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Enzyme Electrochemical Preparation of a 3-Keto Derivative of 1,5-Anhydro-D-Glucitol Using Glucose-3-Dehydrogenase

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Abstract

A novel enzymatic organic synthesis was reported, utilizing glucose-3-dehydrogenase (G3DH) and its regeneration via electrochemical methods. We combined the water-soluble G3DH prepared from a marine bacterium, *Halomonas* sp. α-15, and electron mediator with the electrode system in order to regenerate the enzyme. Using this system, the conversion of 1,5-anhydro-D-glucitol (1,5AG), a diabetes marker in human blood, was investigated. The final yield of the product, 3-keto anhydroglucitol (3-ketoAG), which was identified by ^{13}C nuclear magnetic resonance, was 82% based on the initial amount of 1,5AG. The electrochemical yield of the reaction proceeded almost stoichiometrically. The electrochemical conversion rate of 1,5AG was 1.24 mmol/(L·h), and the electrochemical yield of 1,5AG consumption was 80%, whereas that for 3-ketoAG was 60%.

Index Entries: 1,5 Anhydro-D-glucitol; glucose-3-dehydrogenase; electrochemistry; 3-keto sugars.

Introduction

The preparation of sugar derivatives focusing on the specific hydroxy group requires great skill and complicated organic synthesis processes, because of the high polyfunctionality of molecules, as well as the difficulty in differentiation, between the equally reactive hydroxy groups of sugar. In addition, the 3-keto derivatives of sugars have received much attention

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as the precursor for biodegradative detergents and also for bioactive compounds such as antimicrobial reagents *(1)*. Because 3-keto derivatives of sugars have one specific keto group that can be selectively modified by either chemical or enzymatic methods, the preparation of 3-keto derivatives of sugars promises to be a novel synthetic procedure for the preparation of carbohydrate products.

To prepare 3-keto derivatives of sugars, a group of the glucose oxidoreductases that recognizes and catalyzes the third hydroxy group of sugars has been utilized. These oxidoreductases are the D-glucoside 3-dehydrogenases (glucose 3-dehydrogenases; [G3DHs]) (EC 1.1.99.13), which have been found in Gram-negative bacteria as the membrane-bound enzymes *(2,3)* or as the soluble enzymes *(4,5)*. Two types of marine bacterial G3DHs have been reported: a membrane bound type G3DH from *Cytophaga marinoflava (3)*, and a water-soluble G3DH from *Halomonas* sp. α-15 *(5)*. The preparation of several 3-keto derivatives of sugars has been reported, using the whole cells containing G3DH. These procedures utilized either growing bacterial cells *(6)* or resting cells *(7–10)*, achieving the regeneration of redox enzyme via microbial respiratory activity. In such cases, the process can be developed based on the traditional fermentation techniques. However, medium components, cell derivatives, formation of byproducts, and/or the assimilation of the product by the microorganisms are the inherent problems.

In this article, we propose a novel enzymatic organic synthesis, utilizing G3DH and its regeneration via electrochemical methods. We combine the water-soluble G3DH prepared from a marine bacterium, *Halomonas* sp. α-15, and an electron mediator together with the electrode system in order to regenerate the enzyme. We focused on the enzymatic conversion of 1,5-anhydro-D-glucitol (1,5AG) (Fig. 1), the major polyol observed in human blood that has a pyranoid structure. The 1,5AG concentration in human plasma is usually maintained at a constant level independent of food intake, but decreases specifically in diabetes. Therefore, 1,5AG is utilized as a good clinical marker for diabetes *(11)*. However, there have been few studies on the metabolic and synthetic pathway of 1,5AG. Therefore, derivatives of 1,5AG were utilized in our study to understand better its physiological significance.

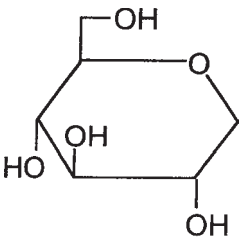
Materials and Methods

Chemicals

5-Methylphenzinium methosulfate (phenazin methosulfate [PMS]), 2,6-dichloro-N-(4-hydroxyphenyl)-*p*-benzoquinoneimine (DCIP), potassium ferricyanide (Kanto, Tokyo, Japan) and 1,5AG (Toronto Research, Toronto, Canada) were purchased for this study. All other chemicals used were reagent grade.

Enzyme Preparation

Enzyme sample was prepared from *Halomonas* sp. α-15 cells, cultivated according to our previous reports *(5)*. The soluble fraction was pre-



1,5AG

Fig. 1. 1,5-Anhdyro-D-glucitol (1,5AG).

pared from the cells by passage through a French pressure cell at 1500 kgf and collected by ultracentrifugation (69,800*g*, for 90 min at 4°C) as a supernatant in a 20 m*M* potassium phosphate buffer (pH 6.0). Ammonium sulfate was added to 40% saturation and the precipitate was discarded. The enzyme sample was then dialyzed against 20 m*M* potassium phosphate buffer (pH 6.0) containing 2 *M* ammonium sulfate applied to a hydrophobic interaction chromatography (Phenyl Toyopearl 650 *M*, 50 mm ID × 450 mm; Tosoh, Tokyo, Japan), equilibrated with 20 m*M* potassium phosphate buffer pH 6.0 containing 2 *M* ammonium sulfate, and eluted with a linear gradient of 2.0–0 *M* ammonium sulfate in 20 m*M* potassium phosphate buffer (pH 6.0). Active fractions were identified with a PMS-DCIP-mediated color development enzyme assay as described previously *(5)*, and preserved at 4°C after being dialyzed in 20 m*M* potassium phosphate buffer (pH 6.0). This prepared fraction was used as the enzyme sample.

Enzyme Electrochemical Conversion of 1,5AG

Figure 2 shows the experimental setup of the enzyme electrochemical conversion of 1,5AG. Pt mesh working electrode (8.75 cm^2), (BAS, IN), Pt wire counter electrode, Ag/AgCl reference electrode (BAS) were put in a reaction vessel equipped with a water jacket containing 15 mL of 100 m*M* potassium phosphate buffer (pH 7.0) with 2.5 U of G3DH and 10 m*M* potassium ferricyanide as an electron mediator, and a potential of 370 mV vs Ag/AgCl was applied using a potentiostat (HA-151 Hokuto-Denko, Tokyo, Japan). After the steady-state current was observed, the reaction was started by adding 20 m*M* of 1,5AG, and the reaction vessel was kept at 25°C under dark conditions during the reaction.

The time course of the reaction was monitored by sampling an aliquot of the reaction mixture every 1 h. After the lyophilization of the sample, 1.0 mL of methanol was added, and protein and potassium ferricyanide were precipitated. Methanol was then evaporated, and the sample was redissolved in distilled water and analyzed for 1,5AG and its enzyme product by high-performance liquid chromatography (Tosoh) using the sugar analyses column (SUGAR SP081, 8 mm ID × 300 mm; Shodex, Tokyo, Japan) equipped with a refractive index detector. The amount of the product was

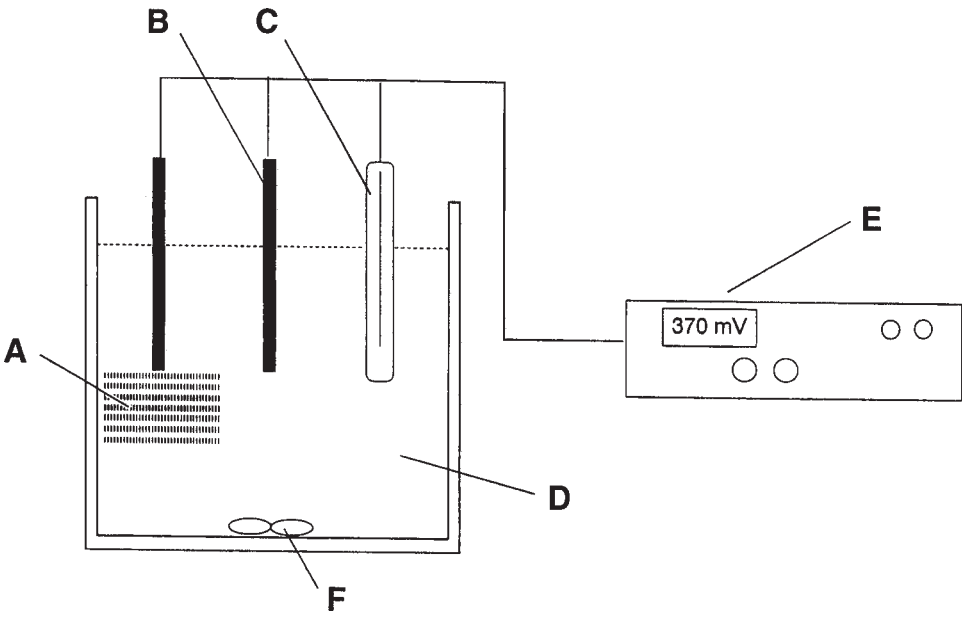


Fig. 2. Experimental setup of enzyme electrochemical conversion of 1,5AG using G3DH. a) Pt mesh working electrode; b) Pt wire counter electrode; c) Ag/AgCl reference electrode; d) 100 m*M* potassium phosphate buffer, pH 7.0, with 2.5 U of G3DH and 10 m*M* potassium ferricyanide; e) potentiostat; f) stirrer bar.

estimated by considering the molecular weight of the 3-keto derivatives of 1,5AG, determined by following nuclear magnetic resonance (NMR) analysis, and calibrating the observed peak height of the product and applied amount of the purified 3-keto derivative. The determination of potassium ferricyanide concentration in the sample was determined by forming Prussian blue color with the addition of ferric sulfate-Dupanol reagent, as was described elsewhere, and by measuring the absorbance at 660 nm.

The product of 1,5AG resulting from enzyme electrochemical conversion using G3DH was purified with silica gel chromatography using acetonitrate as the solvent. Ten milligrams of purified product was dissolved in D_2O. ^{13}C-NMR spectra were recorded with a JEOL ALPHA500 spectrometer (500 MHz) using TMS as the internal probe.

Results and Discussion

Figure 3 shows the time courses of enzyme electrochemical conversion of 1,5AG using G3DH. We also attempted the reaction without applying potential to the electrode, so that no regeneration of the mediator occurred, as the control experiment. Without applying potential, the concentration of potassium ferricyanide immediately decreased. Within 2 h after the addition of 1,5AG, the concentration of ferricyanide was almost nil, and the G3DH catalyzing oxidation of 1,5AG stopped during this period. The final conversion of 1,5AG was 19% under this condition.

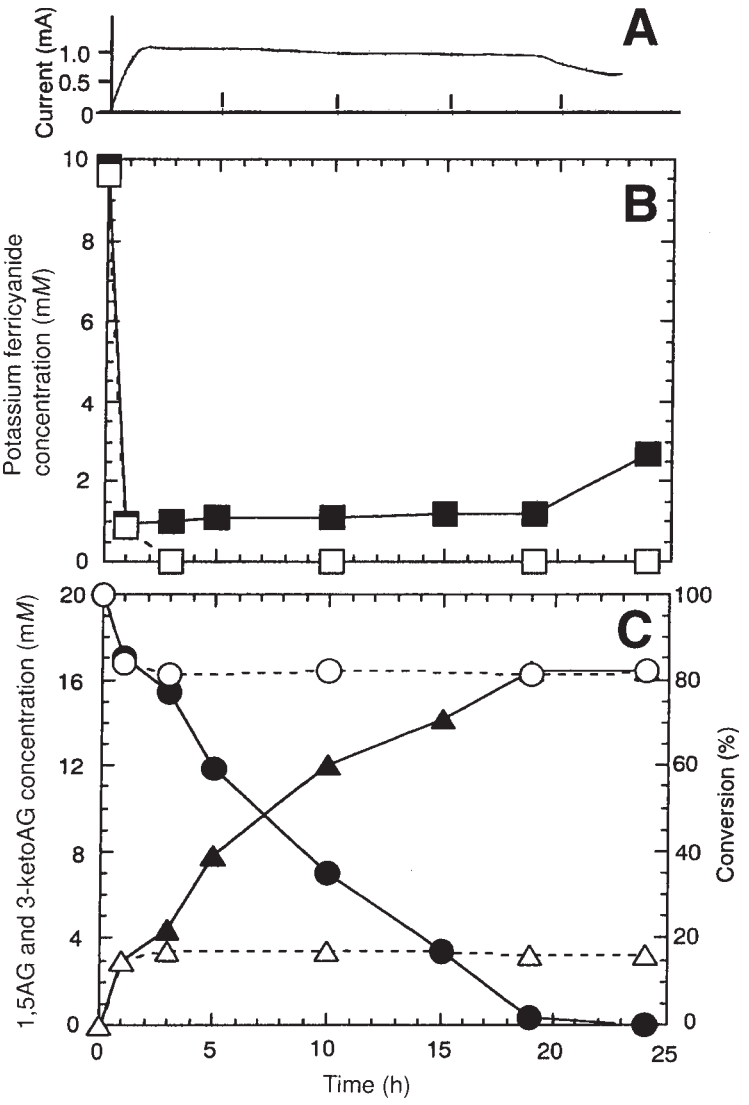
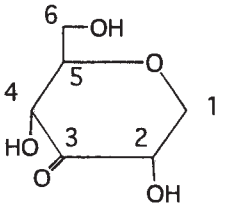


Fig. 3. Time courses of enzyme electrochemical conversion of 1,5AG using G3DH. **(A)** Current; **(B)** potassium ferricyanide concentration; **(C)** concentration of 1,5AG and 3-ketoAG. (—○—), (—●—), 1,5AG concentration (—△—), (—▲—), 3-ketoAG concentration. Closed symbols represent the experiment applying 370 mV vs Ag/AgCl, and open symbols represent the experiment without applying potential.

When the reaction was carried out by applying potential, the current increased by the addition of 1,5AG in the reaction vessel. After 1.5 h, the steady-state current was observed (1 mA). The concentration of potassium ferricyanide decreased after the addition of 1,5AG, as was observed in the aforementioned control experiment. However, after 2 h of reaction, a steady state was observed with a concentration of 1 m*M* potassium ferricyanide. This was owing to the regeneration of the oxidized form of potassium

Table 1
^{13}C NMR Data of 1,5AG and 3-KetoAG

	Assignment chemical shift (Off-resonance multiplicity) (ppm)	
C1	68.68(t)	70.86(t)
C2	69.24(d)	71.83(d)
C3	77.35(d)	**208.6(s)**
C4	69.59(d)	72.27(d)
C5	80.16(d)	82.99(d)
C6	60.78(t)	60.96(t)



product
(3-ketoAG)

s, singlet; d, doublet; t, triplet.

ferricyanide by the electrode reaction. 1,5AG was consumed gradually and simultaneously, and the 3-keto derivative of 1,5AG was accumulated. After 19 h of reaction, 1,5AG was totally consumed and the accumulation of its product reached the maximum. The amount of accumulated 3-keto derivative of 1,5AG was estimated to be 16.5 m*M* (246 μmol).

Table 1 presents the assigned chemical shifts of 1,5AG and its 3-keto derivative, recovered and purified from the reaction vessel after 24 h of reaction. The product lacked the doublet peak at 77 ppm corresponding to the third hydroxy group of 1,5AG, and showed an alternative singlet peak derived from the carbonyl group at 208 ppm. This observation confirmed that the product of enzyme electrochemical conversion of 1,5AG by G3DH was 3-keto anhydro-D-glucitol (3-ketoAG). The purification yield of 3-ketoAG was 68% (32 mg). The 1,5AG derivative, 3-ketoAG, will be further chemically modified and utilized for the preparation of a 1,5AG analog substrate. Such 1,5AG analog compounds will be utilized for the elucidation of the physiological role of 1,5AG.

Table 2 summarizes the overall yield of the reaction. The final yield of 3-ketoAG was 82% based on the initial amount of 1,5AG. The electrochemical yield of the reaction was calculated by using the period when the steady-state current was observed (from 3 to 19 h after the addition of 1,5AG). Based on the oxidative current owing to the regeneration of reduced mediator, the electrochemical conversion rate was 1.24 mmol/(L·h). The electrochemical yield was defined as the ratio of consumed 1,5AG or produced 3-keto AG and the total amount of Coulombs during the process. The electrochemical yield for 1,5AG consumption was 80%, whereas that for 3-ketoAG was 60%. Judging from the electrochemical yield of 1,5AG consumption, the reaction proceeded almost stoichiometrically. The control experiment was carried out in the absence of enzyme but with substrate

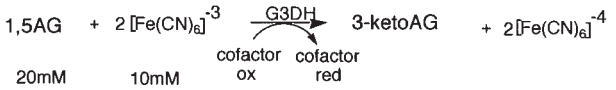
Table 2
Electrochemical Yield of 3-KetoAG Preparation

	initial 1,5AG (μmol)	consumed 1,5AG* (μmol)	produced 3-ketoAG* (μmol)	3-ketoAG* conversion (%) (1)
no potential	300	57	51	17
370 mV (Ag/AgCl)	300	294	246	82

(1) 3-ketoAG conversion (%) = $\frac{\text{produced 3-ketoAG}}{\text{initial 1,5AG}} \times 100$ *(after 19 hours)

$$\text{Electrochemical yield (3-ketoAG) (from 3 hours to 19hours)} = \frac{\text{produced 3-keto-1,5AG}}{\frac{\text{Quantity of electricity}}{96500 \times 2}} \times 100 = \mathbf{60\ (\%)}$$

$$\text{Electrochemical yield (1,5AG) (from 3 hours to 19hours)} = \frac{\text{consumed 1,5AG}}{\frac{\text{Quantity of electricity}}{96500 \times 2}} \times 100 = \mathbf{80\ (\%)}$$



and mediator under potential application, however, no conversion of 1,5AG was observed. We assumed that the lower yield of 3-ketoAG compared with 1,5AG consumption might be owing to the consumption of 3-ketoAG catalyzed by unknown enzyme contaminated in the prepared sample.

Considering that only 10% of existing electron mediator was regenerated during the reaction, an increase in the electrode surface and/or the improvement in both enzyme and mediator localization onto the surface of the electrode will significantly improve the efficiency of the proposed enzyme electrochemical conversion.

Conclusion

We proposed a novel enzymatic organic synthesis, utilizing G3DH with the combination of an electrochemical regeneration system. Using the water-soluble G3DH prepared from a marine bacterium, *Halomonas* sp. α-15, the enzyme electrochemical conversion of and 1,5AG, a novel diabetes marker in human blood, was investigated. The electrochemical yield of the reaction proceeded almost stoichiometrically. By optimizing the electrode surface area and combining of the enzyme and electron mediator immobilization techniques, the yield and efficiency of the system will be further improved. The product 3-ketoAG will be utilized for the starting material for the synthesis of 1,5AG analog compounds, which will be essential for the investigation of the physiological role of 1,5AG.

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