An Enzymatic Fluorometric Assay for Pyridoxal with High Specificity and Sensitivity

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An enzymatic fluorometric assay for pyridoxal with pyridoxal dehydrogenase was developed. The detection limit was about 10 pmol: the calibration curve of pyridoxal showed high linearity ($r = 0.993$). The values obtained by this method correlated well with those by the HPLC method. The enzyme had a high specificity for pyridoxal, and thus animal samples could be directly analyzed without separation of pyridoxal 5'-phosphate by column chromatography.

Key words: pyridoxal; enzymatic assay; pyridoxal dehydrogenase; Microbacterium luteolum

Vitamin B$_6$ is essential in over 100 enzymatic reactions involved in the metabolism of amino acids, carbohydrates, neurotransmitters and lipids. There are six natural vitamin B$_6$ compounds: pyridoxine, pyridoxal, pyridoxamine, pyridoxine 5'-phosphate, pyridoxal 5'-phosphate, and pyridoxamine 5'-phosphate. Pyridoxal 5'-phosphate and, to a lesser extent, pyridoxamine 5'-phosphate are coenzyme forms. Plant materials contain pyridoxine 5'-β-D-glucoside as a storage form of vitamin B$_6$.

Methods for measurement of vitamin B$_6$ compounds are available based on fluorometric HPLC in addition to the classical microbiological assay with yeasts and enzymatic and chemical assays. The enzymatic assays with apoenzymes of vitamin B$_6$-dependent enzymes are especially well suited for measurement of the coenzyme forms. The microbiological assay has several drawbacks but is still used practically for measurement of vitamin B$_6$ compounds in food materials. The HPLC methods permit the individual measurement of the six forms of the vitamin B$_6$ and pyridoxine 5'-β-D-glucoside, and have been widely used in various areas of research. However, the HPLC methods are still troublesome when samples contain various fluorescent contaminants: it is difficult to identify a specific peak component as one of the six natural vitamin B$_6$ compounds.

So far, no sensitive direct enzymatic method for measurement of pyridoxal has been reported. A sensitive assay for pyridoxal is a chemical method, in which it is oxidized to a highly fluorescent product, 4-pyridoxolactone, by the catalytic action of cyanide. However, the reaction is not specific for pyridoxal. Thus, pyridoxal 5'-phosphate in the samples has to be removed by ion-exchange chromatography before the reaction. The detection limit of the chemical method is about 100 pmol of pyridoxal in the sample solution to be carried in the assay mixture. Recently, we have homogeneously purified and characterized a pyridoxal dehydrogenase (PLDH, EC 1.1.1.107) from Microbacterium (= Aureobacterium) luteolum (IFO 16738). The enzyme catalyzes dehydrogenation of pyridoxal to 4-pyridoxolactone concomitant with reduction of NAD$^+$ to NADH. The enzyme showed a high hydrogen donor substrate specificity. In this paper, an enzymatic assay for pyridoxal with PLDH is described. This method had high specificity and sensitivity (the detection limit, 10 pmol in the sample solution to be carried in the assay mixture) without pretreatment by any column chromatography.

Standard 4-pyridoxolactone was prepared by heating a stock solution of 4-pyridoxic acid (Sigma) in 1 N HCl at 100°C for 15 min and then adjusting to pH 4.0 with KOH. Pyridoxal·HCl was purchased from Wako Chemicals. M. luteolum cells were prepared as described previously. The enzymatic fraction eluted from the Blue A column was dialyzed against 20 mM sodium phosphate buffer containing 1 mM DTT, and then the dialyze was concentrated with a small QAE-cellulose column (0.5 × 2.0 cm). The specific activity of the enzyme preparation was 25.8 units/mg protein. Since the dye affinity chromatography worked for purification of the enzyme, the preparation of the enzyme fraction was easy.

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2 Abbreviations: PLDH, pyridoxal 4-dehydrogenase; DTT, dithiothreitol
Enzymatic Assay for Pyridoxal

PLDH in the enzyme sample for this method was not homogeneous: faint bands of contaminating protein were observed on a SDS-PAGE gel. However, the contaminants caused no trouble in the assay of pyridoxal. The enzyme could be stored without loss of activity at 4°C for at least one month.

The standard conditions for measurement of pyridoxal are as follows. The sample (5 ~ 150 µl) was added to the mixture (195 ~ 50 µl) containing 10 µmol of sodium phosphate buffer (pH 8.0), 0.2 µmol of NAD⁺, and 40 mU of PLDH. The reaction mixture (final volume, 200 µl) was incubated at 30°C for 2 h, and then 10 µl of 20% (w/v) of SDS was added to the reaction mixture to stop the enzyme reaction. To the reaction mixture, 800 µl of 50 mM sodium phosphate buffer (pH 8.0) was added, and then the fluorescence intensity (excitation at 356 nm and emission at 432 nm) was measured with a Hitachi 650-10S spectrofluorometer. The control experiment was done with the reaction mixture without PLDH. The enzyme reaction was done for 2 h because the fluorescence intensity increased linearly through 2 h.

Effects of various compounds on the fluorescence intensity of 1 µM 4-pyridoxolactone in 50 mM sodium phosphate buffer (pH 8.0) were measured. β-Cyclodextrin, which is a well-known increaser of fluorescence of various compounds,¹⁰ did not increase the fluorescence intensity of 4-pyridoxolactone. However, SDS, Tween 40, Triton X-100, and deoxycholate increased the intensity it by 43%, 39%, 39%, and 39%, respectively. DMSO (50%, v/v) increased by 88%, although some precipitate was formed. Thus, we have used SDS to stop the reaction and to increase the fluorescence intensity.

Figure 1, A shows that 4-pyridoxolactone dissolved in the buffer containing 1% (w/v) SDS can be measured sensitively in a wide range of concentrations. The linearity of the curve was excellent: r = 0.999. Pyridoxal could be measured sensitively under the standard assay conditions (Fig. 1, B). The calibration curve showed high linearity (r = 0.993): the production of 4-pyridoxolactone plateaued at each concentration of pyridoxal in this end-point assay. The lower detection limit of the assay was about 10 pmol: NAD⁺ and additives in the enzyme solution increased the fluorescence intensity of the control experiment and interfered with the assay of lower amounts of pyridoxal. The higher detection limit was 1000 pmol: the calibration curve showed the same high linearity through 1000 pmol.

Concentrations of pyridoxal were measured by both the PLDH and HPLC methods (Fig. 1, C). Reversed-phase isocratic HPLC with a fluorescence monitor was done as described previously.¹¹ Results agree well over the concentration range (r = 0.98).

Specificity of the PLDH method was examined. No increase in fluorescence intensity was observed when 100 pmol of pyridoxal 5' phospho- phosphate, pyridoxamine 5'-phosphate, pyridoxine, or pyridoxamine was measured by the standard assay conditions. Thus, this assay is quite specific for pyridoxal among the natural forms of vitamin B₆.

The inhibitory effects of various compounds were examined. The other natural forms of vitamin B₆ examined showed no effect on the sensitivity of the PLDH method (Table 1). Among pyridoxal-related aldehydes with a pyridine ring, p-nitrobenzaldehyde and pyridine-3-aldehyde slightly but significantly increased the fluorescence intensity. Succinimide semialdehyde, isatin, and formaldehyde decreased the sen-
sitivity because of their inhibition of PLDH.

The concentration of pyridoxal in mouse organs was measured by the PLDH method. Organs of a mouse were treated for preparation of samples to be measured by the method with perchloric acid. The amount (nmol/g, wet weight) and yield obtained from the internal standard method were 3.9 ± 1.4 and 98% for liver, 3.0 ± 1.4 and 92% for muscle, 3.5 ± 0.98 and 92% for brain, and 6.7 ± 0.1 and 91% for kidney, respectively. These values were consistent with those of corresponding rat organs. This method was conveniently applicable for the animal samples. However, it was not applicable for plant samples such as spinach or green onion. The fluorescent intensity of the control reaction mixture was very high when they were measured. The HPLC method also could not be used for analysis of plant samples: fluorescent compounds were coeluted with pyridoxal. Thus, further investigations are in progress to establish a preliminary step for selective concentration of pyridoxal in plant samples. A combination of pyridoxine oxidase (EC 1.1.3.12) and glyoxylic acid may allow measurement of pyridoxine and pyridoxamine, respectively, by this method.

References


Table 1. Effects of Various Compounds on Sensitivity of Pyridoxal Measurement

<table>
<thead>
<tr>
<th>Compounds (each, 200 pmol)</th>
<th>Relative fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxal only (control)</td>
<td>100 ± 0.8 (%)</td>
</tr>
<tr>
<td>+ Pyridoxine 5'-phosphate</td>
<td>103 ± 4.0</td>
</tr>
<tr>
<td>+ Pyridoxamine 5'-phosphate</td>
<td>101 ± 0.0</td>
</tr>
<tr>
<td>+ Pyridoxine</td>
<td>97 ± 2.3</td>
</tr>
<tr>
<td>+ Pyridoxamine</td>
<td>105 ± 3.3</td>
</tr>
<tr>
<td>+ p-Nitrobenzaldehyde</td>
<td>118 ± 8.0*</td>
</tr>
<tr>
<td>+ 2-Carboxybenzaldehyde</td>
<td>92 ± 8.0</td>
</tr>
<tr>
<td>+ Pyridine-3-aldehyde</td>
<td>125 ± 0.0*</td>
</tr>
<tr>
<td>+ Pyridine-4-aldehyde</td>
<td>101 ± 13.3</td>
</tr>
<tr>
<td>+ Succinic semialdehyde</td>
<td>92 ± 1.3*</td>
</tr>
<tr>
<td>+ Isatin</td>
<td>82 ± 13.3*</td>
</tr>
<tr>
<td>+ Formaldehyde</td>
<td>76 ± 2.7*</td>
</tr>
</tbody>
</table>

Each value shows mean ± S.D. from duplicate experiments.

* Values which are significantly different from the control value for p < 0.05.