Journal of Chromatography, 526 (1990) 25–33
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5093

Simple isocratic high-performance liquid chromatographic method for measuring pyridoxine kinase activity in crude biological extracts

CHRIS J. ARGOUDELIS

Department of Food Science, University of Illinois, 580 Bevier Hall, 905 S Goodwin, Urbana, IL 61801 (U.S.A.)

(First received July 26th, 1989; revised manuscript received October 24th, 1989)

SUMMARY

To measure the enzymatic activity of pyridoxine kinase (EC 2.7.1.35) when pyridoxine or pyridoxamine are the substrates an additional enzymatic step is usually required. The products of the kinase activity, pyridoxine 5′-phosphate or pyridoxamine 5′-phosphate, are oxidized, enzymatically, to pyridoxal 5′-phosphate (co-enzyme) which is then measured either spectrophotometrically or using apo-enzymes. In this report the enzymatic activity of pyridoxine kinase, in crude biological extracts, is assayed by a simple high-performance liquid chromatographic method which determines the amount of pyridoxine 5′-phosphate formed when pyridoxine is the substrate. The same method could be used when pyridoxamine is the substrate.

INTRODUCTION

In our studies on the biosynthesis of vitamin B_6 in yeast [1], it was speculated [2] that the increased quantity of pyridoxine secreted, in the culture medium, by the yeast mutant was due to its reduced pyridoxine kinase (EC 2.7.1.35) activity. All three B_6 vitamers, pyridoxine (PN), pyridoxamine (PM) and pyridoxal (PL) are phosphorylated by a single kinase [3,4] according to the reaction:

$$\text{PL (PN, PM)} + \text{ATP} + \text{Zn}^{2+} \text{ (or } \text{Mg}^{2+}\text{)} \underset{\text{kinase}}{\rightleftharpoons} \text{PL (PN, PM) 5′-phosphate} + \text{ADP} + \text{Zn}^{2+} \text{ (or } \text{Mg}^{2+}\text{)}$$

0378-4347/90/$03.50

The enzymatic assay for measuring the activity of this kinase is fairly simple when PL is the substrate since the pyridoxal 5′-phosphate (PLP) (co-enzyme) formed can be determined either spectrophotometrically for purified kinase preparations [4,5] or enzymatically using the apotryptophanase assay [6] or apotyrosine decarboxylase [7].

However, when PN or PM are the substrates an additional enzymatic step is required in order to transform the products of the kinase activity pyridoxine 5′-phosphate (PNP) or pyridoxamine 5′-phosphate (PMP) into the co-enzyme form PLP. To accomplish this additional step, liver pyridoxamine (pyridoxine) 5′-phosphate oxidase is used [6].

Another method, a very sensitive one, to determine the kinase activity and avoid the additional enzymatic step when PN is the substrate is by measuring the formation of [^{3}H]PNP, which can be easily separated from [^{3}H]PN using DEAE-cellulose columns [8] or paper disks [9]. The [^{3}H]PN is available commercially or can be synthesized by reduction of PLP with NaB^3H_4 [10] to give [^{3}H]PNP, then the phosphate group can be removed enzymatically [11]. We have shown [12] that PN, labelled by exposing it to $^3H_2$ gas, contains approximately 14% of 4- and 5-deoxypyridoxine as radioactive impurities which might interfere with the kinase assay.

Recently [13], a high-performance liquid chromatographic (HPLC) assay was reported for measuring erythrocyte enzyme activity levels in vitamin B_6 metabolism. However, the substrate for the B_6-kinase activity was PL and the method suffers from the disadvantage of not using an internal standard. One additional assay has been reported for measuring pyridoxine kinase activity irrespective of the B_6 vitamer used as substrate by taking advantage of the formation, during the enzymatic reaction, of adenosine 5′-diphosphate (ADP) which is coupled to diphosphopyridine nucleotide (reduced form) (DPNH) enzymatic oxidation [5].

In this paper a simple, sensitive HPLC method is described for measuring the enzymatic activity of pyridoxine kinase, using PN as substrate, in crude biological extracts.

EXPERIMENTAL

Reagents

Phenylmethylsulfonyl fluoride (PMSF), glutathione, bovine serum albumin, rabbit liver acetone powder, bakers yeast (YSC-1), adenosine 5′-triphosphate (ATP), ADP, PN, PM and PMP were obtained from Sigma (St. Louis, MO, U.S.A.). The synthesis of PNP [14] and that of isopyridoxal, the internal standard, have been reported previously [15]. All other chemicals used were of reagent grade.

Preparation of biological extracts

Rabbit liver acetone powder, 5 g, was suspended in 20 ml of 50 m*M* potassium phosphate pH 6.9, 0.05 m*M* in PMSF and 0.1 m*M* in glutathione. The suspension was left at 4°C for 2 h and then centrifuged for 45 min at 4°C (100 000 *g*). The supernatant was distributed in small vials and kept frozen at −20°C till used. No appreciable loss in pyridoxine kinase activity was observed after two months of storage.

Bakers yeast, 1 g, was suspended in 4 ml of 100 m*M* potassium phosphate pH 6.9, 0.05 m*M* in PMSF. The suspension was left at 30°C overnight, mixed and centrifuged (2000 *g*). The supernatant was centrifuged again for 10 min in a Beckman microfuge E. The supernatant was kept frozen at −20°C till used.

The protein concentration in the extracts was determined by a modification of the Lowry method [16], using bovine serum albumin as standard.

The concentrations of stock solutions of ATP and PN were verified spectrophotometrically at 260 nm [17] and 290 nm [18], respectively.

Enzymatic assays

The assay system for the liver extract was a slight modification of the one reported by Merrill and Wang [19] and was 20 m*M* in potassium phosphate pH 5.75, 0.08 m*M* in $ZnCl_2$, 0.06 m*M* in KCl, 0.02 m*M* in isopyridoxal (internal standard), 1.2 m*M* in ATP, 0.1 m*M* in PN, an appropriate amount of extract, and water to a volume of 1 ml. A blank without PN was included for each assay. For the yeast cell extract assay, $ZnCl_2$ was replaced with 0.1 m*M* $MgCl_2$ and KCl was not included. The solutions were placed in small screw-capped test tubes. The assay was started by adding the extract and the test tubes were incubated in the dark at 37°C for 90 min. The reaction was stopped by immersing the test tubes in a boiling water bath for 3 min. The precipitated protein was centrifuged off in a Beckman microfuge E for 10 min and an aliquot from the supernatant was injected into HPLC system.

High-performance liquid chromatography

The conditions for the HPLC were the same as those reported previously [15] except that the column had been in use for three months.

To determine the quantity of PNP formed, the internal standard plot method was used (peak-area ratios of PNP over isopyridoxal versus the corresponding ratios of their respective picomoles, at five different ratios). The correlation coefficient of the straight line obtained was better than 0.998.

All experiments were carried out under conditions of subdued light.

RESULTS AND DISCUSSION

In quantitative work with HPLC, the advantages of using an internal standard are obvious. When the internal standard is added at the beginning of the

assay it accounts for any volume changes during the preparation of the sample and for variations in the volume injected into HPLC system. Also, if the internal standard is chemically similar to the analyte that is determined it corrects for their occlusion in the precipitated protein and accounts for instrumental variations during the HPLC analysis.

In the assay reported here, 4′-deoxypyridoxine could not be used as internal standard since it is apparently phosphorylated by yeast pyridoxine kinase and ATP [20]. However, isopyridoxal, the other internal standard we have used [15], at a concentration ten times higher than the one used in this assay, was either very weakly inhibitory or without effect on the kinase from rat liver [21]; therefore, it was considered a suitable internal standard.

In preliminary experiments, when the assay was carried out under the conditions described by Merrill and Wang [19] (1 m*M* ATP, 0.03 m*M* PN) using our crude rabbit liver extract, the quantity of PNP formed was very small. However, when the concentration of PN was increased to 0.1 m*M* [9] a large quantity of PNP was formed.

In Fig. 1A is presented the effect of increasing concentrations of one of the substrates, PN, on PNP formation by the kinase in the liver extract while the concentrations of the other two substrates were kept constant (ATP = 1.1 m*M*;

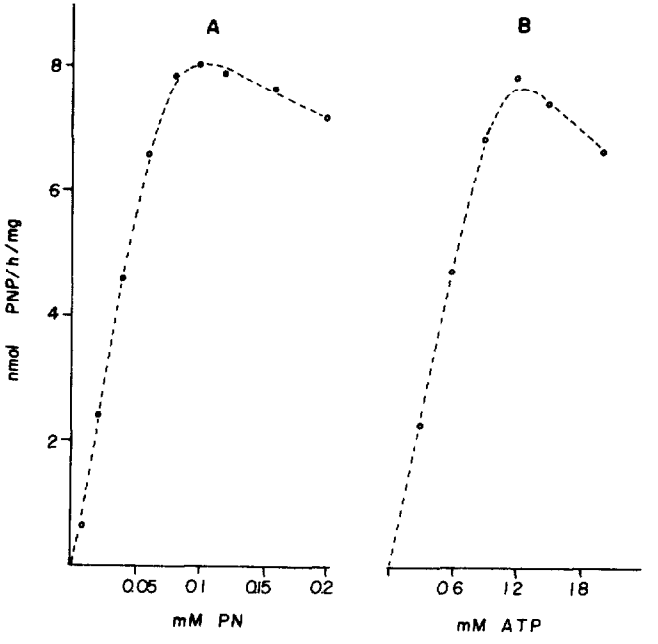

Fig. 1. Effect of substrate concentration PN (A) or ATP (B) on the rate of formation of PNP. Assays were conducted as described in the Experimental and Results and discussion sections.

Zn^{2+} = 0.08 m*M*). In Fig. 1B is shown the effect of varying the concentration of ATP on PNP formation while the concentrations of the other two substrates were kept constant (PN = 0.1 m*M*; Zn^{2+} = 0.08 m*M*). Under the conditions of the assay, substantial inhibition was observed at PN concentrations higher than 0.12 m*M* and at ATP concentrations higher than 1.2 m*M*. Inhibition of the kinase activity by its substrates has been reported [3,8,22]. Maximal activities were in the area of 0.08–0.12 m*M* for PN and around 1.2 m*M* for ATP, i.e. at a PN-to-ATP ratio of 1 : 10. A similar PN-to-ATP ratio of 1 : 10 was found as optimum by Loo and Whittaker [23] for brain pyridoxal kinase when PL was the substrate and by Karawya and Fonda [9] for sheep liver pyridoxine kinase when [^{3}H]PN was the substrate.

Double reciprocal plots were not linear for either PN or ATP under the assay conditions described.

The rate of formation of PNP was linear for at least 90 min. The relationship between the concentration of protein in the crude liver extract and the formation of PNP was linear up to 1.85 mg which produced from 0 to more than 13 nmol of PNP per h (Fig. 2).

In Fig. 3A is depicted a chromatogram obtained from the supernatant of the kinase assay, from liver extract, used as blank (PN was not added). A small peak with retention time a little longer than that of PNP is present. When a small amount of PNP was formed by the enzyme those two peaks could be resolved. However, when an appreciable amount of PNP was formed the two peaks, of course, could not be resolved. Therefore, from the area of the PNP peak was subtracted the small area of the unknown peak given by the blank.

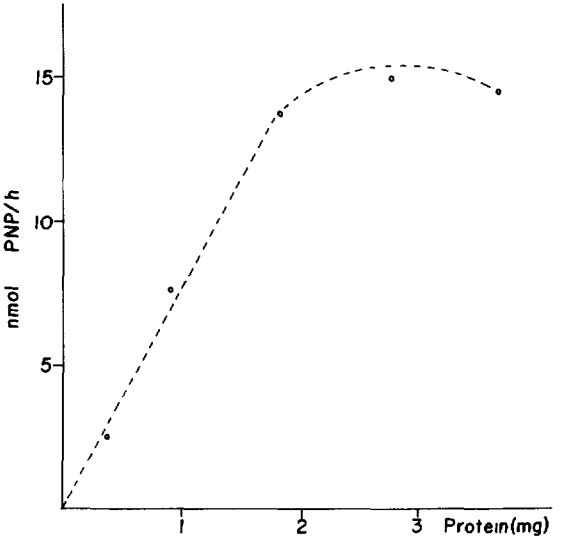

Fig. 2. Relation of PNP formation to the concentration of pyridoxine kinase in the crude liver extract (total protein).

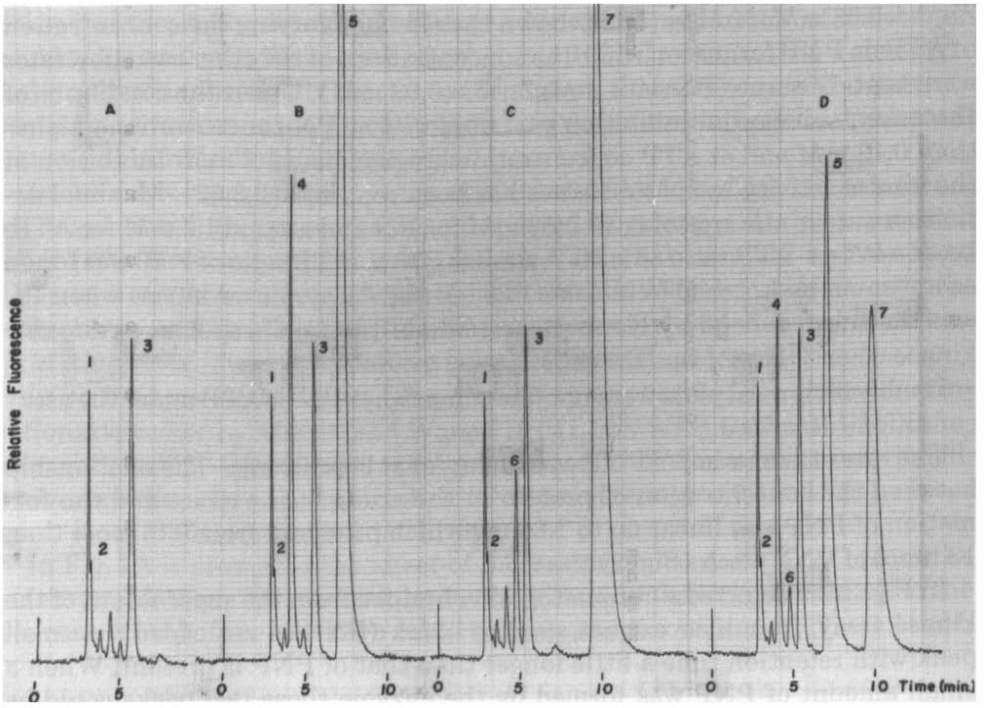

Fig. 3. HPLC tracings on a Partisil-10 SCX (250 mm × 4.6 mm I.D.) column (Whatman, Clifton, NJ, U.S.A.) of pyridoxine kinase assays from crude liver extract (A) Blank (PN was not added); (B) PN was added; (C) PM was the substrate; (D) both PN and PM were added in the assay medium. Assays were stopped after 90 min incubation and 4 μl from the 1 ml of supernatant were injected. The mobile phase was 0 1 *M* $NH_4H_2PO_4$, pH 4.0, at a flow-rate of 1.0 ml/min. Peaks: 1 = ATP; 2 = ADP; 3 = isopyridoxal (internal standard); 4 = PNP; 5 = PN; 6 = PMP; 7 = PM.

This small unknown peak present in the blank was also present when PN and enzyme were added and the test tube was immersed immediately in the boiling water bath. In Fig. 3B, PN had been added to the assay mixture and PNP was formed. In Fig. 3C, PM was added and PMP was formed and in Fig. 3D, both PN and PM were added each at half the concentrations of those in Fig. 3B and 3C. If PM is the substrate, an excitation wavelength at 320 nm, instead of 290 nm as shown in Fig. 3C and 3D, will increase the fluorescence intensity of both PM and PMP by a factor of 2 under the HPLC conditions described.

In Fig. 4A is presented a chromatogram obtained from the supernatant of the kinase assay from the yeast cell extract, used as blank (PN was not added) and in Fig. 4B when PN was added. Again, in the blank the small peak with a retention time a little longer than that of PNP can be seen.

The specific activity of pyridoxine kinase in the crude liver extract was calculated as 8 nmol of PNP formed per h per mg of total protein while in the yeast cells extract it was 3 nmol.

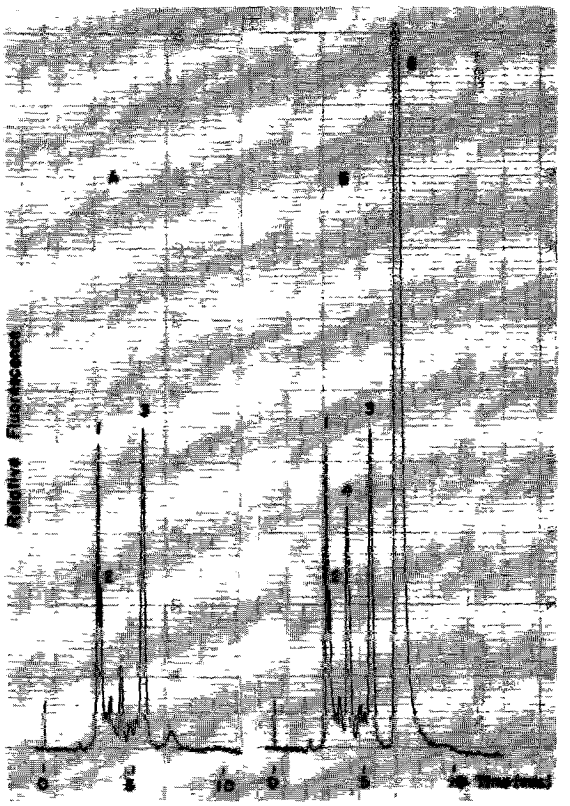

Fig. 4. HPLC tracings of pyridoxine kinase assay from crude yeast cells extract. (A) Blank (PN was not added); (B) PN was added. Chromatographic conditions and peak identification as in Fig. 3.

In all the chromatograms a small peak with the same retention time as ADP is present. This peak was not present in the assay that was placed in a boiling water bath immediately after the addition of the crude extract.

Under the conditions of the kinase assay from yeast cell extract a little more PNP was formed when Mg^{2+} instead of Zn^{2+} was used for activation of the enzyme, in agreement with the report of Hurwitz [24] but in disagreement with that of McCormick et al. [3]; also, less amount of PNP was formed when the pH of the assay was 6.9 instead of 5.75 in disagreement with both reports. However, it has been observed [3] that the optimum pH for the kinase assay depends on the purity of the enzyme and it is possible to depend also on the substrate used.

This B_6-kinase assay can not be used when PL is the substrate due to close retention times between PL and the internal standard [15]. However, it could be used without an internal standard or with a different one.

For measuring true initial velocities of the B_6-kinase reactions in purified enzyme this HPLC assay has a drawback because of stopping the reaction with heat which takes 2–5 min. [5]. However, in that case the reaction can be stopped by adding concentrated H_2SO_4 [4] or even trichloroacetic acid and extracting the excess with diethyl ether. In one experiment the reaction was stopped by adding to the assay tube three drops, with a Pasteur pipet, of concentrated H_2SO_4 and removing the precipitated protein by centrifugation. The quantity of PNP measured by HPLC was, as expected, the same as that found by stopping the reaction with heat, since the assay was run for 90 min.

The within-assay precision of the method (coefficient of variation, %) for measuring PNP at various concentrations has been reported [15].

It is believed that this simple HPLC assay for measuring the enzymatic activity of B_6-kinase will be used by researchers interested in its distribution in various biological tissues and by enzymologists to study the properties of the enzyme and find the optimum assay conditions for its various substrates. This assay can also be used to measure B_6 phosphatase enzymatic activities.

Unfortunately, this method could not be used to measure B_6-kinase activity in cell extracts of the yeast mutant [1] since a large peak with a retention time a little longer than that of PNP was present in the extracts, masking the PNP peak that could be formed.

ACKNOWLEDGEMENT

This study was a part of Project No. 50-0330 of the Agricultural Experiment Station, College of Agriculture, University of Illinois at Urbana-Champaign.

REFERENCES

1 R.S. Pardini and C.J. Argoudelis, J. Bacteriol., 96 (1968) 672.
2 R.S. Pardini, Ph.D. Thesis, University of Illinois, Urbana, IL, 1965.
3 D.B. McCormick, M.E. Gregory and E.E. Snell, J. Biol. Chem., 236 (1961) 2076.
4 J.T. Neary and W.F. Diven, J. Biol. Chem., 245 (1970) 5585.
5 R.S. White and W.B. Dempsey, Biochemistry, 9 (1970) 4057.
6 H. Wada and E.E. Snell, J. Biol. Chem., 236 (1961) 2089.
7 V.M. Camp, J. Chipponi and B.A. Faraj, Clin. Chem., 29 (1983) 642.
8 A.H. Merrill, Jr., J.M. Henderson, E. Wang, B.W. McDonald and W.J. Millikan, J. Nutr., 114 (1984) 1664.
9 E. Karawya and M.L. Fonda, Anal. Biochem., 90 (1978) 525.
10 A. Stock, F. Ortanderl and G. Pfleiderer, Biochem. Z., 344 (1966) 353.
11 N.T. Meisler and J.W. Thanassi, J. Nutr., 110 (1980) 1965.
12 C.J. Argoudelis and F.A. Kummerow, Biochemistry, 5 (1966) 1.
13 J.B. Ubbink and A.M. Schnell, J. Chromatogr., 431 (1988) 406.
14 C.J. Argoudelis, J. Agric. Food Chem., 34 (1986) 995.
15 C.J. Argoudelis, J. Chromatogr., 424 (1988) 315.
16 G.L. Peterson, Anal. Biochem., 83 (1977) 346.
17 R.B. Hurlbert, Methods Enzymol, 3 (1957) 785.

18 D.E. Metzler and E.E. Snell, J. Am. Chem. Soc., 77 (1955) 2431.
19 A.H. Merrill, Jr. and E. Wang, Methods Enzymol., 122 (1986) 110.
20 J. Hurwitz, J. Biol. Chem., 217 (1955) 513.
21 D.B. McCormick and E.E. Snell, J. Biol. Chem., 236 (1961) 2085.
22 L. Lumeng and T.K. Li, J. Clin. Invest., 53 (1974) 693.
23 Y.H. Loo and V.P. Whittaker, J. Neurochem., 14 (1967) 997.
24 J. Hurwitz, J. Biol. Chem., 205 (1953)935.