Biochimica et Biophysica Acta, 1116 (1992) 67–71

BBAGEN 23647

Formation of β-galactosyl compounds of pyridoxine in growing culture of *Sporobolomyces singularis*

Yukio Suzuki and Kei Uchida

Research Institute for Bioresources, Okayama University Kurashiki (Japan)

(Received 21 October 1991)

Key words: Pyridoxine 5′-β-galactoside; Pyridoxine 4′-β-galactoside; Pyridoxine 4′-β-galactobioside; (*S. singularis*)

Several pyridoxine compounds were found to be formed in a high yield in a growing culture of *Sporobolomyces singularis* containing lactose and pyridoxine. Three compounds, I, II and III, were isolated from cultured broth by Dowex 50W-X8 column chromatography, paper chromatography, lyophilization, and then obtained as needle crystals (m.p. (decomp.): I, 204–206°C; II, 192–194°C; III, 222–223°C. $[\alpha]_D^{16}$: I, −27.7° (c = 3.82, H_2O); II, −1.6° (c = 2.52, H_2O); III, +8.3° (c = 3.98, H_2O)). Compounds I, II and III were identified as 5′-*O*-(β-D-galactopyranosyl)-pyridoxine, 4′-*O*-(β-D-galactopyranosyl)-pyridoxine, and 4′-*O*-(β-D-galactopyranosyl-(1 → 4)-β-D-galactopyranosyl)-pyridoxine, respectively, on the basis of the various experimental results, viz., ultraviolet, infra-red, ^{1}H-NMR, and ^{13}C-NMR spectra, products by hydrolysis with acid and with α- and β-galactosidases, migration on paper electrophoresis, and Gibbs reaction in the presence and absence of boric acid. Also, the yeast produced a remarkable amount of β-glucosyl compounds of pyridoxine in cultured broth, when grown on cellobiose and pyridoxine.

Introduction

5′-*O*-(α-D-Glucopyranosyl)-pyridoxine and 4′-*O*-(α-D-glucopyranosyl)-pyridoxine, new derivatives of vitamin B_6, were first found by Ogata et al., in the culture broth of *Sarcina lutea* which was grown on the medium consisting of sucrose and pyridoxine [1–3]. Subsequently, we reported that 5′-*O*-(β-D-glucopyranosyl)-pyridoxine and 4′-*O*-(β-D-glucopyranosyl)-pyridoxine were formed when cellobiose and pyridoxine were incubated with wheat bran β-glucosidase (cellobiase) [4]. Both β-glucosylpyridoxines were formed in germinating seeds of wheat, barley, and rice cultured in a pyridoxine solution, whereas only 5′-*O*-(β-D-glucopyranosyl)-pyridoxine accumulated in germinating soybean seeds. Also, β-glucosylpyridoxine formation was widely distributed in various plant seedlings cultured in a pyridoxine solution [5]. Furthermore, we reported on the formation of two β-glucopyranosyl-pyridoxines in plant calluses and cells grown on the sucrose media with pyridoxine [6]. On the other hand, β-glucosyl compounds of pyridoxine were found in nature: 5′-*O*-(β-D-glucopyranosyl)-pyridoxine [7,8] and three pyridoxine β-cellooligosaccharides [9] in rice bran and plant-derived foods. Only glucosides of pyridoxine have hitherto been reported. The present paper describes a remarkable formation of two β-galactosylpyridoxines and a β-galactobiosylpyridoxine in a growing culture of *Sporobolomyces*, their isolation and characterization.

Correspondence: Y. Suzuki, Research Institute for Bioresources, Okayama University, Kurashiki, 710, Japan.

Materials and Methods

Materials

Sporobolomyces singularis ATCC 24193 was obtained from American Type Culture Collection, Maryland, U.S.A. Pyridoxine · HCl was obtained from Nacalai tesque, Kyoto. Crystalline β-galactosidase from *Escherichia coli* (Boehringer Mannheim Japan, Tokyo) and α-galactosidase from *Mortierella vinacea* (Seikagaku Kogyo, Tokyo) were purchased through commercial routes. β-Galactosidase from *Aspergillus oryzae* was kindly supplied from Kohjin, Tokyo. Other reagents used were of analytical grade of commercial sources.

Cultivation

Sporobol. singularis was grown at 25°C in the dark with shaking on a culture medium containing 5% lactose, 0.75% yeast extract and 2% pyridoxine · HCl, as pyridoxine, adjusted to pH 3.7. After a 4-day cultivation, a culture broth (2 l) was centrifuged to remove the cells. The supernatant solution was used for the isolation of pyridoxine compounds. The intact cells were prepared as follows. The yeast was grown at 25°C for 4 days with shaking on the culture medium (ad-

justed to pH 3.7) without pyridoxine · HCl. After cultivation, the cells were collected by centrifugation, washed two times with 0.85% NaCl, and suspended in the same solution (0.1 g of cells (as dry weight) per ml).

Analyses

Assay of pyridoxine compounds. A suitable amount of the supernatant fluid was applied as a band on a Toyo filter paper No. 50 (40 × 40 cm), and developed twice by ascent in *n*-butanol-pyridine-water (6:4:4, v/v). After drying, purplish fluorescent bands of pyridoxine and its compounds were detected on chromatogram under an ultraviolet ray lamp (2537 Å filter), cut out, and extracted with 0.1 M phosphate buffer (pH 6.8) for 3 h at 37°C. The amount of pyridoxine compound in each extract was measured by the diazotized *p*-aminoacetophenone method with slight modification [2].

Hydrolysis by α- and β-galactosidases. The reaction mixture containing the isolated pyridoxine compound (4 mg), 5 units of enzyme, and buffer in a total volume of 1 ml was incubated for 20 h. Control experiments were carried out with pyridoxine (2.5 mg). 0.05 M acetate buffer (pH 5.3) was used in the reaction with *Mortierella* α-galactosidase at 25°C, 0.05 M phosphate buffer (pH 7.3) with *E. coli* β-galactosidase at 37°C, and 0.05 M phosphate buffer (pH 5.0) with *Asp. oryzae* β-galactosidase at 37°C. The amounts of pyridoxine and sugar (galactose) released were estimated by the diazotized *p*-aminoacetophenone method [2] and the method of Nelson [10], respectively.

Instrumental analyses. Ultraviolet (UV) absorption spectra were measured with a Hitachi recording spectrophotometer model EPS-3T. Infra-red (IR) absorption spectra were measured in a KBr tablet with a Hitachi IR spectrophotometer model 260-30. ^{1}H-NMR spectra were taken on a Varian spectrometer model VXR-500 at 500 MHz in dimethylsulfoxide-d_6 containing small volume of D_2O as solvents, and with tetramethylsilane (TMS) as an internal standard. ^{13}C-NMR spectra were taken in D_2O with sodium 3-(trimethylsilyl)-propanesulfonate as internal standard. $[\alpha]_D^{16}$ was measured on a Nippon Bunko digital polarimeter model DIP-360.

Results and Discussion

The formation of galactosyl compounds of pyridoxine from lactose and pyridoxine was investigated with various microorganisms. Screening showed that a remarkable amount of the compounds accumulated in the cultured broth of *Sporobol. singularis* when grown on a lactose medium containing pyridoxine. The compounds were not formed in a galactose medium. The isolation and identification of the compounds were attempted as follows.

Isolation of three pyridoxine compounds

After the addition of 1 vol. of methanol to the supernatant culture fluid, pH adjustment to 4.8, and heat treatment for 10 min in a boiling water bath, the mixed solution was centrifuged. The supernatant solution was concentrated below 30°C in vacuo, adjusted to pH 2.0, and put on a Dowex 50W-X8 (H^+ type) column (56 × 120 cm) at 4°C. After washing the column successively with 0.01 M HCl and water to remove sugars, pyridoxine and its compounds were eluted with 1% NH_4OH at 4°C. The eluates containing the compounds were concentrated, and applied to the 1st paper chromatography (PPC) with Toyo filter paper No. 50 in *n*-butanol-pyridine-water (6:4:3, v/v). Each of three fluorescent bands of compounds (I, II and III) having lower R_F values than that of pyridoxine (R_F 0.80) on chromatogram was cut out, and extracted with acetic acid aqueous solution, pH 4.8. Each extract was applied to the 2nd PPC with a solvent system of *n*-butanol/pyridine/water (6:4:4, v/v), in order to separate completely compound I from compound II. After appropriate sectioning and elution, each compound solution was concentrated, decolorized with a small amount of active charcoal, and lyophilized. Each purified preparation of compounds I and III was crystallized several times from ethanol, and that of compound II from the mixture of ethanol/benzene (1:3, v/v) to give fine needle crystals. All crystalline preparations were dried in vacuo on P_2O_5 (Yield: I, 2.97 g; II, 11.25 g; III, 2.21 g). Their melting points and $[\alpha]_D^{16}$ were as follows. m.p. (decomp.): I, 204–206°C; II, 192–194°C; III, 222–223°C. $[\alpha]_D^{16}$: I, −27.7° (c = 3.82, H_2O); II, −1.6° (c = 2.52, H_2O); III, +8.3° (c = 3.98, H_2O). The results of elementary analyses of the compounds were as follows. I, Found: C, 50.18; H, 6.29; N, 4.13%. Calcd. for $C_{14}H_{21}NO_8$: C, 50.75; H, 6.39; N, 4.23%. II, Found: C, 50.27; H, 6.44; N, 4.13%. Calcd. for $C_{14}H_{21}NO_8$: C, 50.75; H, 6.39; N, 4.23%. III, Found: C, 47.96; H, 6.54; N, 2.93%. Calcd. for $C_{20}H_{31}NO_{13}$: C, 48.68; H, 6.33; N, 2.84%.

Identification of three compounds

R_F values (I: 0.18, 0.53; II: 0.16, 0.47; III: 0.04, 0.29) of the isolated three compounds I, II, and III on PPC in two solvent systems (*n*-butanol saturated with 0.2 M acetate buffer (pH 5.0), and *n*-butanol/pyridine/water (6:4:3, v/v)) were different from those of pyridoxine (0.69, 0.80), pyridoxal (0.55, 0.75) and pyridoxamine (0.14, 0.31). On acid hydrolysis with 0.25 M H_2SO_4 at 100°C for 2 h in the dark, all of three compounds gave a sugar and a blue-violet fluorescent substance, which showed R_F values identical to those of pyridoxine on paper chromatograms. The sugar component in the hydrolyzate was confirmed as galactose on a Kieselgel 60 plate developed with a solvent system of *n*-propanol/2% NH_4OH (2:1, v/v) and also by high-perfor-

mance liquid chromatography using Water's carbohydrate analysis column developed with acetonitrile-water (90:10, v/v). Compounds I and II were completely hydrolized by either *E. coli* β-galactosidase or *Asp. oryzae* β-galactosidase to pyridoxine and galactose, but *Mortiella* α-galactosidase had no effect on compounds I and II. Compound III was readily hydrolyzed by *Asp. oryzae* β-galactosidase, and very weakly by *E. coli* β-galactosidase, but not by *Mortiella* α-galactosidase. The molar ratio of pyridoxine and galactose liberated was around 1:1 in compounds I and II, and 1:2 in compound III. No reducing activity for any of the compounds was found by the Nelson method. Ultraviolet absorption spectra of three compounds at acidic, neutral and alkaline pH, showed the position of maxima and minima in the resemblance with those of pyridoxine. But the λ_{max} values of three compounds were slightly higher than those of pyridoxine as follows. Pyridoxine, I, II, and III: $\lambda_{max}^{0.1\,M\ HCl}$ nm: 291, 291, 292, 292; $\lambda_{max}^{pH\,7.0}$ nm: 254 and 325, 256 and 325, 254 and 328, 254 and 328; $\lambda_{max}^{0.1\,M\ NaOH}$ nm: 245 and 309, 246 and 311, 246 and 314, 246 and 314. The examination of ^{1}H-NMR spectra (in $DMSO_4$-d_6, δppm) showed the following signals. I: a methyl signal in pyridoxine moiety (2.3451, 3H, s), signals of ring protons of the sugar moiety (3.2–3.8 ppm), one anomeric proton signal of β-galactosidic linkage (4.1155, 1H, d, $J = 7.33$ Hz), signals of 4′-methylene protons (4.7451, 2H, d, $J = 4.12$ Hz) and doublets of 5′-methylene protons (H_A, 4.5543, 1H, d, $J = 11.99$ Hz; H_B, 4.7964, 1H, d, $J = 11.94$ Hz), and a methyne signal of H–6 (7.9055, 1H, s) in pyridoxine moiety. II: a methyl signal in pyridoxine moiety (2.3621, 3H, s), signals of ring protons of the sugar moiety (3.2–3.8 ppm), one anomeric proton signal of β-galactosidic linkage (4.2350, 1H, d, $J = 7.60$ Hz), signals of 58′-methylene protons (4.5334, 2H, d, $J =$ 4.40 Hz) and doublets of 4′-methylene protons (H_A, 4.6942, 1H, d, $J = 11.71$ Hz; H_B, 4.9821, 1H, d, $J =$ 11.71 Hz), and a methyne signal of H–6 (7.9455, 1H, s) in pyridoxine moiety. III: a methyl signal in pyridoxine moiety (2.3612, 3H, s), signals of ring protons of the sugar moiety (3.2–3.9 ppm), two anomeric proton signals of β-galactosidic linkage (4.2390, 1H, d, $J = 7.70$ Hz) and β-galactosidic linkage of non-reducing residue in galactobiosyl moiety (4.2448, 1H, d, $J = 7.76$ Hz), signals of 5′-methylene protons (4.5322, 2H, d, $J = 4.78$ Hz) and doublets of 4′-methylene protons (H_A, 4.6942, 1H, d, $J = 11.61$ Hz; H_B, 4.9414, 1H, d, $J = 11.64$ Hz), and a methyne signal of H–6 (7.9454, 1H, s) in pyridoxine moiety. These data suggested that three compounds were two β-D-galactopyranosylpyridoxines and a β-D-galactobiosylpyridoxine. Their infra-red spectra supported this. In paper electrophoresis at 400 V for 2 h with 0.01 M phosphate buffer (pH 7.0), all of three compounds migrated at a similar rate to pyridoxine toward the cathode. All compounds gave a reddish

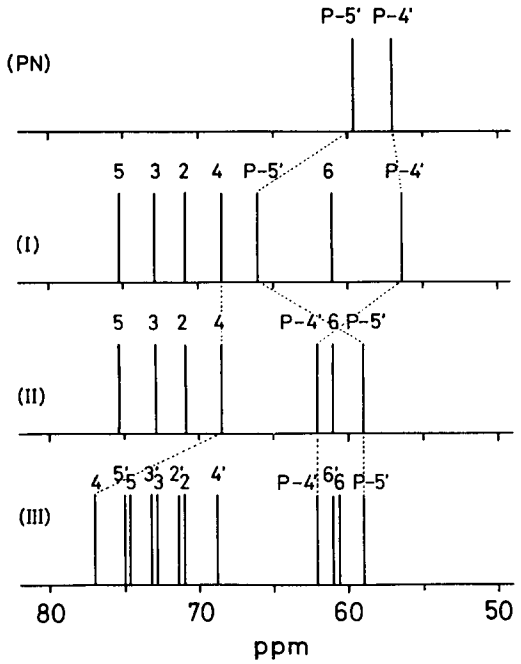

Fig. 1. Relevant ^{13}C-NMR resonances of pyridoxine and compounds I, II and III. PN: pyridoxine. Carbon No. P–4′ and P–5′: C–4′ and C–5′ in pyridoxine moiety. Carbon No. 2 ~ 6: C–2 ~ C–6 in galactose moiety which was linkaged to pyridoxine moiety. Carbon No. 2′ ~ 6′: C–2′ ~ C–6′ in non-reducing end galactose moiety in compound III.

orange color with diazotized *p*-aminoacetophenone. Compound I gave the positive Gibbs color reaction with 2,6-dichloroquinone chloroimide, but the reaction was negative in the presence of boric acid. While, compounds II and III gave the positive Gibbs reaction both in the presence and absence of boric acid. These qualitative tests showed that 3- and 4′(5′)-hydroxyl groups and N–1 in pyridine ring of three compounds were unsubstituted. To confirm their fine structures, carbon-13 chemical shifts of three compounds in D_2O were compared with those of pyridoxine (Fig. 1). Signal assignments were based upon the data reported for methyl-β-D-galactopyranoside [11]. Galactosylation of pyridoxine in compounds I and II caused a large chemical shift of C-5′ and C-4′ in pyridoxine moiety, respectively. In compound III, carbon-13 chemical shifts of the pyridoxine moiety were very similar to those of compound II, not compound I. The formation of compound III by galactosylation of compound II caused a large chemical shift of C–4 in galactose carbon which was linkaged to pyridoxine moiety. From these results, it was apparent that the galactosylation site in compounds I and II was present at C–5′ and C–4′ in pyridoxine moiety. In compound III (galactosylated compound II), its site was present at C–4′ in pyridoxine moiety and also at C–4 in galactose carbons linkaged to pyridoxine moiety. Thus, compounds I, II and III were identified as 5′-*O*-(β-D-galactopyranosyl)-pyridoxine, 4′-*O*-(β-D-galactopyranosyl)-pyridoxine and

4′-*O*-(β-D-galactopyranosyl-(1 → 4)-β-D-galactopyranosyl)-pyridoxine.

Fluctuation of pyridoxine and its compounds I, II and III during fermentation

As shown in Fig. 2, the formation of compound II commenced in the earlier stage of fermentation and its accumulation attained a maximum value after a 4-day incubation (yield being 71% on pyridoxine used), and thereafter gradually decreased, while compound III was successively increasing during decreasing of compound II. Compound I was formed at the later stage of fermentation, followed by the gradual increase, and the formation of compound IV (5′-*O*-(β-galactobiosyl)-pyridoxine-like compound) was also observed after a 10-day incubation. These fluctuations seem to show that compound II once formed was consumed gradually in order to form compound III by receiving β-galactosyl residue again from lactose; i.e., galactosylpyridoxine (compound II) played the role as the second acceptor in the successive galactosyl transfer, as was already demonstrated with sucrose and riboflavin by the action of dextransucrases from *Leuconostoc mesenteroides* and *Streptococcus bovis* [12–14]. After a 4-day incubation, the combined yield of galactosyl and galactobiosyl compounds of pyridoxine was about 90% on pyridoxine added.

Moreover, *Sporobol. singularis,* when grown on a culture medium (adjusted to pH 3.7) containing cellobiose, yeast extract and pyridoxine, formed both 5′-*O*-(β-D-glucopyranosyl)-pyridoxine (compound I′) and

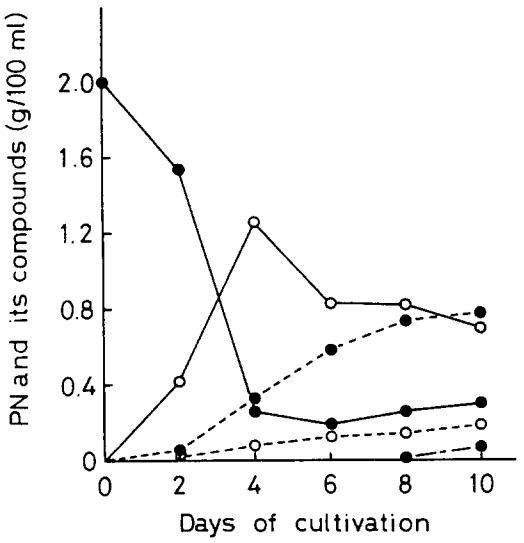

Fig. 2. Formation of β-galactosyl compounds of pyridoxine during fermentation. Fermentation was carried out at 25°C with the culture medium (adjusted to pH 3.7) containing 5% lactose; 0.75% yeast extract, and 2% pyridoxine·HCl as pyridoxine, for 10 days in the dark on a reciprocal shaker. Pyridoxine (PN), ●———●; compound I, ●------●; compound II, ○———○; compound III, ○------○; compound IV, ●– – –●.

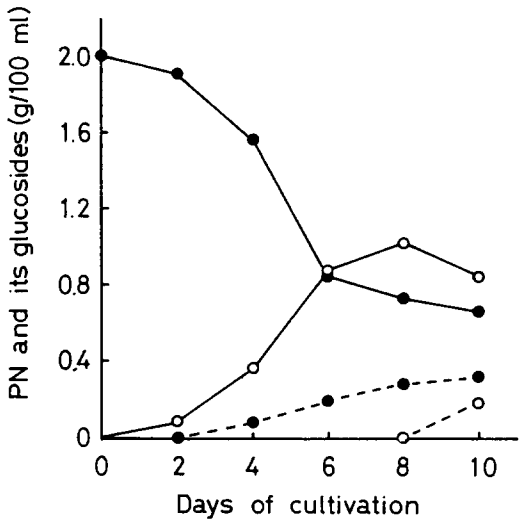

Fig. 3. Formation of β-glucosyl compounds of pyridoxine during fermentation. Pyridoxine (PN), ●———●; compound I′, ●------●; compound II′, ○———○; compound III′, ○------○.

4′-*O*-(β-D-glucopyranosyl)-pyridoxine (compound II′) as the major metabolites, and 4′-*O*-(β-D-cellobiosyl)-pyridoxine-like compound (compound III′) as the minor one at the later stages of fermentation (Fig. 3). These β-glucosyl compounds of pyridoxine were identified by comparing with the characteristics of 4′-*O*-(β-D-glucopyranosyl)-pyridoxine and 5′-*O*-(β-D-glucopyranosyl)-pyridoxine synthesized by the action of wheat bran β-glucosidase [4].

Formation of compounds I and II by intact cells

As shown in Fig. 4, the maximum activity was observed at pH 4.0–4.5. The formation of only compound II occurred in the earlier stage (3 h) of incubation, and compound I also was formed at the later stage (18 h) of incubation. A higher ratio of compound II to compound I was obtained at a low pH, after an 18-h incubation. Lactose, *o*-nitrophenyl β-D-glactopyranoside, cellobiose, salicin, phenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-glucopyranoside were effective on the formation of glycosylpyridoxine, but galactose, glucose, glucose 1-phosphate, melibiose, raffinose, maltose, and sucrose were quite ineffective. Most of the glycosylpyridoxine-forming activity existed in the cells, whereas no activity in cultured filtrate.

It has been already pointed out that *Micrococcus* α-glucosidase [15] and wheat bran β-glucosidase [4] catalyzed the transfer of a glycosyl residue from disaccharides to 5′- and 4′-hydroxymethyl groups of pyridoxine, and the resultant compounds formed were 5′- and 4′-glycosylpyridoxines. On the other hand, uridine 5′-diphosphoglucuronyltransferase from rabbit liver catalyzed the formation of a β-glucuronylpyridoxine from uridine 5′-diphosphoglucuronate and pyridoxine [16]. Also, a particulate enzyme in seedlings of *Pisum*

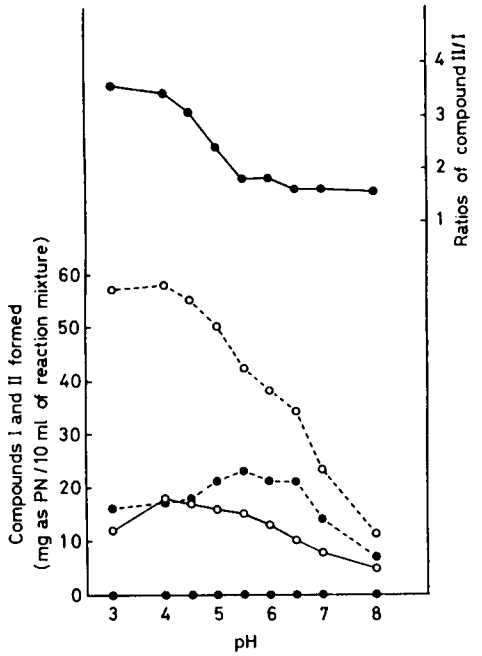

Fig. 4. Effect of pH on the formation of two compounds I and II by intact cells. Incubation time 3 h: compound I, ●———●; compound II, ○———○. Incubation time 18 h: compound I, ●------●; compound II, ○------○. Reaction mixture containing 500 mg lactose, 206 mg pyridoxine·HCl (neutralized to pH 7.0), 5 ml, McIlvaine's buffer (pH 3.8–8.0), 1.3 ml cell suspension (127 mg of cells as dry weight) and deionized water to a final volume of 10 ml was incubated at 25°C for 3 and 18 h in the dark under shaking conditions.

sativum L. Kinusaya was reported to catalyze the transfer of glucose from uridine diphosphoglucose to the 5′-, but not 4′-hydroxymethyl group of pyridoxine [17]. These data on the enzymes catalyzing the formation of glycosyl-pyridoxines suggest that two β-galactosyl- and two β-glucosyl-pyridoxines may be formed by the action of β-galactosidase and β-glucosidase in the cells of *Sporobol. Singularis.*

Kabir et al. reported that 5′-*O*-(β-D-glucopyranosyl)-pyridoxine was a common component of vitamin B_6 of plant-derived foods, but not detected in animal-derived foods including meats, human milk, and cow's milk [18]. And, the bioavailability of 5′-*O*-(β-D-glucopyranosyl)-pyridoxine in the rat was observed to be less than 40% of that of pyridoxine [19]. Its availability for the growth of *Saccharomyces carlsbergensis* was not more than 10% of that of equivalent mole of pyridoxine after a 16 h incubation (unpublished data). The physiological role of these pyridoxine glycosides is still not clear.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (No. 62560108) from the Ministry of Education, Japan, and by a research fund from Asahi Chemical Industry Co. Ltd. We wish to thank the SC-NMR Laboratory of Okayama University for the measurements of the ^{1}H- and ^{13}C-NMR spectra, and Miss H. Kushida for her technical assistance.

References

1 Ogata, K., Tani, Y., Uchida, Y. and Tochikura, T. (1968) Biochim. Biophys. Acta 165, 578–579.
2 Ogata, K., Tani, Y., Uchida, Y. and Tochikura, T. (1969) J. Vitaminol. 15, 142–150.
3 Ogata, K., Uchida, Y., Kurihara, N., Tani, Y. and Tochikura, T. (1969) J. Vitaminol. 15, 160–166.
4 Suzuki, Y., Uchida, K. and Tsuboi, A. (1979) Nippon Nogeikagaku Kaishi 53, 189–196.
5 Suzuki, Y., Inada, Y. and Uchida, K. (1986) Phytochemistry 25, 2049–2051.
6 Suzuki, Y., Ishii, H., Suga, K. and Uchida, K. (1986) Phytochemistry 25, 1331–1332.
7 Yasumoito, K., Tsuji, H., Iwami, K. and Mitsuda, H. (1977) Agric. Biol. Chem. 41, 1061–1067.
8 Gregory, J.F. and Ink, S.L. (1987) J. Agric. Food Chem. 35, 76–82.
9 Tadera, K., Kaneko, T. and Yagi, F. (1988) J. Nutr. Sci. Vitaminol. 34, 167–177.
10 Nelson, N. (1944) J. Biol. Chem. 153, 375–380.
11 Voelter, W., Breitmaier, E., Rathbone, E.B. and Stephen, E.M. (1973) Tetrahedron 29, 3845–3848.
12 Suzuki, Y. and Katagiri, H. (1963) J. Vitaminol. 9, 293–298.
13 Suzuki, Y. and Uchida, K. (1967) Vitamins (Japan) 35, 27–30.
14 Suzuki, Y. (1987) Jpn. J. Dairy Food Sci. 36, A-303–A-310.
15 Kawai, F., Yamada, H. and Ogata, K. (1971) Agric. Biol. Chem. 35, 184–190.
16 Suzuk, Y. and Uchida, K. (1977) Vitamins (Japan) 51, 507–511.
17 Tadera, K., Yagi, F. and Kobayashi, A. (1982) J. Nutr. Sci., Vitaminol. 28, 359–366.
18 Kabir, H., Leklem, J. and Miller, L.T. (1983) J. Food Sci. 48, 1422–1425.
19 Ink, S.L., Gregory, J.F. and Sartain, D.B. (1986) J. Agric. Food Chem. 34, 857–862.