Bioelectrochemically Accelerated Microbial Conversion of Nicotinic Acid to 6-Hydroxynicotinic Acid on Microorganism-immobilized Column Electrolytic Flow System

Masaki Torimura, Hideto Yoshida, Kenji Kano,* Tokuji Ikeda,* Toru Nagasawa,† and Teruhisa Ueda††
Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-01
†*Department of Biomolecular Science, Faculty of Engineering, Gifu University, Gifu 502*
††*Analytical Instruments Division, Shimadzu Corporation, Nakagyo-ku, Kyoto 604*

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Nicotinic acid (NA) is efficiently hydroxylated into 6-hydroxynicotinic acid (6HNA) by *Pseudomonas fluorescens* TN5-immobilized column electrolytic method using $K_3Fe(CN)_6$ as an extracellular electron transfer mediator, in which the conversion rate was sufficiently accelerated compared with the aerobic oxidation. The NA conversion system was applied to the continuous-production of 6HNA in 100% yield and the absolute determination of NA in flow-injection analysis.

Variety of microorganisms have been utilized for the production of useful materials and the detection of specific substrates. A membrane-bound NA dehydrogenase (which is unstable in the isolated state) in *Pseudomonas fluorescens* TN5 (*P.f.*) catalyzes the hydroxylation of nicotinic acid (NA) into 6-hydroxynicotinic acid (6HNA),[1] an important compound as a starting material for the synthesis of pesticides.[2] The electrons from NA is transported to O_2 via the cytochrome respiratory chain.[1] The authors (T. I. and coworkers) have shown that *P.f.*-immobilized electrodes exhibit bioelectrocatalytic oxidation of NA in the presence of electron transfer mediators.[3] On the other hand, continuous-flow column electrolysis is very useful in achieving rapid and quantitative bulk electrolysis.[4] In this work, we have constructed a *P.f.*-immobilized electrochemical reactor in continuous-flow column electrolysis using $K_3Fe(CN)_6$ as a mediator as illustrated in Figure 1 for the purpose of the 6HNA production and the NA analysis.

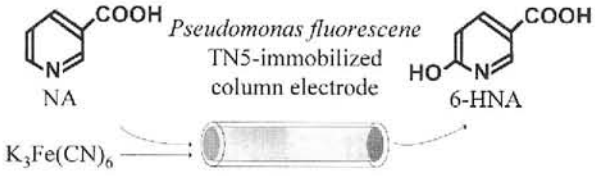

Figure 1. Schematic view of *P.f.*-immobilized column electrolysis of NA.

P.f. cells cultured according to a literature[1c] were harvested, washed, and suspended in a saline solution (0.85% NaCl) before use. Prior to column electrolytic experiments, the NA oxidation in *P.f.* suspensions was monitored by subjecting an aliquot of the cell suspension to an HPLC analysis on a Shimadzu LC-10A HPLC system coupled with a Shimadzu SPD-M10Avp photodiode array detector using a Develosil ODS-A-5 column (4.6 mm × 15 cm), pH 2.5 phosphate buffer containing 30% methanol and 5 mmol dm^{-3} sodium 1-octanesulfonate as a mobile phase (1.0 mL min^{-1}), and caffeine as an internal standard.

Under aerobic conditions ($[O_2]$ = 0.25 mmol dm^{-3}), NA is oxidized into 6HNA with O_2 as the final electron acceptor,[1] as shown by Figure 2 (A). The oxidation rate was not affected by an increase in $[O_2]$, probably due to a low apparent Michaelis constant (K_M) for O_2 (< 20 μmol dm^{-3}) in the *P.f.*-catalyzed NA oxidation.[3c] The NA oxidation proceeds also under anaerobic conditions in the presence of $K_3Fe(CN)_6$ as an extracellular electron acceptor (Figure 2 (B)), during which 6HNA and $Fe(CN)_6^{4-}$ were generated stoichiometrically (1:2 in a molar ratio). Interestingly, the rate of the anaerobic oxidation with $Fe(CN)_6^{3-}$ was faster than that of the aerobic oxidation and was enhanced by an increase in $[Fe(CN)_6^{3-}]$ since an apparent K_M for $Fe(CN)_6^{3-}$ is as large as 7 mmol dm^{-3}.[3c] This result suggests that the aerobic oxidation of NA into 6HNA is regulated by some reduced intermediate accumulated in the normal metabolic pathway. Thus the electron leak into $K_3Fe(CN)_6$ can be expected to accelerate the NA oxidation under aerobic conditions. This is clearly evidenced as demonstrated by Figure 2 (C).

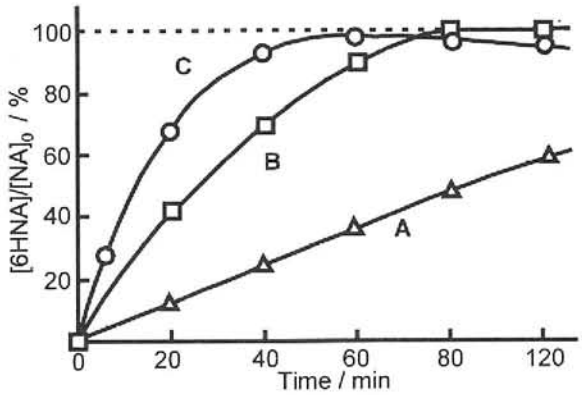

Figure 2. Time course of the 6HNA production from NA (1 mmol dm^{-3}) in *P.f.* suspension of 0.1 g (dry weight) dm^{-3} at pH 7.0 in the presence of (A): 0.25 mmol dm^{-3} $[O_2]$, (B): 5 mmol dm^{-3} $K_3Fe(CN)_6$ (anaerobic), and (C): 0.25 mmol dm^{-3} $[O_2]$ + 5 mmol dm^{-3} $K_3Fe(CN)_6$.

In the presence of an increased amount of the *P.f.* cells, all NA was quickly converted into 6HNA under aerobic conditions (Figure 3 (A)), but after then [6HNA] decreased gradually . The decrease in [6HNA] is reasonably ascribed to the subsequent oxidative metabolism of 6HNA.[5] In contrast, the generated 6HNA was quite stable when $Fe(CN)_6^{3-}$ was used as an electron acceptor (Figure 3 (B)). This means that $Fe(CN)_6^{3-}$ cannot serve as an electron acceptor for the enzymes concerning the oxidative metabolism of 6HNA. The membrane permeability of $Fe(CN)_6^{3-}$ and the location of the related enzymes seem to be responsible for the difference in the 6HNA oxidation activity. Judging from the above results, $Fe(CN)_6^{3-}$ is superior to O_2 as an electron acceptor from the standpoint of the 6HNA production and the control of the reaction time is essential to prevent the subsequent oxidative metabolism of 6HNA in the aerobic NA oxidation.

Benzoquinone and related compounds can work as better electron acceptors than $Fe(CN)_6^{3-}$,[3] but the quinones were not suitable in this work because non-enzymatic reaction between NA and the quinones could not be ignored.

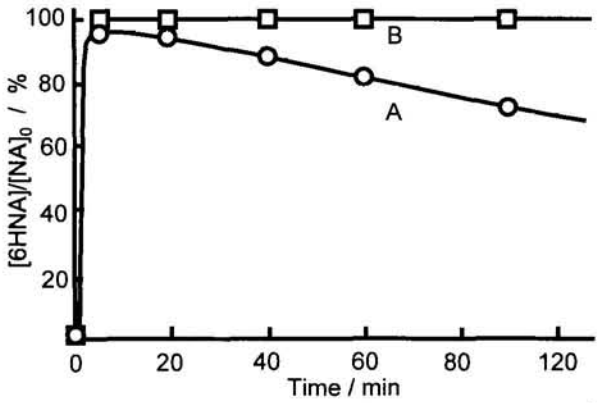

Figure 3. Time course of the 6HNA production from NA (1 mmol dm^{-3}) in *P.f.* suspension of 1.2 g (dry weight) dm^{-3} at pH 7.0 in the presence of (A): 0.25 mmol dm^{-3} [O_2] and (B): 5 mmol dm^{-3} $K_3Fe(CN)_6$ (anaerobic).

Considering these factors, we constructed a *P.f.*-immobilized electrochemical bioreactor (Figure 1). *P.f.* cells (7 mg dry weight) were immobilized on the carbon fiber in a Hokuto Denko HX-110 column electrolysis cell (i.d. 8 mm, length 50 mm) by passing a *P.f.* suspension (pH 7.0) containing 0.05% glutaraldehyde thorough the column electrode. NA solutions (pH 7.0, ionic strength 15 mmol dm^{-3} with NaCl) containing 1 mmol dm^{-3} $K_3Fe(CN)_6$ were continuously flowed through the *P.f.*-immobilized column electrode with a Shimadzu LC-6A pump. The electrode potential was fixed at 0.4 V vs. Ag/AgCl/sat. KCl to re-oxidize $Fe(CN)_6^{4-}$ into $Fe(CN)_6^{3-}$.

At a flow rate of 0.1 mL min^{-1}, NA at the concentration up to 5 mmol dm^{-3} was completely converted into 6HNA without any by-product. The maximum conversion rate was evaluated as 0.83 mmol min^{-1}. The rate was 15 times faster than that of the simple aerobic conversion in the *P.f.*-immobilized column reactor at open circuit in the absence of $K_3Fe(CN)_6$. The result clearly indicates the bioelectrochemical acceleration of the microbial conversion of NA. The absence of by-product under aerobic conditions means that the flow rate was sufficiently large to prevent the subsequent aerobic oxidation of 6HNA. The *P.f.*-immobilized electrochemical reactor exhibited stable performance during 4 days. For practical use, some improvement in the immobilization of *P.f.* cells and mediator on column electrodes would be required.

Column electrolytic detection coupled with flow-injection analysis (FIA) allows for the absolute determination of redox samples. NA can be bioelectrocatalytically oxidized using *P.f.* as a catalyst and $Fe(CN)_6^{3-}$ as a mediator. Thus, the *P.f.*-immobilized column electrolytic method was applied to the absolute determination of NA as a pharmaceutical importance. A pH 7 phosphate buffer as a mobile solution and a $K_3Fe(CN)_6$-containing buffer as a reagent solution were pumped at 0.1 mL min^{-1} and mixed before the *P.f.*-immobilized column electrolytic detector. [$K_3Fe(CN)_6$] in the reagent solution was increased up to 20 mmol dm^{-3} to compete the aerobic oxidation of NA.

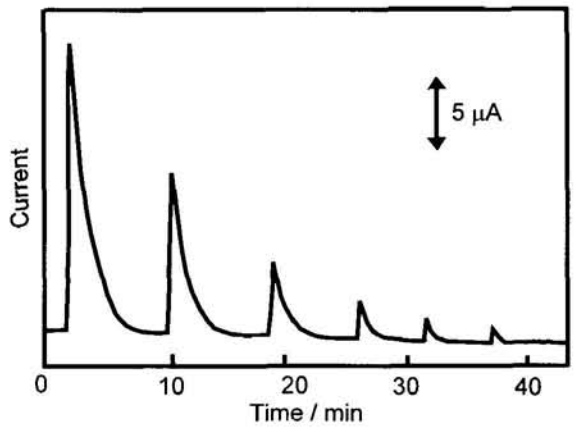

Figure 4. Typical current responses on the successive injection of NA samples at 20, 10, 4, 2, 1 and 0.4 pmol (left to right) in *P.f.*(7 mg dry weight)-immobilized column electrolytic FIA system.

Figure 4 shows the oxidative current response upon the successive injections of NA solutions. This system is rapid enough to analyze one NA sample within a few minute.[6] The peak area was in a linear relation to the injected amount of NA in the range from 0.2 to 20 pmol with a correlation coefficient of 0.995 and a relative standard deviation of 2.5% for a 10 pmol NA sample (n = 5). Furthermore, the electric charge calculated from the peak area attained 98% of the calculated value for NA and then the present FIA method can serve as an absolute determination of NA. In this system, the aerobic oxidation was almost completely inhibited by the fast anaerobic oxidation at increased [$Fe(CN)_6^{3-}$].[7] Selectivity of the system against related pyridine derivatives is well guaranteed owing to the high specificity of NA dehydrogenase and *P.f.*[1c,3]

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References and Notes

1 a) B. Hurh, M. Ohshima, T. Yamane, and T. Nagasawa, *J. Ferment. Bioeng.*, **77**, 382 (1994). b) B. Hurh, T. Yamane, and T. Nagasawa, *J. Ferment. Bioeng.*, **78**, 19 (1994). c) T. Nagasawa, B. Hurh, and T. Yamane, *Biosci. Biotech. Biochem.*, **58**, 665 (1994).
2 K. Moriya, K. Shibuya, Y. Hattori, S. Tsuboi, S. Shiokawa, and S. Kagabu, *J. Pesticide Sci.*, **18**, 119 (1993).
3 a) K. Takayama, T. Kurosaki, T. Ikeda, and T. Nagasawa, *J. Electroanal. Chem.*, **381**, 47 (1995). b) K. Takayama, T. Ikeda, and T. Nagasawa, *Electroanalysis*, **8**, 765 (1996). c) T. Ikeda, T. Kurosaki, K. Takayama, and K. Kano, *Anal. Chem.*, **68**, 192 (1996).
4 T. Fujinaga, *Pure Appl. Chem.*, **25**, 709 (1971); S. Okazaki, *Rev. Polarogr. (Kyoto)*, **15**, 154 (1968); S. Kihara, *Bunseki*, **1980**, 850; S. Kihara, Z. Yoshida, and H. Aoyagi, *Bunseki Kagaku*, **40**, 309 (1991).
5 J. P. Kaiser, Y. Feng, and J. M. Bollag, *Microbiol. Rev.*, **60**, 483 (1996).
6 The peak tailing might be resulted from extremely large diameter of the column electrode compared with the solution guide line (i.d. 0.8 mm) and also heterogeneous distribution of the immobilized cells in the column.
7 When [$Fe(CN)_6^{3-}$] in a reagent solution was decreased down to 5 mmol dm^{-3}, completely anaerobic conditions in a glove box filled with N_2 gas were required to realize such absolute determination of NA.