APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2003, p. 7058–7062 Vol. 69, No. 12
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.12.7058–7062.2003

Use of Borate To Control the 5′-Position-Selective Microbial Glucosylation of Pyridoxine

Koichi Wada* and Yasuhisa Asano

Biotechnology Research Center, Toyama Prefectural University, Kosugi, Toyama 939-0398, Japan

Received 26 March 2003/Accepted 23 September 2003

Nearly 100% 5′-position selectivity of transglucosylation from maltodextrin to pyridoxine (PN) by cells of *Verticillium dahliae* TPU 4900 was observed when the reaction was carried out with borate. The same effect of borate was observed not only during synthesis of pyridoxine 5′-α-D-glucoside by partially purified enzyme of this strain but also during synthesis of this compound by other microorganisms and with other enzymes (α-glucosidase and cyclomaltodextrin glucanotransferase). The effect was thought to be caused by the formation of a borate complex with 3- and 4′-position hydroxyl groups of PN. A decrease in the formation of pyridoxine 5′-α-D-glucoside was observed in the reaction with borate, but this decrease was overcome by optimizing the pH and increasing the amount of cells in the reaction mixture.

Pyridoxine 5′-α-D-glucoside (PN-5′-α-Glc) has nutritional importance as vitamin B_6 and exhibits stability in the presence of light irradiation (6, 7, 25). Recently, we found that some fungi (e.g., *Verticillium* and *Coriolus*) enzymatically synthesize PN-5′-α-Glc selectively from pyridoxine (PN) and maltodextrin (Fig. 1) (2). We selected *Verticillium dahliae* TPU 4900 (Toyama Prefectural University, Kosugi, Toyama, Japan), which produced PN-5′-α-Glc with a 35% molar yield from 5 mM PN and had 5′-position selectivity, by cultivation. The ratio of PN-5′-α-Glc to pyridoxine 4′-α-D-glucoside (PN-4′-α-Glc), one of the position isomers of pyridoxine α-D-glucoside (PN-α-Glc), in the culture broth was 96:4. Moreover, the productivity of *V. dahliae* TPU 4900 was markedly increased by optimization of the culture and reaction conditions (28). Intact cells of *V. dahliae* TPU 4900 produced PN-5′-α-Glc with a 41% molar yield from 600 mM PN with a ratio of PN-5′-α-Glc to PN-4′-α-Glc of 85:15 in the preparative reaction. The decrease in the ratio of PN-5′-α-Glc to PN-4′-α-Glc in the reaction with intact cells was caused by the decrease in the rate of PN-5′-α-Glc synthesis in the later stage of the reaction, whereas the rate of PN-4′-α-Glc synthesis was depressed little. During purification of PN-5′-α-Glc from a mixture of PN-α-Glc by preparative high-performance liquid chromatography or ion-exchange chromatography, some PN-5′-α-Glc was lost when all of the PN-4′-α-Glc was removed, because the physical characteristics of these compounds are similar. Thus, we preferred to minimize the amount of PN-4′-α-Glc during practical production of PN-5′-α-Glc from a high concentration of PN. In this paper, we describe the effect of borate on enhancing 5′-position selectivity of enzymatic transglucosylation to PN and the possibility that this effect could be applied to practical formation of PN-5′-α-Glc. This is the first report showing that an inorganic anion enhances the selectivity of the enzyme.

* Corresponding author. Present address: Daiichi Fine Chemical Co., Ltd., 530 Chokeiji, Takaoka, Toyama 933-8511, Japan. Phone: 81-766-26-4409. Fax: 81-766-26-4462. E-mail: k-wada@daiichi-fcj.co.jp.

MATERIALS AND METHODS

Chemicals. Pyridoxine hydrochloride (PN-HCl) was provided by Daiichi Fine Chemical Co., Ltd. (Takaoka, Toyama, Japan). Maltodextrin (TK-16; average degree of polymerization, 6; prepared from tapioca starch) was purchased from Matsutani Chemical Industries Co., Ltd. (Itami, Hyogo, Japan). Soluble starch, Polypeptone, boric acid (H_3BO_3), and sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); yeast extract was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan); and Esusan meat (flour of defatted soybeans) was obtained from Ajinomoto Co. Inc. (Tokyo, Japan). α-Glucosidase (from rice) was purchased from Sigma Aldrich Fine Chemicals (St. Louis, Mo.). Cyclomaltodextrin glucanotransferase (CGTase) (from *Paenibacillus macerans*) was provided by Amano Enzyme Inc. (Nagoya, Japan). All other chemicals used were from commercial sources and were analytical grade.

Microorganisms and medium. *V. dahliae* TPU 4900, *Bacillus cereus* TPU 5504, *Edwardsiella hoshinae* TPU 6101, *Ochrobactrum anthropi* TPU 6850, and *Xanthobacter flavus* TPU 7601 were preserved in our laboratory. *V. dahliae* JCM 9510 was purchased from the Japan Collection of Microorganisms, Tokyo, Japan. *V. dahliae* IFO 9765, *Coriolus fibula* IFO 4949, and *C. pubescens* IFO 9782 were obtained from the Institute of Fermentation, Osaka, Japan. *Schizophyllum commune* IAM 9006 was obtained from the Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan.

Medium I contained 2.0% (wt/vol) maltodextrin, 2.0% (wt/vol) sucrose, 1.0% (wt/vol) Polypeptone, 0.05% (wt/vol) yeast extract, 0.5% (wt/vol) K_2HPO_4, 0.1% (wt/vol) KH_2PO_4, 0.02% (wt/vol) $FeSO_4 \cdot 7H_2O$, 0.02% (wt/vol) $MgSO_4 \cdot 7H_2O$, 0.01% (wt/vol) $MnSO_4 \cdot 5H_2O$, and 0.1% (wt/vol) PN-HCl in tap water (pH 7.0). Medium II contained 4% (wt/vol) soluble starch, 1% (wt/vol) Esusan meat, 0.1% (wt/vol) KH_2PO_4, 0.05% (wt/vol) KCl, 0.05% (wt/vol) $MgSO_4 \cdot 7H_2O$, 0.001% (wt/vol) $FeSO_4 \cdot 7H_2O$, and 0.1% (wt/vol) PN-HCl in tap water (pH 7.0).

Analysis of PN-α-Glc. Analysis of PN, PN-5′-α-Glc, and PN-4′-α-Glc was done by high-performance liquid chromatography with a Cosmosil 5C18MS-II column (4.6 by 150 mm; Nakalai Tesque, Kyoto, Japan) monitored at 325 nm. The mobile phase was 1% (vol/vol) methanol, and the flow rate was 1.0 ml/min at 35°C. The retention times of PN, PN-4′-α-Glc, and PN-5′-α-Glc were 7, 10, and 16 min, respectively. The 5′-position selectivity of PN (expressed as a percentage) was determined as follows: [(amount of PN-5′-α-Glc)/(amount of PN-5′-α-Glc + amount of PN-4′-α-Glc)] × 100. Identification and quantification of PN-α-Glc were done by comparison with authentic samples, which were prepared as described previously (2).

Synthesis of PN-5′-α-Glc by cells or enzyme from *V. dahliae* TPU 4900. The basic reaction mixture (total volume, 1.2 ml) consisted of 100 mM potassium phosphate buffer (pH 7.0) containing 0.12 mmol of PN-HCl, 24 mg of maltodextrin, and cells or enzyme from *V. dahliae*. In the assay for PN-5′-α-Glc-forming activity, the reaction mixture was incubated at 40°C for 2 h with shaking in the dark. One unit of PN-5′-α-Glc-forming activity was defined as the ability to produce 1 μmol of PN-5′-α-Glc per min under the assay conditions. For the reaction with cell extract and a partially purified enzyme, the reaction mixture was incubated at 40°C for 8 h in the same volume of the basic reaction mixture

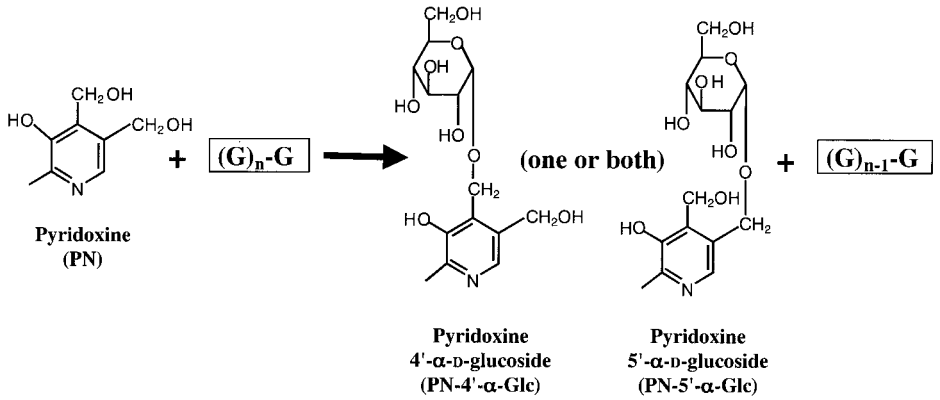

FIG. 1. Enzymatic synthesis of PN-α-Glc. (G)n-G, maltodextrin.

(100 mM potassium phosphate buffer [pH 7.0] or 25 mM $Na_2B_4O_7 \cdot 10H_2O$ [pH 7.0]) containing wet cells of *V. dahliae* TPU 4900 harvested from 1.0 ml of culture broth or 0.11 U of enzyme in solution.

Partial purification of PN-5′-α-Glc-forming enzyme from *V. dahliae* TPU 4900. The PN-5′-α-Glc-forming enzyme from *V. dahliae* TPU 4900 was purified and stored at 0 to 5°C. *V. dahliae* TPU 4900 was cultivated aerobically in 150 ml of medium II in a 500-ml flask at 20°C for 8 days. All of the cells harvested (about 770 g [wet weight] or 131 g [dry weight]) from 8 liters of culture were suspended in 100 mM potassium phosphate buffer. The cells were disrupted twice with a DYNO-Mill KDL (Willy A. Bachofen AG, Basel, Switzerland). The disrupted cells were removed by centrifugation at 10,600 × *g* for 20 min. As shown in Table 1, 35% of the activity of the wet cells was in the cell extract. Next, ammonium sulfate was added to the extract to 30% saturation to remove inactive residue and then brought to 80% saturation. The precipitate was recovered on filter paper, dissolved in 10 mM potassium phosphate buffer (pH 7.0), and dialyzed against the same buffer. The dialyzed enzyme solution was applied to a DEAE-Toyopearl 650 M column (2.5 by 33 cm; Tosoh, Co., Tokyo, Japan) equilibrated with 10 mM potassium phosphate buffer (pH 7.0). After the column was washed with the same buffer containing 100 mM NaCl, the active enzyme was eluted with the buffer containing 200 mM NaCl. The active fractions were combined, dialyzed, and applied to a hydroxyapatite column (2.5 by 17.5 cm). The active fractions were eluted with 200 mM potassium phosphate buffer (pH 7.0), concentrated by ultrafiltration, and applied to a column of Superdex 200 HR 26/60 (Amersham Bioscience, Piscataway, N.J.) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 150 mM NaCl. The active fractions were collected and concentrated by ultrafiltration. The partially purified enzyme was obtained with an overall yield of 18%, and the specific activity was 29-fold greater than that of the original extract. The enzyme was not purified to homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Synthesis of PN-5′-α-Glc by intact cells of regioselective microorganisms. *V. dahliae* TPU 4900 was cultured in medium II for 9 days at 20°C. Other 5′-position-selective microorganisms were cultured in the same medium for 7 days at 25°C. 4′-Position-selective microorganisms were cultured in medium I for 3 days at 30°C. The reaction was performed at 40°C for 18 h, with shaking in the dark, in the basic reaction mixture (total volume, 1.2 ml) consisting of 100 mM potassium phosphate buffer (pH 7.0) or 25 mM $Na_2B_4O_7 \cdot 10H_2O$ (pH 5.0 or 7.0) containing cells harvested from 1.0 ml of culture broth (5′-position-selective microorganisms) or from 3.0 ml of culture broth (4′-position-selective microorganisms). Twenty-four milligrams of maltodextrin was added to the reaction mixtures at 8 h.

TABLE 1. Partial purification of PN-5′-α-Glc-synthesizing enzyme

Step	Activity (U)	Protein (mg)	Sp act		Yield (%)
			U/mg	Fold	
Wet cells (770 g)	834				
Cell extract	290	2,000	0.15	1.0	100
Ammonium sulfate	160	350	0.45	3.0	55
DEAE-Toyopearl	89	39	2.3	15	31
Hydroxyapatite	61	32	1.9	13	21
Superdex 200	51	12	4.4	29	18

Synthesis of PN-5′-α-Glc by α-glucosidase and CGTase. A reaction in which 20 U of α-glucosidase or 120 U of CGTase was used was carried out at 30°C for 70 h by using a reaction mixture (total volume, 1.0 ml) consisting of 180 mM potassium phosphate buffer (pH 7.0) or 45 mM $Na_2B_4O_7 \cdot 10H_2O$ (pH 7.0) containing 0.18 mmol of PN-HCl, 80 mg of maltodexrin, and 0.07 mg of $CaCl_2 \cdot 2H_2O$. One unit of α-glucosidase was defined as the amount of enzyme required to convert 1 μmol of maltose to 2 μmol of D-glucose per min at pH 4 and 37°C, and 1 unit of CGTase was defined by Tilden-Hudson method (24).

Preparative synthesis of PN-5′-α-Glc with intact cells of *V. dahliae* TPU 4900 and borate. *V. dahliae* TPU 4900 was cultured in 150 ml of medium II in a 500-ml flask on a rotary shaker (200 rpm) at 20°C for 7 days. The cells were harvested by centrifugation (10,600 × *g* for 20 min) and then washed with distilled water. The reaction mixture for PN-5′-α-Glc synthesis (total volume, 400 ml) contained 15.3 g (160 mmol as borate) of $Na_2B_4O_7 \cdot 10H_2O$, 32.8 g (160 mmol) of PN-HCl, 8 g of maltodextrin, and the cells harvested from 800 ml of culture broth (15.5 g [dry weight]) and was placed in a 500-ml flask. The reaction was carried out at pH 4.9 to 5.1 (adjusted with NaOH) and 55°C for 48 h in the dark with stirring. Eight grams of dextrin was added six times, at 2, 4, 6, 20, 25, and 30 h.

Maltodexrin hydrolysis by PN-5′-α-Glc-synthesizing enzyme. The reaction mixture for maltodexrin hydrolysis by PN-5′-α-Glc-synthesizing enzyme (total volume, 120 μl) consisted of 100 mM potassium phosphate buffer (pH 7.0) or 25 mM $Na_2B_4O_7 \cdot 10H_2O$ (pH 7.0) containing 2.4 mg of maltodextrin enzyme. The reaction mixture was incubated at 40°C for 60 min in the dark. The glucose released was measured with a glucose determination kit (Glucose B-test; Wako).

RESULTS AND DISCUSSION

Effects of borate on the PN-5′-α-Glc-forming reaction of *V. dahliae* TPU 4900. We investigated the effects of inorganic or organic anions on the 5′-position selectivity of the formation of PN-α-Glc by intact cells of *V. dahliae* TPU 4900 at pH 7. Borate was the only anion among the anions tested that affected the regioselectivity of glucosylation. The reaction mixtures (0.12 mmol of PN-HCl, 24 mg of maltodextrin, and 100 mg of cells in 1.2 ml [total volume]) were incubated at 40°C for 8 h in the dark with either K_2HPO_4, Na_2SO_4, $NaNO_3$, NaCl, sodium acetate, or sodium citrate at a concentration of 100 mM, and the pH was adjusted to 7 with NaOH or HCl, as needed. All of the mixtures produced the same yield of PN-α-Glc (29 to 30%) that was 92 to 93% selective for the 5′ position. Reactions carried out with 25 mM potassium or sodium tetraborate resulted in lower yields (7 to 8% instead of 29 to 30%), but the regioselectivity was 98%.

Next, we partially purified the PN-5′-α-Glc-synthesizing enzyme from *V. dahliae* TPU 4900 as described in Materials and

TABLE 2. Effect of borate on the production of PN-α-Glc by regioselective microorganisms and two enzymes[a]

Microorganism or enzyme	Reaction buffer[b]	pH	Yield of PN-α-Glc (%)		Position selectivity (%)
			PN-5′-α-Glc	PN-4′-α-Glc	
V. dahliae TPU 4900	P	7	40	6.8	86 (5′)
	B	7	14	0.3	98 (5′)
	B	5	28	0.5	98 (5′)
V. dahliae JCM 9510	P	7	36	4.1	90 (5′)
	B	5	22	0.4	98 (5′)
V. dahliae IFO 9765	P	7	32	4.6	87 (5′)
	B	5	17	0.3	98 (5′)
C. fibula IFO 4949	P	7	28	2.7	91 (5′)
	B	7	6.1	ND[c]	>98 (5′)
C. pubescens IFO 9782	P	7	31	2.6	92 (5′)
	B	7	11	0.2	99 (5′)
S. commune IAM 9006	P	7	22	6.4	77 (5′)
	B	5	12	0.3	98 (5′)
B. cereus TPU 5504	P	7	8.4	27	76 (5′)
	B	7	1.9	1.3	59 (4′)
	B	5	ND	ND	
E. hoshinae TPU 6101	P	7	5.6	22	80 (4′)
	B	7	0.3	0.5	64 (4′)
	B	5	ND	ND	
O. anthropi TPU 6850	P	7	7.3	21	74 (4′)
	B	7	0.5	0.5	52 (4′)
	B	5	ND	ND	
X. flavus TPU 7601	P	7	1.8	6.6	78 (4′)
	B	7	1.3	0.5	63 (4′)
	B	5	ND	ND	
α-Glucosidase from rice	P	7	8.5	3.5	71 (5′)
	B	7	3.1	0.3	91 (5′)
CGTase from *P. macerans*	P	7	10.3	6.5	62 (5′)
	B	7	1.1	0.2	83 (5′)

[a] The reactions were performed as described in Materials and Methods.
[b] P, reaction with 100 mM potassium phosphate buffer; B, reaction with 25 mM sodium tetraborate.
[c] ND, not detected.

Methods. The enzyme purification results are summarized in Table 1. Similar borate effects were observed for transglucosylation with cell extract and for transglucosylation with purified enzyme. Reactions performed as described above with 100 mM potassium phosphate (pH 7) in which the cells were replaced with 0.11 U of activity as either crude extract or partially purified enzyme resulted in yields of PN-α-Glc (26 to 28%) that were 92% 5′-position selective, whereas parallel reactions with 25 mM sodium tetraborate resulted in 6 to 8% glucosylation but 98% 5′-position selectivity.

The conclusions of this experiment are as follows: (i) addition of borate caused an increase in 5′-position selectivity and a decrease in the synthesis of PN-5′-α-Glc intra- and extracellularly; (ii) one enzyme probably catalyzed the PN-α-Glc synthesis 5′-position selectively in *V. dahliae* TPU 4900; and (iii) the position selectivity of one enzyme was changed by the addition of borate.

Effect of addition of borate on the regioselective transglucosylation with other microorganisms and enzymes. We tested a number of other PN-α-Glc-synthesizing organisms (2) with both phosphate and borate. The results are summarized in Table 2. The reactions of other 5′-position-selective strains (*V. dahliae* JCM 9510 and IFO 9765, *C. fibula* IFO 4949, *C. pubescens* IFO 9782, *S. commune* IAM 9006) were similar to those of *V. dahliae* TPU 4900; borate was moderately inhibitory to the glucosylation reactions, but it increased the regioselectivity. The reactions of strains selective for 4′-position glucosylation (*B. cereus* TPU 5504, *E. hoshinae* TPU 6101, *O. anthropi* TPU 6850, *X. flavus* TPU 7601) were >90% inhibited by borate, and 4′-position glucosylation activities were almost lost. We tested α-glucosidase from rice and CGTase from *P. macerans*, which were previously reported to be able to synthesize PN-α-Glc (22; T. Hosokawa, T. Yamamoto, and S. Kishihara, Abstr. Annu. Meet. J. Soc. Biosci. Biotechnol. Agrochem. 1999, p. 27) in the presence of both phosphate and borate, as shown in Table 2. The results suggested that while borate inhibited both 5′- and 4′-glucosyltransferase activities in all the systems tested, the inhibition of 4′-glucosyltransferase activity was more severe, so that borate enhanced regioselectivity.

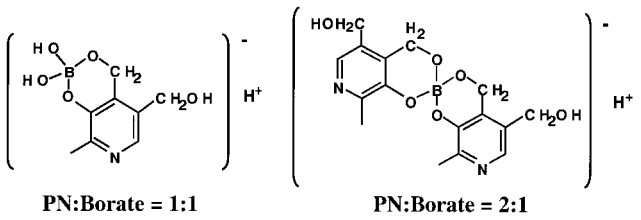

FIG. 2. Structure of PN-borate complexes.

Mechanism of borate effect. The remarkable increase in 5′-position selectivity in the enzymatic transglucosylation to PN is probably caused by the formation of a borate complex with the position 4′ and 3 hydroxyl groups. It is well known that PN and borate form a specific complex (19), and this phenomenon was utilized in some applications, including (i) detection of PN (19, 26), (ii) separation or purification of PN (4), and (iii) stabilization of PN in solution (7, 9, 20). The structure of the complex was proposed by Scudi et al. (Fig. 2) (19). Borate is linked to one or two molecules of PN through the oxygen atoms at positions 3 and 4′. It has also been reported that compounds modified at the 5′ position, including PN-5′-α-Glc (11), pyridoxine 5′-β-D-glucoside (29), and pyridoxine 5′-phosphate (1), form complexes with borate, whereas PN-4′-α-Glc does not (11).

Inhibition of some enzymes by borate has been described previously. Iinhibition of cytochrome b_5 reductase (21) and alcohol dehydrogenase (18) by the formation of an NAD^+-borate complex, inhibition of xanthine oxidase (17) by the formation of a flavin adenine dinucleotide-borate complex, and inhibition of γ-glutamyl transpeptidase (23) by the formation of a complex with serine and borate have been reported previously.

Maltodextrin hydrolysis has been reported for other PN-α-Glc-synthesizing enzymes (11, 22; Hosokawa et al., Abstr. Annu. Meet. J. Soc. Biosci. Biotechnol. Agrochem. 1999). We therefore tested the activity of our enzyme preparation on maltodextrin, as described in Materials and Methods. We found that maltodextrin hydrolysis was not sensitive to borate (100 mM); the rate observed with 0.0064 U of the enzyme was 0.011 μmol of glucose formed per min. Thus, it appears that maltodextrin, unlike PN, does not form a complex with borate in a way that inhibits the enzyme.

Improvement of reaction conditions for PN-5′-α-Glc synthesis with borate. In an examination of the effect of pH on the transglucosylation to PN by intact cells of *V. dahliae* TPU 4900, we found PN-5′-α-Glc, but not PN-4′-α-Glc, in a reaction

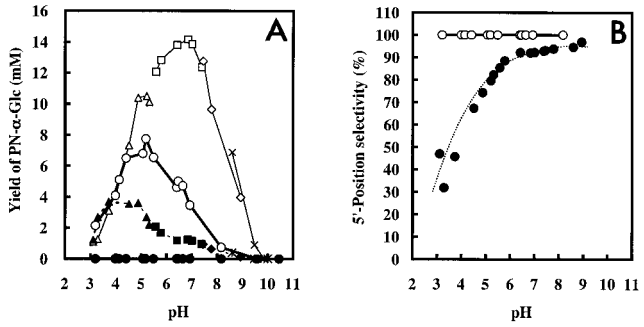

FIG. 3. Effects of pH on the yield of PN-α-Glc (A) and on 5′-position selectivity (B). We used harvested cells of *V. dahliae* TPU 4900 that were cultured aerobically in 150 ml of medium II in a 500-ml flask at 25°C for 7 days. The assay for PN-α-Glc-forming activity was carried out at various pHs for 2 h at 40°C by using a reaction mixture consisting of 25 mM $Na_2B_4O_7 \cdot 10H_2O$ (the pH was adjusted with HCl or NaOH) or a 100 mM buffer as described Materials and Methods. (A) Symbols: ○, yield of PN-5′-α-Glc in 25 mM $Na_2B_4O_7 \cdot 10H_2O$; △, yield of PN-5′-α-Glc in 100 mM sodium acetate buffer; □, yield of PN-5′-α-Glc in 100 mM potassium phosphate buffer; ◇, yield of PN-5′-α-Glc in 100 mM Tris-HCl buffer; ×, yield of PN-5′-α-Glc in 100 mM sodium carbonate buffer; ●, yield of PN-4′-α-Glc in 25 mM $Na_2B_4O_7 \cdot 10H_2O$; ▲, yield of PN-4′-α-Glc in 100 mM sodium acetate buffer; ■, yield of PN-4′-α-Glc in 100 mM potassium phosphate buffer; ◆, yield of PN-4′-α-Glc in 100 mM Tris-HCl buffer; ×, yield of PN-4′-α-Glc in 100 mM sodium carbonate buffer. (B) Symbols: ○, 5′-position selectivity in 25 mM $Na_2B_4O_7 \cdot 10H_2O$; ●, 5′-position selectivity in other buffers.

mixture containing 100 mM borate within 2 h at all pH values. Furthermore, the optimal pH for synthesis of PN-5′-α-Glc changed from 6.4 to 7.0 in the absence of borate to 4.5 to 5.5 in the presence of borate (Fig. 3). Thus, the 5′-position selectivity was controlled at a level of 98% by addition of borate at all pH values, whereas without borate this selectivity was affected significantly by pH.

The optimal temperature was around 50 to 60°C in the presence of 100 mM borate, a result similar to the results obtained for the reaction without borate. However, rapid inactivation of the enzyme by 100 mM borate at a high temperature (65°C) was confirmed by a rapid decrease in the transglucosylation rate for 3 h when intact cells were used, whereas the reaction rate decreased slowly without borate at the same temperature.

The effects of the concentrations of PN and borate are summarized in Table 3. All of reactions were carried out with the same amount of cells (200 mg [wet weight] of cells in a 1.2-ml reaction mixture) and for the same reaction time (48 h). As shown in Table 3 (experiments 1 to 3), a change in the reaction pH from 7 to 5 minimized the decrease in conversion. Experiments 4 to 6 showed that 4′-position glucosylation was almost eliminated (5′-position selectivity, 95% or more) in the presence of 200 mM borate, one-half the concentration of PN (400 mM). Moreover, at pH 5, the concentration of PN-5′-α-Glc formed and the 5′-position selectivity increased gradually (from 99 to 137 mM and from 71 to 99%, respectively) with the increase of the concentration of borate (experiments 7 to 9). The decrease in the amount PN-5′-α-Glc was almost overcome with high 5′-position selectivity (99%) by the change in reaction conditions, as shown in experiments 4 and 9.

TABLE 3. Increase in the yield of PN-5′-α-Glc and 5′-position selectivity after improvement of the reaction conditions[a]

Expt	PN concn (mM)	Borate concn (mM)	Reaction pH	Yield of PN-5′-α-Glc		5′-Position selectivity (%)
				% Conversion	Concn (mM)	
1	100	0	7	51	51	85
2	100	200	7	24	24	98
3	100	200	5	37	37	95
4	400	0	7	36	144	83
5	400	200	7	32	128	95
6	400	400	7	29	115	99
7	400	0	5	25	99	71
8	400	200	5	33	132	97
9	400	400	5	34	137	99

[a] We used harvested cells of *V. dahliae* TPU 4900 that were cultured aerobically in 150 ml of medium II in a 500-mL flask at 20°C for 7 days. The concentration of $Na_2B_4O_7 \cdot 10H_2O$ ranged from 0 to 100 mM (0 to 400 mM as borate), and the concentration of PN was 100 mM (experiments 1 to 3) or 400 mM (experiments 4 to 10) in the basic reaction mixture (total volume, 1.2 ml) consisting of 100 mM potassium phosphate buffer (pH 7) (experiments 1, 2, and 4 to 9) or 100 mM acetate buffer (pH 5) (experiments 3 and 7 to 9) containing cells harvested from 2.0 ml of culture broth. The reaction mixtures were incubated at 55°C for 48 h in the dark. Twenty-four milligrams of maltodextrin was added to the reaction mixtures at 4, 8, and 18 h.

Preparative synthesis of PN-5′-α-Glc with high 5′-position selectivity by using borate. We performed preparative-scale (400 ml) synthesis of PN-5′-α-Glc using intact cells of *V. dahliae* TPU 4900 under the optimal conditions (Fig. 4). The concentrations of PN-HCl and borate were fixed at 400 mM. After incubation for 48 h at pH 5 and 55°C, the concentration of PN-5′-α-Glc was 161 mM (53.2 g/liter), while only 1.3 mM PN-4′-α-Glc was formed, so the 5′-position selectivity was very high (99.2%).

The total amount of by-products other than PN-4′-α-Glc in a reaction mixture with borate was 1.3 times higher than the total amount in a reaction mixture without borate and could reach levels that were 24% of the level of PN-5′-α-Glc. The by-products that formed were thought to be pyridoxine 5′-α-maltoside and pyridoxine diglucoside, because these by-products were converted to PN via PN-5′-α-Glc by glucoamylase

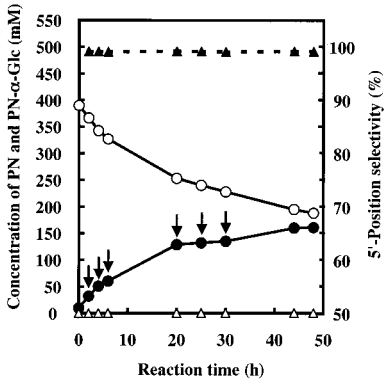

FIG. 4. Time course of PN-5′-α-Glc synthesis by *V. dahliae* TPU 4900 at a preparative scale. The reactions were performed under the conditions described in Materials and Methods. Symbols: ○, PN; ●, PN-5′-α-Glc; △, PN-4′-α-Glc; ▲, 5′-position selectivity. The arrows indicate when maltodextrin was added.

(from *Rhizopus niveus*) and α-glucosidase (from *Saccharomyces cerevisiae*) (data not shown).

Moreover, we found that the borate was easily removed from PN and PN-5′-α-Glc by cation-exchange chromatography with Dowex 50WX8 (Dow Chemical Company, Midland, Mich.) in the first step of purification of PN-α-Glc, as described by Suzuki et al. (22). PN and PN-5′-α-Glc were absorbed in the cation-exchange resin under acidic conditions (pH 3 or below), whereas borate eluted first. The eluent including PN-5′-α-Glc was obtained with 100 mM ammonium formate (pH 3). Borate was not detected in the eluent by using Azomethine H, a borate-specific color-producing reagent.

Effect of borate as an enhancer of regioselectivity. As described above, the advantages of adding borate as an enhancer of 5′-position selectivity can be summarized as follows: (i) the ease of addition at a reasonable cost at levels that are equal to the levels of PN and (ii) the ease of removal during purification of PN-5′-α-Glc by cation-exchange column chromatography.

There have been no reports about the effect of borate on increases in regioselectivity in an enzymatic reaction, although arylboronate was used for regiospecific chemical modification of sugar (14, 15). In addition, to our knowledge there are not inorganic additives that enhance enantio-, stereo-, or regioselectivity, except for some cations; thus, $CaCl_2$ (5), NaCl (27), LiCl (12, 13), and $MgCl_2$ (13) enhance the *E* value of lipase, $FeCl_2$ and $FeCl_3$ enhance the *E* value of alkylsulfatase (16), and $MgCl_2$ enhances the enantiomeric excess of asymmetric reduction by baker's yeast (10). It is known that borate forms complexes with various polyhydroxy compounds, such as polyols (3) (e.g., mannitol, xylitol, and sorbitol), phenols (3) (e.g., catechol and pyrogallol), sugars (3, 8) (e.g., glucose and fructose), and α-hydroxy acids (3) (e.g., 2-hydroxyisobutyric acid, salicylic acid, and *cis*-2-hydroxycyclopentanecarboxyric acid). Consequently, borate has potential for use as an additive to enhance regio- or stereoselectivity in enzymatic modification of many other compounds.

REFERENCES

1. **Argoudelis, C. J.** 1986. Preparation of crystalline pyridoxine 5′-phoshate and some of its properties. J. Agric. Food Chem. **34:**995–998.
2. **Asano, Y., and K. Wada.** 2003. Regioselective glucosylation of pyridoxine by microorganisms. Biosci. Biotechnol. Biochem. **67:**499–507.
3. **Böeseken, J.** 1949. The use of boric acid for determination of the configuration of carbohydrate. Adv. Carbohydr. Chem. **4:**189–210.
4. **Fujiwara, Y., and T. Shinjutsu.** October 1977. Japan patent 52128379.
5. **Holmbarg, E., M. Holmquist, E. Hedenström, P. Berglund, T. Norin, H.-E. Högberg, and K. Hult.** 1991. Reaction conditions for the resolution of 2-methylalkanoic acids in esterification and hydrolysis with lipase from *Candida cylindracea*. Appl. Microbiol. Biotechnol. **35:**572–578.
6. **Joseph, T., H. Tsuge, Y. Suzuki, and D. B. McCormick.** 1996. Pyridoxine 4′-α- and 5′-α-D-glucosides are taken up and metabolized by isolated rat liver cells. J. Nutr. **126:**2899–2903.
7. **Kawai, F., H. Yamada, and K. Ogata.** 1971. Properties of pyridoxine glucoside. J. Vitaminol. **17:**121–124.
8. **Khym, J. X., and L. P. Zill.** 1952. The separation of sugars by ion exchange. J. Am. Chem. Soc. **74:**2090–2094.
9. **Koide, M., N. Kojima, and Y. Yano.** January 1993. Japan patent 05017355.
10. **Nakamura, K., Y. Kawai, S. Oka, and A. Ohno.** 1989. A new method for stereochemical control of microbial reduction. Reduction of β-keto esters with bakers' yeast immobilized by magnesium alginate. Tetrahedron Lett. **30:**2245–2246.
11. **Ogata, K., Y. Uchida, N. Kurihara, Y. Tani, and T. Tochikura.** 1969. Studies on transglycosidation to vitamin B_6 by microorganisms. II. Chemical structure of pyridoxine glucoside. J. Vitaminol. **15:**160–166.
12. **Okamoto, T., and S. Ueji.** 1999. Drastic enhancement of enantioselectivity of lipase-catalysed esterification in organic solvents by addition of metal ions. Chem. Commun. **1999:**939–940.
13. **Okamoto, T., and S. Ueji.** 2000. A new method for improving the enantioselectivity of lipase-catalyzed hydrolysis in organic solvent containing a small amount of water in the presence of metal ions. Bio/Technology Lett. **22:** 1169–1171.
14. **Oshima, K., and Y. Aoyama.** 1999. Regiospecific glycosigation of unprotected sugars via arylboronic activation. J. Am. Chem. Soc. **121:**2315–2316.
15. **Oshima, K., E. Kitazono, and Y. Aoyama.** 1997. Complexation-induced activation of sugar OH groups. Regioslective alkylation of methyl fucopyranoside via cyclic phenylboronate in the presence of amine. Tetrahedron Lett. **38:**5001–5004.
16. **Pogorevc, M., U. T. Strauss, T. Riermeier, and K. Faber.** 2002. Selectivity-enhancement in enantioselective hydrolysis of *sec*-alkyl sulfates by an alkylsulfatase from *Rhodococcus ruber* DSM 44541. Tetrahedron Asymm. **13:** 1443–1447.
17. **Roush, A., and E. R. Norris.** 1950. The inhibition of xanthine oxidase by borates. Arch. Biochem. **29:**344–347.
18. **Roush, A. H., and B. B. Gowdy.** 1961. The inhibition of yeast alcohol dehydrogenase by borate. Biochim. Biophys. Acta **52:**200–202.
19. **Scudi, J. V., W. A. Bastedo, and T. J. Webb.** 1940. The formation of a vitamin B_6-borate complex. J. Biol. Chem. **136:**399–406.
20. **Shiroishi, M., and A. Hayakawa.** 1961. Effects of sunlight irradiation on vitamin B_6 relating compounds. Vitamins **22:**138–141. (In Japanese.)
21. **Strittmatter, P.** 1964. Reversible direct hydrogen transfer from reduced pyridine nucleotides to cytochrome b_5 reductase. J. Biol. Chem. **239:**3043–3050.
22. **Suzuki, Y., Y. Doi, K. Uchida, and H. Tsuge.** 1997. Enzymatic preparation of pyridoxine 4′- and 5′-α-D-glucosides. Methods Enzymol. **280:**66–71.
23. **Tate, S. S., and A. Meister.** 1978. Serine-borate complex as a transfer-state inhibitor of γ-glutamyl transpeptidase. Proc. Natl. Acad. Sci. USA **75:**4806–4809.
24. **Tilden, E. B., and C. S. Hudson.** 1942. Preparation and properties of the amylases produced by *Bacillus macerans* and *Bacillus polymyxa*. J. Bacteriol. **43:**527–544.
25. **Tsuge, H., M. Maeno, T. Hayakawa, and Y. Suzuki.** 1996. Comparative study of pridoxine-α,β-glucosides, and phosphopyridoxyl-lysine as vitamin B_6 nutrient. J. Nutr. Sci. Vitaminol. **42:**377–386.
26. **Tsuji, K.** 1968. Automated analysis of pyridoxine (vitamin B_6) using diethyl-*p*-phenylenediamine and boric acid. Ann. N. Y. Acad. Sci. **153:**446–455.
27. **Tsukube, H., A. Betchaku, Y. Hiyama, and T. Itoh.** 1994. Lipase-catalyzed hydrolysis of crown ester substrates: metal cation-enhanced reactivity and enantioselectivity of 12-crown-4 ester. J. Org. Chem. **59:**7014–7018.
28. **Wada, K., and Y. Asano.** 2003. Significant improvement of 5′-position selective glucosylation of pyridoxine by *Verticillium dahliae* TPU 4900. Biosci. Biotechnol. Biochem. **67:**508–516.
29. **Yasumoto, K., H. Tsuji, K. Iwami, and H. Mitsuda.** 1977. Isolation from rice bran of a bound form of vitamin B_6 and its identification as 5′-*O*-(β-D-glucopyranosyl)pyridoxine. Agric. Biol. Chem. **41:**1061–1067.