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Purification of Pyridoxamine and Pyridoxamine 5´-Phosphate from Culture Broth of *Rhizobium* sp. 6.1C1

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Abstract

Vitamin B6 is an essential micronutrient for the metabolism of proteins, lipids and carbohydrates. Recently, pyridoxamine shows a potent antioxidant for cellular well-being. The Gram negative bacterium, *Rhizobium* sp. 6.1C1 produces extracellular pyridoxamine (PM) and pyridoxamine 5´-phosphate (PMP) in synthetic media. In this work, PM and PMP from the cultured broth of *R*. 6.1C1 were purified with strong acid cation exchange chromatography (DOWEX 50W × 8). The amount of vitamin B6 was measured by agar diffusion assay using *S. carlsbergensis* TISTR 5345 (ATCC 9080) as the test organism. The elution profile showed 7 peaks (P1-P7) which responded to the growth of *S. carlsbergensis*. To determine the forms of vitamin B6, each peak was examined by reversed-phase HPLC. The retention time of P5 and P7 corresponded to that of PMP and PM standards, respectively. The retention time of P1-P4 and P6 corresponded to that of the synthetic medium. Both fluorescence spectra and dephosphorylation confirmed that P5 and P7 were PMP and PM, respectively. This purification method can be used as a model for the development of large scale purification of vitamin B6 from fermentation broth.

Keywords: *Rhizobium* sp. 6-1C1, purification, pyridoxamine, pyridoxamine 5´-phosphate, agar diffusion assay

Introduction

Vitamin B6 is a water soluble and photosensitive vitamin. It is a derivative of 2-methyl-3-hydroxypyridine, which consists of 6 compounds, pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM) and the 5´ phosphorylated derivatives (**Figure 1**). They differ in group present at their C4-position with PN carrying a hydroxymethyl group, and PL and PM having an aldehyde and an aminomethyl group, respectively [1].

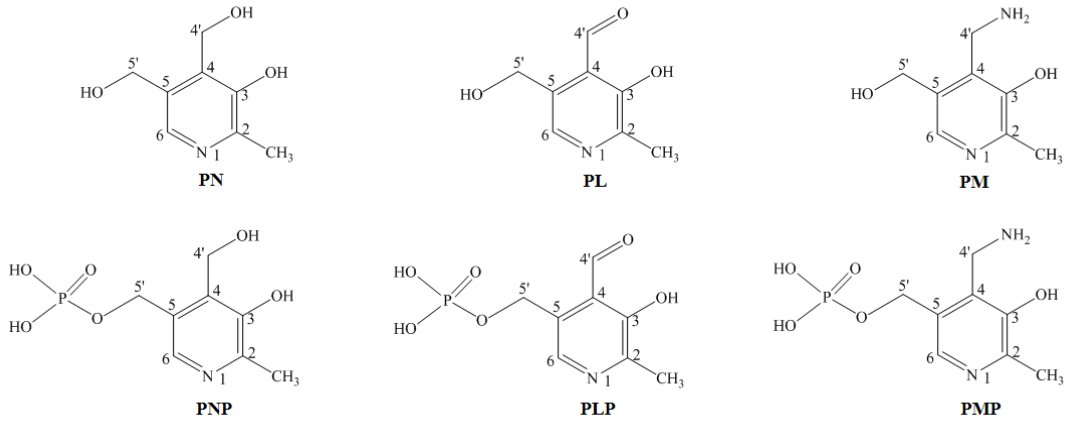

Figure 1 Structure of vitamin B6: PN, pyridoxine; PL, pyridoxal; PM, pyridoxamine, PNP, pyridoxine 5'-phosphate; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate.

The importance of vitamin B6 as a cofactor is well established. Both pyridoxal 5'- phosphate (PLP) and pyridoxamine-5'-phosphate (PMP) have been reported to function as cofactors [1,2]. PM is a critical transient intermediate in catalysis of transamination reactions by vitamin B6-dependent enzymes. In the last decade, PM has also emerged as a promising pharmacological agent for protecting against progressive tissue damage that occurs in a number of diseases [3]. It has been shown to inhibit several oxidative and glycoxidative pathways that can cause protein damage. Furthermore, PM is a prospective drug for treatment of diabetic nephropathy [4,5].

To date, all forms of vitamin B6 have been manufactured by a chemical process. The biotechnological synthesis of vitamin B6 is attractive as it might reduce waste and provide economic benefits. Many studies have showed microorganisms can produce vitamin B6 [6-10]. Previously, only recombinant microorganisms of the genus *Sinorhizobium*, have been used to give extracellular vitamin B6 in PN form from culture broth [11].

In our previous studies, we found the Gram negative bacterium, *Rhizobium* sp. 6.1C1 could produce extracellular vitamin B6 in both PM and PMP forms [12]. In this study, the method for separation of vitamin B6 from plasma according to Lumeng *et al*. [13] was modified using the culture broth *R*. 6.1C1.

Materials and methods

Culture conditions

The glycerol stock of *R*. 6.1C1 was thawed and streaked onto a synthetic medium (SM) agar plate pH 5.8 (2 % glucose, 2 % peptone, 0.1 % KH_2PO_4, 0.05 % $MgSO_4 \cdot 7H_2O$ and 0.0005 % $FeSO_4 \cdot 7H_2O$ and 2 % (w/v) agar) and incubated at 37 °C for 16 h [12]. A loopful of *R*. 6.1C1 was inoculated in 25 ml SM broth at 37°C, 160 rpm until OD_{600} equal to 0.4. Cells were aseptically transferred into 100 ml SM in a 2 L flask and incubated at 37 °C for 24 h in the dark. The cultured medium was centrifuged at 8,000 *g* for 30 min to remove the cells. Supernatant was collected and frozen at –20 °C until used.

Determination of vitamin B6

Measurement of the amount of extracellular vitamin B6 was determined by agar diffusion assay (ADA), which was modified from a microbiological assay [14]. This procedure used a Pyridoxine Assay Medium (PAM; Difco) and *S. carlsbergensis* TISTR 5345 (ATCC 9080) for testing. The yeast was streaked onto yeast malt (YM) agar and incubated at 30 °C for 18 h. The yeast cells were transferred into sterile distilled water, and their turbidity adjusted at OD_{660} equal to 1. The suspension was added to PAM

agar in the ratio of 1:100 and incubated at 30 °C for 6 h. Samples were assayed for vitamin B6 after acid hydrolysis according to AOAC [15]. Nine wells (0.33 mm diameter per well) were punched on the PAM agar plate. Samples were applied into each well and incubated at 30 °C for 12 h. For vitamin B6 determination, the diameter of the zone around the well was measured and compared with that of standard vitamin B6 (1 - 5 ng).

Purification of vitamin B6

The separation of vitamin B6 was performed by a modification of Lumeng *et al.*'s method [13]. The supernatant was deproteinized by adding 7.5 % (w/v) trichloroacetic acid (TCA; Carlo Erba). The treated sample was mixed at 4 °C for 10 min and then warmed in a water bath at 37 °C for 10 min in the dark. The clear supernatant was obtained by centrifugation at 32,800 g for 30 min at 4 °C and was used as the starting material for the vitamin B6 purification.

The strongly acidic cation exchange resin (Dowex 50 W-X8 200 - 400 mesh; Sigma) was used for column preparation. The resin was washed in 1 N HCl and excess acid removed by washing with double distilled water, and then washed with 1 N NaOH and rinsed with double distilled water until the pH was neutral. The washed resin was packed in a column (1.5 × 11.4 cm) and equilibrated with 0.1 M of formic acid, then 0.01 M formic acid, until the pH was 3.2. The resin was finally equilibrated with 0.01 M ammonium formate pH 3.2.

All steps were done at 4 °C in the dark. The clear supernatant was applied to a column and eluted under a stepwise condition by increasing the pH and ionic strength [13]. Elution buffers were performed as follows: 100 ml of 0.01 M ammonium formate (pH 3.2), 300 ml of 0.1 M ammonium formate (pH 3.2), 400 ml of 0.095 M sodium acetate (pH 4.2), 200 ml of 0.1 M sodium acetate (pH 5.2), 300 ml of 0.12 M sodium acetate (pH 5.6), 300 ml of 0.95 M sodium phosphate (pH 6.1), 300 ml of 0.1 M sodium phosphate (pH 6.5) and 300 ml of 0.12 M sodium phosphate (pH 7.5) at a flow rate 1 ml/min. Five ml fractions were collected and the amount of vitamin B6 measured by ADA. The pool fractions with the desired peaks were lyophilized and stored at –20 °C.

Protein concentration was determined by the dye-binding method [16]. Increase of absorbance at 595 nm was compared with the standard, bovine serum albumin.

High performance liquid chromatography (HPLC)

Vitamin B6 compounds were investigated by the reversed-phase isocratic HPLC method described by Argoudelis [17]. The sample was diluted with DI water which was adjusted to pH 1 with 70 % perchloric acid. Then the diluted sample was filtered using a 0.2 μm cellulose acetate membrane filter. The filtrate was used for HPLC. A 4.6 mm ID × 25 cm Hydrosphere C18 column (YMC, Japan) was used in conjunction with a 3 mm ID × 4 cm SecurityGuard C18 column (Phenomenex, UK). The pre-column buffer was 0.15 M sodium dihydrogen phosphate, pH 2.5. The post-column buffer (1 g/L sodium bisulfite) was used for the derivatization of PLP at a flow rate of 0.1 ml/min. The column effluent was monitored with a Shimadzu spectrofluorometer detector RF-10A at an excitation and emission wavelength of 290 and 389 nm, respectively. The HPLC system was operated at ambient temperature and the column flow rate was 1 ml/min. Retention times of standard PMP, PM, PLP, PL, and PN were 3.514, 3.726, 5.917, 6.221 and 7.818 min, respectively.

Fluorescence spectroscopy

Fluorescence spectra of PMP and PM were recorded using a SpectraMax M5 microplate reader (Molecular Devices, USA). Samples and 1 μM of PMP and PM standards were diluted by 0.05 M sodium dihydrogen phosphate (pH 7.0) and filtered using 0.2 μm cellulose acetate membrane filters in the 96-well microplate. The emission spectra were recorded covering a spectral range of 340 - 500 nm with an excitation wavelength at 324 nm. The excitation spectra (250 - 350 nm) were recorded with an emission wavelength at 388 nm to determine PM and PMP [18].

Results and discussion

Production of vitamin B6 by *Rhizobium* sp. 6-1C1

The growth curve and vitamin B6 production at 37 °C by *R*. 6-1C1 was determined as shown in **Figure 2**. The optical density at 600 nm of the growth of *R*. 6-1C1 slightly increased after 20 h. The amount of vitamin B6 gradually increased until 18 h of culture when it reached a plateau, which remained constant until the end of the culture after 24 h. The highest amount of vitamin B6 production was 2.14 mg/L after 22 h of cultivation. The increase in vitamin B6 coincided with the increase in optical density at 600 nm, indicating primary metabolite kinetics of vitamin B6. So, vitamin B6 was harvested in the 18 - 22 h of growth curve to give optimal results.

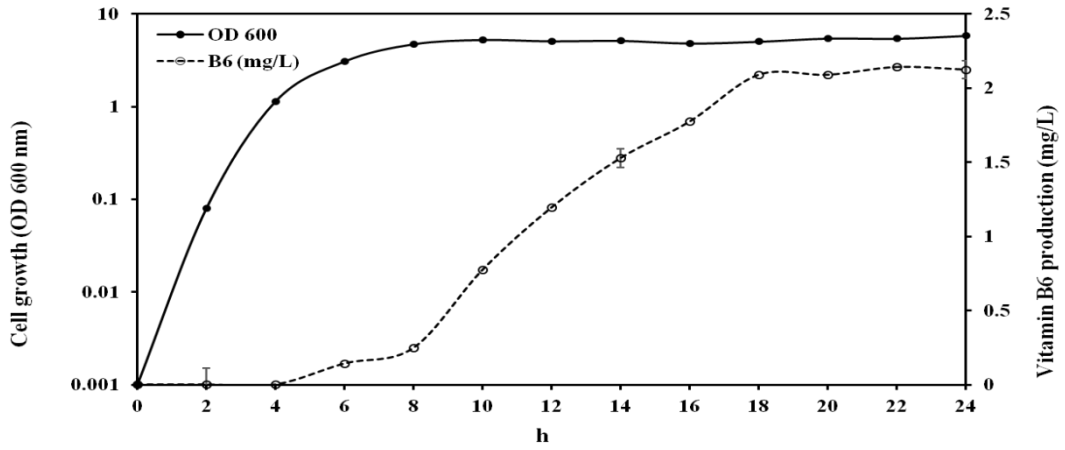

Figure 2 Kinetic of growth and vitamin B6 production (mg/L) by *R*. 6-1C1.

Prior to the study of the purification of vitamin B6 from cultured broth of *R*. 6-1C1, forms of vitamin B6 were analyzed by reversed-phase HPLC. **Figure 3** shows chromatograms of excreted vitamin B6 by *R*. 6-1C1 with the major component PM (74.02 %) and smaller amount of PMP (25.98 %). This result agrees with previous studies that this bacterium produces the same PM/PMP ratio, although it was in different batches of growth [12]. It suggests that *R*. 6-1C1 has genotypic stability.

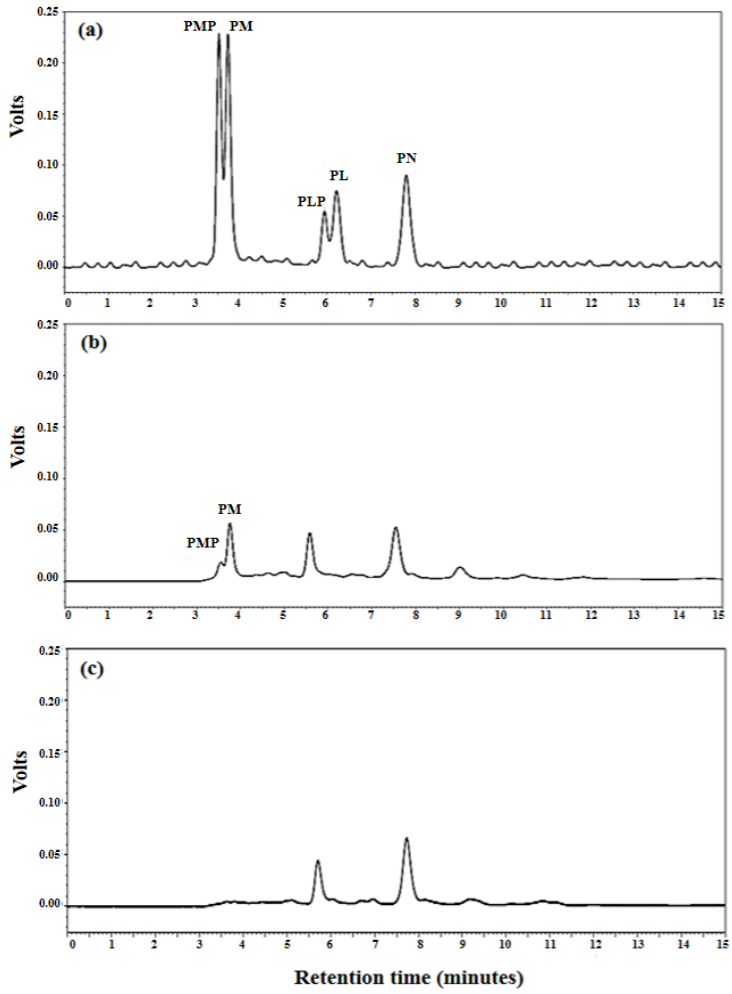

Figure 3 HPLC chromatograms of vitamin B6 in the supernatant of *R*. 6-1C1. (a) Standard solution of vitamin B6 (400 nM of each form of vitamin B6). (b) The supernatant of cultured broth of *R*. 6-1C1 after 24 h growth at 37 °C and (c) the SM broth.

Purification of vitamin B6

Cell-free medium was precipitated with 7.5 % TCA. The protein in the supernatant was removed as shown in the data on the UV of deproteinized sample after adjusting the pH to 7 with 1M NaOH (**Figure 4**). There was no absorption at 280 nm in the UV spectrum, indicating that a high amount of protein was removed from culture broth.

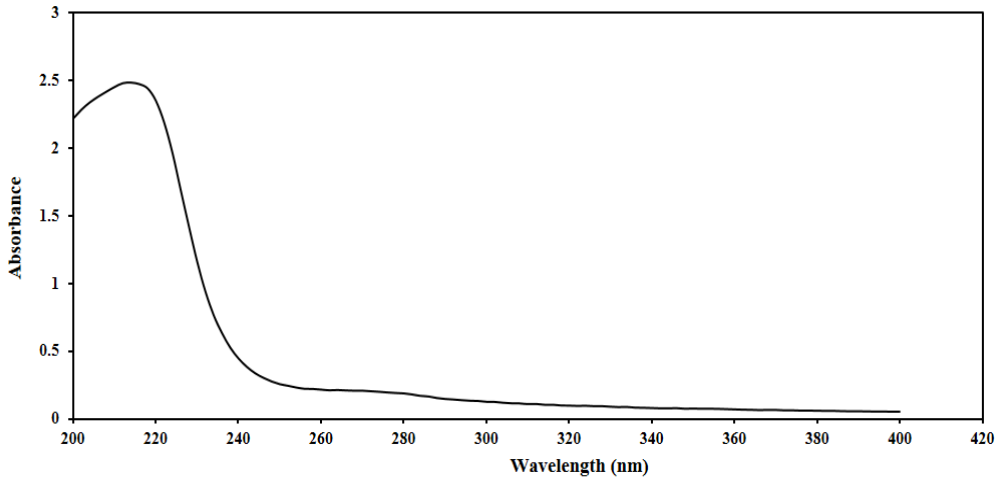

Figure 4 UV-vis absorption spectra of the deproteinized supernatant from cultured broth of *R*. 6-1C1.

Purification steps were summarized in **Table 1**. The purification fold and yield after TCA precipitation were 14.88 and 41.45 %, respectively. This result is in disagreement with what was reported by Lumeng *et al*. [13] that the use of 7.5 % TCA did not chemically alter added vitamin B6 in the extraction procedure. Usually, TCA has been largely used for removing protein and extraction in the measurement of vitamin B6 [19]. The protein precipitation efficiency of TCA is due to the 3 chlorine atoms in the molecule [20]. It can be removed from the aqueous phase by organic solvent extraction with diethyl ether [19]. Normally, using the concentration of TCA ranges between 5 - 50 % (w/v) and depends on the binding of vitamin B6 with substances in the samples [21,22]. From our result, a decrease in the percent recovery may occur from partially lability of the produced vitamin B6 in free form with a high concentration of acid.

After TCA precipitation, the supernatant was applied to the Dowex-50 W-X8 column. There were 7 peaks (P1-P7) which were eluted stepwise by increasing the pH and ionic strength and responded to the growth of *S. carlsbergensis* (**Figure 5**). The column resin contained a negative functional group that allowed the positive charge of vitamin B6 to bind under acidic conditions.

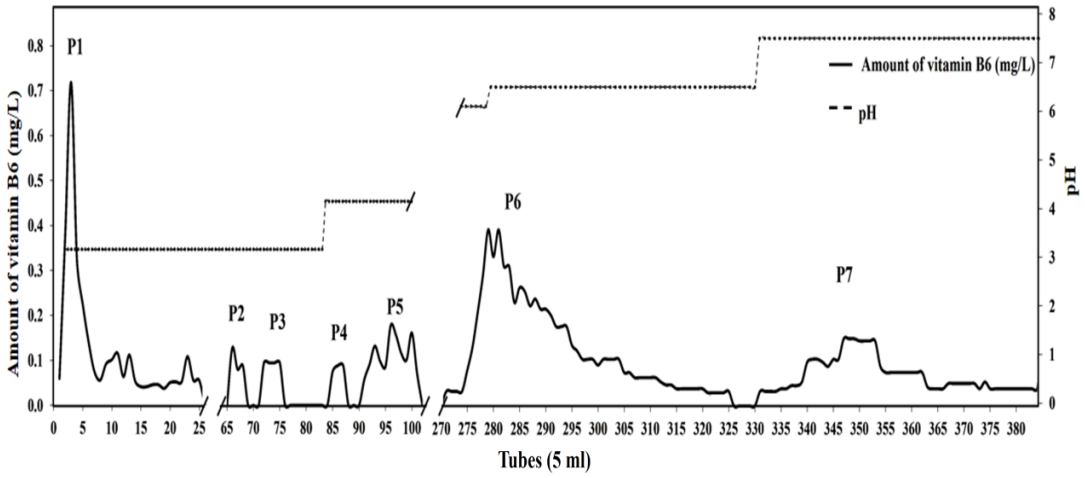

Figure 5 Elution profile of the deproteinized supernatant from cultured broth of *R*. 6-1C1 by Dowex 50W-X8 cationic-exchange chromatography. Amount of vitamin B6 was determined by ADA. The vitamin B6 fractions were eluted stepwise by increasing the pH and ionic strength.

The major components of SM were 2 % peptone and 2 % glucose; peptone was removed by TCA precipitation. Glucose caused the microbial growth. Each fraction of the Dowex-50 W-X8 column was determined for the glucose content by the phenol sulfuric acid method (data not shown). The result showed that the total amount of glucose was co-eluted with P1 and could not be measured in the 6 other peaks. Since glucose is a non-ionized substance, it could not bind with the negative charge column resin, so it was co-eluted with the 0.01M ammonium formate (pH 3.2), the equilibrium and wash buffer. To determine the effect of glucose on the growth of *S. carlsbergensis*, the concentrations of glucose (2 - 50 %) were examined on ADA. The result showed that it did not support the growth of *S. carlsbergensis*. Thus, P1 should be vitamin B6 or other substances in the culture broth that could support the growth of *S. carlsbergensis*.

As shown in **Figure 5**, P2 and P3 were separately eluted with 0.1 M ammonium formate (pH 3.2) after increase of the ionic strength. When the pH of the eluted buffer increased, P4 and P5 were separately eluted with 0.095 M sodium acetate (pH 4.2). No peaks responding to the growth of *S. carlsbergensis* were observed at pH values of 5.2 - 6.1. The elution of P6 and P7 at increased pH values of 6.5 and 7.5, respectively.

Table 1 Summary of purification of vitamin B6 from the cultured broth of *R*. 6-1C1.

Steps	Volume (ml)	Total vitamin B6 (mg)	Total protein (mg)	Total vitamin B6 (mg)/ Total protein (mg)	Purification fold	Yield (%)
Crude	20 ml	0.043	1.212	0.035	1.000	100.00
Deproteinized sample Dowex 50W- X8	20 ml	0.018	0.034	0.526	14.88	41.45
P5	55 ml	0.006	0.001	6.231	176.41	14.56
P7	275 ml	0.019	0.001	19.276	545.72	45.04

Identification of produced vitamin B6 forms

To identify the forms of vitamin B6, all peaks were examined by HPLC. The retention time of 5 peaks (P1-P4 and P6) were different from that of standard vitamin B6 but they corresponded to that of the SM broth (data not shown). The retention times of P5 and P7 corresponded to that of PMP and PM standards, respectively (**Figure 6**). To confirm the retention time of P5 and P7, each 100 nM internal standard PMP and PM were added in the P5 and P7, respectively. Both peak heights of the P5 and P7 increased with an increasing amount of internal PMP and PM as shown in **Figure 6**, respectively.

Five peaks (P1-P4 and P6) could support the growth of *S. carlsbergensis*, which the retention time of 5 peaks corresponding to that of the SM broth. To determine the effect of the ingredients in SM broth, all ingredients were examined on ADA. The results showed only peptone could support the growth of *S. carlsbergensis* among all ingredients (data not shown). The peptone is made from hydrolysis of animal protein, used as an organic nitrogen source. It contains a variety of free amino acids, peptides and also vitamins. So, the substance supported the growth of yeast might be unknown and found in the peptone.

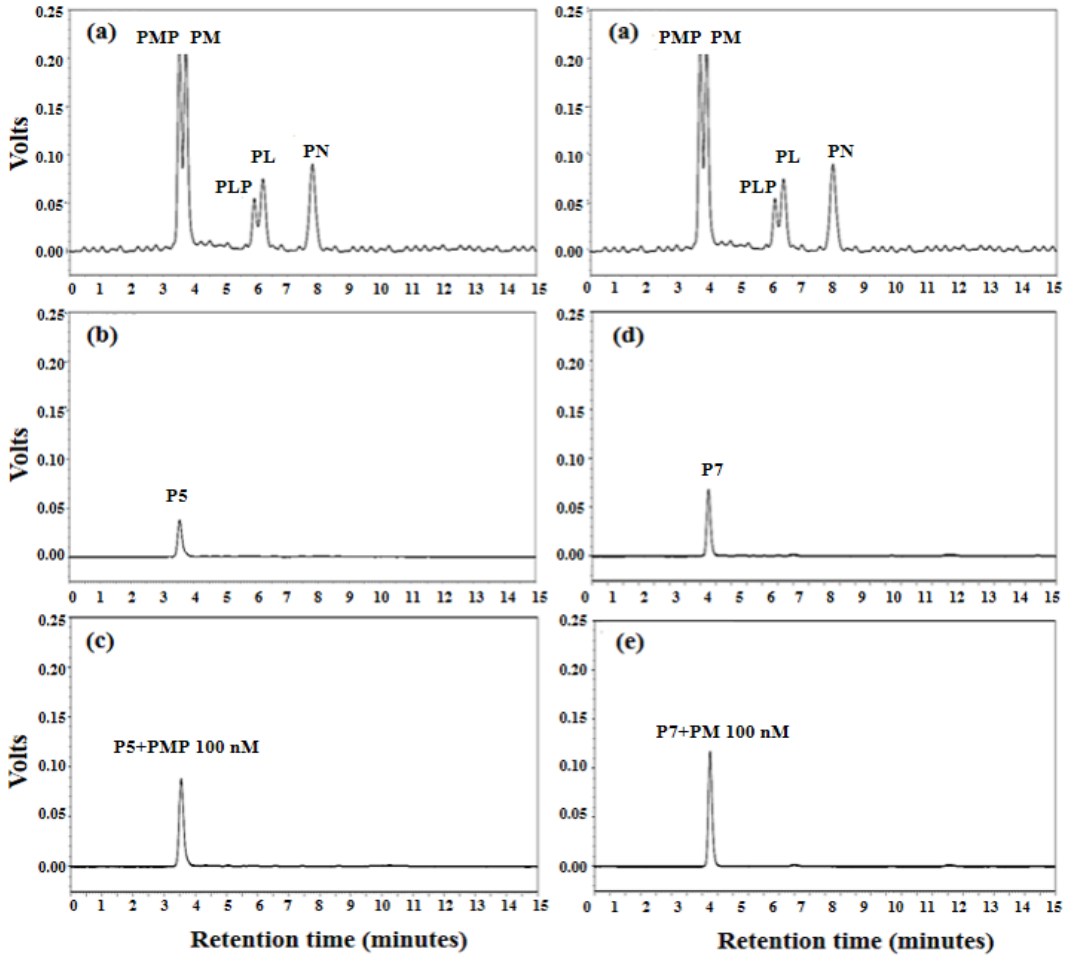

Figure 6 HPLC chromatograms of *R*. 6-1C1 vitamin B6 fractions. (a) Standard solution of vitamin B6 (400 nM of each form of vitamin B6). (b) P5 (eluted with 0.095 M sodium acetate, pH 4.2). (c) P5 with 100 nM internal Standard PMP. (d) P7 (eluted with 0.12 M sodium phosphate, pH 7.5). (e) P7 with 100 nM internal standard PM.

Their phosphorylated derivatives of the P5 and P7 were determined by ADA with and without a dephosphorylation step. The yeast *S. carlsbergensis* (ATCC 9080) has been widely accepted as an organism for the microbiological assay of vitamin B6 in food and other biological materials. This yeast only responds to 3 free forms of vitamin B6. When P5 was tested by ADA without dephosphorylation, the growth zone was not observed (**Figure 7a**). After P5 was dephosphorylated and examined by ADA, the growth zone was observed. This result confirmed that P5 is PMP. When P7 was examined by ADA with and without dephosphorylation, both of the growth zones were observed (**Figure 7b**). This result confirmed that P5 is PM.

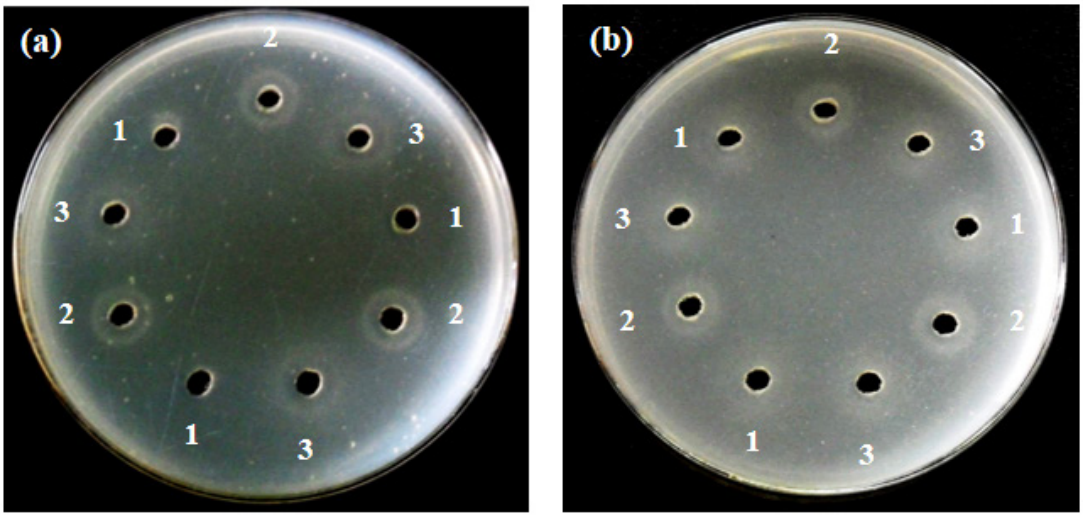

Figure 7 Determination of their phosphorylated derivatives of *R*. 6-1C1 vitamin B6 fractions by ADA. (a) P5, (b) P7; (1) without dephosphorylation; (3) with dephosphorylation; (2) standard PN (2 ng) as the reference zone.

Fluorescence spectra of PMP and PM

The fluorescence spectra of P5 and P7 were compared with that of standard PMP and PM as shown in **Figure 8**. The excitation and emission spectra of both peaks synchronized with those of standard PMP and PM. All substances showed excitation maxima at 327 nm and their emission maximum were at 393 nm. The different tautomeric forms of vitamin B6 in the different solution show that vitamin B6 has fluorescence properties [23]. These data confirmed that P5 and P7 purified from cultured broth of *R*. 6-1C1, were PMP and PM, respectively.

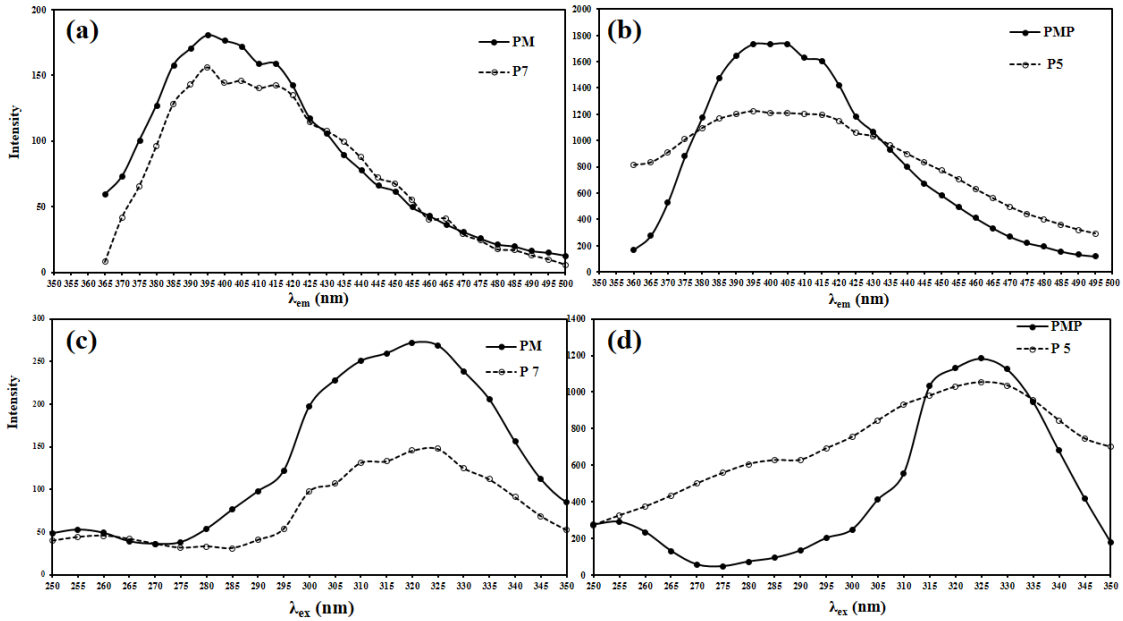

Figure 8 Fluorescent spectra of P5 and P7 comparing with standard PMP and PM in 0.05 M NaH_2PO_4 pH 7.0. (a and c) Excitation spectra with an emission wavelength at 388 nm of P5 and P7. (b and d) Emission spectra with an excitation wavelength at 324 nm of P5 and P7.

Conclusions

This is the first study of the purification of PM and PMP from cultured broth of *R.* 6-1C1. Protein from the supernatant was removed by TCA precipitation and then purified using a Dowex-50 W-X8 column into 7 peaks. Reversed-phase HPLC, fluorescence spectroscopy and dephosphorylation were used for identification. Five peaks (P1-P4 and P6) belonged to ingredients in SM broth. P5 and P7 were PMP and PM, respectively. However, the produced PM and PMP from cultured broth of *R.* 6-1C1 were further characterized.

Acknowledgements

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