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

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Naturally, the paper discusses the use of borate to control the 5′-position-selective microbially catalyzed glucosylation of pyridoxine (PN). The study was conducted with the fungus *Verticillium dahliae* TPU 4900, and the reaction was carried out in the presence of borate. The effect of borate on the formation of 5′-α-D-glucoside was investigated, and it was found that borate enhanced the selectivity of the enzyme. The reaction mixture was prepared by incubating the substrate with the fungal cells or enzyme in the presence of borate. The results showed that the use of borate improved the selectivity of the microbial glucosylation, contributing to the efficient production of the desired 5′-α-D-glucoside.

**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 (H3BO3), and sodium tetraborate (Na2B10O10·5H2O) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, 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.). Cylomaltodextrin 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.** *Verticillium 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. *Verticillium dahliae* IAM 9510 was purchased from the Japan Collection of Microorganisms, Tokyo, Japan. *V. dahliae* IFO 9765, *Coriolus flavus* IFO 4949, and *C. pustulatus* IFO 9782 were obtained from the Institute for Fermentation, Osaka, Japan. *Schizopyllum commune* IAM 10906 was obtained from the Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan.

**Medium.** 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) KH2PO4, 0.1% (wt/vol) KH2PO4, 0.02% (wt/vol) FeSO4·7H2O, 0.02% (wt/vol) MgSO4·7H2O, 0.01% (wt/vol) MnSO4·5H2O, 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) KH2PO4, 0.05% (wt/vol) KCl, 0.05% (wt/vol) MgSO4·7H2O, 0.001% (wt/vol) FeSO4·7H2O, and 0.1% (wt/vol) PN-HCl in tap water (pH 7.0).

**Analysis of PN-α-Glc.** Analysis of PN, PN-α-Glc, and PN-4′-α-Glc was done by high-performance liquid chromatography with a Cosmosil 5C18-MS-II column (4.6 by 150 mm; Nakalai Tesque, Kyoto, Japan) monitored at 254 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-α-Glc, and PN-4′-α-Glc were 7.10, and 16 min, respectively. The 5′-position selectivity of PN (expressed as a percentage) was determined by calculating the area ratios of PN-α-Glc and PN-4′-α-Glc. The selectivity was calculated as the ratio of the area of PN-α-Glc to the total area of PN-α-Glc plus PN-4′-α-Glc.

**Synthesis of PN-5′-α-Glc by cells or enzyme.** The reaction mixture was prepared by incubating the substrate with the fungal cells or enzyme in the presence of borate. The reaction mixture was incubated at 40°C for 2 h with shaking in the dark. The ratio of PN-5′-α-Glc to PN-4′-α-Glc was determined by high-performance liquid chromatography. The production of PN-5′-α-Glc was calculated as the percentage of the total amount of PN-5′-α-Glc formed.

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Position-selective microorganisms were cultured in the same medium for 7 days.

\( \text{V. dahliae} \) TPU 4900 was cultured in medium II for 9 days at 20°C. 

Dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed after electrophoresis, the original extract. The enzyme was not purified to homogeneity by sodium sulfate. 

The partially purified enzyme was obtained with a preparative gel filtration, 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 wet cells. 

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Activity (U)</th>
<th>Protein (mg)</th>
<th>Sp act</th>
<th>Fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet cells (770 g)</td>
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<td></td>
<td></td>
<td></td>
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<td>2,000</td>
<td>0.15</td>
<td>1.0</td>
<td>100</td>
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<tr>
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<td>39</td>
<td>2.3</td>
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<tr>
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<td>21</td>
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<tr>
<td>Superdex 200</td>
<td>51</td>
<td>12</td>
<td>4.4</td>
<td>29</td>
<td>18</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

The 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 K₂HPO₄, Na₂SO₄, NaNO₃, 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 Methods.
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) 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. pube- scens 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.

**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. Inhibition 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, 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.

**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. 2. Structure of PN-borate complexes.](image-url)
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 trans-glucosylation 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.

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-maltside and pyridoxine diglucoside, because these by-products were converted to PN via PN-5'-α-Glc by glucoamylase.
(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, CaCl₂ (5), NaCl (27), LiCl (12, 13), and MgCl₂ (13) enhance the E value of lipase, FeCl₂ and FeCl₃ enhance the E value of alkylsulfatase (16), and MgCl₂ enhances the enantionic 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-hydroxycyclopentanecarboxylic acid). Consequently, borate has potential for use as an additive to enhance regio- or stereoselectivity in enzymatic modification of many other compounds.

**REFERENCES**