active formyl group on the immobilized PLP (**6**) with an amine titration assay using N_{α}-acetyl-L-lysine (Ac-Lys-OH)(Fig. 2A). The reaction of the primary amine of Ac-Lys-OH and the formyl group of immobilized PLP (R = AHS, (**6**)), or the reference compound MRS 2159 (R = O^{-}), was detectable by a change in UV–Vis absorbance (Fig. 2B, C). The UV–Vis spectrum of MRS 2159 (0.3 mM) in the presence of 80-fold molar excess Ac-Lys-OH showed

increased absorbance at 393 nm upon reaction (Fig. 2B, orange lines). It has previously been reported that the formyl group of PLP undergoes photodegradation upon exposure to visible light resulting in the formation of several undesired products including 4-pyridoxic acid 5′ phosphate and a PLP dimer [19-21]. As additional confirmation that the observed UV–Vis absorption shift was caused by Schiff base formation, we also incubated MRS 2159 which had been exposed to white light for 48 h at room temperature with Ac-Lys-OH (Fig. 2B, gray spectra). The light exposure shifted the local absorbance maximum from 393 nm to 358 nm and significantly lowered the absorbance in that region of the spectrum. Furthermore, the light-exposed MRS 2159 no longer showed a change in absorbance upon adding Ac-Lys-OH. Similar to the reference compound, our immobilized PLP resin (**6**) showed an increase in absorbance upon Ac-Lys-OH addition when the resin had been protected from light (Fig. 2C, orange spectra). Light exposure of the resin resulted in reduced overall absorbance and a lack of absorbance change in the 393 nm range upon Ac-Lys-OH addition (Fig. 2C, gray spectra). Although the extent of the spectral changes was insufficient to quantify the formyl groups, these results indicate the presence of formyl groups in the immobilized PLP (**6**) that are accessible to react with primary amines.

In summary, the methods described here used facile and benign NHS ester chemistry to prepare Sepharose 4B resin with p-diazobenzoyl groups that can be used to immobilize PLP at its C-6 position or potentially other molecules such as other cofactors or nucleic acids [22]. We demonstrated that immobilization of 2.6 μmol PLP per mL of resin could be obtained comparable with other commercially available affinity resins. The immobilized PLP retained the reactive formyl group for Schiff base formation. It should be possible to use the immobilized PLP resin described here for general PLP enzyme separation via PLP affinity chromatography. Furthermore, the resin should facilitate directed evolution campaigns of existing or *de novo* PLP-binding proteins using *in vitro* selection methods [7]. Considering the versatility and the importance of PLP-dependent enzymes, *de novo* PLP binding proteins hold much potential for interesting chemistry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Yari Cabezas and Dr. Deniz Yüksel-Yurt for their general guidance. Dr. Suresh Pujari for help with vacuum dryer equipment and Dr. Dimitri Perusse and Dr. Adeline Espinasse for assistance with NMR experiments.

Funding sources

This work was supported in part by grants from the Simons Foundation (340762 (B.S.)), the National Aeronautics and Space Administration (80NSSC18K1277, 80NSSC21K0595 (B.S.)), the National Institutes of Health (R35GM141853 (M.D.D.)) and the Office of the VP of Research at the University of Minnesota (Grant-in-Aid).

Data availability

Data will be made available on request.

Abbreviations:

PLP	Pyridoxal 5′-phosphate
NHS	*N*hydroxysuccinimide
pNB-Cl	p-nitrobenzoyl chloride
pNBOS	p-nitrobenzoyl-oxy-succinimide
TNBS	Sodium 2,4,6-trinitrobenzenesulfonate

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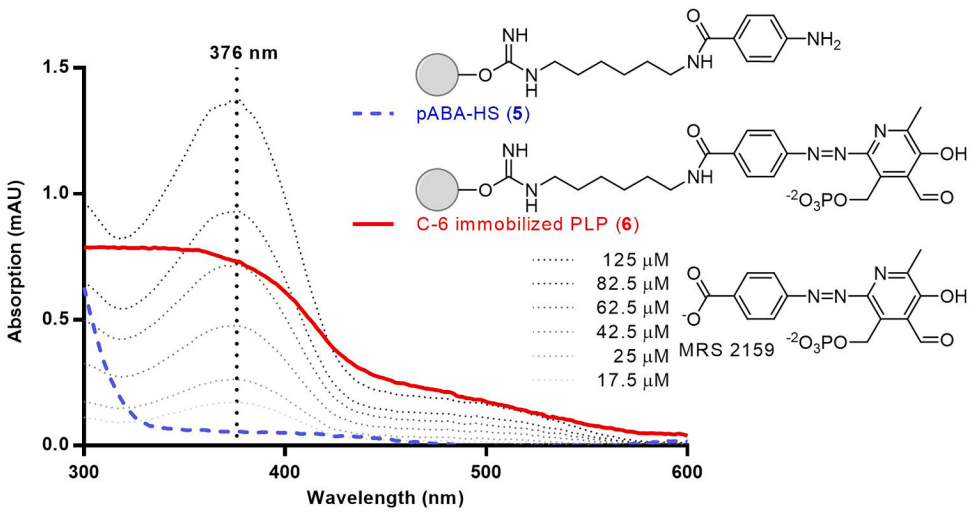


Fig. 1.

Quantification of C-6 immobilized PLP Sepharose 4B. UV–Vis absorption of the C-6 immobilized PLP (6, solid red line) and pABA-HS (5, dashed blue line) were measured by resuspending washed resin in 0.1 M HCl with 75% glycerol. The absorption spectra of reference compound MRS 2159 at varying concentrations (17.5, 25, 42.5, 62.5, 82.5, 125 µM) were also measured in 0.1 M HCl with 75% glycerol (dotted lines in shades of gray). The absorbance at 376 nm was used to quantify the PLP loading of the resin (2.6 µmol/mL).

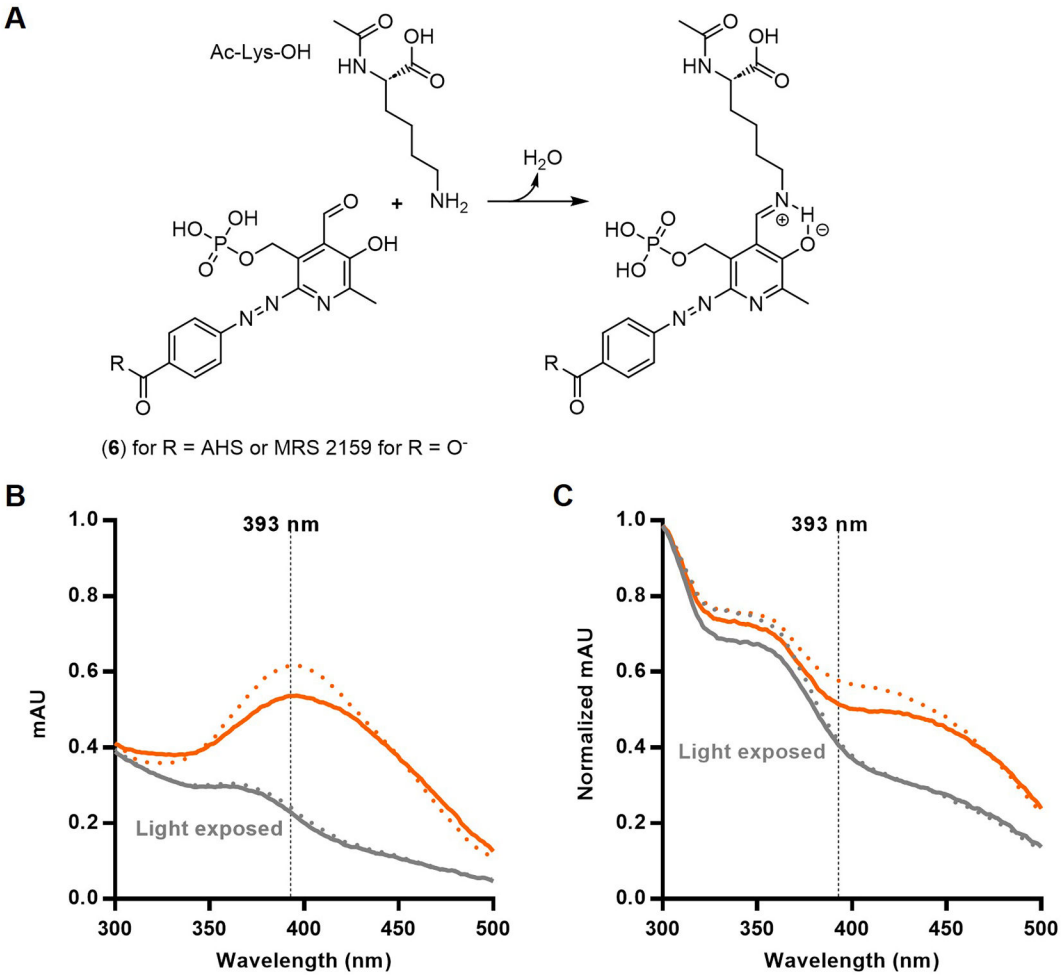
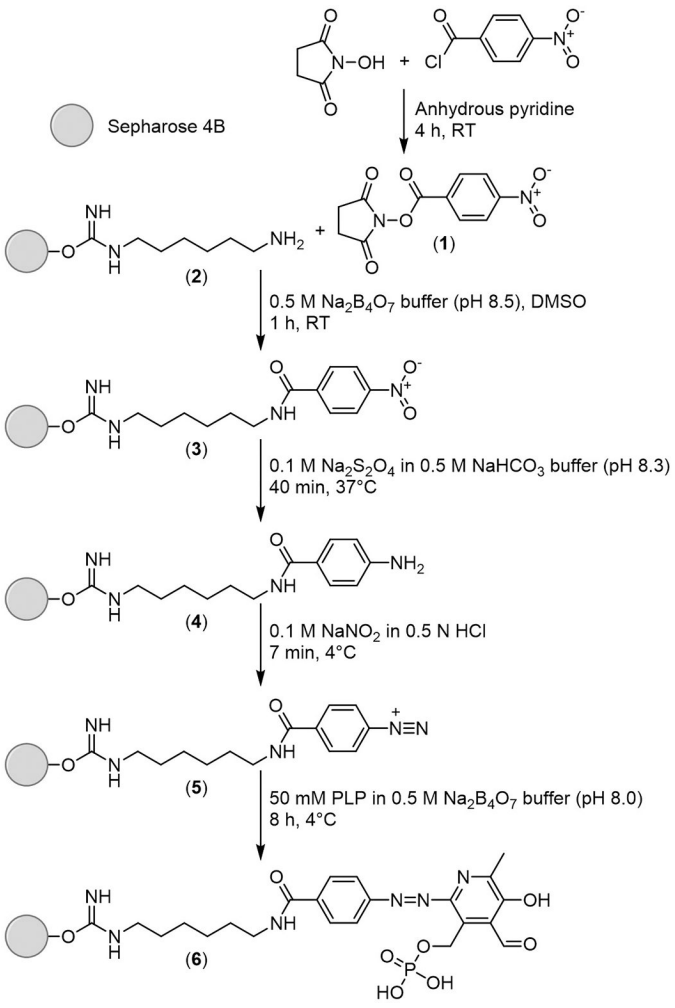


Fig. 2.

Aldehyde reactivity of immobilized PLP (6) and PLP reference compound MRS 2159 with Ac-Lys-OH in 0.1 M HEPES buffer (pH 7.5) and 80% glycerol to keep the resin in suspension. (A) Schiff base formation reaction of the formyl group of immobilized PLP (R = AHS) or MRS 2159 (R = O^-) with the primary amine of Ac-Lys-OH. (B) MRS 2159 without Ac-Lys-OH showed an absorbance maximum at 393 nm (solid orange line). The addition of Ac-Lys-OH led to an increase in absorbance at 393 nm, indicating the Schiff base formation (dotted orange line). The light-exposed MRS 2159 showed an absorbance shift from 393 nm to 358 nm with reduced overall absorbance (solid grey line). The Ac-Lys-OH did not affect the absorbance of light-damaged MRS 2159 (dotted gray line). (C) The immobilized PLP (6) upon addition of Ac-Lys-OH showed increased absorbance around the 393 nm range (dotted orange line) compared to the resin without Ac-Lys-OH (solid orange line). The resin exposure to light also led to a decrease in overall absorbance (solid grey line. The subsequent addition of Ac-Lys-OH did not affect the absorbance at 393 nm range (dotted grey line).

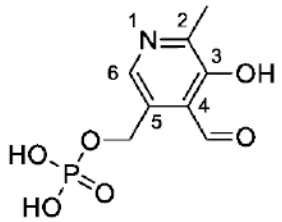


Scheme 1.

Reaction scheme for C-6 immobilization of PLP to p-diazobenzoyl derivatized Sepharose 4B (5).

Table 1

PLP structure and previously reported immobilization points of PLP.



Pyridoxal 5′-phosphate

Coupling position	Reactive group/solid support	Reference
N-1	Bromo-acetamidohexyl Sepharose	
Hydroxy-3		Ikeda et al., 1974
C-6	p-Diazobenzamidohexyl Sepharose	
Formyl-4	1,6-Diaminohexane linker on Sepharose	Collier et al., 1971
	Hydrazido cellulose	Junowicz et al., 1976